Characterization of the heterooligomeric red-type rubisco activase from red algae

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**CHARACTERIZATION OF THE HETERO-
OLIGOMERIC RED-TYPE RUBISCO
ACTIVASE FROM RED ALGAE**

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A thesis submitted to the Nanyang Technological University in partial fulfillment of the requirement for the degree of Doctor of Philosophy

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ABBREVIATIONS

2PG	2-phosphoglycolate
3PG	3-phosphoglycerate
4- hb	4- helix bundle subdomain
8102Rca	Rubisco activase of <i>Synechococcus sp. WH8102</i>
AAA+	ATPase associated with various cellular activities
ADP	Adenosine diphosphate
ATP	Adenosine 5'-triphosphate
b6f	Cytochrome b6f complex
C tail	Red-type Rubisco large subunits's C terminal extension
CA1P	2-carboxy-D-arabinitol-phosphate
CABP	2-carboxyarabinitol-1,5-bisphosphate
CBB	Calvin-Benson-Bassham
cfxQ	Alternative name for CbbX
Cm, <i>C. merolae</i>	<i>Cyanidioschyzon merolae</i>
CmECM/CmER/CmECM-C	<i>C. merolae</i> activated/RuBP inhibited/CABP inhibited Rubisco
CmN	<i>Cyanidioschyzon merolae</i> Nuclear encoded CbbX activase
CmN ^A or CmP ^A	Nuclear or Plastid isoform with K80A mutation (Walker A)
CmN ^B or CmP ^B	Nuclear or Plastid isoform with E138Q mutation (Walker B)
CmN ^{BS} or CmP ^{BS}	Nuclear or Plastid isoform with E138Q and R239A mutation
CmN ^{K123A} or CmP ^{R123A}	Nuclear or Plastid isoform with K123A mutation (surface-exposed lysine)
CmNP	<i>Cyanidioschyzon merolae</i> hetero oligomeric Rubisco activase
CmN ^R or CmP ^R	Nuclear or Plastid isoform with R194A mutation (arginine finger)
CmN ^{RS} or CmP ^{RS}	Nuclear or Plastid isoform with R194A and R239A mutation
CmN ^S or CmP ^S	Nuclear or Plastid isoform with R239A mutation (RuBP sugar binding)

CmN ^Y or CmP ^Y	Nuclear or Plastid isoform with Y114A mutation (Pore loop)
CmP	<i>Cyanidioschyzon merolae</i> Plastid encoded CbbX activase
CO ₂	Carbon dioxide
DMF	N, N–dimethylformamide
E	Decarbamylated/ uncarbamylated form of Rubisco
E.C.M	Carbamylated form of Rubisco
<i>E.coli</i>	Escherichia coli
E.I	Uncarbamylated-Inhibited complex
ECM	Carbamylated or activated Rubisco
ECM-C	Tightly inhibited Rubisco complex
ECMI	Carbamylated-inhibited complex
EM	Electron microscopy
ER	RUBP inhibited Rubisco
Fd	Ferredoxin
FNR	Ferredoxin-NADP ⁺ oxidoreductase
GAP	GTPase-activating protein
GroEL/ES	Chaperonin GroEL and GroES
H ⁺ ions	Hydrogen ions
H ₂ O	Water
His ₆ Ub	Hexa-histidine tagged Ubiquitin
hr	Hour
HRP	Horseradish peroxidase
IMAC	Immobilized metal ion affinity chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
K _{1/2}	Concentration of substrate to reach half maximum velocity
K _d	Dissociation constant
K _m	Michaelis-Menton constant
l	Litre
Mg ²⁺	Magnesium ion
min	Minute
MIT9313	<i>Prochlorococcus marinus</i> sp. MIT9313
n	Hill constant

NADP+	Nicotinamide adenine dinucleotide phosphate oxidized form
NADPH	Nicotinamide adenine dinucleotide phosphate
NBD	Nucleotide binding α/β - subdomain
Ni-NTA	Nickel-Nitrilotriacetic acid
NtRca	Tobacco plant activase
O ₂	Oxygen
OEC	Oxygen evolving complex
PC	Plastocyanin
PCR	Polymerase chain reaction
PDBP	D-glycero-2,3-pentodiulose-1,5-bisphosphate
P-loop	Phosphate binding loop
PMSF	Phenylmethylsulfonylfluoride
PQ/PQH ₂	Plastoquinone
PSI	Photosystem I
PSII	Photosystem II
Rbc	Rubisco
RbcL	Rubisco Large Subunit
RbcS	Rubisco Small Subunit
Rca	Rubisco activase
R-finger	arginine finger
Rs	Frictional coefficient/ Stokes radius
Rs, <i>R. sphaeroides</i>	<i>Rhodobacter sphaeroides</i>
RsE90A/RsP91A	E90A/P90A mutation in <i>R. sphaeroides</i> large subunit
RsECM\RsER\RsECM-C	<i>R. sphaeroides</i> deactivated/RuBP inhibited/CABP inhibited Rubisco
RsECM-C	Bacterial CABP Rubisco complex
RsER	RuBP inhibited complex
RsEX\WH8102EX	XuBP inhibited Rubisco of <i>R. sphaeroides</i> and WH8102
RsRca	Rubisco activase of <i>Rhodobacter sphaeroides</i>
Rs Δ 2	<i>R. sphaeroides</i> large subunit with last 2 residues of C terminus deleted
Rs Δ 4	<i>R. sphaeroides</i> large subunit with last 4 residues of C-

	terminus deleted
RT	Room temperature
Rubisco	Ribulose 1,5-bisphosphate carboxylase/oxygenase
RuBP	Ribulose 1,5-bisphosphate
s, sec	Second
$S_{c/o}$	Ratio of the catalytic efficiencies (V_{max}/K_m) for CO ₂ and O ₂
SDS-PAGE	Sodiumdodecylsulfate polyacrylamide gel electrophoresis
TAE	TRIS-acetate-EDTA
Tris	Tris-(hydroxymethyl)-aminomethane
TV	Pgem-T Vector
Ub	Ubiquitin
USP2	Ubiquitin specific-protease 2
V_{max}	Maximum velocity
vWA domain	von Willebrand Factor A domain
WH8102	<i>Synechococcus sp. WH8102</i>
WH8102RsC	WH8102 large subunit engineered with <i>R. sphaeroides</i> C-terminus extension
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside
XuBP	D-xylulose 1,5-bisphosphate
ϵ	Extinction coefficient

In this thesis, the indicated residue numbers of the Rubisco is according to Spinach Rubisco sequence.

ABSTRACT

The key photosynthetic CO₂ fixing enzyme Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) is prone to be inhibited by its own substrate RuBP and other sugar phosphates. In diverse organisms, various molecular chaperones termed the Rubisco activases have been recruited to remodel inhibited Rubisco complexes leading to the release of the inhibitors. Although red-type Rubiscos are a potential target in plant biotechnology to enhancing photosynthetic efficiency, their regulation is poorly studied. Here we biochemically characterized the two red-type Rubisco activase isoforms encoded by the thermophilic red algae *Cyanidioschyzon merolae*, as well as a homologue found in α -cyanobacteria. The individual algal isoforms are inactive in isolation, but when mixed form a functional hetero-oligomeric activase. Mutagenesis of key functional motifs indicates that ATP hydrolysis of the subunits is highly coordinated and that the nuclear-genome encoded isoform is more critical for activase function than the plastid-genome encoded counterpart. Our findings also suggest broad substrate compatibility among red-type Rubisco activases.

1.INTRODUCTION

Food and consequently the biological energy fuelling virtually all living organisms is provided by the process of photosynthesis. Plants, algae and a wide variety of photosynthetic microorganisms capture the sun's light energy and use it to convert carbon dioxide into organic compounds, which is the primary source of energy for other living organisms on earth. Besides energy, the process of oxygenic photosynthesis evolves oxygen gas, which is vital to the survival of multicellular organisms. Thus photosynthesis can be argued to be the most important biological process, which drives life on this planet.

With a burgeoning population which is expected to grow to over 9.7 billion by 2050 (UN 2015), an increased efficiency of primary productivity is essential. But a shortage of land for agriculture and changing climates pose an additional problem for plant growth and in turn food security (Gornall et al. 2010). The combination of all these problems makes it difficult to keep pace with the food demand. Hence a primary challenge in agricultural research is to increase crop productivity. Although efforts like green revolution, selective breeding, genetic engineering, etc. in the recent past have helped to improve the agricultural production, the gains from these approaches have begun to plateau and are yielding diminishing returns. Strategies to overcome this apparent yield ceiling are now increasingly aiming to enhance the photosynthetic efficiency of crops, which is currently limited to about 1% (Zhu et al. 2010).

The key player of photosynthesis is the enzyme Rubisco (Ribulose 1, 5-bisphosphate carboxylase/oxygenase). The vast majority of photosynthetic organisms use this enzyme to fix carbon dioxide during photosynthesis. The reaction catalysed by Rubisco represents the rate limiting step of photosynthesis under most conditions (Andrews and Whitney 2003, Parry et al. 2007, Whitney et al. 2011). Despite its fundamental role in autotrophs and being the most abundant protein on earth (Ellis 1979), the enzyme has many shortcomings. Compared to other enzymes the catalysis rate of Rubisco is very slow, with only a few turnovers per second (Spreitzer and Salvucci 2002). Due to the similarity in structures and electrostatic potential of CO₂ and O₂ (Kannappan and Gready 2008), Rubisco can catalyse reactions utilizing both gasses. Due to its inability to precisely differentiate between CO₂ and O₂ as a substrate, the reaction with O₂ lowers the rate of photosynthesis and the efficiency. This negatively affects the crop yield, nitrogen and water use efficiency (Bauwe et al.

(ATPases associated with various cellular activities) motor. This common module is ubiquitously found across biology and is involved in mechanically remodeling macromolecular substrates such as proteins and polynucleotides (Hanson and Whiteheart 2005). Although the basic function of the three activase classes is the same, they vary dramatically by sequence, regulation and mechanism. This indicates that during evolution different organisms have recruited different nano-machines for the same role to remodel inhibited Rubisco complexes but tweaked their properties to meet the particular needs of the organism (Mueller-Cajar et al. 2013). Recently a growing number of AAA+ systems are being carefully characterized (Arlt et al. 1996, Mogk et al. 2003, Siddiqui et al. 2004, Snider et al. 2008, Ciniawsky et al. 2015). Consequently, a flood of knowledge about their working mechanism is being generated. By carefully considering their molecular mechanisms, structure-function relationships and the design of appropriate experiments, a dissection of the various Rubisco activase mechanisms is now feasible. Recent genomic studies have revealed that many key photosynthetic organisms encode different activases. CbbX genes appear to be found in association with all red-type Rubiscos including all phytoplankton of the red plastid lineage (Fujita et al. 2008). CbbX genes are also found predominantly in α -cyanobacterial and in some β -cyanobacterial genomes encoding a green-type Rubisco (Zarzycki et al. 2012) which also additionally encode a plant-like Rca in some cases. Understanding the structure and function of these different activases should facilitate efforts in biotechnology to improve the efficiency of plants and microorganisms to convert more CO₂ into valuable biomass. Since some red algae that survive at a very high temperature encode CbbX (Barbier et al. 2005), this provides a possible avenue for finding a more thermostable Rubisco activase for use in biotechnological applications.

2.1. Photosynthesis

Photosynthesis in plants, algae or specialised bacteria is a fundamental but complex biochemical process of synthesizing organic compounds (sugars) by utilizing light energy from the sun (Nelson et al. 2008). Photosynthesis not only fuels the autotrophs but all living organisms that cannot produce organic compounds from inorganic sources depend directly or indirectly on this process for their survival.

In a photosynthetic eukaryotic cell, photosynthesis takes place in specialized organelles called chloroplasts, which are evolutionarily derived from an ancestral endosymbiotic cyanobacterium. The chloroplast encloses membrane-bound flattened sacs (thylakoids) which are folded upon themselves forming stacks or disks (grana) in the chloroplast aqueous matrix (stroma). The inner surface enclosed by the thylakoids is referred to as lumen (Nelson et al. 2008). Photosynthesis is separated into two processes, the light-dependent reactions and carbon reactions. First, the energy from light is converted into chemical energy (NADPH and ATP), the latter is then utilised to drive a metabolic cycle that produces useful sugar molecules. The necessary machinery and apparatus for light-dependent reactions are embedded in the thylakoid membranes. The enzymes utilized for the carbon assimilation reactions are soluble stromal proteins (Nelson et al. 2008) (Figure 1.1).

Figure 1.1| Schematic illustration of the basic process of photosynthesis that takes place in the chloroplast. Modified from (Campbell and Reece 2005)

2.1.1. The Light-Dependent Reactions

The light-driven reactions of photosynthesis take place on the thylakoid membranes of the chloroplast. For a green algal chloroplast (from *Dunaliella tertiolecta*) it has been estimated that the thylakoid membrane houses 24×10^6 PSI and 17×10^6 PSII reaction centers. Assuming 200-300 chlorophyll molecules per reaction center (Anna et al. 2014) this would correspond to $\sim 12.3 \times 10^9$ chlorophyll molecules (Suknik et al. 1987), which capture the energy from the sunlight under ambient light intensity. The pigment numbers are observed to be reduced by an order of 10^3 with increasing light intensities. Light energy trapped from absorbed photons is utilised to excite electrons from chlorophyll and other pigments of the photosystems. The excited electrons flow from photosystem II (PSII) to photosystem I (PSI) through an electron transport chain to finally reduce NADP^+ to NADPH in the stroma. The electron flow also drives the generation of a chemiosmotic gradient that is utilized to phosphorylate ADP to form ATP (Figure 1.2A).

Photosynthesis initiates with the simultaneous excitation of electrons from pigment molecules in the reaction centers of PSI and PSII. Chlorophyll pigments P680 and P700 together with other accessory pigments and proteins forms the reaction centers of PSI and PSII respectively that perform the photochemical electron transfer from water to NADPH. The chlorophyll molecules in the reaction center are uniquely positioned in the reaction centers in such a way that it mediates an efficient electron transfer to the next electron carrier. The energy cascaded by the pigments in PSI is used by the P700 chlorophyll, which absorbs energy from the red region of the spectrum ($\lambda \sim 700\text{nm}$), to excite an electron to a higher energy level (P700^* in Figure 1.2B), which enables an adjoining electron acceptor molecule to capture the excited electron. Electrons derived from P700 are eventually passed down to adjacent lower energy carrier molecules until reaching the ultimate electron carrier NADP^+ , creating an electron transport chain. Two electrons transported in the system combine with H^+ ions and NADP^+ to form NADPH (Figure 1.2). The electron deficient P700 is replenished by electrons originating from PSII. Similar to PSI, light energy excites an electron of the P680 chlorophyll molecule so as to be absorbed by the neighboring electron acceptor molecule. The electrons are cascaded through a series of carrier molecules in a different electron transport chain to be finally transferred to the P700^+

molecule of the PSI and making it functional for the next cycle (Nelson et al. 2008). Like the electron flow in PSI, the electron transport chain of PSII results in the release of energy and establishes a proton gradient across the thylakoid membrane which fuels the production of ATP from ADP by chloroplast F₀F₁ ATP synthase (Duysens et al. 1961, Graciet et al. 2004) (Figure 1.2A). This closely resembles the ATP synthesis by mitochondrial F₀F₁ ATPase (Boyer 1997). The electrons donated by P680 to electron deficient P700 is replaced by the electrons generated by splitting of water into hydrogen ions (H⁺) and oxygen gas (O₂) (Leslie 2009). These electrons are used to regenerate P680 of PSII. Electron transport between two photosystems through electron transport chain complex to reduce NADH⁺ is also referred as the “Z-scheme” (Figure 1.2B). Protons are pumped into the thylakoid lumen to generate the proton gradient powering ATP synthesis. This is driven by cyclic or non-cyclic electron transport across the photosystems (Cruz et al. 2005, Merchant and Sawaya 2005).

Structural and biophysical studies of PSI and PSII are currently flourishing providing an atomic level understanding of the light reactions. Hence the light reactions of photosynthesis can be considered as an example of an extremely well-studied biochemical process, the details of which are beyond the scope of this introduction (Nelson and Junge 2015).

Figure 1.2| Electron and proton flow in the light reactions of photosynthesis.

(A) Proton and electron circuits in thylakoids. Transfer of electrons from (orange arrows) H_2O to PSII, PSI and intermediate carrier chains to NADP^+ (see B), yields NADPH ($\text{NADP}^+ + \text{H}^+ + 2\text{e}^- \rightarrow \text{NADPH}$). As a consequence of cyclic and noncyclic electron transfer through the electron carriers linking PSII and PSI (see B), protons (blue arrows) are pumped into the lumen. Additionally, protons are generated in the thylakoid lumen by the light-driven splitting of H_2O in the oxygen-evolving complex. Due to the proton gradient across the thylakoid membrane, protons re-enter the stroma through the CF_0 subunits of ATP-synthases. The proton motive force is used for the synthesis of ATP from ADP by the CF_1 subunit ($\text{ADP} + \text{P}_i + 3 \text{H}^+ \text{ thylakoid lumen} \rightarrow \text{ATP} + 3 \text{H}^+ \text{ stroma}$). Both NADPH and ATP are utilized by the light-independent reaction of photosynthesis for carbohydrate synthesis in the Calvin cycle.

(B) Schematic representation of the “Z scheme” presenting the linear electron transfer orange-green arrows from H_2O to NADP^+ through photosystems (PS) I and II in the chloroplasts. The electrons are generated when the oxidation of two water molecules occurs at the Mn4-containing oxygen-evolving complex (OEC) as a result of four absorbed photons ($2\text{H}_2\text{O} \rightarrow 4\text{H}^+ + 4\text{e}^- + \text{O}_2$). These electrons are transferred to the reaction centre of PSII, P680. The excited P80 (P680*) by the absorbed light energy, transfers electrons over pheophytin to plastoquinone. Half of the electrons pass over a chain of intermediate carriers (cytochrome b6f complex and plastocyanin), to the reaction centre of PSI, P700. While the other half of the electrons return to the PQH2 pool. Upon absorbing light energy, the electrons transfer from P700 through a series of electron carriers to ferredoxin and finally to reduce NADP^+ to NADPH. The electron flow through plastoquinone and the cytochrome b6f complex drives protons from the stroma into the thylakoid lumen. Together with the protons generated at the OEC a proton gradient is generated across the membrane and the proton-motive force provides the energy for ATP synthesis by an ATP synthase (see A). Modified from Cruz et al. (Cruz et al. 2005, Nelson et al. 2008).

2.1.2. The Carbon Fixation Reactions

The carbon reactions are a series of enzyme-controlled oxidation-reduction reactions to convert CO_2 and H_2O into carbohydrates utilizing the chemical energy from the by-products of the light-independent reactions, namely ATP and NADPH (Nelson et al. 2008). The CO_2 enters the plant through pores in the leaf known as stomata and/or the membrane of the autotrophic eukaryotes and then diffuses through the cell towards the site of fixation, which is the chloroplast stroma. The carbon assimilation reactions then take place within the stroma of the eukaryotes or the bacterial cytosol which contain all the essential enzymes. The cyclic pathway mediating this process is formally known as the Calvin-Benson-Bassham cycle (also known as Calvin cycle, reductive pentose phosphate pathway or photosynthetic carbon reduction cycle) (Raines 2003). All plants, algae and many autotrophic bacteria remove CO_2 from the environment and reduce it to carbohydrates by the Calvin cycle. This pathway encompasses essentially three phases: carbon fixation, reduction and regeneration of the substrate as shown in Figure 1.3. The fixation of carbon phase is the carboxylation of the 5-carbon sugar ribulose-1, 5-bisphosphate (RuBP) with a CO_2 molecule to form a 6-carbon molecule (unstable) which is subsequently cleaved to form two 3-carbon molecules, 3-phosphoglycerate (3PG). This key reaction is catalysed by the enzyme Ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco), a large water soluble protein complex and reportedly the most abundant enzyme on the planet (Ellis 1979). The reduction phase involves several intermediate reactions catalysed by different enzymes that consume the end products

from the light-dependent reactions of photosynthesis (ATP and NADPH) to reduce 3-phosphoglycerate to glyceraldehyde-3-phosphate (Figure 1.3). In the regeneration phase, the high energy precursor RuBP is regenerated from glyceraldehyde 3-phosphate (Figure 1.3). This is accomplished by a series of carbon reshuffling reactions that resemble the non-oxidative portion of the pentose phosphate pathway. In a final reaction, the precursor ribulose 5-phosphate is phosphorylated by phosphoribulokinase to regenerate RuBP in an ATP-dependent manner. The unused glyceraldehyde 3-phosphate molecules for regeneration are used for the synthesis of glucose and organic molecules (e.g., proteins, nucleic acids). Altogether, thirteen enzymes are required to catalyse the reactions in the Calvin cycle. As a net result of the Calvin cycle, the carboxylation of six 5-carbon RuBP molecules with six molecules of CO₂ yields one 6-carbon molecule (glucose-P or fructose-P) and regeneration of six 5-carbon RuBP molecules at the expense of 18 ATP and 12 NADPH molecules (Nelson et al. 2008).

Rubisco is a bifunctional enzyme that contributes to two opposing metabolic pathways: photosynthetic carbon fixation and the competing process of photorespiration (Spreitzer and Salvucci 2002). In addition to catalysing addition of CO₂ to RuBP, it can also catalyse addition of O₂ to the same. As a consequence of oxygenation of RuBP, a 3-carbon molecule (3-phosphoglycerate) that is used in the Calvin cycle and a 2-carbon molecule (2-phosphoglycolate, 2PG) that is not useful for the organism (Foyer et al. 2009) are formed. 2PG can be considered a damaged metabolite (Linster et al. 2013), that needs to be repaired via a complicated and energy requiring series of reactions known as the photorespiratory pathway that leads to regeneration of 3-PG. During photorespiration previously fixed CO₂ is released again (Bauwe et al. 2010).

The significant drain in metabolic energy associated with running a high-flux photorespiratory pathway has resulted in a strong evolutionary selection pressure to minimize the oxygenation reactions. This has led to multiple independent events, where organisms have evolved dedicated mechanisms to concentrate the substrate CO₂ at the active site of Rubisco. These so-called carbon concentrating mechanisms can be of a biochemical or biophysical nature. Cyanobacteria have evolved Rubisco containing micro-compartment known as carboxysomes (Badger et al. 2002, Rae et

al. 2013). Many single-celled algae concentrate their Rubisco in a distinct stromal region designated as pyrenoids (Badger et al. 1980, Badger et al. 1998, Kaplan and Reinhold 1999, Giordano et al. 2005). In both cases, inorganic carbon in the form of bicarbonate is pumped into the cells by active transport, and at the site of Rubisco, is converted to gaseous CO₂ by co-localized carbonic anhydrases. This process saturates Rubisco with CO₂ and minimizes the competing oxygenation reaction. In some higher plants biochemical CCMs, known as the C₄ and CAM pathways, have evolved to reduce the oxygenation reaction (Keeley and Rundel 2003).

Figure 1.3| Calvin Cycle.

RuBP(5-carbon molecule) with the addition of CO₂ forms an unstable 6-carbon molecule (Carbon fixation phase). This 6-carbon molecule formed immediately breaks down into two 3-carbon molecules and is reduced to form 1,3-bisphosphoglycerate molecules (Reduction phase). Some of the glyceraldehyde-3-phosphate formed is used to produce glucose (Carbohydrate formation) and other, more complex organic molecules. The remaining glyceraldehyde-3-phosphate is used to regenerate the 5-carbon ribulose (Regeneration phase). Modified from concepts in biology (Chapter 7)

and http://media1.shmoop.com/images/biology/biobook_photosyn_3.png.

2.2. Rubisco structure

The fundamental structural unit of all Rubiscos is a dimer of 52 kDa large subunits (RbcL₂). Rubisco is classified into four major forms namely Form I-IV (Figure 1.4). Only Form I-III perform catalysis of carboxylation and oxygenation of RuBP. Only Form I has small subunits (RbcS ~15 kDa) and exists as a hexadecameric complex of four antiparallel RbcL₂ arranged around a 4-fold axis, capped at each end by a RbcS (RbcL₈S₈) (Knight et al. 1990, Tabita et al. 2008). This is the most common form of Rubisco which is found in higher plants, cyanobacteria and autotrophic proteobacteria and this is further classified into four sub groups: Form IA-D. Form IA and Form IB are commonly termed as green-type Rubisco (Tabita et al. 2008), which is found in plants, green algae, cyanobacteria and some proteobacteria (Badger and Bek 2008).

In plants and green algae, the RbcL is encoded by the chloroplast genome, whereas RbcS is encoded by the nuclear genome (Blair and Ellis 1973). Correlated with the form of Rubisco present (Form IA and IB), cyanobacteria are classified as α - and β - cyanobacteria. In contrast to plants and green algae, cyanobacterial Rubisco subunits are encoded in the same operon. Form IC and Form ID are known as red-type Rubisco (Tabita et al. 2008) which are present in certain proteobacteria and eukaryotes of the red-plastid lineage (non-green algae). In these organisms subunits, RbcL and RbcS are encoded in the same operon (Hartman and Harpel 1994). Several red-type Rubiscos have much higher CO₂/O₂ specificity values ($S_{c/o}$ – defined as the ratio of the catalytic efficiencies (V_{max}/K_m) for CO₂ and O₂) than their green-type counterparts (Read and Tabita 1994, Uemura et al. 1997, Whitney et al. 2001). For instance Rubisco from the red algae *Griffithsia monilis* shows three fold higher affinity towards CO₂ than O₂ compared to all plant Rubisco (Whitney et al. 2001). Form II Rubisco is found in proteobacteria and dinoflagellates, and is active as RbcL₂. This form possesses a poor CO₂/O₂ specificity. Form III enzymes are found in some thermophilic archaea, which lack the Calvin cycle but appear to generate RuBP via an alternative pathway (Sato et al. 2007). It is active as a RbcL₂₋₅ (Kitano et al. 2001). Form IV Rubiscos do not catalyse CO₂ fixation and are known as Rubisco-like proteins (RLP) (Hanson and Tabita 2001, Tabita et al. 2008). Some of them have been

implicated to be functioning in other metabolic pathways, such as the methionine salvage pathway (Ashida et al. 2003).

Figure 1.4 | Representative structures of different forms of Rubisco.

All forms are comprised of dimers of large catalytic subunits. Side-view and top-view of Form I from *Synechococcus* PCC6301 (PDB: 1RBL), comprised of four dimers with small subunits decorating the top and bottom of the RbcL₈ octameric core. Only Form I has small subunits. Form II from *Rhodospirillum rubrum* is comprised of dimers of RbcL (PDB: 5RUB). But it varies from RbcL₂₋₈ depending on the source. Form III is found only in some Archaea and is comprised of dimers of RbcL in either a RbcL₂ or Rbc(L₂)₅ arrangement. Side-view and top-view of Form III Rubisco from *Thermococcus kodakarensis* in Rbc(L₂)₅ is shown (PDB: 1GEH). Form IV (the Rubisco-like Protein or RLP) seems to have a RbcL₂ structure. Form IV Rubisco from *Chlorobium tepidum* is shown (PDB: 1YKW). (Large subunit monomers are shown in purple and green while small subunits are shown in violet).

2.2.1. Activation (Carbamylation) and catalysis of Rubisco

In order to become catalytically competent, the Rubisco active site needs to bind two cofactors. The active site for carboxylation is present at the interface between the N-terminal domain of one subunit and the C-terminal domain of the second subunit of the antiparallel RbcL₂ (Schneider et al. 1986). The activation step is initiated by the reaction of a non-substrate CO₂ molecule with the ε-amino group of lysine 201 located at the catalytic sites to form a carbamate (EC). The formation of carbamate is slow, but this form is stabilized by rapid binding of Mg²⁺ to form the active complex (ECM) (Lorimer et al. 1976, Cleland et al. 1998). The activated state is also referred as carbamylated form (Lorimer et al. 1976), and the mechanism is illustrated using the Figure 1.5. These spontaneously occurring reversible reactions are only possible in the ‘open state’ of the enzyme, which allows solvent access to the binding pockets. The addition of Mg²⁺ cofactor at the Rubisco active site is important for positioning the substrate RuBP. Accompanied by ligand binding several loops of the enzyme form an ordered arrangement to isolate the ligand from the solvent (Schreuder et al. 1993, Taylor and Andersson 1996) forming a “closed state” and bound ligands stabilize this conformation by interacting with one of the loops (loop 6). The active site opens and closes during catalysis, which involves the movement of loop 6 (Duff et al. 2000).

Figure 1.5| Mechanism of Carbamylation.

Modified from (Nelson et al. 2008) and <http://www.wikiwand.com/fr/Rubisco>.

During catalysis, in the 'closed state,' loop 6 forms a latch- like structure on top of the substrate and reaction intermediates that are deeply buried in the active site of the complex. Carboxylation is initiated when carbamylated lysine 201 (residue numbering is according to Spinach Rubisco) abstracts a proton from RuBP to form enediol isomer of RuBP (enolisation) at the active site and this isomer is responsible for carboxylation, as it makes an electrophilic attack on the substrate CO₂ molecule to form a ketonic acid form. This unstable 6-carbon intermediate is subsequently hydrated and cleaved to produce the two 3-carbon molecules, 3-phosphoglycerate (3PG) (Houtz and Portis 2003) (Figure 1.3 and 1.6A). Due to the catalytic property of the Rubisco in the inability to distinguish O₂ molecules, Rubisco enzyme oxygenates RuBP. The oxygenation reaction takes place when O₂ molecule competes at the active site instead of CO₂ molecules. The mechanism is similar to CO₂ fixation; enediol isomer makes an electrophilic attack on the O₂ molecule resulting in an intermediate, which is hydrolysed to form a 3-carbon (3-phosphoglycerate) and a 2-carbon (2-phosphoglycolate) molecule (Figure 1.6B). At the completion of catalysis, the binding energy released from the cleavage of connection between two phosphate substituents, is thought to be needed for the phosphates to separate and the active site to open to release the products (Andrews 1996).

A

B

Figure 1.6| Catalytic mechanism of Rubisco.

Rubisco catalyzes two types of reactions carboxylation and oxygenation. **(A)** Carboxylation activity of Rubisco. Rubisco is activated after carbamylation of the ϵ -amino group of Lys201 by a non-substrate CO_2 (indicated in red) followed by the binding of Mg^{2+} (indicated as green sphere) to the active site (Figure 1.5). Mg^{2+} stabilizes binding of RuBP and substrate CO_2 to be fixed at the active site. At the active site, binding of RuBP is followed by proton extraction and rearrangement (green arrows) to form 2,3-enediolate intermediate. With the addition of substrate CO_2 (orange), Mg^{2+} ion polarizes the CO_2 which subsequently undergoes nucleophilic attack by the enediolate. The reaction of CO_2 with enediolate (carboxylation) generates a branched C_6 intermediate. 2'-carboxy-3-keto-D-arabinitol 1,5-bisphosphate. Hydration at C-3 of this intermediate by a water molecule (blue) forms a β -keto acid intermediate. The cleavage between C-2 and C-3 yields one molecule of 3-phosphoglycerate (3PG) that leaves the active site of the enzyme and a carbanion of the other 3-carbon. Protonation of the carbanion by the side chain of Lys175 forms a second molecule of 3PG which completes the carboxylation catalytic cycle. **(B)** Oxygenation activity of Rubisco. Reaction of O_2 with enediolate form of RuBP results in an unstable C_5 hydroperoxide intermediate. Analogous to carboxylation reaction, the C_5 intermediate undergoes hydration, cleavage and protonation to give rise to 2-phosphoglycolate (2PG) and 3-phosphoglycerate (3PG). The 2PG is useless to the organism but can be recycled to 3PG via energy consuming photorespiratory pathway, the formed 3PG can re-enter the Calvin cycle. The Rubisco residue numbering is according to spinach Rubisco. (Reproduced from (Nelson et al. 2008).

2.2.2. Inhibition and Regulation of Rubisco by phosphorylated sugars

Ligands can bind to the uncarbamylated active site, leading to an unactivated complex that is catalytically inactive (Jordan and Chollet 1983, Portis Jr 1992). This shows that loop movements that characterize the closed state depend only on ligand binding (to carbamylated and uncarbamylated) and not carbamylation and a metal ion. Catalytic cleavage necessary for reopening closed complexes happens only when the ligand is RuBP and only when Rubisco is properly carbamylated with the Mg^{2+} in place. However with the absence of catalysis in the uncarbamylated complexes, there is no quick way of escaping the closed state to release the ligands, this leads to an unproductive complex. Moreover measured rate of release for various inhibitors is often very slow, varying from hours to days (Jordan and Chollet 1983, Berry et al. 1987, Schloss 1988, Cardon and Mott 1989, Pearce 2006).

A number of phosphorylated sugar compounds bind to substrate binding pockets (Rubisco active sites) to form a tight binding complex making it catalytically inhibited (Paech et al. 1978) (Figure 1.7A). Some of the known inhibitors of Rubisco are, 2-carboxy-D-arabinitol 1,5-bisphosphate (CABP) (Wishnick et al. 1970, Pierce et al. 1980), a non-physiological transition state analogue; 2-carboxy-D-arabinitol-1-phosphate (CA1P) (Servaites 1985, Gutteridge et al. 1986, Berry et al. 1987), a naturally occurring nocturnal inhibitor; and D-xylulose 1,5-bisphosphate (XuBP) (Edmondson et al. 1990, Edmondson et al. 1990), a stereoisomer of RuBP, formed by misfiring of Rubisco during catalysis (Figure 1.7B). Including other misfired products that tend to inhibit the activity of Rubisco, interestingly its own substrate RuBP also interacts tightly with the substrate binding pockets of decarbamylated apo Rubisco (E). Thus the apo enzyme is unable to catalyse either the oxygenation or the carboxylation reaction and this results in blocked complexes (Jordan et al. 1983). This is consistent with the kinetic characteristics of Rubisco, at carbamylated state the affinity for RuBP is low (McNevin et al. 2006) because RuBP is rapidly cleaved and released at the carbamylated active site, conversely at uncarbamylated state the affinity for RuBP is higher ($K_d \sim 20$ nM for spinach Rubisco (Jordan and Chollet 1983)). Thus binding of RuBP to uncarbamylated sites makes the Rubisco complex inactive.

Both carbamylated (E.C.M) and decarbamylated forms (E) of Rubisco are prone to form tightly inhibited complexes (Figure 1.7A). Different inhibitors bind with different affinities to different states of the Rubisco. In addition to RuBP, XuBP and other misfired isomers of RuBP are known to favour binding to the uncarbamylated state (E) of Rubisco to form an uncarbamylated-Inhibited complex (EI), while CA1P and CABP bind to carbamylated state (ECM) of Rubisco to form carbamylated-inhibited complex (ECMI) (Pierce et al. 1980, Seemann et al. 1985). The CABP-Rubisco complex is similar to the intermediate transition state during carboxylation. CABP binds to Rubisco with high affinity (Pierce et al. 1980) and forms the most tightly bound complex because K_d (dissociation constant) of the CABP was comparatively low relative (0.2 pM for spinach Rubisco) to other inhibitors (Schloss 1988). The half-life for the release of CABP from the inhibited complex was significantly more (530 days) than other inhibitors (Schloss 1988), in spinach Rubisco making the binding almost irreversible (Pierce et al. 1980). Rubiscos with ligands

occupying the substrate binding sites in carbamylated (Knight et al. 1990) and uncarbamylated state (Newman and Gutteridge 1994) are structurally similar, indicating that cofactors do not play a critical role in substrate binding to the active sites. Irrespective of whether RuBP, a non- or slow cleavable analogue of RuBP, or XuBP binds to the uncarbamylated active site or if CA1P, CABP binds to carbamylated active sites, no rapid path exists whereby the active sites can open up to release the ligand (Andrews 1996).

Binding by its own substrate RuBP (Cardon and Mott 1989) and CA1P at the active site to make Rubisco a catalytically inhibited complex is part of the physiological process of regulation of Rubisco *in vivo* in higher plants (Servaites 1985). Additionally activated Rubisco enzyme in the presence of saturating levels of CO₂ and RuBP shows non-linear catalysis. This slow time-dependent reversible inactivation of Rubisco is popularly termed as “fall over” phenomenon (Andrews and Hatch 1971, Sicher et al. 1981, Parry and Gutteridge 1984, Mott and Berry 1986, Robinson and Portis 1989, Edmondson et al. 1990). This phenomenon is known to occur by the accumulation of isomerized and misfired/misprotonated RuBP products during catalysis (oxygenation and carboxylation) and thereby results in a decline of Rubisco activity. If non-cleavable ligands are present in significant amounts, they will also sequester Rubisco active sites in both states. Though these inhibitors are very small in fraction compared to its substrate RuBP (Paech et al. 1978), they cause the formation of slow potent tight binding inhibited complexes (Zhu and Jensen 1991, Zhu and Jensen 1991, Zhu et al. 1998). So during active photosynthesis, inactive Rubisco complexes would thus accumulate and block carbon fixation (Mueller-Cajar et al. 2013). Although the rate of production of misfire products increases with temperature, some inhibitors may not bind Rubisco tightly at a higher temperature resulting in a less severe fall over phenomenon (Schrader et al. 2006). This reduced inhibition could be an outcome of increased flexibility of the Rubisco structure at higher temperatures, resulting in the prompt release of the inhibitors from the catalytic site. The inclination to inhibition by these mechanisms varies between Rubisco enzymes from diverse sources (Uemura et al. 1998, Pearce and Andrews 2003, Pearce 2006).

Extensive comparisons of kinetic properties of Rubisco from diverse species have shown inverse proportionality between substrate selectivity and catalytic rate (Tcherkez et al. 2006). Rubisco forms compromise low catalytic rate with high substrate selectivity and less selective enzymes compromise by their fast catalytic rate (Zhu et al. 2004, Tcherkez et al. 2006). A prominent model proposed by Tcherkez to explain the substrate discrimination by Rubisco predicts that enzymes exhibiting a high specificity for CO₂ over O₂ favour tight binding of transition state intermediates of the carboxylation reaction and consequently the cleavage of tightly bound bisphosphate ligand intermediate becomes difficult, resulting in a slower carboxylation rate. This tight binding favours carboxylation over oxygenation but slower rate, unfortunately, favours increased misfire product formation (Tcherkez et al. 2006). This model together with the study by Pearce (Pearce 2006) showing high S_{c/o} enzymes forming slow tight binding complexes with inhibitors indicate that the active site of high S_{c/o} enzymes is more rigid. Table 1.1 briefly summarizes the relation between the specificity for CO₂ and the inhibition properties of the Form I Rubiscos from various sources. Form I Rubiscos of plants possessing high specificity forms inhibited complexes with RuBP and displayed fall over. Form I Rubisco of red algae with high S_{c/o} apparently do not exhibit fall over inhibition, but when inhibited complexes are formed they are extremely stable (Pearce and Andrews 2003, Pearce 2006). The enzymes of green algae with intermediate S_{c/o} form ER but do not fallover (Uemura et al. 1998), while low S_{c/o} enzymes of cyanobacteria form neither inhibited ER complexes nor do they display fallover (Andrews and Abel 1981, Lee et al. 1991, Uemura et al. 1998). It thus appears that evolution of high S_{c/o} enzymes with a low catalytic turnover was inevitably accompanied by an increasing propensity to form inhibited dead-end complexes.

Table 1.1. Comparison of inhibition properties of Rubiscos from different sources.

Source	Rubisco	Specificity (S _{c/o})	Inhibition		Reference
			Fall over	ER	
Plants	Form IB	High	Yes	Yes	Pearce and Andrews 2003, Pearce 2006
Red algae	Form ID	Very high	No	Yes	
Green algae	Form IB	Intermediate	No	Yes	Uemura et al. 1998
Cyanobacteria	Form IB	low	No	No	Andrews and Abel 1981, Lee et al. 1991, Uemura et al. 1998

The evolution of Rubisco enzyme improved its ability to distinguish better the CO₂ over O₂ as its substrate, but together with this positive outcome their susceptibility to get inhibited by the sugar phosphates in the cellular environment also increased. The more evolved Rubisco enzymes of plants and red algae with higher specificity for CO₂ substrate showed more inhibition than the cyanobacterial Rubisco.

This situation was addressed during evolution, by the recruitment of dedicated helper proteins, called the Rubisco activase (Rca). (Salvucci et al. 1985, Robinson and Portis 1988, Robinson et al. 1988) (Figure 1.7). Three classes of Rubisco activases have been described so far. They are the green-type Rubisco activase (Rca) found in higher plants (Salvucci et al. 1985, Stotz et al. 2011), red-type Rubisco activase (CbbX) from α -proteobacteria (Mueller-Cajar et al. 2011), and CbbQO-type found in chemoautotrophic bacteria (Tsai et al. 2015). Chemoautotrophic proteobacteria uses sulphur and other inorganic compounds as a substrates as opposed to the light by the photoautotrophic organisms to make the energy molecules. Although chemoautotrophs perform dark reactions to fix CO₂ using the Rubisco (Swan et al. 2011), their genomes do not encode either of the previously studied activases (Rca or CbbX). However, gene products of the *cbbQ* and *cbbO* which were found directly downstream of the Rubisco was characterized to be a different class of Rubisco activase (Tsai et al. 2015). In all cases the energy required to remodel inhibited Rubisco complexes is generated by an AAA+ protein module. In order to provide the framework for understanding the function of the various Rubisco activases, we summarize our understanding of this large group of molecular chaperones in the following section.

Figure 1.7| Regulation of Rubisco activity and inhibition by sugar phosphates.

(A) The catalysis of Rubisco is initiated when decarbamylated Rubisco (E) binds to CO_2 and Mg^{2+} forming an active carbamylated form (E.C.M), which then actively fixes CO_2 by carboxylation of its substrate RuBP. In the absence of CO_2 (activator) and Mg^{2+} , E form can bind to the substrate RuBP or other sugar phosphates to form a dead inhibited complex (E.I). Active E.C.M form can also be inhibited when it binds to RuBP substrate analogues (like 2-carboxy-arabinitol-1-phosphate (CA1P) or D-glycero-2,3-pentodiulose-1,5-bisphosphate (PDBP) and the transition state analogue CABP etc.), denoted as E.C.M.I in figure. Helper enzyme, Rubisco activase is necessary to remodel the inhibited Rubiscos (E.I and E.C.M.I), by removing the inhibitory sugar-phosphate compound from the active site and thus maintaining the active sites for catalysis. (B) Structures of Rubisco inhibitors. Except for CABP, which is artificially synthesized (indicated in blue), other sugar inhibitors are formed naturally during carboxylation reaction (indicated in green). RuBP-Ribulose-1,5-bisphosphate; XuBP-Xylulose-1,5-bisphosphate; CA1P-2-carboxy-D-arabinitol 1-phosphate; CABP-2-carboxyarabinitol-1,5-bisphosphate; PDBP-D-glycero-2,3-pentodiulose-1,5-bisphosphate; KABP-3-ketoarabinitol-1,5-bisphosphate; CTBP-2-carboxytetritol-1,4-bisphosphate. Phosphate groups are indicated as a yellow sphere. Modified from (Parry et al. 2008).

2.3. All known Rubisco activases belong to the family of AAA+ Proteins

The AAA+ superfamily of enzymes (ATPases Associated with a variety of cellular Activities) (Neuwald et al. 1999) is defined by the presence of a conserved region of 200-250 amino acids known as AAA fold or module (Confalonieri and Duguet 1995).

AAA+ proteins are found in various cellular locations and participate in diverse cellular processes ranging from protein unfolding and degradation or disaggregation, DNA replication and RNA unwinding, micro tubular transport and severing, membrane fusion, to transcriptional activation (Ogura and Wilkinson 2001, Hanson and Whiteheart 2005). Multiple AAA+ proteins are found in all living organisms. While most eukaryotes have 50-80 AAA+ proteins, *Arabidopsis thaliana* had the maximum of around 140 family members (Beyer 1997, Frickey and Lupas 2004).

Although the AAA+ module of the superfamily is similar, some family members contain two modules or domains while others have only one. So on the basis of the number of AAA+ domains the superfamily is divided into two distinct classes (Schirmer et al. 1996). Class I proteins (e.g. ClpA, ClpB, ClpC and ClpE) contain two highly conserved AAA domains, referred to as AAA-1 (or D1) and AAA-2 (or D2), separated by a variable length linker sequence. Class II proteins (e.g. ClpX, HslU), including all known Rubisco activases, contain only a single AAA domain (Hanson and Whiteheart 2005, Erzberger and Berger 2006).

Conserved features of the AAA+ domain are shown in Figure 1.8A, with the helices and strands as designated by (Iyer et al. 2004). AAA+ domain has a conserved P-loop (phosphate binding loop) fold forming the N-terminal subdomain and a less conserved C-terminal subdomain. α/β Rossman Fold with β -sheet of parallel strands, arranged in a $\beta 5-\beta 1-\beta 4-\beta 3-\beta 2$ fashion at N-terminal and several α -helices forming a bundle at C-terminal that partially covers the open end of N subdomain are the distinctive conserved features of AAA+ proteins (Figure 1.8A, B and C) (Iyer et al. 2004).

All members of the AAA+ family are Mg^{2+} dependent ATPases (Wendler et al. 2012, Sysoeva 2016) with most members forming ring-shaped oligomer structure (mostly homo- or hetero-hexamer oligomeric rings), with a spherical or cylindrical central pore (Sysoeva 2016) (Figure 1.8C). A common theme in the function of AAA+ ATPases is their ability to remodel the conformation of macromolecules. Multiple cycles of ATP binding and hydrolysis cause conformational changes that enable the AAA+ ring to achieve mechanical work on macromolecular substrates (Hanson and Whiteheart 2005, White and Lauring 2007). Though these proteins perform a similar core function (i.e. energy-dependent remodeling of structural bodies) to achieve their biological function, nevertheless there is diversity in their domain architecture. Though their domain composition is seen to be usually similar, however, the diversity largely comes from the number of the α -helices that links the β -strand core of N domain and in some cases the AAA+ domain is ornamented with accessory domains that offer specialised function (Bochtler et al. 2000, Sousa et al. 2000, Ramachandran et al. 2002).

2.4. Active site architecture of AAA+ proteins

The Walker A and Walker B sequences encode structural elements that are necessary for nucleotide binding (Walker A) and hydrolysis (Walker B) within the AAA+ domain (Walker et al. 1982). The P-loop of Walker A is preceded by the β 1 strand and succeeded by the α 1 helix (Figure 1.8A). The Walker A motif is a conserved catalytic site motif in all AAA+ proteins that comprises the consensus sequence GXXXXGK[T/S] (where X is any amino acid) and has been implicated to directly interact with the phosphate moiety of bound ATP and magnesium ion (Figure 1.8B,C). The lysine residue (K) in the consensus sequence is essential in coordinating the β and γ phosphates of the ATP (Walker et al. 1982). So a mutation in this motif at the conserved lysine usually results in the abolition of ATPase activity in the AAA+ proteins and often influences oligomerization due to the failure to bind ATP molecules (reviewed in (Wendler et al. 2012)). Following nucleotide binding Walker A motif, nucleotide hydrolysis Walker-B motif extends from the β 3 strand to the start of the α 3 helix with a consensus sequence hhDEXX (h represents a hydrophobic amino acid) (Figure 1.8A). The Walker B motif also establishes contacts with nucleotide and it is very crucial for the ATP hydrolysis. The aspartic acid residue (D)

coordinates Mg^{2+} that is required for the stable ATP binding and the glutamate residue (E) is responsible for β - γ bond breakage during ATP hydrolysis (Walker et al. 1982, Iyer et al. 2004). Mutation in Walker B motif, usually glutamate residue in homomeric and heteromeric proteins often resulted in defective or loss of ATP hydrolysis (Augustin et al. 2009, Wendler et al. 2012, Gardner et al. 2015). In most cases a Walker B mutation does not affect ATP binding to the active site, so largely does not influence the oligomerization but mostly the activity of the complex.

Not a well-conserved sequence, but placed at the C-terminal of Walker-B motif, is the so-called second region of homology (SRH Figure 1.8A,B). Part of β 4, all of the α 4 and the loop to β 5 spans the SRH motif. The SRH induces conformational changes between subunits and coordinates ATP hydrolysis with Walker B (Ogura and Wilkinson 2001). The functionality of the SRH is constituted by two structural elements, Sensor1 and arginine finger at its N and C terminals respectively. Sensor1, a highly conserved motif in AAA+ superfamily, is formed by the loop connecting β 4 to α 4 (Figure 1.8A,B). It functions as a sensor of the ATP molecule bound to the pocket by interacting with the Walker B element and γ phosphate of the ATP bound to Walker A element generally via conserved polar residues (His, Asn, Ser or Thr) structurally positioned between both the Walker motifs. Mutational studies with Sensor1 resulted in impairment of ATP hydrolysis (Karata et al. 1999, Liu et al. 2000, Steel et al. 2000). Following Sensor1 the loop between α 4 and β 5, the C-terminal of the SRH is the putative arginine finger (Figure 1.8A,B). It is named because the arrangement of Arginines is similar to that found in GTPase activator promoting nucleotide hydrolysis (Scheffzek et al. 1997). Conserved Arginine residues in GAP (GTPase activating protein) are crucial for interaction with GTP-binding proteins (Ras or Rho) for enhancing its GTPase activity (Scheffzek et al. 1996). The arrangement of the invariant arginine residues “arginine fingers” in the nucleotide binding pocket of small GTPases are intrinsic for Ras-mediated GTP hydrolysis by stabilizing and developing the charge formed in the transition state of the reaction (Ahmadian et al. 1997, Scheffzek et al. 1997). Equally, one of the significant features of AAA+ ATPases is the presence of one or more arginine residues in nucleotide binding domain that potentially functions as an arginine finger as previously described for GTPases. In most hexameric AAA+ structures, nucleotide binding pocket rests in the interface between two subunits. The arginine finger from

one subunit is contributed to the active site in *trans* by positioning it in close proximity to the γ phosphate of the bound ATP molecule of the neighbouring subunit (Ogura et al. 2004). In various AAA+ proteins, mutation of these residues often resulted in a complete loss of *in vivo* and/or *in vitro* activity of the complex (Karata et al. 1999, Putnam et al. 2001, Wang et al. 2005) similar to GAPGTPases, indicating that these residues are necessary for catalytic activity. Studies on NtrC1, FtsH and ClpB elucidated that the arginine finger residues are not involved in ATP binding but hydrolysis (Karata et al. 1999, Rombel et al. 1999, Werbeck et al. 2009). On the other hand, a recent study using covalently linked ClpB dimers suggested that arginine fingers are essential for nucleotide binding (Zeymer et al. 2014). Hence the arginine finger acts as a crucial *trans* acting element at the catalytic site of the neighbouring subunit to bind ATP and stimulate ATPase activity. Additionally, arginine finger mutations in HslU, p97 VCP, ClpB D1, ClpC D1 and Hsp104 D1 impair oligomer formation even in the presence of ATP unlike GAPGTPases, where such substitutions do not interfere with complex formation. This suggests arginine fingers evolved with an added role in hexamer stability of the AAA+ proteins (Wendler et al. 2012). Overall, it is likely that the arginine fingers participate in ATP hydrolysis and account for subunit interface contacts or for the structural integrity of the binding pocket. Most AAA+ members usually possess two consecutive or spaced out, over few amino acids, arginine fingers. It is not clear why some other AAA ATPases have only one arginine finger residue (Ogura et al. 2004, Wendler et al. 2012).

The final nucleotide binding element, Sensor 2 maps to the third helix ($\alpha 7$) of the α -helical domain of the C-terminal and contains a conserved arginine residue (Ammelburg et al. 2006) (Figure 1.8A, B). Similar to arginine fingers, sensor 2 also acts in *trans* (Moreau et al. 2007) by interacting with the γ -phosphate of bound ATP substrate of the adjacent subunit and it is associated with a range of different roles, including nucleotide binding/hydrolysis and both inter- and intra-subunit communication and movement of the C-terminal subdomain (Joshi et al. 2004, Snider et al. 2008, Wendler et al. 2012).

All hexameric AAA+ proteins also have a central cavity or pore that is lined by loop residues from each subunit (Figure 1.8C). The surface of the pore is mostly constituted by the conserved residues between $\beta 2$ and $\alpha 2$ which define a loop (Figure 1.8A, B) (Wang et al. 2001). The Pore loop motif is essential for the biological function of many AAA+ proteins. The conserved aromatic-hydrophobic-glycine motif is essential for substrate interactions. For several AAA+ proteins, including the red-type Rubisco activase CbbX, the substrate is threaded through the central pore (Hanson and Whiteheart 2005).

B

C

Figure 1.8| Structure and organization of AAA+ proteins.

(A) The linear diagram (top) is a schematic representation of the secondary structures annotated from N to C terminal of the AAA+ domain. α helices are depicted by cylinders and β strands are depicted by arrows. All the important functional elements are highlighted in different colors along the annotated secondary structure. The consensus sequences of these elements/motifs are indicated below the respective sites of the elements and in the same color. α helices (α 5-8) with orange outline constitute the α helical subdomain. Schematic representation of a topology of the AAA+ fold is shown in the bottom panel. The distinguishing feature of AAA+ fold is the C-terminal α -helical bundle domain (formed by orange bordered cylinders). The β strand core is surrounded by N-terminal helices. The secondary elements are highlighted in the same color code as top panel. (B) Structure of an AAA+ subunit of NSF protein (PDB:1D2N) is shown in a cartoon representation. α helices are colored in red while the β strands are in yellow and loops are in green. This structure clearly depicts the two subdomains (α helical and nucleotide binding). The bound AMP-PNP (nucleotide analogue) at the nucleotide binding site (between the subdomains) is represented in stick and Mg^{2+} is indicated as sphere. The secondary structures, α helices and β strands are annotated as α 0-8 and β 1-5. Approximate sites of the key elements are indicated in the structure. (C) The subunits of NSF AAA+ protein are arranged to form a hexameric ring structure with a central pore. α helices that form the α helical subdomain are represented in red while the α helices of the nucleotide binding subdomain are colored in dark, light blue, magenta, teal and orange. The nucleotide binding pocket formed by the interface of two subunits is represented with the nucleotide analogue (AMP-PNP represented in stick) and coordinated by Mg^{2+} (represented as grey sphere).

It seems evident that studies on ClpX, RFC, Orc1 and other AAA+ proteins had shown the engagement of these AAA+ ATPase with its substrate frequently regulate (generally stimulate) the ATPase rate of AAA+ proteins (Zhang and Wigley 2008, Moggi et al. 2009). In many cases, this regulation appears to be due to a hydrogen bond “switch” between a conserved glutamic acid in the Walker B to an asparagine or serine/threonine at the C-terminus of β 4, known as Glutamate Switch. In the absence of substrate the switch is “on” with the intact hydrogen bond whereby inhibiting the ATP hydrolysis. Upon substrate binding switch is “off”, a conformational change is predicted to break this hydrogen bond stimulating ATP hydrolysis (Zhang and Wigley 2008, Moggi et al. 2009).

2.5. Models of subunit coordination

As established in the previous section, AAA+ proteins tend to form hexameric assemblies that bind and hydrolyze ATP to power conformational changes that are then transduced to a macromolecular substrate. A question under intense investigation is whether the six active sites function independent of each other, or whether ATP hydrolysis is a strictly coordinated process with the different subunits communicating

with each other. Different workers studying this issue have come up with quite different answers for diverse and even the same AAA+ proteins (Martin et al. 2005, Lyubimov et al. 2011, Lyubimov et al. 2012).

Suggested models for hexameric AAA+ ATPase are synchronized, rotational, sequential and probabilistic as illustrated in Figure 1.9. It is predicted if ATP binds to all six subunits at the same time and hydrolysed simultaneously the model is synchronized. ATP binding and hydrolysis is synced for all the subunits.

In rotational model, only half of the subunits are ATPase active while the other subunits are inactive. Similar to hexameric F₁-ATPase with $\alpha_3\beta_3$ stoichiometry, where the ATPase activity is observed only in the β subunits, structures of HslU were observed with nucleotide bound only to three alternative binding sites (Bochtler et al. 2000). The sequential model is an extension of the rotational model where all subunits are ATPase active. The binding pattern observed in HslU (Bochtler et al. 2000) and T7 gp4 DNA helicase (Singleton et al. 2000) with four nucleotides, may correspond to a sequential model. Although HslU and helicase act on different types of substrate, both were speculated to have a sequential mechanism of action (Ogura and Wilkinson 2001). Finally, in the probabilistic model, all subunits actively bind ATP and hydrolyse randomly, they don't follow a sequence or order in ATP hydrolysis (Martin et al. 2005).

Synchronized Model

Rotational Model

Sequential Model

Probabilistic Model

Figure 1.9| The cycle of ATP hydrolysis.

Four different models are presented for the ATP firing cycles in AAA⁺ proteins. The subunits cycle through the three ligation states namely ATP-bound state, ADP and Pi bound state and unbound state. Synchronized model: all the subunits are active and symmetry is preserved throughout the cycle. Rotational model: every other subunit is inactive, the three active subunits are always at different phases of the reaction cycle. Sequential model: all the subunits are active with pairs of subunits on opposite sides of the ring in phase with one other but out of phase with the remaining subunits. Probabilistic model: sequential firing is possible but not obligatory. Adopted from (Ogura and Wilkinson 2001, Martin et al. 2005).

It is currently mostly unresolved and an active area of debate whether one of these models prevails or whether multiple different models exist among the plethora of AAA+ proteins. Using standard biochemical methods it is extremely challenging to confidently distinguish between the different models, and it is likely that highly sophisticated biophysical methods (such as single particle approaches) will be required to resolve these questions (Stinson et al. 2013).

2.6. Substrate Recognition and remodelling in various AAA+ proteins

Clearly AAA+ proteins must be able to recognize a specific protein (or other macromolecular) substrate from the vast bulk of cellular proteins. A number of mechanisms have been described in detail. In some cases substrates are recognised by a short sequence motif and sometimes small adaptor proteins help deliver the substrate to AAA+ enzymes (Dougan et al. 2002, Kirstein et al. 2009). One of the widely studied targeting sequence to the ClpXP protease is an 11 amino acid (AANDENYALAA) tag popularly known as SsrA tag. This tag is added to the target substrate by a small 10S RNA ssrA (Keiler et al. 1996).

Adaptor proteins play a key role in delivering the substrate generally with no targeting tags to the AAA+ proteins. SspB, SspA and RssB are some of the adaptor proteins that mediate selective targeting of substrates to ClpX (Levchenko et al. 2000, Zhou et al. 2001). Different adaptor proteins aid in substrate targeting to p97 and NSF complexes. Interestingly, regarding Rubisco activases, the hexameric AAA+ ATPase CbbQ forms a complex with a large adaptor protein CbbO (which acts as a substrate adaptor for Rubisco via a von Willebrand Factor A (vWA) domain) only then it enables the complex to recognise the Rubisco and activate (Tsai et al. 2015).

The location of the targeted substrate binding to the AAA+ protein is essential to understand how the AAA+ proteins handle their substrates. So far there is no clear evidence from structural data to elucidate the elements or motifs in the AAA+ involved in substrate binding. Lon protease appears to use C-domain known as SSD domain (sensor and substrate discriminator domain) for substrate binding while substrate binding to Yme1p protease was mapped to the N-domain (~65 residues) of the AAA+ module. For ClpX and ClpA protease, flexible N-terminal was believed to selectively deliver the substrates (Sauer et al. 2004), but it was later demonstrated that

lack of N-domain in both proteases did not affect the substrate targeting or its function. The N-terminal domain of plant (green-type) Rubisco activase is likely also involved in substrate binding (Esau et al. 1996). Other elements in AAA+ domains have also been implicated in substrate binding. As mentioned previously, mutagenesis data indicate that hydrophobic residues in the conserved pore loop 1 (YVG) have a key role in substrate engagement (Wang et al. 2001, Siddiqui et al. 2004), and this is particularly relevant to the red-type Rubisco activase CbbX as detailed in subsequent sections.

AAA+ proteins make productive interactions with proteins in various states, native, complexed, denatured and aggregated. Molecular and structural details of the substrate recognition and binding in most AAA+ proteins remain elusive and need to be studied in detail. Here we believe that detailed study of the various Rubisco activase systems can play a critical role in understanding such issues.

2.7. The axial pore translocation mechanism utilized by many AAA+ proteins

ClpA and ClpX fully unfold their substrates by translocating them through the channel at the center of the oligomeric ring (Weber-Ban et al. 1999, Kim et al. 2000, Ortega et al. 2000, Reid et al. 2001). This mechanism represents the best understood mechanical model of action of AAA+ ATPases and a variation appears to be utilized by the red-type Rubisco activase CbbX (Mueller-Cajar et al. 2011). For these types of systems, the main function of the ATPase appears to be the induction of substrate unfolding by energy-driven threading through the narrow pore at the center of the oligomeric ring.

Protein translocation by threading has been intensely studied using the ClpA and ClpX protease systems (Sauer et al. 2004, Olivares et al. 2015). These proteases recognise the SsrA tagged substrates (Keiler et al. 1996) and engage them using the central pore motifs. Following binding of the substrate with peptide tag, the protease begins to translocate this tag through its central axial pore, which is formed by the pore-loop1 elements with the tyrosines lining the pore (Sauer et al. 2004). Attempting to pull the attached protein through this small aperture would generate a denaturation force that destabilizes the substrate polypeptide (Martin et al. 2008a, Martin et al. 2008b). With the tagged substrate clamped in the pore, concerted changes in the

polarity and the size of the central pore promoted by ATP binding and hydrolysis may facilitate processive translocation. The operation of translocation was shown clearly using structural snapshots of HslU protease system. The pore loop tyrosine changes its position in the channel as the ATPase switches between different conformational states (Wang et al. 2001) which suggest that channel loop movement could directly drive the translocation of a clamped/engaged substrate. The interaction site of the threaded polypeptide with the loop or how the loop mediates such interaction with the substrate is not properly understood (Zolkiewski 2006), however, conservative aromatic residues at the loop tip are proposed to bind unfolded hydrophobic substrates (Sousa 2014). Mutation of the loop glycine impairs not only substrate translocation but also ATPase of HslU indicating it might reduce loop flexibility. This demonstrates a tight conformational coupling between the ‘engine’ (ATP-binding sites located between the sub-units of the oligomeric ring) and the ‘drive’ (flexible loops inside the central channel) (Zolkiewski 2006). It has been speculated that the function of other AAA+ ATPases that form ring-shaped oligomers might be similarly linked to threading and unfolding of their substrates (Zolkiewski 2006).

2.8. The green-type Rubisco Activase

Having discussed structural and mechanistic insights into AAA+ proteins that have been described by a voluminous literature, we will focus on summarizing current relevant knowledge on the Rubisco activases.

Green-type Rubisco activase was the first discovered class of activases, derived from large scale screens of *Arabidopsis* mutants that required high CO₂ for growth (designed as a screen for photorespiratory mutants) (Somerville et al. 1982). From the *in vivo* characterization of the *Arabidopsis* Rca mutant strain, it was observed that the strain was unable to grow at ambient CO₂ level due to lack of activation of the Rubisco but survived in high CO₂ levels. This altered regulation of Rubisco in the Rca mutant of *Arabidopsis* indicates the importance of the absence of the activator enzyme (Somerville et al. 1982). Biochemical and genetic analysis confirmed that the two missing polypeptides from *rca* mutant were responsible for the observed phenotype. This protein was identified to be responsible for activation of Rubisco and was named Rubisco activase (Salvucci et al. 1985). A very important feature of the activase is that it causes the Rubisco to exhibit its maximal catalytic activity at physiological

concentrations of CO₂ (Portis et al. 1986) by promoting the release of bound inhibitors from both carbamylated and uncarbamylated forms (Robinson and Portis 1988, Robinson and Portis 1989, Edmondson et al. 1990, Edmondson et al. 1990, Lilley and Portis 1990, Wang and Portis 1992, Mate et al. 1993). The presence of Rca was identified in soluble protein extracts of various C₃ and C₄ plant species, confirming the fact that a Rubisco activase dependent regulation of Rubisco was ubiquitous in almost all higher plant species (Salvucci et al. 1987). In the same year it was also discovered that the remodelling of inhibited Rubisco to release the bound sugar phosphate inhibitors from its active site to enable to be carbamylated again is effected by Rubisco activase in an ATP-dependent manner (Streusand and Portis 1987). The addition of Rca, to uncarbamylated Rubisco, showed no activity loss or to carbamylated Rubisco showed no fallover (Robinson and Portis 1989). However, the Rubisco activase was not directly involved in the carbamylation or binding of Mg²⁺ at the active site (Robinson et al. 1988). In 1999 it was recognized that Rca belongs to the superfamily of AAA+ ATPases (Neuwald et al. 1999, Lupas and Martin 2002). This provided a mechanistic framework for the activation of Rubisco by Rubisco activase, which is required to engineer its function. Importantly many early mutational studies provided valuable evidence for the direct interaction between the activase and its substrate Rubisco (Spreitzer and Salvucci 2002, Parry et al. 2008). Studies by Esau et al. (Esau et al. 1998) with chimeric activases lead to the conclusion that the C-terminal subdomain of Rubisco activase held elements for Rubisco preference and is important in Rubisco recognition. Subsequent site-directed mutagenesis studies in the sensor2 domain of the activase revealed that residue 311 and 314 were essential for substrate discrimination in spinach, tobacco and cotton (Li et al. 2005). This complemented with the charge switch mutation on the surface of *Chlamydomonas* Rubisco at Arg/Lys 89 or Lys/Asp 94 residues which changed the preference for the activase as well and clearly suggested a Rubisco activase docking site (Larson et al. 1997, Ott et al. 2000).

Recently, three Rca X-ray structures have become available. A 1.9 Å model of the *Creosote* C-domain (residues 250–351 out of 379 total) (Henderson et al. 2011), a 3.0 Å model of the *Nicotiana tabacum* (tobacco) activase (residues 68–360 out of 384 total) (Stotz et al. 2011) and a 2.9 Å model of the *Arabidopsis thaliana* (residues 65–362 out of 416 total) (Hasse et al. 2015) revealed the organisation of the apo state and

architecture of the AAA+ domain. These models suggested a conformational plasticity in the specificity-determining region that was responsible for differentiation between *Solanaceae* (e.g. Tobacco) and *non-Solanaceae* (e.g. Spinach) Rubiscos (Li et al. 2005).

Rubisco Activase is ~45 kDa protein with a small amino-terminal adapter domain, a central AAA+ module and a C-terminal regulatory region. However, no consensus exists regarding the oligomeric state of the enzyme (structural organisation is not well established due to its polydisperse nature). Oligomer size of the wildtype activase is observed to vary with concentration of the activase, from monomer to hexamer (Wang et al. 1993, Barta et al. 2010) and even higher oligomers (Chakraborty et al. 2012, Henderson et al. 2013). However a mutant, (R294A) formed stable hexamers in the presence of ATP analogue ATP γ S (Blayney et al. 2011). A similar mutant (R294V) of the tobacco enzyme was fully functional and could be visualised as hexamer using negative-stain electron microscopy (Stotz et al. 2011). The mechanism of activation is not characterized as well as the activase of the red-type Rubisco. As opposed to full threading and translocation of the substrate protein by the ClpX, red-type activase is presumed to only partially thread (or pull) the extended C-terminus (~12 amino acids) of the red-type Rubiscos large subunit through its pore. The C-terminus (C-tail) of the Rubisco refers the amino acids following the highly conserved residue D473 (spinach Rubisco numbering) which is known as the latch site (Duff et al. 2000). However, the green-type Rubiscos don't have the extended C-tail (~ 2-6 amino acid long) (Figure 5.1C). Additionally the central pore ~36 Å wide and lack classical pore loop 1 sequence, makes it unlikely for Rca to engage single polypeptide motif of Rubisco for activation following the previously discussed threading mechanism (Siddiqui et al. 2004, Martin et al. 2008a, Martin et al. 2008c), but are more likely to involve partial threading of a more complex secondary structure (Mueller-Cajar et al. 2013). So the mechanism of activation by green-type activase is unlikely to involve the C-tail of their respective Rubisco. But instead we presume that these activases could recognise and partially thread a loop or other secondary structures on the surface of the Rubisco during the process of activation. In addition the pore diameter of the green-type Rca is larger than the red-type activase which makes us to assume that green type has the possibility to thread a larger structure than the red-type activase.

In many higher plants, Rubisco activase encoded by a single gene exists in two isoforms as a result of alternative splicing (Werneke et al. 1989). The isoforms differ in length at the C-terminus, long ~46 kDa and short ~42 kDa. The activase is constitutively ATPase active and in contrast to many other AAA+ proteins, the ATPase of activase is not stimulated by its protein substrate (Robinson and Portis 1989). The long isoform is more prone to ADP inhibition. ATP/ADP ratio in the stroma regulates the Rubisco activase. Its propensity to be inhibited by ADP is modulated by thioredoxin-mediated disulfide bond formation in the C-terminal extension of the long isoform, which in turn is regulated *in vivo* by light (Zhang et al. 2002, Carmo-Silva and Salvucci 2013).

Rubisco activase displays heat inactivation. It shows optimum activity at a temperature below 40 °C (Salvucci and Crafts-Brandner 2004). Extensive evidence supports the finding that thermal inactivation of Rubisco activity arises primarily from temperature-dependent denaturation of Rubisco activase (Portis 2003).

Proteins with sequence homology to Rubisco activase have also been identified in the filament forming bacteria (heterocystous cyanobacteria) (Li et al. 1993) and subsequently called Rubisco activase-like proteins (rca-like). Since the Rubisco from this species was not able to be inactivated *in vitro* by known sugar-phosphates the functional role of rca-like protein is uncertain (Mueller-Cajar et al. 2013). However, some functions of the rca-like were consistent with the observed *in vivo* functions of the Rubisco activase. Rca-like gene of cyanobacteria showed a diurnal expression pattern *in vivo* (Li and Tabita 1994) and a knockout strain of the rca-like gene in *Anabaena variabilis* required a higher level of CO₂ to grow (Li et al. 1999).

2.8.1. Mutational Studies in Rubisco Activase

To understand the mechanistic insights about the Rubisco remodelling by Rubisco activase extensive mutagenesis and structural studies have been conducted. Some of the critical residues involved in ATP binding and hydrolysis and activation were identified by several mutational and biochemical studies (Figure 1.10). This work has been reviewed by (Portis 2003) and (Mueller-Cajar et al. 2013).

Figure 1.10| Domain Architecture of Rubisco Activase.

(Adapted from Mathias Stotz 2011)

2.9. The red-type Rubisco activase (CbbX)

Given that RuBP and other non-substrate sugar phosphates can lead to the formation of inactivated Rubisco complexes in higher plants and green algae, Rubisco can be restored back to activation by Rubisco activase (Rca). However, an Rca homologue was absent in organisms with the red lineage Rubiscos, although these Rubiscos were early shown to form an inhibited complex with RuBP (Gibson and Tabita 1979, Read and Tabita 1994). Further, Rubisco from red algae was also observed to show the fallover phenomenon (Uemura et al. 1998).

The gene *cbbX* in the plastid genome encoding a small protein of 35 kDa was early hypothesized to play a key role in the carbon fixation, as it was always found in the downstream of Rubisco (small subunit *cbbS*) in proteobacteria (Meijer et al. 1991, Kusian et al. 1992).

Genetic approaches demonstrated that *cbbX* lesions in *R. sphaeroides* and *Ralstonia eutropha* impaired photoautotrophic growth (Gibson and Tabita 1997, Bowien and Kusian 2002). These results implicated CbbX role in photosynthesis. Since *cbbX* always associated with Rubisco genes in the plastid genome, it was believed to be a chaperone or involved in the production of Rubisco. After unsuccessfully examining CbbX for Rubisco assembly chaperone activity it was investigated for Rubisco activase activity. Indeed, Mueller-Cajar discovered that CbbX was involved in the carbon fixation in the proteobacterium *R. sphaeroides*, functioning as a Rubisco activase by activating the inhibited Rubisco in an ATPase-dependent manner (Mueller-Cajar et al. 2011). This discovery was counter to another function reported for CbbX, as a transcriptional regulator of Rubisco in red algae *Cyanidioschyzon merolae* (Fujita et al. 2008). Although CbbX showed no sequence homology with Rca, CbbX was able to activate the inhibited Rubisco complex suggesting that CbbX is a novel red-type Rubisco activase. Surprisingly, proteins related to CbbX were also found in non-photosynthetic organisms associated with very different functions. For example, SpoVK was found to be associated with sporulation in *Bacillus subtilis* (Foulger and Errington 1991, Fan et al. 1992) and Rv3868 was involved in the ESX-1 secretion pathway of *Mycobacterium tuberculosis* (Luthra et al. 2008, DiGiuseppe Champion et al. 2009).

cbbX genes were always found in photosynthetic organisms, downstream of red-type Rubisco genes in the genome of proteobacteria (Form IC) and the plastid genome eukaryotic red algae (Form ID) (Maier et al. 2000, Fujita et al. 2008). In some organism like diatoms, the *cbbX* gene was not found directly associated with the Rubisco genes but encoded elsewhere in the chloroplast genome (Oudot-Le Secq et al. 2007). In eukaryotes, additional to the CbbX expression from plastid genome, an isoform was also found encoded by the nuclear genome of red algae (Matsuzaki et al. 2004) and the nucleomorph of cryptophytes (Maier et al. 2000, Fujita et al. 2008). In contrast to its complete association with red-type Rubisco genes, genome-wide screening identified that CbbX was also found in many α -cyanobacterial species including many *Prochlorococcus* strains (eg. *MIT9313*) and *Synechococcus* strains (eg. *Synechococcus sp. WH8102*) containing Form IA green-type Rubisco (Roberts et al. 2012) and also some β -cyanobacterial species (Zarzycki et al. 2012).

Red-type Rubisco activase, like green-type Rubisco activase, also belongs to the AAA+ family of proteins consisting of a short N-terminal region and a single AAA+ domain constituting a small and a large domain with essentially all conserved motifs and elements (Figure 1.11A). However, the proteins are clearly not homologues with <10% sequence identity (Hasse et al. 2015). The segment which contains the specificity helix in Rca is lacking in CbbX (Mueller-Cajar et al. 2013). Biochemical characteristics and mechanism of Rubisco activation by CbbX is very different from Rca (Robinson and Portis 1989). As a distinguishing feature, CbbX was not constitutively ATPase active like its plant counterpart but interestingly shown to require an allosteric regulator for its activity (Mueller-Cajar et al. 2011). The allosteric regular for CbbX was determined to be none other than the substrate of Rubisco, RuBP. CbbX showed no ATPase activity in the absence of RuBP whereas ATPase activity was increased to 8 min⁻¹ in the presence of RuBP. Moreover, the ATPase activity of CbbX was shown to increase tenfold with the addition of its protein substrate, inhibited/activated Rubisco complex similar to other AAA+ proteins but different to the plant counterpart (Robinson and Portis 1989).

Consistent with biochemical studies, CbbX negatively stained for Electron microscopy (EM) in the presence of ATP formed a fibrillar structure. Only in the presence of both ATP and RuBP, it formed a six-fold symmetric ring structure with a central pore of ~ 25 Å wide, similar to the pore dimension of other AAA+ proteins. The crystal structure of full length RsCbbX was solved at 3.1 Å resolution (Figure 1.11B). Structural dissection of CbbX revealed that the RuBP binding pocket is located in the highly conserved inter-subunit interface of the hexamer. The residues R239, S250, N253, R257 and R261 of the C-terminal α -helical subdomain were hypothesized to interact with the RuBP molecule. Mutational studies have shown that these residues were important for RuBP binding and ATP hydrolysis. CbbX has a single α/β subdomain with a conserved N-terminal extension. The N-terminal α/β subdomain contains canonical Walker A and B motifs and pore loop elements. Mutational studies demonstrate that the Walker A and B motifs regulate ATP hydrolysis and Rubisco activation. During active conformation in the presence of ATP and RuBP, CbbX assumes a hexameric structure; α -helical subdomain is rotated toward the α/β subdomain which is necessary for accommodating the ATP in its nucleotide binding pocket. A sequence conservation score showed that the top surface around the central pore (including the pore loop helices $\eta 1$, $\alpha 5$, $\alpha 6$) is highly conserved, implicating the surface in protein-protein interactions with Rubisco. The bottom surface was much less conserved, excepting the RuBP binding pocket.

Rubisco remodelling is expected to involve the opening of the closed, inhibitor-bound substrate binding pocket. Sequence and structural studies identified a conserved, flexible C-terminal extension element in the Rubisco large subunit (RbcL) of all representatives of the red-type lineage but missing in the green-type counterparts. The mutational studies with 8 ($\Delta 8$), 4 ($\Delta 4$) and 2 ($\Delta 2$) residues of the large subunit truncated at the C-terminus of the Rubisco did not stimulate the ATPase activity of the CbbX. Moreover, CbbX activated only $\Delta 2$ mutant with 20 % efficiency whereas it was not able to activate $\Delta 4$ and $\Delta 8$ mutants. Additionally, mutants of the conserved tyrosine residue in the pore loop and surface lysine in close proximity to the central pore at positions 114 and 123 respectively failed to activate the ER complex although the ATPase activity was unaffected. Hence it is believed that elongated C-terminal element and the conserved pore loop1 of the CbbX lined by the tyrosine residues (Y114) are important to mediate the activation of the Rubisco.

Distantly related ClpX and other AAA+ proteins were shown to remodel their substrate by threading into the pore loop (Kirstein et al. 2009). Mutation in their conserved pore loop residues or in their recognition tag severely affects the substrate processivity. Due to the similarities in the mutational phenotypes of the ClpX and CbbX, the mechanism of activation of Rubisco by CbbX was hypothesised to be threading of the C-terminal tail but only partially. Consistent with the partial threading model it was shown that there is no Rubisco disassembly taking place. Based on the other AAA+ models it was hypothesised that force exerted by CbbX helps to crack open the active site releasing the RuBP from the inhibited Rubisco and making it active for CO₂ fixation. In contrast, the mechanism of Rubisco remodelling by plant activase is understood in much less detail. This elongated C-terminal element was shown to be essential for recognition by CbbX (Figure 1.11C). The conserved pore loop1 of the CbbX lined by the tyrosine residues (Y114) is believed to mediate the pulling of the C-tail through its central pore (Figure 1.11C). The force exerted by CbbX helps to crack open the active site releasing the RuBP from the inhibited Rubisco and making it active for CO₂ fixation. Mutants of the conserved tyrosine residue in the pore loop and surface lysine in close proximity to the central pore at positions 114 and 123 respectively failed to activate the ER complex although the ATPase activity was unaffected. This mechanistic hypothesis was supported by additional evidence from mutational studies with 8, 4 and 2 residues of the large subunit truncated at the C-terminus. The respective mutants $\Delta 8$, $\Delta 4$ and $\Delta 2$ did not stimulate the ATPase activity. CbbX activated only $\Delta 2$ mutant with 20 % efficiency whereas it was not able to activate $\Delta 4$ and $\Delta 8$ mutants, indicating that the elongated C-terminal of red-type Rubisco is important for recognition of the Rubisco by CbbX. Mechanism of activation by CbbX appears to be similar to that of ClpX and other AAA+ proteins where the substrate is remodelled by threading into the pore loop (Kirstein et al. 2009). However, it was also shown that there is no Rubisco disassembly taking place. In contrast, the mechanism of Rubisco remodelling by plant activase is understood in much less detail.

Hence both green-type Rca and red-type CbbX can rescue the dead-end inhibition of Rubisco complexes, but the working mechanism, regulation and structure of CbbX are entirely different to that of the plant activase (Mueller-Cajar et al. 2013). So in conjunction with the non-homologous nature of their primary sequence, it is believed that these characteristic differences of Plant Rca and CbbX are a result of convergent evolution in green and red lineage organisms where different molecular chaperones of the AAA+ type were recruited to remodel inhibited Rubisco active sites.

Figure 1.11| Structure and mechanism of CbbX from proteobacteria *R. sphaeroides*.

(A) Subunit architecture of the CbbX in ribbon representation (PDB: 3SYK). The α/β (large subdomain) subdomain indicated in blue has the key conserved motifs, the Walker A and B motifs shown in dark blue and magenta respectively. The sensor I and pore loop indicated in yellow and orange are also presented in the α/β subdomain. The α -helical (small subdomain) subdomain is shown in teal and sensor II region indicated in green is presented in this subdomain. The nucleotide and the RuBP binding site are indicated by the bound sulphates shown in ball and stick. The simple domain structure is schematically shown as a linear bar at the bottom. The large (α/β subdomain) and small domain (α -helical subdomain) of the AAA+ modules are colored teal and light blue respectively. The amino acid positions of the start and

terminal of the domain is indicated in numbers. **(B)** Side and top views of averaged negative stain envelope showing the ring dimensions including the pore width. The subunits in the hexamer are shown in alternate light and dark blue (Mueller-Cajar et al. 2011). **(C)** CbbX activation model. The CbbX hexamer docks onto inactive Rubisco and transiently pulls the extended C-terminal sequence of red-type RbcL into the central pore of the hexamer. This action is mediated by the stimulation of ATPase of the CbbX which apparently destabilize the Rubisco active site thereby releasing the inhibitory sugar. CbbX and Rubisco are shown in surface representation. The positions of the extended C-terminal RbcL tails (red), the Rca pore loops (red) and the RuBP allosteric sites (red) on Rca are indicated (modified from (Hauser et al. 2015)).

2.9.1. CbbX from red lineage Phytoplankton

Most photosynthesis in the ocean and fresh water bodies is performed by phytoplankton, which is an important source of food for marine life. With almost less than 1% of Earth's photosynthetic biomass, these contribute for more than 45% of net primary production (Falkowski et al. 2004).

In some green algae, which are a constituent of phytoplankton, green-type Rubisco activase is encoded where it is required for maintaining optimal photosynthesis (Pollock 2003). However, the vast majority of eukaryotic phytoplankton, especially in the oceans, is represented by organisms of the red-plastid lineage. In all of these organisms, instead of green-type Rca, *cbbX* homologues are encoded (Maier et al. 2000, Hovde et al. 2015). With the discovery of CbbX, as a novel activase of the inhibited red-type Rubisco in proteobacteria (Mueller-Cajar et al. 2011) we assumed that the CbbX encoded by the phytoplankton of red lineage (e.g. red algae and diatoms) would have the same function. Furthermore, algae belonging to the red plastid lineage such as *Nanochloropsis* and some diatom species have become economically important as they have potential applications in the biofuel industry (Radakovits et al. 2012). However improving photosynthesis in these organisms is difficult because the biochemical understanding is missing and genetic tractability of these organisms is very limited.

The plant activase is known to become non-functional at high temperature which leads to Rubisco deactivation and cessation of CO₂ fixation (Crafts-Brandner and Salvucci 2000). Hence keeping in mind the aim to improve the thermal efficiency of the Rubisco activase, which is a major problem in agriculture, we chose

Cyanidioschyzon merolae (*C. merolae*), a club-shaped unicellular red algae that live in challenging environments (pH 1.5 and 45 °C) as a model organism. *C. merolae* has a single chloroplast and mitochondria but lacks vacuole and cell wall. The *C. merolae* genome was the first full algal genome to be sequenced (Matsuzaki et al. 2004). Like all other red-lineage phytoplankton species examined, *C. merolae* has two isoforms of CbbX in its genome (Meyer and Griffiths 2013). One is encoded by the nuclear genome and the other is encoded in the plastid (Fujita et al. 2008). The plastid CbbX is always found in an operon with the Rubisco subunits similar to *R. sphaeroides* complementing the evolutionarily conserved role in Rubisco activation. At the outset of this project no biochemical data regarding red algal (or any red-lineage phytoplankton) Rubisco activation systems had been generated. To clarify these issues we intended to biochemically characterize the function of both the isoforms of CbbX encoded in *C. merolae*.

2.9.2. Cyanobacterial CbbX

Cyanobacteria are prokaryotic photosynthetic organisms that evolve O₂ (Bryant, 1994). To reduce Rubisco oxygenation, all cyanobacteria possess a powerful carbon dioxide concentrating mechanism (Badger et al. 2006). The polyhedral protein microcompartment known as the carboxysome encapsulates the Rubisco and maintains a high CO₂ concentration around its active sites to enable carbon fixation and avoid wasteful oxygenation reaction. Based on the Rubisco type it holds and protein composition associated with the carboxysomes, it is phylogenetically classified into two groups. α -cyanobacteria have α -carboxysome that encloses Form IA Rubisco and β -cyanobacteria have β -carboxysome that encloses Form IB Rubisco (Tabita 1999, Badger et al. 2002, Rae et al. 2013). The two carboxysomes types have arisen through convergent evolution, and α -cyanobacteria and β -cyanobacteria occupy different ecological niches (Whitehead et al. 2014). From genome-wide sequencing of a large number of α and β -cyanobacteria it was shown that α -cyanobacteria with green-type Rubisco (Form IA) were often found associated with *cbbX* genes (Zarzycki et al. 2012). As discussed, the mechanistic model for red-type Rubisco activation was shown to involve the extended C-terminus of the large subunit (Mueller-Cajar et al. 2011) but in contrast α -cyanobacteria's large subunit (Form IA Green lineage Rubisco) does not contain the extended C-terminus element.

Additionally, proteobacterial CbbX was not able to activate tobacco's Form I green-type Rubisco (Mueller-Cajar unpublished data). This instigated us to understand if the CbbX in these organisms functions via a different mechanism or alternatively has evolved to interact with the short C-terminus of the large subunit. Studying the cyanobacterial system would aid us to understand the mechanistic basis of CbbX function with green-type Rubisco which ultimately could provide insight for adapting and engineering the CbbX system for crop plants. Here we chose *Synechococcus sp. WH8102* of α -cyanobacteria as a model organism for characterizing CbbX activation system because its genomic DNA was made available to us by Prof. Dean Price.

In summary, our biochemical approach of characterizing homologues of an extremely well-studied red-type activase has provided a deeper knowledge of understanding of the regulatory requirement of these biotechnologically interesting Rubiscos (due to their superior catalytic properties relative to the green-type counterparts) which was previously elusive. Also our work indicates the necessity of co-introduction of compatible activases for the optimal regulation of such Rubiscos in the ongoing biotechnological approach. We hope to convince the reader that we were able to generate a significant volume of data that will guide future biotechnological approaches (incorporating cyanobacterial carboxysome or red algal Rubisco) regarding the engineering of photosynthetic carbon dioxide fixation in target organisms.

3.MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals

All the chemicals used in the study were of the at least analytical grade. Chemicals other than the ones listed below were purchased from Sigma-Aldrich:

Table 3.1. Chemicals

Item	Brand
Glycerol Sodium Phosphate, Monobasic, 98-102%, ACS Reagent Grade Yeast Extract	Affymetrix
Coomassie Brilliant Blue, G-250	Applied Chemistry
Bacto Agar Tryptone	BD, Difco
30% Acryl/ Bis solution 37.5:1 EDTA disodium salt dihydrate Ethidium Bromide (10mg/ml Sol.) TEMED Tris	Bio-Rad
dNTP Set 100 mM solutions X-Gal	Fermentas
Bleach 5x concentrate	GB bleach
Kanamycin sulfate	Gibco
Ampicillin IPTG	Gold Biotechnology
SDS, Sodium Dodecyl Sulphate	Hoefer
SYBR Safe® DNA gel stain	Invitrogen
Acetic Acid(Glacial) 100% anhydrous Ethanol 99.5-99.8%, Absolute, GR Grade for Analysis Methanol, GR, min 99.8 % N, N-Dimethyl formamide AR Sodium Chloride Sodium hydroxide, Analysis grade pellets, 99%	Merck
Magnesium chloride hexahydrate	MP Biomedicals

Item	Brand
Ammonium Persulphate (Molecular Grade) Proteinase K	Promega
Phosphoenolpyruvate (PEP)	Roche
DTT, Cleland's Reagent HisPur™ Ni-NTA Resin	Thermo Scientific
Agarose (Mol Grade)	Vivantis
Polyethylene glycol (PEG) 3350 20% (v/v)	Sigma

3.1.2. Enzymes & Kits

Table 3.2. Enzymes and Kits

Enzymes	Supplier
Pfu and Taq DNA Polymerase	Fermentas
Restriction enzymes - SacII, Hind III, XbaI, NdeI, BamHI, NotI	New England Biolabs
T4 DNA Ligase	
calf intestinal phosphatase (CIP)	
ATPase assay	
Pyruvate kinase/ Lactate dehydrogenase from Rabbit muscle	Sigma
Rubisco assay	
Creatine P-kinase from rabbit muscle	Roche
Glyceraldehyde-3-P dehydrogenase	Sigma
Triose-P Isomerase/Glycerol-3-P dehydrogenase	Sigma
Kits	Supplier
pGem-T vector system	Promega
Wizard® Plus SV MiniPreps DNA purification kit	
Wizard SV Gel and PCR Clean-Up System Kit	

3.1.3. Instruments

Table 3.3. Instruments

Instruments	Supplier
Chromatography	
Akta Purifier Frac900, Akta Micro Ion exchange MonoQ HR 16/10 MonoQ 10/10 GL Resource Q Source 30Q Gel filtration Hiload Superdex 200 16/600 Pg Superdex 200 Increase 10/300 GL Superdex 200 PC 3.2/30	GE Healthcare
Other Instruments	
TB2 Thermo block Professional TRIO thermocycler	BioMetra
Avanti J-25 (rotor JA 10, JA25.5)	Beckman Coulter
Mini-protean tetra system Power pac Basic Power pac HC	BioRad
Thermo mixer comfort	Eppendorf
PL-600 pH/mV/Temp meter	Ezodo
Minicentrifuge	Green liv
Z300K refrigerated Bench top centrifuge	Hermle
Safe imager2.0 Trans-Blot Semi dry Transfer cell	Invitrogen
Bench top Microcentrifuge	Kintaro
Excella E25 Incubator Innovo 44R	New Brunswick
Rocker 400	Rocker
Vortex genie2	Scientific Industries

Instruments	Supplier
Uv-1800 spectrophotometer Cps controller	Shimadzu
Sorval Micro centrifuge Nanodrop 2000 Forma 900(-80°C) MaxQ 6000 incubator Cimarec stirrer	Thermo Scientific
Sonics Sonicator	Vibra cell

3.1.4. Buffers and Media

Media were prepared with deionised, double-distilled water and subsequently autoclaved.

LB medium:

Tryptone	10 g l ⁻¹
Yeast extract	5 g l ⁻¹
NaCl	10 g l ⁻¹
agar for solid medium	15 g l ⁻¹

Modified Allan's media

NH ₄ SO ₄	2.62 g l ⁻¹
KH ₂ PO ₄	0.54 g l ⁻¹
MgSO ₄ .7H ₂ O	0.5 g l ⁻¹
CaCl ₂ .2H ₂ O	0.14 g l ⁻¹

Trace Metals was prepared in 100 ml MilliQ water by adding the following salts

H ₂ BO ₃	285 mg
MnCl ₂ .4H ₂ O	180 mg
ZnCl ₂	10.5 mg
Na ₂ MoO ₄ .2H ₂ O	39 mg
CoCl ₂ .6H ₂ O	4 mg
CuCl ₂ .2H ₂ O	4.3 mg

This was autoclaved and diluted to 100 times. Finally, 100 μ l of the diluted solution was added to 1 l medium.

250X Fe was prepared in 100 ml MilliQ water by adding the following components

2Na EDTA	0.7 g
FeCl ₃	0.4 g

3.1.5. Buffers composition

Table 2.4. Buffers Composition

Buffers	Composition
Coomassie destaining solution	10% ethanol, 7% acetic acid
Coomassie staining solution	0.1% (w/v) Serva CoomassieBlue R-250, 40% ethanol, 8% acetic acid
5X DNA loading dye	0.1 M EDTA, 0.1% (w/v) SDS, 0.025% (w/v) Bromo phenol blue, 40% glycerol
TAE	40 mM Tris-acetate, pH 8.5, 2 mM EDTA
PonceauS staining solution	0.1% (w/v) PonceauS, 5% Aceticacid
Lysis Buffer A	50 mM Tris-HCl pH8.0, 50 mM NaCl, 10 mM imidazole.
Lysis Buffer B (elution buffer)	50 mM Tris-HCl pH8.0, 50 mM NaCl, 200 mM imidazole.
Lysis Buffer A_CmP	50 mM Tris-HCl pH10.0, 300 mM NaCl, 10 mM imidazole.
Lysis Buffer B_CmP (elution buffer)	50 mM Tris-HCl pH10.0, 300 mM NaCl, 200 mM imidazole.
Purification Buffer A	20 mM Tris-HCl pH8.0, 50 mM NaCl
Purification Buffer B	20 mM Tris-HCl pH8.0, 1 M NaCl
Purification Buffer A CmP	20 mM Tris-HCl pH10.0, 300 mM NaCl
Assay Buffer A	100 mM Tricine/NaOH pH8.0, 5 mM MgCl ₂
Assay Buffer B	200 mM Tricine/NaOH pH8.0, 10 mM MgCl ₂
EM Buffer	20 mM Tris-HCl pH8.0, 50 mM NaCl, 10 mM MgCl ₂
TBS	50 mM Tris-HCl pH 8.0, 137 mM NaCl, 2.7 mM KCl
Western blot transfer buffer	50 mM Tris-HCl, 20% methanol, 192 mM glycine
5x SDS-PAGE loading buffer	225 mM Tris-HCl pH 6.8, 50% (w/v) glycerol, 5% (w/v) SDS, 5% (w/v) β-mercaptoethanol, 0.01% (w/v) Bromphenolblue
SDS-PAGE running buffer	3 g Tris, 14.4 g Glycine, 0.1% (w/v) SDS, 1 l deionized water

*Buffers used for FPLC were vacuum filtered and degassed.

3.1.6. Oligonucleotides

Oligonucleotides in this study were purchased from IDT (Singapore, Singapore). To amplify the *cbbX* from the coding region from *WH8102*, oligonucleotides primers incorporated with SacII and Hind III restriction site (underlined) were designed. To amplify the *rbcL* and *rbcS* from the coding region from *WH8102*, oligonucleotides primers incorporated with NdeI and Hind III restriction site (underlined) were designed. For introducing CmN into co-expression plasmid, oligonucleotides primers incorporated with KpnI and NdeI restriction site (underlined) were designed.

Table 2.5. Primers

Purpose	Name	Sequence
Amplification of WH8102Rca	WH8102Rca_SacII	CT <u>CCGCGG</u> TGGTATGCCCTCCTCGGTT
	WH8102Rca_HindIII	TTA <u>AAGCTT</u> TACCCACCGCTCGCCAT
Amplification of WH8102RbcLS	WH8102RbcL_NdeI	AAC <u>CATATG</u> AGCAAGAAGTACGA
	WH8102RbcS_HindIII	ATA <u>AAGCTT</u> TAGCGTCCTTCGAA
For introducing KpnI site at CmN 5'	CmN_NdeI	AAC <u>CATATG</u> AGTATCCCTGACGAC
For introducing KpnI site at CmN 5'	CmN_KpnI	TT <u>GGTACCT</u> TACTCCACAATCGC
To introduce Walker A mutation	CmPK80A_for	GGAACAGGTGCAACCACAGTAGCT
	CmPK80A_rev	AGCTACTGTGGTTGCACCTGTTCC
	CmNK80A_for	GGTACGGGCGCGACGACTGTT
	CmNK80A_rev	AACAGTCGTCGCGCCCGTACC
To introduce Walker B mutation	CmPE138Q_for	CTGTTTATTGACCAAGCATACTATCTC
	CmPE138Q_rev	GAGATAGTATGCTTGGTCAATAAACAG
	CmNE138Q_for	CTTTTCATCGACCAGGCGTACTACCT
	CmNE138Q_rev	AGGTAGTACGCCTGGTCGATGAAAAG
To introduce arginine finger mutation	CmPR194A_for	GGACTAGCTTCTGCTGTAGCAAATCAT
	CmPR194A_rev	ATGATTTGCTACAGCAGAAGCTAGTCC
	CmNR194A_for	GGCATCAGCTCCGCAATCGGAAAT
	CmNR194A_rev	ATTTCCGATTGCGGAGCTGATGCC
To introduce RuBP binding site mutation	CmPR239A_for	ATTCAACGTGCAATGGAACAACCACAT
	CmPR239A_rev	ATGTGGTTGTTCCATTGCACGTTGAAT
	CmNR239A_for	ATCGAGAAGGCTATGCAGATGCCATAC
	CmNR239A_rev	GTATGGCATCTGCATAGCCTTCTCGAT
To introduce pore loop mutation	CmPY114A_for	TTAGTAGGACAAGCTATAGGACATACA
	CmPY114A_rev	TGTATGTCCTATAGCTTGTCTACTAA
	CmNY114A_for	TCGTGCGACAAGCCGTGGGTCATA
	CmNY114A_rev	TATGACCCACGGCTTGTCCGACGA

Purpose	Name	Sequence
To introduce surface exposed Lysine mutation	CmPK123A_for	GCACCAAAAACCGCACAAAGTATTAAAG
	CmPK123A_rev	CTTTAATACTTGTGCGGTTTTTGGTGC
	CmNK123A_for	GCACCGAAAACCGCGGAGGTAATCAA
	CmNK123A_rev	TTGATTACCTCCGCGGTTTTTCGGTGC

3.2. Methods

3.2.1. Molecular biology methods

3.2.1.1. DNA analytical methods

UV spectroscopy (Nanodrop 2000 (Thermo scientific)) was used to determine the DNA concentrations. The measurements were carried out at 260 nm where one absorption unit equals to 50 ng/μl of DNA. Additionally, the purity of the DNA was determined by the ratio of absorbance at 260 nm and 280 nm using Nanodrop 2000. The ratio for pure DNA is 1.85.

DNA samples were subjected to horizontal agarose gels electrophoresis for separation according to size. The DNA samples to be applied on the gel were mixed with 5X DNA loading dye. Agarose gel electrophoresis was performed in 1 % (w/v) TAE-agarose gels supplemented with SYBR-SAFE (1:10000) for DNA visualization in 1X TAE buffer. The gels were run at constant 100 V and DNA fragments were visualized by blue light (Invitrogen). Cloned constructs were verified using a restriction enzyme digest analysis with suitable restriction enzymes and DNA sequencing by 1st base (Singapore). For one sequencing reaction, 1 μg of plasmid was needed. Sequencing results were analysed using the programs BioEdit (Ibis Biosciences, USA) and MultAlin (<http://multalin.toulouse.inra.fr/multalin/>) for comparative sequence alignment analysis.

3.2.1.2. Competent *E. coli* cells

Chemically competent DH5α and BL21 cells were prepared by the CaCl₂ method (Sambrook and Russell 2001). Cells from glycerol stock were revived by streaking onto an agar plate and growing overnight (8-16 hr) at 37 °C. A single

colony was inoculated in LB media and grown overnight in INNOVA 44R incubator shaker (New Brunswick Scientific) at 37 °C, 220 rpm. The overnight grown culture was used to inoculate 1 l of media and grown to mid-log phase (OD 600 = 0.5) at 37 °C, 220 rpm. After chilling the cells on ice for 20 min, the culture was centrifuged for 30 min at 4000 rpm at 4 °C and pelleted cells were resuspended gently in 20 ml ice-cold 0.1 M CaCl₂, 15 % glycerol (autoclaved). The stock culture was prepared as stated above and stored at -80 °C until used.

3.2.1.3. *E.coli* Transformation

For transformation, 100 µl of chemically competent cells were thawed on ice and mixed with ~100 ng plasmid DNA. After incubation on ice for 30 min, cells were heat shocked at 42 °C for 90 s and allowed to cool down before 400 µl recovery media (LB media without antibiotics) were added. Cells were incubated with shaking at 37 °C and 1200 rpm for 45 min to 1 hr. Cells were spun down at 8000 rpm for 30 sec, 300 µl supernatant media was discarded and the pellet was resuspended in the remaining supernatant to spread on to selection plates i.e. LB-Agar plates supplemented with respective antibiotics (200 µg/ml Ampicillin, 34 µg/ml Chloramphenicol, 30 µg/ml Kanamycin) and incubator overnight at 37 °C. A single colony of the transformants was picked and inoculated in liquid medium for further analysis.

3.2.1.4. Plasmid and DNA-fragment purification

For the amplification of plasmid DNA, *E. coli* DH5α were grown for 8-16 hr in LB medium containing the appropriate antibiotics at 37 °C and 220 rpm. Plasmids were subsequently purified via anion exchange chromatography using the Wizard® Plus SV Miniprep DNA Purification System (Promega Corporation, USA), according to the manufacturer's instructions. Plasmid concentrations were measured using Nanodrop 2000 Spectrophotometer (Thermo-scientific, Singapore) at 260 nm. Restriction digestion (Table 2.6) followed by agarose gel electrophoresis was performed to verify the presence of insert and plasmid DNA. In order to purify or isolate DNA-fragments after agarose gel electrophoresis, the Wizard® SV Gel and PCR Clean-Up system (Promega) was used according to the manufacturer's instructions.

Table 2.6. Components of restriction enzyme digestion reaction

Component	Amount
10x NEB Buffer	2 µl
Restriction enzyme 1	0.3 µl
Restriction enzyme 2	0.3 µl
Plasmid/DNA	1 µg
Autoclaved water	Variable µl
Total volume	20 µl

3.2.1.5. Polymerase chain reaction (PCR)

Amplification of DNA fragments with point mutations and insertions was achieved by PCR. Both amplification of genes from plasmids and site-directed mutagenesis was achieved by using high fidelity polymerase with proof reading capacity, Pfu polymerase (Thermo Scientific, Singapore).

Plasmid/Genomic DNA was used as a template for PCR, set up in a total vol. of 20 µl on ice with the appropriate concentration of the constituents as mentioned below (Table 2.7). DNA amplification was carried out as manufacture's instruction.

Table 2.7. PCR reaction mix with *Pfu* polymerase.

Components	Amount
<i>Pfu</i> 10x buffer with MgSO ₄	2 µl
2 mM dNTP	2 µl
(1.2 U) <i>Pfu</i> Polymerase enzyme	0.5 µl
template DNA	1 ng
10 µM forward primer	0.5 µl
10 µM reverse primer	0.5 µl
Autoclaved water	Variable µl
Total volume	20 µl

All reagents were pipetted into a PCR tube kept on ice all the time, mixing of the reagents was done by short centrifugation. PCR thermo cycler (BioMetra) was

pre heated to 99 °C before reaction tubes were placed inside the machine. The following PCR thermocycler program was used (Table 2.8). Primers used are listed in Table 2.5.

Table 2.8: Temperature cycles for DNA for amplification by PCR

Cycle Steps	Temperature	Time	Cycle
Lid	95 °C		-
Initial denaturation	95 °C	2 min	1
Cycle denaturation	95 °C	20 sec	35
Annealing	50 °C	30 sec	
Extension	72 °C	(1 mins for every 1 kb)	
Final Extension	72 °C	10 min	1
End	16 °C	∞	-

For cloning into a pGem-T vector, polyA tail amplification was done by the addition of 0.2 µl of Taq polymerase and 2 µl of 2 mM dATP to the amplified DNA followed by incubation at 72 °C for 30 min in the PCR thermocycler to introduce a deoxyadenosine (dA) tail via ligating with the deoxythymidine overhang. dA-tailed PCR product was mixed with 6x DNA loading dye (Thermo Scientific, Singapore) prior to loading into 1% TAE-agarose gel (40 ml) supplemented with 4 µl of SYBR Safe DNA Gel Stain (Invitrogen, Singapore). 5 µl of GeneRuler 1 kb DNA Ladder (Thermo Scientific, Singapore) was loaded to serve as a marker. Agarose gel electrophoresis was performed in 1xTAE buffer (40 mM Tris-acetate, pH8.5, 2 mM Ethylene diaminetetraacetic acid) at 100 V for 30-45 min and DNA fragments were then purified from the gel, using the Wizard SV Gel and PCR clean-Up system (Promega, USA) according to the manufacturer's protocol. The product was finally eluted in 50 µl of nuclease free water.

3.2.1.6. Site-directed mutagenesis

Site-directed mutagenesis was used to introduce point mutations and insertion in existing plasmids. Site-directed mutagenesis of existing plasmids was done using QuikChange protocol (Stratagene) according to manufacturer's instructions (Table 2.9 and Table 2.10). Forward and reverse primers were self-complementary and contained the desired mutation. All mutagenesis of plastid and nuclear were carried out in pHueCmN and pHueCmP. After the PCR 0.5 μ l DpnI were added to 10 μ l of the reactions and incubated at 37 °C for 1 hr to digest the methylated template plasmid. The treated PCR product was subsequently transformed into DH5 α .

Table 2.9. PCR reaction mix for site-directed mutagenesis

Components	Amount
Pfu 10x buffer with MgSO ₄	2 μ l
2 mM dNTP	2 μ l
(1.2 U) Pfu Polymerase enzyme	0.38 μ l
template DNA	30 ng
10 μ M forward primer	0.72 μ l
10 μ M reverse primer	0.72 μ l
Total volume	20 μ l

Table 2.10. Temperature cycles for DNA amplification for site-directed mutagenesis

Cycle Steps	Temperature	Time	Cycle
Lid	95 °C		-
Initial denaturation	95 °C	2 min	1
Cycle denaturation	95 °C	30 sec	18
Annealing	variable	1 min	
Extension	68 °C	15 min	
Final Extension	68 °C	15 min	1
End	16 °C	∞	-

3.2.1.7. Restriction digest and ligation

Expression vector for cloning or vector containing gene of interest or amplified PCR product (1 µg DNA) were digested with appropriate restriction enzymes (20-60 U) in 20 µl reactions with respective buffer according to manufacturer's recommendations. The digestion reaction was carried out at 37 °C for 3 hr or overnight (Table 2.6). Digested plasmid vector DNA was incubated for 1 hr at 37 °C with calf intestinal phosphatase (CIP) for dephosphorylation. Restriction digestion of plasmid produce linearized plasmids (vector) and DNA fragments flanked with respective overhangs. The digests, plasmid and inserts, were separated by 1% agarose gel electrophoresis and the respective DNA bands were extracted for purification by the Wizard Plus SV gel clean DNA Purification System. Concentrations of the purified insert and plasmid DNA were measured using a Nanodrop2000 (Thermo-scientific, Singapore).

Following purification, ligation of purified DNA fragments (insert with the vector) was carried out at room temperature (RT) for 1 hr or overnight at 4 °C. The ligation reaction was performed in a total reaction mix of 10 µl containing ~100 ng of digested plasmid DNA and ~500 ng DNA insert incubated with 200 U of T4 DNA ligase (Thermo-Scientific). The reaction was conducted in T4 DNA ligase reaction. The pGem-T vector system (Promega) was used according to manufacturer's instructions, for ligation of dA tailed PCR products. Followed by the ligation the reaction mix was either heat shock transformed into *E. coli* DH5α cells or DNA precipitation was carried out to prepare the ligated vectors for sequential restriction enzyme digestion. Appropriate constructs were verified by purifying the plasmid DNA from the transformed colonies grown overnight at 37 °C on the selective agar plates and subjecting to restriction digestion followed by sequencing the digest using 1st base (Singapore). The sequencing results were validated by Multalin and BioEdit programs.

3.2.1.8. General cloning strategy

The PCR product was polyadenylated at both the 5' and 3' ends to allow cloning of PCR product into a vector. The polyadenylated PCR product was run on 1% agarose gel and the DNA fragments were purified from the gel using the Wizard SV Gel and PCR Clean-Up system according to the manufacturer's instructions. Ligation of the polyadenylated PCR product into pGem-T was carried out according to manufacturer's instruction. The ligation mixture was transformed into DH5 α and plated on ampicillin LB plate (200 μ g/ml ampicillin) supplemented with 25 μ l of 40 mg/ml X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) in N,N-dimethylformamide (DMF). X-gal enables blue white screening and only white colonies containing insert was inoculated into 6 ml LB medium supplemented with ampicillin. This culture was grown overnight in a shaking incubator at 37°C, 220 rpm. The plasmids were purified using Wizard Plus SV Miniprep DNA Purification System (Promega Corporation, USA) and their concentrations were measured using Nanodrop 2000 Spectrophotometer (Thermo-scientific, Singapore) at 260 nm. The integrity of the plasmids with inserts was checked by 1st base (Singapore) sequencing. The protein coding insert from the validated pGem-T vector was isolated by restriction digestion with respective restriction enzymes and subsequently purified for ligation into the pHue expression vector digested complementarily with the respective enzymes and dephosphorylated by CIP. In the pHue vector proteins are expressed with an N-terminal fusion to hexa-histidine tagged ubiquitin (His₆-Ub), which improves solubility and protein purification by immobilised metal-ion affinity chromatography (IMAC) (Catanzariti et al. 2004). The resulted pHue vector was transformed into *E. coli* DH5 α and screened for ampicillin resistant colonies, which was eventually picked and grown in LB medium supplemented with ampicillin and the correct insert was confirmed by sequencing. Mutations in the inserts were generated by site directed mutagenesis. Cleavage of the tag by the deubiquitinating enzyme USP2c leaves the native N-terminus of the protein of interest (Baker et al. 2005).

3.2.1.8.1. Construction of WH8102 Rca plasmid

The *Synechococcus* sp. *WH8102* cyanobacterial genomic DNA was a kind gift from Dr. Dean Price (Australian National University). The coding sequence of *WH8102cbbX* for Rubisco activase (Uniprot: Q7TTT9) was amplified from the genomic DNA with the flanking 5'SacII and 3'HindIII restriction site using the primer pair WH8102 SacII for and WH8102 HindIII (Table 2.5). The PCR product was subsequently polyA tailed, cloned into the vector pGem-T and subsequently digested with SacII and HindIII. Linearized insert and vector fragments were separated by agarose gel electrophoresis and purified using Wizard Plus SV Gel and PCR Clean-Up System. Target insert and pHue vector were ligated at RT and transformed to DH5 α . The expression plasmid was named pHue8102Rca and confirmed for the correct insert by sequencing (1st Base, Singapore).

3.2.1.8.2. Construction of WH8102 Rubisco plasmid

The coding sequence of *WH8102rbcLS* encoding WH8102 Rubisco (Uniprot RbcL: Q7U5I8; Uniprot RbcS: P0A4S5) was amplified from the genomic DNA with the flanking 5'NdeI and 3'HindIII restriction site using the primer pair WH8102 RbcL_NdeI and WH8102 RbcS_HindIII (Table 2.5), cloned into the vector pGem-T and sequentially digested with NdeI and HindIII and inserted into the pET30b vector. The expression plasmid was named pET30WH8102RbcLS and confirmed for the correct insert by sequencing (1st Base, Singapore).

3.2.1.8.3. Construction of MIT9313 Rubisco plasmid

The coding sequence of *MIT9313rbcLS* encoding MIT9313 Rubisco (Uniprot RbcL: Q7V6F8; Uniprot RbcS: Q7V6F9) was synthesised by Genscript (USA) with flanking NdeI and BamHI restriction sites at 5' and 3' housed in pUC plasmid. *MIT9313RbcLS* was purified by sequential digestion of BamHI followed by NdeI and cloned into a pET24b vector. The expression plasmid was named pET24bMIT9313RbcLS and confirmed for the correct insert by sequencing (1st Base, Singapore).

3.2.1.8.4. Construction of *C. merolae* Plastid encoded CbbX plasmid

The coding sequence of the *Cyanidioschyzon merolae* plastid genome *cbbX* (Gene symbol: cfxQ; geneID: AB002583) encoding Rubisco activase isoform (CmP) (Uniprot: O22025) was synthesised by Genscript (USA) with flanking SacII and HindIII restriction sites at 5' and 3' was cloned into pUC57. CmP insert was purified by double digesting pUCCmP plasmid with SacII and HindIII and subsequently cloned the insert into the pHue vector. The expression plasmid was named pHueCmP and confirmed for the correct insert by sequencing (1st Base, Singapore).

3.2.1.8.5. Construction of *C. merolae* Nuclear encoded CbbX plasmid

The coding sequence of the *Cyanidioschyzon merolae* nuclear genome *cbbX* (Gene symbol: CYME_CMT172C; geneID: 16997649) encoding Rubisco activase isoform (CmN) (Uniprot: M1UXG8) was synthesised by Genscript (USA) with flanking SacII and HindIII restriction sites at 5' and 3' sites housed in pUC57 plasmid. CmN insert was purified by double digesting the pUC57CmN plasmid with SacII and HindIII and subsequently cloning the insert into the pHue vector. The expression plasmid was named pHueCmN and confirmed for the correct insert by sequencing (1st Base, Singapore).

3.2.1.8.6. Construction of *C. merolae* Plastid and Nuclear encoded CbbX mutant plasmids

Mutations in pHueCmN and/or pHueCmP were generated by site-directed mutagenesis (section 2.2.1.6) with the appropriate forward and reverse primer pairs (Table 2.5). The respective vectors with mutated insert were confirmed by sequencing (1st Base, Singapore). The expression plasmids of CmN and CmP mutants are listed in Table 2.11.

3.2.1.8.7. Construction of CmNP co-expression plasmid

With pHueCmN as a template, KpnI restriction site at 5' and NdeI restriction site at 3' was added to the CmN by PCR amplification (Section 2.2.1.5) using the respective primers listed in Table 2.5. CmN insert from the amplified pHueCmN was purified after double digestion with KpnI and NdeI. The CmP insert including the

ubiquitin-encoding sequence at its 5' was purified from pHueCmP after double digestion with XbaI and HindIII. These linearized inserts were cloned into a pETDuet-1 vector (Novagen), each isoform expression is under control of T7 promoter. The CmP-Ub expression sequence was cloned into multiple cloning sites one and CmN cloned into multiple cloning site two. The expression plasmid was named pETDuetCmNP and confirmed for the correct insert by sequencing (1st Base, Singapore).

3.2.1.8.8. Construction of Concatamer plasmid

The coding sequence of CmN and CmP connected by a 20 amino acids sequence (ASGAGGSEGGGSEGGTSGAT) designated as L20 by (Martin et al. 2005) was synthesised by Genscript (USA) with flanking SacII and HindIII restriction sites at 5' and 3' and cloned into pUC57 (pUCCmN-Pcon and pUCCmP-Ncon) plasmid. The two concatamer sequences were L20 linked to the C-terminus of CmN and N-terminus of CmP (CmN-L20 (linker)-CmP), and L20 connected to the C-terminus of CmP and N-terminus of CmN (CmP-L20(linker)-CmN). The concatamer inserts were purified by double digesting pUCCmN-P plasmid with SacII and HindIII and subsequently cloned into pHue vector. The expression plasmids were named pHueCmN-Pcon and pHueCmP-Ncon which was confirmed for the correct insert by sequencing (1st Base, Singapore).

The final expression vectors were transformed into both chemically competent BL21 cells and BL21 harbouring pBADESL (Ewalt et al. 1997) (expressing chaperonin GroELES for enhanced folding of proteins). A single colony of transformed cells was grown in 2 ml of LB media with respective antibiotics for overnight at 37 °C and 220 rpm for making glycerol stock of the competent BL21 cells with respective expression vectors. 400 µl of overnight cell suspension and 200 µl of 80% glycerol were mixed/vortexed and frozen with liquid nitrogen and stored at -80 °C.

Table 2.11. List of Plasmids

Plasmid	Protein expressed	Reference
Wild-type activase		
pHueRsRca	RsRca	(Mueller-Cajar et al. 2011)
pHue8102Rca	WH8102 Rca	This study
pETDuetCmNP	Co-expressed CmNP	
pHueCmN	Nuclear encoded isoform	
pHueCmP	Plastid encoded isoform	
CmN and CmP Mutants		
pHueCmN(K80A)	Walker A defective CmN	This study
pHueCmP(K80A)	Walker A defective CmP	
pHueCmN(E138Q)	Walker B defective CmN	
pHueCmP(E138Q)	Walker B defective CmP	
pHueCmN(R194A)	Arginine finger defective CmN	
pHueCmP(R194A)	Arginine finger defective CmP	
pHueCmN(R194A)	Arginine finger defective CmN	
pHueCmP(R194A)	Arginine finger defective CmP	
pHueCmN(Y114A)	Pore loop defective CmN	
pHueCmP(Y114A)	Pore loop defective CmP	
pHueCmN(R239A)	Sugar binding defective CmN	
pHueCmP(R239A)	Sugar binding defective CmP	
pHueCmN(K123A)	Surface exposed Lysine mutated CmN	
pHueCmP(K123A)	Surface exposed Lysine mutated CmP	
pHueCmN(R239A +R194A)	Sugar binding and Arginine finger defective CmN	
pHueCmP(R239A +R194A)	Sugar binding and Arginine finger defective CmP	
pHueCmN(R239A +E138Q)	Sugar binding and Walker B defective CmN	
pHueCmP(R239A +E138Q)	Sugar binding and Walker B defective CmP	
Concatamer		
pHueCmN-Pcon	N-P concatamer	This study
pHueCmP-Ncon	P-N concatamer	

Plasmid	Protein expressed	Reference
Rubisco		
pBAD30RsRbcLS	Rs Rubisco	(Mueller-Cajar et al. 2011)
pET30RsRbcLS Δ 2	Rs Rubisco with last two C-terminal residues of large subunit deleted	
pET30RsRbcLS Δ 4	Rs Rubisco with last four C-terminal residues of large subunit deleted	
pET30WH8102RbcLS	WH8102 cyanobacterial Rubisco	This study
pET24MIT9313RbcLS	MIT9313 cyanobacterial Rubisco	This study
Other Plasmids		
pBADESL	For the expression of chaperonin GroES and GroES	

3.2.2. Protein Biochemistry

3.2.2.1. Protein analytical methods

Protein sequence alignment - Protein sequences were aligned using the program MultAlin (Chenna et al. 2003).

Phylogenetic Tree - MEGA6 and 7 for Molecular evolutionary genetics analysis (Tamura et al. 2013)

Structure analysis – Pymol (DeLano 2002)

3.2.2.2. Protein quantification

Protein mixtures were quantified by a Bradford (BioRad protein assay) assay using bovine serum albumin as a standard. 2-10 μ g of Bovine serum albumin (BSA) were added to a mix of 500 μ l autoclaved H₂O and 500 μ l Bradford reagent and absorbance of the samples were measured at 595 nm on a spectrophotometer. Pure proteins were quantified by their absorbance at 280 nm by Nanodrop 2000 (Thermo-scientific, Singapore) and the concentration was calculated using the extinction coefficients listed in Table 2.12 Extinction coefficients of the proteins were calculated

with the online program, ProtParam (<http://web.expasy.org/protparam/>) (Gasteiger et al. 2005). The final concentration of the protein was calculated using extinction coefficient and molecular weight.

$$C = (A_{280} / \epsilon) \times \text{Molecular weight}$$

C-concentration of the protein in mg/ml

A₂₈₀-absorbance measured by Nanodrop at 280nm

ϵ – Extinction coefficient M⁻¹cm⁻¹

Table 2.12. Extinction co-efficient and Molecular weight

Protein	Extinction coefficient (M⁻¹cm⁻¹)	Molecular weight (kDa)
RsRca	16390	34684.2
CmN	19370	34606.8
CmP	17880	33587.6
CmNP	18625	34097.2
8102Rca	17880	33532.1
RsRbcLS	109250	68774.5
RsRbcLSΔ2	109250	68572.2
RsRbcLSΔ4	109250	68386.0
CmRbcLS	105575	70340.3
WH8102RbcLS	100895	65734.4
WH8102RsC	100895	66295.1
MIT9313RbcLS	101020	65545.3

3.2.2.3. SDS-PAGE gel electrophoresis

Protein analysis was performed under reducing, denaturing conditions by SDS-PAGE (sodiumdodecylsulfate polyacrylamide gel electrophoresis). In this method the proteins were resolved based on their molecular weight and electrophoretic mobility. Gels were casted according to Table 2.13. Samples were mixed with 5X SDS-sample buffer, boiled for 5 min at 100 °C, and centrifuged for 2 min at RT at 16000 xg. Centrifuged samples were loaded into their respective wells and gel electrophoresis was performed in Mini Protean (Bio-rad) electrophoresis chambers in SDS-electrophoresis migration buffer (with SDS) at constant voltage of 100 V for 20 min followed by 140-180 V for the rest of the run. Thus the proteins are electrophoretically separated in denatured conditions.

Table 2.13. Composition of gel

For 4 gels	12.5% SDS-PAGE gel	
	Resolving gel	Stacking gel
Autoclaved H ₂ O	4.27 ml	3.05 ml
1.5 M Tris-HCl pH 8.8	3.33 ml	-
0.5 M Tris-HCl pH 6.8	-	1.25 ml
10% (w/v) SDS	133.33 µl	50.00 µl
30% (w/v) Acrylamide	5.55 ml	0.65 ml
10 % (w/v) Ammonium persulfate	66.67 µl	25.00 µl
Tetramethylethylenediamine (TEMED)	4 µl	5 µl

3.2.2.4. Coomassie brilliant blue staining of SDS

Proteins that were separated by electrophoresis were briefly washed in water before staining by incubation of the gel in Coomassie Brilliant Blue (40 % methanol, 10 % acetic acid, 0.1 % Coomassie Brilliant Blue R-250). Coomassie blue is a triphenylmethane coloring agent that absorbs to proteins. Coomassie blue dye integrates with cationic, nonpolar, and hydrophobic side chains of a polypeptide. Hence, Coomassie blue staining was used for the visualization of protein bands in polyacrylamide gels. Protein bands were fixed and stained by incubating the gels for 1 hr with Coomassie blue staining solution. Consequently, the gel was continually washed with or incubated in destaining solution for 1 hr to remove the background staining.

3.2.2.5. Western blotting and immunodetection

After SDS-PAGE, proteins were transferred onto a nitrocellulose membrane using transfer buffer (50 mM Tris-HCl pH 8.0, 20% methanol, 192 mM glycine) in a semi-dry western blot apparatus (BioRad). This transfer was performed at 15 volts for 15 min. The membrane would be stained with Ponceau S staining solution (0.1% (w/v) Ponceau S, 5% acetic acid) to check the efficiency of transfer and the staining solution was rinsed off with Milli-Q water. The membrane was blocked with 5% (w/v) skimmed milk for 1 hour at room temperature. Primary antibody, anti-Rs (Rabbit, polyclonal) were diluted to 1:5000 in TBS (50 mM Tris-HCl pH8.0, 137 mM NaCl, 2.7 mM KCl). The primary antibody was incubated with the membrane for one hour at room temperature and subsequently washed three times with TBST (TBS, 0.1% (v/v) TWEEN 2.0) with 10 min incubation time between each wash. The secondary antibody, horseradish peroxidase (HRP) conjugated, anti-rabbit was diluted to 1:10000 in TBS and incubated with the membrane for 1 hr at RT followed by another three times of wash. The HRP coupled antibody was detected by enhanced chemiluminescence (ECL) staining with a 1:1 mix of ECL detection reagent 1 and 2 (Thermo Scientific) on an LAS-3000 Imaging system (Fuji).

3.2.2.6. Test expression of proteins

Glycerol stock of the BL21 cells carrying the activase and Rubisco constructs were inoculated in LB media supplemented with respective antibody and grown overnight at 37 °C with shaking (220 rpm) in an Innova44 incubator (New Brunswick Scientific). 50 µl of this overnight culture was subcultured into a couple of fresh 5 ml LB media supplemented with the same antibody. This culture was grown at 37 °C with shaking until the optical density reaches ~0.6 at a wavelength of 600 nm (OD₆₀₀). Two different temperatures (37 °C and 23 °C) were tested for the better expression of proteins. One culture was moved 23 °C with shaking while the other was kept at 37 °C. Both cultures were induced with 0.5 mM isopropyl-D-thiogalactopyranoside (IPTG). For co-expression of construct GroELES, initially 0.2% L-arabinose was added and 30 min later 0.5 mM of IPTG was added to express the interested protein in the construct. Cells were harvested by centrifugation at 4000 xg for 10 min after 4 hr or 12 hr of induction. After removing the supernatant media, the cells were resuspended in 1 ml of lysis buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl). Cells were lysed on ice by sonication at 30% amplitude for 50 sec using 10 sec pulses alternated with 50 sec of pause. 50 µl of the lysate was added with 12.5 µl 5X SDS sample buffer for analysis of total protein expression (total lysate sample). 300 µl of the lysate were clarified by centrifugation at 6000 xg and 4 °C for 15 min and 50 µl of the supernatant were added with 12.5 µl 5X SDS sample buffer for analysis of soluble protein expression (soluble sample). After discarding the remaining supernatant the pellet was washed with 300 µl MilliQ water and centrifuged at 16000 xg and 4 °C for 15 min. The pellet was redissolved in 72.5 µl 2X SDS sample buffer for analysis of insoluble protein expression (insoluble sample). The collected samples were heat denatured using a heat block at 100 °C and centrifuged at 16000 xg and RT for 3 min prior to loading on 12.5% SDS polyacrylamide gel. 5 µl of pre-stained marker (PageRuler Plus Prestained Protein Ladder 10-250 kDa; Thermo Scientific) was loaded alongside the samples. SDS-PAGE gel electrophoresis was performed at a starting voltage of 120 V followed by 160 V for the rest of the run.

3.2.2.7. Expression of His₆Ub-fusion constructs

All the activase proteins and its variants used in this study were expressed in BL21 strain with/without overexpression of the *E. coli* chaperonin using pBADESL (Ewalt et al. 1997), as a His₆Ub-fusion constructs using pHue and pETDuet (only for wild-type CmNP) expression vectors. BL21 cells carrying the respective plasmid construct were grown in LB supplemented with respective antibiotics at 37 °C in an Innova44R incubator (New Brunswick Scientific) at moderate shaking (200 rpm) to an optical density at a wavelength of 600 nm OD₆₀₀=0.6. The culture was then cooled down to 23 °C for 30 min and induced with 0.2% w/v L-arabinose (for expression of GroELES) and 30 min later induced with 0.5 mM IPTG (for expression of the protein of interest). In the absence of chaperonin overexpression, following cooling of cells to 23 °C it was induced with 0.5 mM IPTG alone. Following induction, the culture was allowed to grow for ~16 hr at 200 rpm at 23 °C. After incubation, *E.coli* cells were harvested by centrifugation in JA-10 centrifuge with JA 10 rotor (Beckman Coulter) at 4000 rpm for 20 min at 4 °C. Cell pellets were either stored at -20 °C or directly lysed as described.

3.2.2.8. Purification of His₆Ub-fusion constructs

His₆Ub-fusion constructs were captured by immobilised metal affinity chromatography (IMAC) and followed by ubiquitin moiety cleavage by the ubiquitinase the construct was further purified by ion exchange chromatography and size exclusion. Harvested cells from 1 l of bacterial culture were resuspended in 40 ml Lysis Buffer A (50 mM Tris-HCl pH8.0, 50 mM NaCl, 10 mM imidazole) supplemented with 1 mM PMSF for protease inhibition and 0.3mg/ml lysozyme to aid lysis. The cells were lysed by ultrasonication on ice for 5 min with a 15 s bursts and 30 s rest to limit sample heating. After lysis, the lysate was centrifuged at 20000 rpm for 40 min at 4°C in an Avanti J-25 with JA25.5 rotor (Beckman Coulter) to pellet down the cell debris. The supernatant was bound in batch to an affinity column packed with Ni-NTA resin (Thermo Scientific) equilibrated with Lysis Buffer A (Table 2.4) about 3 times (1 hr/binding) and later washed ~3 times with wash buffer (Table 2.4). The His₆Ub-fusion protein was eluted in 1.5 ml Eppendorf tubes using Lysis buffer B (50 mM Tris-HCl pH 8.0, 50 mM NaCl, and 200 mM imidazole). The eluted fractions were checked for protein concentration using Bradford assay at 595

nm and subsequently, the protein fractions with $OD_{595nm} > 0.1$ were pooled together in a 15ml falcon tube. The ubiquitin moiety was cleaved overnight at 4 °C using the deubiquitylating enzyme USP2c as detailed in Table 2.14 (Catanzariti et al. 2004). The Ubiquitin cleaved protein solution was applied to an anion protein exchanger column (GE Healthcare) (Table 2.3).

Table 2.14. Components for overnight cleavage of Ubiquitin

Component	Amount added
80% (w/v) glycerol	416.6 µl per 10 ml of total pool
β-meptoethanol	3.33 µl per 10 ml of total pool
USP2c	1 mg per 200 mg of protein

2.2.2.8.1 Anion exchange chromatography

MonoQ 10/10, ResourceQ or MonoQ HR 16/10 columns were utilized with an Akta Purifier system (GE Healthcare). Proteins were eluted into fractions using a linearized salt gradient by gradual substitution of 0 to 100% Purification Buffer A (20mM Tris-HCl pH8, 20mM NaCl) with Purification Buffer B (20mM Tris-HCl pH8, 1M NaCl). Samples from Ni-NTA chromatography and corresponding fractions from anion-exchange chromatography were analysed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) to determine the presence of the protein of interest and its purity. Pure samples were then concentrated using Amicon Ultra-15 (50 or 100 kDa MW cut off) Centrifugal Filter Units (Millipore, USA). Subsequently, size exclusion chromatography was performed for further purification.

2.2.2.8.2 Gel filtration chromatography

For further purification, protein after Anion exchange purification was loaded onto SuperDex 200 16/600 Highload or Superdex 200 increase 10/300 GL (GE Healthcare) column. Proteins were eluted with Purification Buffer A (Table buffer 2.4). The appropriate fractions for the protein peak was tested by SDS-PAGE analysis and the confirmed protein fractions were pooled and concentrated in an Amicon Ultra-15 (50 or 100 kDa cut) Centrifugal Filter Units (Millipore, USA),

supplemented with 5% v/v glycerol and flash-freezing in liquid N₂ for the protein to be stored in aliquots at -80°C

3.2.2.9. Purification of *C. merolae* Nuclear encoded isoform (CmN)

His₆Ub-fusion construct of CmN was housed in pHue plasmids and expressed in BL21 *E.coli* cells with pBADESL (Ewalt et al. 1997). The expression and purification of *C. merolae* Nuclear encoded isoform was performed in the same way as explained in earlier section 2.2.2.8 (Expression and purification of His₆Ub-fusion constructs).

3.2.2.10. Purification of *C. merolae* Plastid encoded isoform (CmP)

For the purification of pure CmP protein, His₆Ub-fusion construct of CmP was housed in pHue plasmid and expressed in *E.coli* BL21 cells. The cells were grown in LB supplemented with ampicillin and chloramphenicol to the OD₆₀₀ of 0.5. After achieving the desired OD₆₀₀, the cells were induced with 0.2% v/v L-arabinose followed by 0.5 mM IPTG, and cells were grown at 23 °C, 200 rpm overnight. The cells were harvested and the pellet was resuspended in 40 ml of lysis Lysis Buffer A_CmP (pH10, high salt) (Table 2.4), 1 mM PMSF and 0.3 mg/ml lysozyme to aid lysis. The cells were then sonicated for 5 mins (15 sec bursts with 30 sec rest to limit sample heating). Later the lysate was cleared at 20000 rpm for 40 min at 4°C. The supernatant was loaded onto the Ni-NTA column equilibrated with of Lysis buffer A_CmP (pH10, high salt). Following 3 hr of rebinding and 3 times washing with Lysis buffer A_CmP (pH10, high salt), the protein was eluted in Lysis buffer A_CmP (Table 2.4) The protein fractions with OD₅₉₅ > 0.1 was confirmed by Bradford assay and was pooled together. The pooled protein fractions were cleaved for Ub over night at 4 °C using the protease USP2c (Baker et al. 2005) (Table 2.14). The cleaved protein solution was directly applied onto Superdex 200 16/600 Highload or increase 10/300 column (GE Healthcare) equilibrated with Purification Buffer A_CmP (Table 2.4) and the proteins were eluted with the same buffer. The fraction were analysed for CmP protein using SDS-PAGE and combined. The pooled fraction containing CmP was applied onto anion exchange column MonoQ 10/10 GL (GE Healthcare) equilibrated with Purification Buffer A_CmP to capture some impurities because CmP does not bind the column and elutes in the flowthrough. The flow-through was

concentrated in an Amicon Ultra-15 (50kDa MW cutoff) Centrifugal Filter Units (Millipore, USA), supplemented with 5% v/v glycerol and flash-freezing in liquid N₂ for the protein to be stored in aliquots at -80 °C. For producing pure CmP we did not use pBADESL coexpressed BL21 cells because the protein was soluble at 300 mM salt and pH10. Alternatively, for the co-purification with CmN in the pH8 buffer, we used CmP co-expressed with pBADESL in BL21 cells.

3.2.2.11. Purification of CmNP activase

CmN and His₆Ub-fusion constructs of CmP were housed in pETDuet and expressed in BL21 cells without overexpression of GroELES. The expression and purification of CmNP activase was performed in the same way as explained in earlier section 2.2.2.8 (Expression and purification of His₆Ub-fusion constructs).

3.2.2.12. Purification of CmNP mutants and wild type

For producing CmNP wild type or CmNP mutants by co-purification method, pHue vector harbouring CmN mutants or wild type and pHue vector harbouring CmP mutants or wild type were expressed in BL21 cells with pBADESL (Ewalt et al. 1997) (Table 2.11). The CmN (wild type or respective mutant) and CmP (wild type or respective mutant) cells were grown in 1 l LB with ampicillin and chloramphenicol independently and harvested as described in Expression of His₆Ub-fusion constructs (section 2.2.2.7). Harvested cells were resuspended in Lysis Buffer A (Table 2.4) and mixed together before sonication. Purification of the variants was performed the same as described for purification of His₆Ub-fusion constructs section 2.2.2.8. The concentrated protein with 5% glycerol was flash frozen in N₂ and stored in -80 °C.

3.2.2.13. Purification of *Cyanidioschyzon merolae* Rubisco

The *C. merolae* cells cultured as described in section 2.2.5 were spun down at 4500 rpm at 4 °C for 30 min. Harvested cells from 1 l were resuspended in about 10 ml of Lysis Buffer A (Table 2.4). Cells were lysed initially by performing 5 cycles of freeze/thaw. The cells were aliquoted into Eppendorf tubes which were flash frozen using liquid Nitrogen and thawed at 50 °C (using the heat block) and was repeated five times. To this cell suspension, 0.1% TritonX 100 and 1 mM PMSF was added. Subsequently, the cells were homogenised using Potter-Elvehjem homogenizer

(Sigma-Aldrich). Pestle and glass tube was used to homogenise or grind the cells by manually pulling the pestle up and down about 45 times or 15 min. The homogenised cells were pelleted by centrifugation at 24500 rpm at 4 °C for 30 min using JA 25.5 rotor. The supernatant was filtered using a 0.2 µm syringe filter (Minisart, Sartorius stedim) before loading onto Anion exchange column (MonoQ 10/10 or ResourceQ) (GE Healthcare) equilibrated with Purification Buffer A (Table 2.5). Proteins were eluted into fractions using a linearized salt gradient by gradual substitution of 0 to 100% Purification Buffer A with Purification Buffer B (Table 2.5). Fractions from anion-exchange chromatography were analysed by 12.5% SDS-PAGE to confirm the presence of *C. merolae* Rubisco (large and small subunit). The fraction containing Rubisco were concentrated to about 5 ml using Amicon Ultra-15 (100 kDa MW cutoff) Centrifugal Filter Units (Millipore, USA) and loaded onto a size exclusion column (SuperDex 200 16/600 Hiload). Protein fractions eluted with Purification Buffer A (Table 2.4) was tested by 12.5% SDS-PAGE analysis of Rubisco protein. Confirmed protein fractions were pooled and concentrated in an Amicon Ultra-15 (100 kDa MW cutoff) Centrifugal Filter Units (Millipore, USA), supplemented with 5% v/v glycerol and flash-freezing in liquid N₂ for the protein to be stored in aliquots at -80 °C.

3.2.2.14. Purification of cyanobacterial Rubiscos

1 l of BL21 cells harbouring pET24bMIT9313RbcLS, pET30WH8102RbcLS was grown in LB media supplemented with 30 µg/ml of kanamycin. Harvested cells from 1 l of bacterial culture were resuspended in 40 ml Purification Buffer A (50 mM Tris-HCl pH8.0, 50 mM NaCl) supplemented with 1 mM PMSF and 0.3 mg/ml lysozyme. The cells were disrupted by ultrasonication with a Sonicator (15 sec bursts with 30 sec cooling on ice between bursts to limit sample heating). Cell debris was removed by centrifugation in an Avanti J-25 with JA25.50 rotor (Beckman Coulter) for 40 min at 4 °C at 20000 rpm. The supernatant was applied to equilibrated column Source30Q (GE Healthcare) with Purification buffer A (Table 2.5). Proteins were eluted using Purification buffer B (Table 2.5). Appropriate fractions of the prominent peak were analysed by SDS-PAGE. Fractions containing Rubisco were combined and concentrated. To further purify the proteins, size exclusion chromatography using SuperDex 200 16/600 was performed for the Rubisco protein. Proteins were eluted

with Purification buffer A (Table 2.5) and analysed by SDS-PAGE. If the proteins are not pure then the fractions containing the Rubisco were applied to MonoQ 10/10 GL or Resource Q column (GE Healthcare) equilibrated with Purification buffer A (Table 2.5). Proteins were eluted with a linear salt gradient up to 500 mM NaCl and subsequently, the fractions were analysed using SDS-PAGE. Fractions containing the protein were pooled and concentrated by Amicon Ultra-15 (150 kDa) Centrifugal Filter Units (Millipore, USA), 5 % glycerol was added, flash-frozen in liquid N₂ and stored in aliquots at -80 °C until further use.

3.2.2.15. *Rhodobacter sphaeroides* Rubisco and activase

RsRca and RsRbcLS were purified as described previously (Mueller-Cajar et al. 2011).

3.2.3. Enzymatic Assays

3.2.3.1. Rubisco Assay

Rubisco activity was assayed by coupling generation of 3-phosphoglycerate by Rubisco to Nicotinamide adenine dinucleotide (NADH) oxidation (Kubien et al. 2011). Rubisco Assay was performed as described by (Kubien et al. 2011) with minor modifications. In this assay, we measured the oxidation of NADH spectrophotometrically using a UV-1800 spectrophotometer (Shimadzu, Japan) at 340 nm in a 100 µl reaction volume of Assay buffer B (200 mM Tricine pH 8.0, 10 mM MgCl₂) with 3 µl coupling enzymes mixture (Creatine P-kinase (2.5 units/ml), Glyceraldehyde-3-P dehydrogenase (2.5 units/ml), 3-phosphoglycerate kinase and Triose-P isomerase/Glycerol-3-P dehydrogenase (20/2 units/ml)) (Kubien et al. 2011), 10 mM NaHCO₃, 0.5 mM NADH, 1 mM ATP, 10 mM Creatine-P, 1 mM DTT, 1 mM RuBP, with a final concentration of 0.5 or 0.3 µM Rubisco active sites and 5 or 1 µM activase protomer. The rate of NADH oxidation was converted to the amount of 3PG produced (Tsai et al. 2015).

Fully carbamylated Rubisco (ECM) complex was made in Assay buffer B by incubating 100 µM Rubisco in the presence of 40 mM NaHCO₃ and 10 mM MgCl₂. RuBP inhibited Rubisco (ER) complex was made in Assay Buffer B by incubating 100 µM Rubisco with 4 mM EDTA at 25 °C. After 10 min, 1 mM RuBP was added to

make the inhibited complex. CABP inhibited (ECM-C) complex was made in Assay buffer B by incubating 100 μ M Rubisco with 40 mM NaHCO_3 , 10 mM MgCl_2 at 25 $^\circ\text{C}$ for 10 min and four fold molar excess of carboxypentitol biphosphate (1:1 mixture of the tight binding inhibitor CABP and the loose binding stereoisomer carboxyribitol-1,5-bisphosphate) was added incubated Rubisco. XuBP inhibited (EX) complex was made in Assay buffer B by incubating 100 μ M Rubisco with 4 mM EDTA at 25 $^\circ\text{C}$. After 10 min, 12 times the concentration of Rubisco (1.2 mM) XuBP was added to make the inhibited complex (Bracher et al. 2015).

The activity of Rubiscos (ECM, ER and ECM-C) was calculated by the 3PG generated and activation rate of inhibited Rubiscos (ER and ECM-C to ECM) complexes is measured by 3PG produced with the addition of the respective activase. The 3PG produced was plotted against time to determine the activity of Rubisco in the presence and absence of activase. The rate of 3PG produced is directly proportional to the activity of the Rubisco. For quantification analysis of CmNP variants, the assays were performed with limiting concentration of Rubiscos (0.3 μ M active sites) and activases or its variants (1-2 μ M protomer) to calculate the rate of 3PG produced or the rate ECM converted from ER and compared with its wild-type activase.

Figure 2.1| The spectrophotometric Rubisco assay.

For each carboxylation of RuBP, two molecules of 3-Phosphoglycerate (3-PGA) were produced by Rubisco which were phosphorylated by 3-PGA kinase (PGK). Glyceraldehyde-3-P dehydrogenase (GAPDH) reduces the bis-PGA to an aldehyde (Ga-3-P). Triose-phosphate isomerase (TIM) produces di-hydroxyacetone (DHAP) from Ga-3-P, glycerol-p dehydrogenase (GlyPDH) reduces DHAP to Glycerol 3-Phosphate (Gly 3-P). Four NADH are being oxidized per RuBP, leading to a decrease in absorbance at 340nm.

3.2.3.2. ATPase Assay

ATPase activity of the activases was measured spectrophotometrically by monitoring NADH oxidation using a UV-1800 spectrophotometer (Shimadzu, Japan) at 340 nm. This ATP regenerating system is driven by pyruvate kinase (PK) which catalyses the conversion of a phosphoenol pyruvate (PEP) to a pyruvate, by transferring the phosphate to an ADP to generate an ATP molecule. In the process, the resulting pyruvate is converted to lactate by the Lactate dehydrogenase (LDH) at the expense of NADH which is oxidised to NAD⁺. NADH shows strong absorbance peak at 340 nm owing to the presence of the adenine moiety. Spectrophotometric measurement of the reducing population of NADH at 340 nm, which is the consequence of its oxidation in this continuous assay, allows monitoring the rate of steady state hydrolysis of ATP in real time. Thus the oxidation of NADH is directly coupled to the amount of ATP used by the activase. The assay was performed in a reaction mix of 100 µl (Table 2.15) at 25 °C. The activity of the enzyme was measured by determining the slope of the linear (initial) part of the reaction.

ATPase activity of the activases was also measured in the presence of inhibited Rubiscos. This was calculated by adding inhibited Rubiscos at various concentrations to the 100 µl reaction mix (Table 2.15).

Table 2.15. Reaction mix of ATPase activity assay (modified from (Stotz et al. 2011))

Component	Final concentration (In 100µl)	Volume
Assay Buffer B	200 mM Tricine pH8.0, 10 mM MgCl ₂	50 µl
Phosphoenolpyruvate (PEP)	2 mM	4.5 µl
Pyruvate kinase	20 U/ml	3 µl
Lactate dehydrogenase	30 U/ml	
NADH	0.5 mM	0.7 µl
ATP (stock = 0.2M)	1 mM	0.5 µl
RuBP	1 mM	variable
Activase	5 µM	variable
Inhibited Rubisco complex (only for stimulation measurements)	3 µM	variable

Figure 2.2| Representation of the reactions that occurred during an ATPase activity assay.

A decrease in absorbance indicates oxidation of reduced Nicotinamide adenine dinucleotide (NADH), which is proportional to ATP hydrolysis rates.

3.2.3.3. Variation of Enzymatic assays

To investigate effects of temperature on the ATPase activity and Rubisco activation by activases, reaction mixes were incubated at the respective temperatures in a heat block for 10 min prior to measurement. The UV-1800 spectrophotometer (Shimadzu, Japan) was also heated using its thermostat. Also the quartz cuvette used for measuring the activity was heated in the spectrophotometer to the respective temperatures prior to measurement. Following 10 min heating the assay was initiated by mixing the activase to the reaction mix.

To investigate both the ATPase activity and Rubisco activation rate of *C. merolae* activases and its mutants at its physiological temperature, Cm activases, Rubisco complexes, reaction mix and cuvettes were heated to 45 °C for 10 min before conducting assays at 45 °C.

3.2.3.4. Analytical size exclusion chromatography

Analytical size exclusion experiments were performed on a Superdex 200 PC3.2/30 column using Akta micro system (GE Healthcare) with a flow rate of 0.075 ml/min. UV signal for the proteins were detected at 280nm. All the activases (10-60µg in 50 µl) except CmP were prepared in Buffer A (Table 2.4) and loaded onto the column equilibrated with the same buffer. CmP (10-80µg in 50 µl) was prepared in Buffer A_CmP (Table 2.4) and applied on to the column equilibrated with the same buffer. For reconstitution of the co-purified complex, CmN and CmP (10-60 µg in 50 µl) were mixed in equal or different stoichiometry in Buffer A and incubated at room temperature for about 1 hr prior to loading onto the column. In a variation, to check for the oligomeric state, the activases (20 µg in 50 µl) were made in Purification Buffer A with 0.1 mM RuBP and 1 mM ATP and loaded onto column equilibrated with the respective buffer as activase protein. In the case of CmP Purification Buffer A_CmP with 0.1 mM RuBP and 1 mM ATP was used. For calibration of the column, known globular proteins blue dextran 2000 kDa, ovalbumin 44 kDa, conalbumin 75 kDa, aldolase 158 kDa, ferritin 440 kDa, thyroglobulin 669 kDa from HMW gel filtration calibration kit (GE Healthcare) were used. For analysis, the molecular weight of the standards was plotted against their respective elution volume and the slope of the fitted curve was determined. For Stokes radius analysis (Rs), Stokes

radius of the standards was plotted against their respective elution volume and the slope of the fitted curve was determined (Erickson 2009). Thereby, with the elution volume of the protein, molecular weight and Stokes radius were determined using the slope of the fitted curve for standards.

3.2.4. Negative-stain Electron Microscopy

Protein samples of Rs, CmNP, CmN^{BPB} (Walker B mutant) (0.1 mg.ml⁻¹) were prepared in EM buffer (20 mM Tris-HCl, 50 mM NaCl and 10 mM MgCl₂; pH8). Samples prepared with EM buffer in the presence of 1mM ATP and/or 1mM RuBP, was incubated at room temperature for 10 min prior to negative staining. Protein samples were adsorbed to glow discharged carbon-coated copper grids for 30 seconds. Excess liquid was removed away with filter paper, negatively stained with 2% (w/v) uranyl acetate and air dried after removal of the excess staining solution. Micrographs were recorded in a FEI T12 transmission electron microscope equipped with a 4K CCD camera (FEI) at x 50000 magnification and 50-100 kV.

3.2.5. Growth of *Cyanidioschyzon merolae*

The strain *Cyanidioschyzon merolae* 10D (NIES-1332), was purchased from National Institute of Environmental Studies (Tsukuba, Japan). The cells were grown in modified Allen's media (Minoda et al. 2004). The media was prepared by dissolving the ingredients listed under Allen's Modified medium in MilliQ water and adjusting the pH to 2.5 by sulphuric acid (H₂SO₄). Before autoclaving the medium, 100 µl of 100 times diluted trace metal elements solution (Allen's medium) was added. Finally, Iron chloride (FeCl₃) was added to the autoclaved medium. 1 ml of the subcultured *C. merolae* 10D cells were inoculated for 1 l of the medium. The culture was grown in the presence of white light (24 hr) at 45 °C in 2 l culture flasks. The culture was continuously stirred using magnetic stirrer at a moderate speed of 200 rpm to avoid sedimentation or clumps and continuously aerated with an aquarium pump. OD₆₀₀ was measured to check the growth rate. The cells were harvested when OD₆₀₀ = 0.9-1 was reached and culture was reinoculated in a fresh medium.

4.CHARACTERIZATION OF A RED ALGAL RUBISCO ACTIVATION SYSTEM

The apparent absence of a Rubisco activase encoding gene in organisms containing red-type Rubisco initially led to the assumption that the requirement of the enzyme to be remodelled by motor proteins to release inhibitors was restricted to organisms of the green plastid lineage, such as green algae and higher plants (Pearce 2006). This view changed when the gene product of *cbbX*, encoded directly downstream of the Rubisco genes in the photosynthetic bacterium *Rhodobacter sphaeroides* was identified as the activase of red-type Rubisco (Mueller-Cajar et al. 2011). It had been known for a long time that *cbbX* homologues were also encoded in an identical position in the chloroplast genomes of red lineage organisms (Meijer et al. 1991, Kusian et al. 1992, Reith and Munholland 1995, Gibson and Tabita 1997). In addition a *cbbX* homolog was always detected in the nuclear genome whenever such data was available (Maier et al. 2000, Matsuzaki et al. 2004, Fujita et al. 2008). Consequently, the identity of at least the plastid encoded homologue as the Rubisco activase of red algal Rubisco appeared certain at the outset of this project (Mueller-Cajar et al. 2013), and in this chapter, we set out to formally prove this hypothesis.

The amino acid sequence alignment of selected prokaryotic and plastid encoded CbbX and nuclear encoded CbbX (excluding putative chloroplast transit peptide) indicated that residues that have been demonstrated to be functionally important in the bacterial activase from *R. sphaeroides* (RsRca) are in general highly conserved (Figure 3.1).

Phylogenetic analyses have previously shown that *cbbX* sequences can be separated into three clusters: cluster1 with prokaryotic and cyanobacterial, cluster2 with plastid encoded and cluster3 with nuclear (or nucleomorph) encoded (Figure 3.2) (Fujita et al. 2008, Starkenburg et al. 2014, Hovde et al. 2015). All organisms, prokaryotes and eukaryotes in all three CbbX clusters encode a red-type Rubisco with the exception of the α -cyanobacteria. It is believed in red algae, some plastid encoding genes including *rbcL-rbcS-cbbX* operon originated by horizontal gene transfer from proteobacteria (Delwiche and Palmer 1996) while most other genes are derived from cyanobacteria during the primary endosymbiotic event. It has been suggested that after lateral gene transfer to plastid, a copy of *cbbX* could have been integrated into the nucleus (Maier et al. 2000, Fujita et al. 2008) or alternatively the

nuclear genome acquired a *cbbX* gene in a separate lateral gene transfer event (Hovde et al. 2015). The phylogenetic relationship of plastid encoded *cbbX* genes are closer to proteobacterial sequences (~70% amino acid identity) than to their nuclear counterpart (Hovde et al. 2015) while the nuclear genes have diverged more extensively (~50 % amino acid identity to the other groups). This indicates that because of the selective pressure to maintain the activity, plastid encoded *cbbX* are less diverged than its nuclear homolog. The out group of CbbX-like proteins with significant sequence similarity was reported with alternative functions (e.g. Sporulation factors in some bacteria and hypothetical protein with unknown function in mycobacteria) (Foulger and Errington 1991, Luthra et al. 2008).

In order to understand the significance of the distribution of CbbX genes observed in red lineage phytoplankton and its role in Rubisco regulation, we decided to produce and characterize the gene products of both nuclear and plastid encoded *cbbX* isoforms from the red algal model organism *Cyanidioschyzon merolae* (Matsuzaki et al. 2004).

R_sphaeroides

- 1 R_sphaeroides
S_meliloti
Bradyrhizobium
S_hamburgensis
S_merolae_P
P_tricornutum_P
P_pseudonana_P
E_siliculosus_P
N_gaditana_P
C_merolae_N
T_pseudonana_N
P_tricornutum_N
E_siliculosus_N
N_gaditana_N
- 2 R_sphaeroides
S_meliloti
Bradyrhizobium
S_hamburgensis
S_merolae_P
P_tricornutum_P
P_pseudonana_P
E_siliculosus_P
N_gaditana_P
C_merolae_N
T_pseudonana_N
P_tricornutum_N
E_siliculosus_N
N_gaditana_N
- 3 R_sphaeroides
S_meliloti
Bradyrhizobium
S_hamburgensis
S_merolae_P
P_tricornutum_P
P_pseudonana_P
E_siliculosus_P
N_gaditana_P
C_merolae_N
T_pseudonana_N
P_tricornutum_N
E_siliculosus_N
N_gaditana_N

R_sphaeroides

- 1 R_sphaeroides
S_meliloti
Bradyrhizobium
S_hamburgensis
Syn_8102
C_merolae_P
P_tricornutum_P
T_pseudonana_P
E_siliculosus_P
N_gaditana_P
C_merolae_N
T_pseudonana_N
P_tricornutum_N
E_siliculosus_N
N_gaditana_N
- 2 R_sphaeroides
S_meliloti
Bradyrhizobium
S_hamburgensis
Syn_8102
C_merolae_P
P_tricornutum_P
T_pseudonana_P
E_siliculosus_P
N_gaditana_P
C_merolae_N
T_pseudonana_N
P_tricornutum_N
E_siliculosus_N
N_gaditana_N
- 3 R_sphaeroides
S_meliloti
Bradyrhizobium
S_hamburgensis
Syn_8102
C_merolae_P
P_tricornutum_P
T_pseudonana_P
E_siliculosus_P
N_gaditana_P
C_merolae_N
T_pseudonana_N
P_tricornutum_N
E_siliculosus_N
N_gaditana_N

Walker A

R_sphaeroides

- 1 R_sphaeroides
S_meliloti
Bradyrhizobium
S_hamburgensis
Syn_8102
C_merolae_P
P_tricornutum_P
T_pseudonana_P
E_siliculosus_P
N_gaditana_P
C_merolae_N
T_pseudonana_N
P_tricornutum_N
E_siliculosus_N
N_gaditana_N
- 2 R_sphaeroides
S_meliloti
Bradyrhizobium
S_hamburgensis
Syn_8102
C_merolae_P
P_tricornutum_P
T_pseudonana_P
E_siliculosus_P
N_gaditana_P
C_merolae_N
T_pseudonana_N
P_tricornutum_N
E_siliculosus_N
N_gaditana_N
- 3 R_sphaeroides
S_meliloti
Bradyrhizobium
S_hamburgensis
Syn_8102
C_merolae_P
P_tricornutum_P
T_pseudonana_P
E_siliculosus_P
N_gaditana_P
C_merolae_N
T_pseudonana_N
P_tricornutum_N
E_siliculosus_N
N_gaditana_N

Figure 3.1| Sequence alignment of representative red-type Rubisco activase sequences.

Amino acid sequences of CbbX proteins from five prokaryotes and five eukaryotes (nuclear and plastid-encoded) were aligned using Multalin (Corpet 1988). Functional motifs and key residues as determined previously (Mueller-Cajar et al. 2011) are indicated using coloured bars and triangles. Secondary structure elements of RsRca (PDB: 3SYL) are shown above the sequences. The poorly conserved N-terminal sequence of nuclear isoforms was removed based on sequence alignment to RsRca (and thus will not precisely correspond to the chloroplast transit peptide). Uniprot accession codes of the CbbX sequences are: *Rhodobacter sphaeroides* (P95648), *Bradyrhizobium* sp. strain ORS278 (A4YNR3), *Nitrobacter hamburgensis* (Q1QGD2), *Synechococcus* sp. strain WH8102 (Q7TTT9), *Sinorhizobium meliloti* (F7XIL3), *Cyanidioschyzon merolae* (nuclear: MIUXG8, residues 95-401; plastid: O20225), *Phaeodactylum tricornutum* (nuclear: B7FPB9, residues 198-500; plastid: A0T0M5), *Thalassiosira pseudonana* (nuclear: B8C163, residues 101-411; plastid: A0T0U6), *Ectocarpus siliculosus* (nuclear: D8LPQ0, residues 111-424; plastid: D1J7C8), *Nannochloropsis gaditana* (nuclear: W7TPW8, residues 111-418; plastid: K9ZXG9). The figure was prepared using Esript (Gouet et al. 1999).

Figure 3.2| Phylogenetic tree of selected CbbX protein sequences.

The sequence alignment shown in Figure 3.1 was used to produce a phylogenetic tree by Neighbor-Joining in the MEGA6 software suite (Tamura et al. 2013) using the p-distance model and pairwise gap deletion. Bootstrap values are the percentage from 1000 replicates where a given node was present.

4.1. Production and purification of recombinantly expressed red algal CbbX isoforms

The nuclear genome encoded CbbX (Uniprot: M1UXG8) is coded by the *cbbX* gene (Gene symbol:CYME_CMT172C; geneID:16997649) of *Cyanidioschyzon merolae*. This nuclear gene was found to have two transcription initiation sites within its sequence. ChloroP indicates a chloroplast target peptide at the start of the sequence (Fujita et al. 2008). Since it is difficult to predict correct transit peptides for proteins targeted to red-type plastids we decided to produce a protein encoding residues 94-401 of the nuclear encoded isoform (CmN) based on homology to the functional RsRca and the plastid encoded isoform (Figure 3.1). Nuclear encoded wild-type CbbX (CmN) protein was heterologously expressed in *E.coli* BL21 (DE3) as an N-terminal fusion with a hexa-histidine tagged Ubiquitin (His₆Ub) (Catanzariti et al. 2004, Baker et al. 2005) for improved solubility and facile purification of the proteins. Upon testing expression of the constructs at different temperatures and conditions as explained in methods, we found that CmN was most soluble when expressed at 23 °C in cells overexpressing the chaperonin proteins, GroEL and GroES using the plasmid pBADESL (Ewalt et al. 1997) (Data not shown). Following test expression, larger scale purification (1 or 2 l) was performed. A representative example of the purification of CmN is shown in Figure 3.3. CmN was captured by Immobilized metal ion affinity chromatography (IMAC) and subsequently treated with the ubiquitin-specific protease USP2c, for precise cleavage of His₆-Ubiquitin from the N-terminus of the expressed CmN.

Figure 3.3| Representative purification of CmN isoform.

(A) Purification of CmN using a linear gradient (0-1 M NaCl) by anion exchange chromatography. Successive purification by IMAC (Ni-NTA), followed by USP2c mediated cleavage of N-terminal tag protein was purified using anion exchange chromatography (AIEC). Anion exchange chromatogram representing the eluted proteins from MonoQ 10/100 GL column equilibrated with purification buffer A and eluted with purification buffer B (Materials Table 2.4) (left) are analysed using 12.5 % SDS-PAGE (right). The molecular weight marker bands and location of respective activase band are indicated. (M: Molecular Weight Marker, L: Lysate, S: Soluble, Ni-NTA: Fractions after immobilized metal affinity chromatography (IMAC), U: Unbound, W: Wash, C: Elute after USP2c mediated cleavage of Ubiquitin. The numbers indicate the SDS samples from respective fractions of AIEC) **(B)** Collected CmN after AIEC was further purified by size-exclusion chromatography using a Superdex 200 16/600 column equilibrated with purification buffer A (Materials Table 2.4). Size exclusion chromatogram of CmN showing purified activase with single peak (left) and 12.5% SDS-PAGE gel for the respective fractions along with the molecular marker. (M: Marker, numbers represent the SDS samples from the respective fractions from size exclusion column).

Cleaved protein was further purified using anion exchange chromatography. CmN eluted at around 300 mM NaCl (Figure 3.3A). The positively charged Ubiquitin did not bind to this column and GroEL/ES was effectively separated from the activase isoform as assessed by SDS-PAGE (Figure 3.3A). Eluted fractions containing pure Rca were then exposed to a size exclusion chromatography step using a Superdex 200 16/600 column (Figure 3.3B). The yield of protein from 1 l culture of *E.coli* BL21 (DE3) grown in LB media after induction with 0.5 mM IPTG and 0.02 w/v % L-arabinose at 23 °C grown overnight was measured to be ~10-15 mg of pure protein.

The plastid encoded *cbbX* gene (Gene symbol: cfxQ; geneID:AB002583) of *Cyanidioschyzon merolae* encoding a CbbX homologue (UniProt: O22025), was synthesized by Genscript (USA). Like CmN, CmP was heterologously expressed in *E.coli* BL21 (DE3) as an N-terminal fusion with hexa-histidine tagged Ubiquitin (His₆Ub). As opposed to nuclear encoded activase, plastid encoded activase is a very basic protein with an isoelectric point (pI) of 9.4 (calculated using the ExPASy-Protparam computational tool (Gasteiger et al. 2005)). Significant problems were encountered when attempting to obtain soluble protein. As it was not soluble under standard conditions even when over expressing the GroEL/ES chaperonin extensive test expressions with different temperatures, buffers with a range of NaCl salt concentrations and different pH were tried to improve the solubility,.

Finally, CmP was found to be soluble in a pH 10 buffer containing high salt concentrations of 250 mM NaCl (Data not shown). CmP was purified using IMAC (Ni-NTA) followed by size exclusion chromatography as described (Methods Section 2.2.2.10). Following His₆-Ub cleavage, the activase was directly applied to a SuperDex 200 16/600 column (GE) and the fractions were analysed using SDS-PAGE (Figure 3.4). The anion exchange chromatography with purification buffer A_CmP (Table 2.4) was used (optionally) to remove some impurities since CmP did not bind to the column it was collected in the flow through. The yield of protein from 1 l culture of *E.coli* BL21 grown in LB media after induction with 0.5 mM IPTG for 16 hrs at 23 °C was measured to be ~10-15 mg of pure protein.

Figure 3.4| Representative purification of CmP isoform.

Purification of CmP using gel filtration chromatography. Successive purification by IMAC (Ni-NTA), followed by USP2 mediated cleavage of the N-terminal fusion protein was purified using gel filtration Superdex 200 16/600 column equilibrated with 300 mM NaCl, 50 mM Tris-HCl pH 10 (top). 12.5% SDS-PAGE analysis of the CmP purification (bottom). The molecular weight marker bands and location of respective protein bands are indicated. (L: Lysate, S: Soluble, Ni-NTA: concentrated fraction after IMAC, AC: IMAC fraction cleaved after cleavage of the His₆Ub moiety, SEC: size exclusion chromatography and IEX (FT): flow through from anion exchange column due to positive charge of CmP.

4.2. CmN and CmP interact to form a hetero-oligomeric CmNP Complex

The observed recalcitrance of CmP was unexpected and the purified protein was stable only at pH 10 and high salt concentration (>300 mM NaCl). This behaviour is unusual because the *in vivo* physiological pH of the chloroplast is around 7-8, but when tested at this pH range the activase isoform precipitates. This was consistent with the idea that to be functional in the chloroplast it would have to be associated with another protein or possibly CmN, its homologue encoded by the nuclear genome. We speculated that CmN could be the binding partner for the plastid encoded isoform that prevents its aggregation in the chloroplast. For import of nuclear encoded proteins into chloroplast, it should contain a plastid targeting sequence or a transit peptide as N-terminal extension and followed by the functional protein. Fujita et.al previously suggested that sequence in *cbbX* homologue gene of the nuclear genome contains a plastid-targeting signal as predicted by TargetP 1.1 CBS server (<http://www.cbs.dtu.dk/services/TargetP/>) (Emanuelsson et al. 2007) for import into chloroplast of the red algae (Fujita et al. 2008).

As a consequence of these considerations, we decided to test whether the nuclear encoded isoform CmN forms a hetero-oligomeric complex with the plastid encoded isoform CmP. To assess formation of a heterocomplex, we mixed equimolar amounts of pure CmN and CmP proteins at pH 8 and loaded the mixture onto an analytical gel filtration column. We observed a shift in the elution profile compared to the separately applied isoforms at the same concentration. SDS-PAGE analysis for the respective peaks confirmed the oligomeric transition and indicated the formation of a heterocomplex containing equimolar ratio of both isoforms. Moreover, the complex was stable at physiological pH 8 (Figure 3.5A). This result was the first indication that nuclear encoded activase forms a complex with the plastid encoded isoform, which otherwise would not be stable at physiological conditions of the chloroplast.

In vivo CmP could be associated with the chloroplast specific molecular chaperones (such as homologs of HSP 100, 90, 70, 60 and 40 chaperone family) to prevent aggregation in the absence of the partner (Sjögren et al. 2004, Lee et al. 2007, Olinares et al. 2011). Based on our experimental observations with GroEL/ES co-purification there is probability that CmP could be associated with the chloroplast homolog of GroEL/ES in the chloroplast. The chloroplast chaperone Cpn60 of the HSP60 (Nishio et al. 1999) family is evolutionary homologues to GroEL (Martel et al. 1990) and the co-chaperone of Cpn60 (Cpn10, 21, 23) is homologous to GroES (Martel et al. 1990). Also owing to the higher physiological temperature the organism could constitutively express high amount of chaperones which would prevent CmP from aggregation. So CmP could be maintained by HSP family of chaperones or by an unidentified protein specific to the *C. merolae* with a chaperonin function. Another mechanism could involve anterograde (nucleus to chloroplast) and/or retrograde (chloroplast to nucleus) signalling to regulate the CmP expression (Nott et al. 2006, Singh et al. 2015). Presumably CmN with transit signal would be imported to chloroplast and which would then trigger a signalling for the expression of CmP. Thereby CmP can readily form the hetero complex with its partner and as well the aggregation would be prevented.

Since CmN and CmP appeared to form a stable complex *in vitro*, we decided to co-purify CmN and CmP using two different approaches. We either mixed the cell pellets expressing the isoforms together prior to purification or co-expressed both isoforms in the same vector system (using a pETDuet-1) to obtain the hetero-oligomeric complex.

Figure 3.5| CmN and CmP interact to form a hetero-oligomeric CmNP complex.

(A) Size exclusion chromatography. Elution profiles for the homo and hetero-oligomeric complexes in Apo form measured at 280 nm. Independent run was loaded with 20 μ g of pure CmN and CmP isoforms and 20 μ g reconstituted complex prepared by mixing 10 μ g of each isoform was loaded on to Superdex 200 PC3.2/30 column. CmN and reconstituted complex were eluted with purification buffer A while CmP was eluted with purification buffer A_CmP (Materials Table 2.4). Corresponding fractions under the peak are shown in the 12.5% SDS-PAGE gel (right). (B,C) Purification of co-purified CmNP complex by mixing CmN and CmP pellets. Purification of CmNP using linear gradient of NaCl by anion exchange chromatography (C) Collected CmNP after AIEC was further purified by size exclusion chromatography (Superdex 16/600) equilibrated with purification buffer A (Materials Table 2.4).

4.3. Co-expression and Co-purification of the hetero-oligomeric complex activase (CmNP)

The two *cbbX* isoform inserts, one encoding CmP with hexa-histidine tagged Ubiquitin (His₆Ub) and other encoding CmN were cloned into a pETDuet vector so that they could be co-expressed. Expression of pETDuetCmNP allows the formation of the hetero-oligomeric activase complex (CmNP) *in vivo* (Methods 2.2.2.11). Test expression results showed solubility of heterocomplex was enhanced at 23 °C (Data not shown). CmNP was purified using a similar procedure to that described for CmN. In stark contrast to the individual isoforms, the heterocomplex (CmNP) eluted at around 190 mM NaCl (compared to 300 mM NaCl for CmN) in anion exchange chromatography (Figure 3.6A). It was confirmed from SDS-PAGE gels that the eluted protein fractions contained bands for both isoforms in a 1:1 ratio (Figure 3.6A). The CmNP was further purified by size exclusion column (Figure 3.6B). As an additional evidence for the heterocomplex, densitometric analysis of the co-expressed CmNP complex was consistent with a 1:1 stoichiometry (Figure 3.6C). Fractions containing pure CmNP were concentrated and stored at -80 °C.

In addition to the co-expression method, CmNP complex was also made using a co-purification method, where cells are expressing CmN and CmP separately were mixed before lysis. The CmN and CmP plasmids in *E.coli* BL21 (DE3) cells over expressing chaperonins GroEL and GroES was grown in 1 l LB medium independently induced by IPTG and C-Arabinose at 23 °C overnight. Harvested cells of isoforms were mixed in equal ratio prior to lysis step as explained in Methods section 2.2.2.12 (Figure 3.5C, D co-purification). This mixing of lysates enabled reconstitution of a nuclear isoform with its counterpart to form the CmNP complex during the opening of the cells. This was a crucial step, otherwise CmP would precipitate during purification and no CmNP heterocomplex would be formed. Further purification of the reconstituted complex was very similar to that explained for in the above section (co-expression of CmNP). The formation of the heterocomplex was verified by SDS-PAGE analysis for CmN and CmP in 1:1 ratio and the excess CmN and CmP which did not form a complex with its counterpart eluted in different fractions during chromatographic separation. The reconstituted heterocomplex (CmNP) from co-purification method also eluted at similar salt

gradient as compared to CmNP from the co-expressed method, around 190 mM NaCl in anion exchange chromatography (Figure 3.5C and 1.6A). The average yield of protein from a 1 l culture of *E.coli* BL21 grown in LB media after induction with IPTG at 23 °C was found to be ~10-15 mg of pure protein.

Figure 3.6| Representative purification of CmNP hetero complex obtained by co-expression.

(A) Purification of CmNP using the linear gradient of NaCl on anion exchange chromatography. Successive purification by IMAC (Ni-NTA), followed by USP2 mediated cleavage of N- terminal tag protein was purified using anion exchange chromatography (AIEC). Anion exchange chromatogram representing the eluted proteins (left) are analysed using 12.5 % SDS-PAGE (right). The molecular weight marker bands and location of respective activase band are indicated. (M: Molecular Weight Marker, L: Lysate, S: Soluble, Ni-NTA: Fractions after IMAC, U: Unbound, W: Wash, Cut: Elute after USP2 mediated cleavage of Ubiquitin. The numbers indicate the SDS samples from respective fractions of AIEC. **(B)** Collected CmNP after AIEC was further purified by size exclusion chromatography. Size exclusion chromatogram of CmNP showing single peak purified activase (left) and 12.5% SDS-PAGE gel for the respective fractions along with the molecular marker. (M: Marker, numbers represent the SDS samples from the respective fractions from size exclusion column). **(C)** Densitometric analysis of CmNP. Varying concentrations of CmNP were separated using SDS-PAGE and stained using Coomassie Blue. Densitometric analysis was performed using Quantity One.

4.4. Rubisco activase from *Cyanidioschyzon merolae* function as a hetero-oligomeric complex

4.4.1. Activation of bacterial Rubisco

Rhodobacter sphaeroides is a photosynthetic α -proteobacterium. It has a red-type form IC Rubisco and its activation system is the only one characterized for red-type Rubiscos (Mueller-Cajar et al. 2011). Red algal Rubisco (red-type Form ID) together with the operon *rbcL-rbcS-cbbX* is believed to originate from α -proteobacteria (red-type Form IC) as a product of horizontal gene transfer (Ohta et al. 1997, Maier et al. 2000). Bacterial Form IC Rubisco is easily produced recombinantly in *Escherichia coli*, whereas so far no eukaryotic Rubiscos have been successfully expressed in the organism. Since their Rubiscos are closely related (their large subunits share a primary sequence identity of 74 %), we initially wanted to establish whether the Rubisco from the proteobacterium could be activated by the activase from red algae.

Rhodobacter sphaeroides Form IC Rubisco was recombinantly produced and purified as described previously (Mueller-Cajar et al. 2011). The functional holoenzyme (Enzyme-CO₂-Mg²⁺, ECM) was prepared by incubating the apo-Rubisco in a buffer containing 40 mM NaHCO₃ and 10 mM MgCl₂ as described in Methods

2.2.3.1. In the holoenzyme, lysine-201 at the active site is carbamylated by a non-substrate CO₂, and the resulting carbamate is involved in coordinating the Mg²⁺ ion (Figure 1.5). The RuBP- inhibited complex (ER) was formed by incubating apo-Rubisco with EDTA to chelate free Mg²⁺ ions, followed by addition of 1 mM RuBP (Methods 2.2.3.1). The sugar phosphate then binds to the non-activated complex, which forms a stably inhibited complex (Jordan and Chollet 1983, Mueller-Cajar et al. 2011). The spectrophotometric Rubisco activity assay was then used to assay the activity of the complexes in the presence and absence of activase proteins (Kubien et al. 2011). In this assay, the rate of 3PG production is directly coupled to the oxidation of NADH, allowing continuous monitoring of Rubisco activity through the changes in absorbance at A_{340nm}. The carboxylation activity of the Rubiscos can thus be derived. Importantly, ATP is part of the assay (but its consumption does not influence the readout) and constantly replenished due to the presence of an ATP regeneration system, thus providing chemical energy for activase function if required. The ATP is regenerated when the phosphocreatine in the reaction mix converts the ADP to ATP by donating its phosphate group. This results in the formation of creatine and the creatine kinase (which is the part of the coupling enzyme) in the assay converts the formed creatine to phosphocreatine by utilizing the excess ATP (Lilley and Walker 1974).

The fully activated RsECM fixed CO₂ at a rate of 3.4 s⁻¹ (from Figure 3.7A data). Although we observed some spontaneous activation for the RuBP inhibited complex (RsER), the rate of 3PG production was negligible (Figure 3.7A). These results are consistent with previous studies of the native and recombinant *R. sphaeroides* Form IC Rubisco (Gibson and Tabita 1979, Mueller-Cajar et al. 2011). On testing the activation of the inhibited Rubisco complex by the red algal activases, the nuclear (CmN) and plastid (CmP) encoded isoforms of the *C. merolae* activase were unable to activate the inhibited bacterial Rubisco complex in isolation (Figure 3.7A). In contrast, the red algal heterocomplex (CmNP) efficiently activated RsER (Figure 3.7A), implicating CmNP as the functional Rubisco activase. We then combined pure CmN and CmP at a 1:1 ratio to form a reconstituted heterocomplex (CmN+CmP). The reconstituted complex was also functional in activating RsER (Figure 3.7A).

To quantitatively compare RsRca and CmNP function we titrated down activase and Rubisco concentrations to become limiting. We found that when the activase concentrations were in the range of 0.5-1 μ M, a strong dependence on activase concentration could be observed. Under these conditions, activation rate by CmNP was ~50-60% compared to RsRca (Figure 3.7C). The reduced rate in Rubisco activation could be due to a lower affinity for bacterial Rubisco and/or differences in the biochemical properties of the algal activase as compared to the bacterial one. Importantly, our results show that CmNP can recognize and function on Rubisco across a significant evolutionary distance which indicates a highly conserved nature of the protein-protein interactions involved in binding of the Rubisco.

The transition state analogue CABP is a competitive inhibitor of Rubisco which forms a slow, tight binding inhibited complex with carbamylated Rubisco (ECM) (Pierce et al. 1980). Higher plants, red algae and cyanobacterial Rubiscos showed the negligible exchange of bound CABP in their active sites (Pearce 2006). Spinach plant activase (Rca) cannot activate CABP inhibited Rubisco (Robinson and Portis 1988). So we wanted to test whether the red activase of bacterial and algal are capable of remodelling a tightly inhibited Rubisco complex (ECM-C) by removing CABP. Interestingly, both RsRca and CmNP readily activated the completely inhibited bacterial CABP Rubisco complex (RsECM-C) (Figure 3.7B). A tempting explanation for this result is that red activases can generate more force than green activases during Rubisco remodelling. Alternatively, the red-type ECMC complex may not be as stable as the higher-plant equivalent. At limiting activase concentration the rate of activation by CmNP was ~60% compared to RsRca (Figure 3.7C), which could be due to a lower affinity for bacterial Rubisco. Predictably individual isoforms were non-functional in activating the RsECM-C complex (Figure 3.7B), and the reconstituted CmN+P complex was functional at activating CABP complex (Figure 3.7B).

Figure 3.7| CmNP complex functions as a potential Rubisco activase and inhibited bacterial Rubisco stimulates its ATPase activity.

CmNP complex activates *R. sphaeroides* inhibited Rubiscos. Assays were performed with activated (RsECM) and inactivated Rubiscos (0.5 μM active sites and 10 mM NaHCO_3), RsER (A) and RsECM-C (B) in the absence and presence of bacterial and red algal activases (5 μM protomer). (C) The rate of activation of RsER and RsECM-C (0.2 μM active sites and 30 mM NaHCO_3) by CmNP and RsRca (0.5 and 1 μM protomer) was measured by Rubisco activity assays. (D) ATP hydrolysis rate comparison of CmN, CmP and CmNP with RsRca as a control. ATPase activity rate with 5 μM protomer was measured in the absence (Apo state) and in the presence of RuBP (1 mM) together with inactivated (RsER and RsECM-C) Rubiscos (3 μM active sites). (E) Stimulation of ATPase activity for RsRca and CmNP with different concentrations of RsER and RsECM-C. ATPase rate was measured with the concentration of inactivated (RsER and RsECM-C) Rubisco active sites as indicated in the figure with respective activase (5 μM protomer). (F) ATPase assay of reconstituted CmNP complexes formed by mixing of pure CmN and CmP in the shown ratios but keeping the total protein concentration constant (5 μM protomer). ATPase measurements were made after 30 min incubation of the isoforms to form a complex at 45 °C. All ATPase and Rubisco assays were measured in the presence of 1 mM RuBP and ATP at 25 °C except (F) was measured at 45 °C. Error bars shown are the standard deviation of three independent experiments.

4.4.2. The regulation of ATPase differs between RsRca and CmNP

The energy required to remodel inhibited Rubisco complexes by all Rubisco activase is derived by ATP hydrolysis, which mediates conformational changes in the activase. The ATPase activity of the characterized red-type activase RsRca is under intricate regulation by at least two different factors (RuBP and Rubisco). ATP hydrolysis is strongly stimulated by Rubisco's substrate RuBP (with the apo form being inactive), an allosteric regulator that binds to conserved residues of its α -helical subdomain. Further, significant stimulation was observed when exposing RsRca to its substrate Rubisco in the presence of RuBP (Mueller-Cajar et al. 2011). Consistent with the published data, the ATPase rate for RsRca in the presence of 1 mM RuBP was 10 ATP min^{-1} (activities are given per activase protomer) and in the presence of inhibited Rubiscos (3 μM active site) it was stimulated >3 fold to 38 and 35 ATP min^{-1} for ER and ECM-C respectively (Figure 3.7D). Thus as observed for many other AAA+ proteins, the ATP hydrolysis rates of red-type activases are stimulated by their substrates, which will presumably translate into an increased rate of Rubisco remodelling. In comparison with RsRca, ATPase assay with pure CmN and CmP, with the addition of RuBP and inhibited Rubiscos showed no ATPase activity at 25 °C (Figure 3.7D), suggesting both isoforms were ATPase inactive, which is consistent with the Rubisco activation results (Figure 3.7A, B). Interestingly, CmNP had a small constitutive ATPase activity even in the absence of RuBP, in contrast to RsRca which

is inactive in the Apo form (Figure 3.7D). We also noted a similar ATPase activity for reconstituted CmN+P at all measured conditions (Figure 3.7D). ATPase activity of CmNP was weakly stimulated to $\sim 2 \text{ ATP min}^{-1}$ in the presence of 1 mM RuBP and to about 4 ATP min^{-1} in the presence of inhibited Rubiscos ($3 \mu\text{M}$ active sites) (Figure 3.7D). We increased the Rubisco concentrations and even at $9 \mu\text{M}$ we did not observe any significant change in the stimulation of activity for CmNP contrast to RsRca. The ATPase stimulation by RsER ($9 \mu\text{M}$) and RsECM-C ($9 \mu\text{M}$) for RsRca was sevenfold and fourfold respectively, whereas for CmNP it was four-fold and two-fold respectively (Figure 3.7E).

It is striking that CmNP has robust Rubisco remodelling activity although its stimulated ATPase activity is only a fraction of that of RsRca. With our current data it is difficult to give a plausible explanation for the low ATPase phenotype of the CmNP. More information about kinetics of activation had to be generated to address this question in future. However we suspect that similar amount of ATP would be required by both the activases to completely convert all ER to ECM. Activase efficiency measurements will need to be performed to address this question. However our data suggests the high ATPase activity observed for RsRca is not essential to achieve Rubisco remodelling in red-type activases.

Although the two algal isoforms have highly conserved AAA+ modules, that by comparison with RsRca were predicted to be functional, they are ATPase inactive when separately measured. The absence of ATPase function of isomers justifies its non-functionality. However, heterocomplex formation by the two non-functional isoforms yields ATPase activity and Rubisco activation function (Figure 3.7). The ATP hydrolysis results support our idea that the hetero-oligomer is the biologically relevant form and homomers may only exist transiently *in vivo*.

Co-expression of CmN and CmP resulted in the isolation of the active 1:1 complex (Figure 3.6), and reconstitution of the functional CmNP by combining pure CmN and CmP was also successful (Figure 3.5A). Therefore we wanted to ask whether the 1:1 stoichiometry was obligatory, or whether other stoichiometries also resulted in highly functional algal activase.

To address this question we combined purified CmN and CmP in different ratios and measured the ATPase activity of the respective complexes. This was performed at 45 °C and in the presence of the allosteric regulator RuBP. As expected and consistent with the complex formation from analytical gel filtration profile, these mixed complexes were functional. However, the activity profile was not symmetrical (Figure 3.7F). The change in activity of the isoform complexes with different ratios of the isoforms revealed that increasing CmN concentration was tolerated, but in contrast excess CmP poisoned the activity. Maximum ATPase activity was displayed by the complex when both the CmN and CmP isoforms were mixed together in 1:1 stoichiometric ratio. This indicates that the two ATPase inactive isoforms (CmN and CmP) form a functional heterocomplex activase and the formed complex with equal ratios of its subunits had maximum activity (Figure 3.7F). It is not yet clear if the low activity of the other complexes is due to either lower concentration of the equimolar heterocomplex or formation of defective heterocomplexes with unbalanced subunit composition.

These data suggest that *in vivo* it may be necessary to carefully regulate the relative quantities of both subunits to ensure the 1:1 hetero-oligomer prevails.

3.4.3. The mechanism of activation appears conserved between RsRca and CmNP

The activation mechanism of bacterial red-type Rubisco by its activase is hypothesized to involve transient threading (pulling) of the C-terminal tail of the large subunit through its central pore, triggering the release of the inhibitors from the active site (Mueller-Cajar et al. 2011). This model was inspired by the mechanism of well-studied AAA+ family proteins like ClpX which are known to function by threading (pulling) their substrates through their conserved central pore for aiding in proteolysis of proteins or co-factor insertion to a complex (Kardon et al. 2015). For substrate remodelling by ClpX, substrates have to be tagged by a signal peptide known as the ssrA tag (Gottesman et al. 1998, Flynn et al. 2001, Martin et al. 2008c). Similarly, the 12 amino acid extension at the end of C-terminus of the red-type Rubisco large subunit may act as a recognition signal for red activase. However, its sequence is poorly conserved. Alternatively, the Rubisco may dock via other recognition sites and the length of the C-tail may be the sole determinant. Given the dissimilarity of the C-

tail sequence between red algal and proteobacterium, we wanted to test whether the algal activase also functions via a C-tail dependent mechanism. For this, Rs Δ 2 and Rs Δ 4 (2 and 4 amino acids removed from the C-terminus respectively) (Figure 3.8A) were purified using constructs as described (Mueller-Cajar et al. 2011) to evaluate if CmNP activase can remodel the inhibited Rubisco complex with shortened C termini of large subunits. Further, carbamylated active form (ECM) and RuBP inhibited form (ER) of both the constructs were prepared to verify enzyme functionality.

The carboxylation activity of ECM (3.2 sec⁻¹) and ER (negligible) for the deletion constructs of Rs Rubisco are in fact similar to their wild-type counterpart measured at the same conditions by Rubisco activity assay (from Figure 3.8B, C data). As observed by Mueller-Cajar et.al, the C terminal deletions were found to have no effect on ECM catalysed carboxylation reaction of RuBP or formation of inhibited complex(ER). With the addition of RsRca, the relative rate of Rs Δ 2ER activation was 16% (Figure 3.8B, D), whereas no Rs Δ 4ER activation was observed (Figure 3.8C, D). This strongly suggests that the loss of only 4 amino acids from the C-terminus leads to failure to interact or to be recognised by RsRca. These results are in agreement with the earlier proposed bacterial activase model(Mueller-Cajar et al. 2011). Indeed, red algal CmNP activase merely remodelled Rs Δ 2ER at a rate of ~3% (Figure 3.8B, D) and was unable to activate Rs Δ 4ER in comparison to the rate of activation of RsER (Figure 3.8C, D).

As expected, individual isoforms of nuclear and plastid encoded activase did not remodel the deletion constructs and remained inactive (Figure 3.8B, C).

RsRca ATPase activity was not stimulated by Rs Δ 4 but very weakly by Rs Δ 2ER complexes (Figure 3.8E) (Mueller-Cajar et al. 2011). CmNP and likewise an equimolar mix of CmN and CmP isoforms showed almost no stimulation by C-terminally truncated RsER complexes (Figure 3.8E). The ATPase results of RsRca and CmNP were in agreement with the Rubisco activation data. The individual isoforms showed no ATPase activity in accord with Rubisco activation (Figure 3.8E). Our experimental data suggests that CmNP follows the same mechanistic model as RsRca and also uses the axial pore loop to transiently thread the extended C-tail of the red-type Form I Rubisco. In conclusion, we expect all of the CbbX class activases associated with red-type Rubiscos (from prokaryotic to eukaryotic) to have a

conserved mechanism of operation to target and remodel their substrates. To understand the activating mechanism in detail and the relative role of the subunits, further experiments and studies were conducted by mutating respective pore loop motifs and other essential motifs. These are discussed in the next chapter.

A

B

C

D

E

Figure 3.8| Mechanism of CmNP mediated substrate remodelling is conserved.

(A) Sequence alignment of the large subunit of *Rhodospirillum rubrum* (*R. sphaeroides*), *Cyanidioscyzoon merolae*, *Galdieria sulphuraria*, *Phaeodactylum tricornutum* showing the C-terminal extension and the arrows indicate the residues deleted in the RsRbcΔ2 and RsRbcΔ4 construct. Corresponding Uniprot accession number is provided adjacent to the sequence. CmNP activase can weakly activate RsΔ2 (B) but not RsΔ4 Rubisco (C). (D) The rate of activation of deletion constructs by the activase was compared with the WT Rubisco. Rubisco activity assays were performed with activated (RsECM Δ2/Δ4) and inactivated (RsER Δ2/Δ4) Rubiscos (0.5 μM active sites and 10 mM NaHCO₃) in the absence and presence of bacterial and red algal activases (5 μM protomer). (E) ATPase activity of CmNP was not stimulated by bacterial Δ2 and Δ4 ER. ATPase activity in the presence of RuBP inhibited (RsER Δ2/Δ4) Rubiscos (3 μM active sites) was determined in the presence of bacterial and red algal activases (5 μM protomer). All measurements were performed in the presence of 1 mM ATP and 1 mM RuBP at 25 °C. Error bars shown are the standard deviation of three independent experiments.

3.5. The oligomeric state of the algal activase

Structural analysis of RsRca using negative stain Electron Microscopy (EM) showed that apo enzyme forms largely heterogenous and polydisperse population oligomers, while in the presence of Mg-ATP the oligomers are converted into long fibril structures and finally in the presence of both Mg-ATP and RuBP the functional hexameric oligomers are discernible (Mueller-Cajar et al. 2011). To assess whether these oligomeric transitions are conserved in the algal activase, we performed a series of analytical gel filtration experiments, where the proteins were eluted using buffers containing Mg-ATP and the allosteric regulator RuBP.

In the presence of Mg-ATP alone, RsRca eluted in the void volume, implying that RsRca forms a large structure which correlate to the fibrillar formation (Figure 3.9B) seen using EM (Mueller-Cajar et al. 2011). The addition of ATP and RuBP to RsRca induced a shift in the gel filtration profile, which then eluted as a symmetrical peak at 1.2 ml (Figure 3.9C), suggesting the monodispersity of the protein.

Figure 3.9| Analysis of oligomeric state of *C. merolae* activases.

(A) Elution profiles for the subunits and hetero complex at Apo form measured at 280 nM. Independent run was loaded with 20 µg of protein on to Superdex 200 PC3.2/30 column. The corresponding gel with the collected fraction is displayed on the side. (B) The Oligomeric state of *C. merolae* activases in the presence of 0.2 mM Mg²⁺ATP. Gel filtration analysis of the RsRca variants and *C. merolae* activases in the presence of 0.2 mM ATP, measured at 280 nM. Independent run was loaded with 20 µg of protein on to Superdex 200 PC3.2/30 column. (C) Hetero-hexameric state of CmNP activase in the presence of 0.2 mM Mg²⁺ATP and 0.5 mM RuBP. Gel filtration analysis of the RsRca and *C. merolae* activases in the presence of both 0.2 mM ATP and 0.5 mM RuBP, measured at 280 nM. Independent run was loaded with 20 µg of protein on to Superdex 200 PC3.2/30 column. (D) Standard curve for the determination of Stokes radius (Rs) (left) and (E) molecular weight (right) of the standard proteins measured at 280 nm. Standard proteins used for to generate the standard curves were blue dextran, Thyroglobulin, Ferritin, Aldolase, Covalbumin and Ovalbumin (Erickson 2009). All activases and standard proteins were eluted with purification buffer A except CmP which was eluted in purification buffer A_CmP (Materials Table 2.4).

The molecular weight determined based on the calibration curve of the known standards (Figure 3.9E) was 380 kDa while the expected molecular weight of the hexamer is 208.2 kDa. This is certainly too large to represent a hexamer. If the unknown protein is non-globular (e.g. elongated), it could elute at a position almost twice the molecular weight of a globular protein (Erickson 2009). In principle, gel filtration analysis is not suitable for molecular weight determination of some non-globular proteins since gel filtration depends on their frictional coefficient or Stokes radius (Rs), largely for the elongated proteins (Siegel and Monty 1965). If we assume the hexameric protein is spherical or disk shaped, Rs is the radius of the sphere approximating the size of the protein. The Rs of the standard calibration proteins were determined experimentally from previous studies and this was plotted against its elution volume to obtain a standard curve (Figure 3.9D). Rs of the protein of interest can be found from its elution position on this standard curve. The Rs of RsRca eluted at 1.2 ml was determined to be 6.1 nm and thus a spherical protein would be expected to have a diameter of ~ 120 Å. The diameter of the RsRca ring complex by structural determination was ~ 140 Å (Mueller-Cajar et al. 2011), which is consistent with the estimation by analytical gel filtration. Therefore we conclude that gel filtration

analysis of RsRca in a suitable buffer can recapitulate the oligomeric transitions observed earlier with negative EM (Mueller-Cajar et al. 2011).

Next, the Stokes radius of red algal activase was determined at all conditions tested above and compared to RsRca. The elution profile showed a single peak with the small shoulder at 1.23 ml for CmNP (20 μ g) in apo form. Although there was a small shift in the peak at lower concentrations (5-10 μ g), indicating complex dissociation, CmNP exhibited a monodisperse oligomeric state at higher concentrations (Figure 3.9A and 3.10C). The corresponding Rs for the elution volume of Apo determined using the standard curve was 5.8 nm, a value intermediate to the RsRca hexamer (6.1 nm) and the value measured for the ring-shaped hexameric AAA+ protein AfQ2 (5.4 nm) (Tsai et al. 2015). Hence we conclude that CmNP already forms the hexameric oligomer of similar shape to other AAA+ ATPases in the absence of RuBP and nucleotide. SDS-PAGE analysis of the fractions of the collected peak for CmNP confirmed bands for both isoforms in the complex and in the previously established 1:1 stoichiometric ratio (Figure 3.9A). Subsequently, pure CmN and CmP were mixed in a 1:1 ratio (10 μ g each in 50 μ l) followed by incubation at 25 °C (60 min) prior to loading onto the gel filtration column to allow the formation of the complex. The heterocomplex formed by pure isoforms eluted at 1.27 ml with a minor shoulder (Figure 3.9A) corresponding to a Stokes radius of 5.4 nm, similar to the hexameric co-expressed CmNP complex. Further, SDS-PAGE analysis of the elution profile confirmed that the reconstituted hexameric complex consisted of an equal ratio of CmN and CmP similar to the co-purified heterocomplex (CmNP) (Figure 3.9A).

In striking contrast to the behaviour of RsRca, no detectable shift in the gel filtration profile was observed upon repeating the run in the presence of ATP (Figure 3.9B) or ATP+RuBP (Figure 3.9C). This suggests that the CmNP complex forms a hexamer in solution. Addition of ligands, ATP or RuBP did not result in the oligomeric transitions observed for the bacterial homolog.

In contrast to CmNP, CmN isoform eluted at 1.34 ml (for 20 μ g) and displayed a peak shift towards higher oligomerization when the protein concentration was increased (Figure 3.10B). The bands seen on the SDS-PAGE gel for the fractions eluted under the peak (CmN apo form) corresponds to CmN subunit (34.6 kDa) (Figure 3.9A). However, no detectable difference was noted upon repeating the run in the presence of ATP (Figure 3.9B) or ATP+RuBP (Figure 3.9C). This indicated that ATP or RuBP did not aid in further oligomerization.

At pH=8 condition, elution of CmP was problematic since the protein appeared to interact with the column packing material (the protein eluted very late, data not shown). Since CmP has a high isoelectric point ($pI = 9.3$) and the purified protein was shown to be stable only at high pH and high salt concentration, the protein was eluted at pH 10 and 300 mM NaCl buffer. CmP (20 μ g) eluted at 1.33 ml and the SDS-PAGE analysis confirmed the CmP isoform (33.6 kDa) in the peak volume (Figure 3.9A). But, similar to CmN, no detectable difference in the peak volumes was noted upon repeating the run in the presence of ATP (Figure 3.9B) or ATP+RuBP (Figure 3.9C). On increasing the concentration of the protein, the peak shifted towards higher oligomers (Figure 3.10A).

In summary, CmP and CmN isoform displayed a concentration dependent polydisperse oligomeric state, whereas the hetero complex CmNP eluted as a monodisperse species consistent with the dimensions of a AAA+ hexamer. (Figure 3.10B,C). At equal loading concentrations of 0.4 mg/ml (20 μ g loaded), the size of CmN and CmP was smaller than the hetero-oligomeric CmNP.

Figure 3.10| Individual isoforms display concentration-dependent polydispersity.

(A) Oligomeric state of *C. merolae* plastid isoform. Gel filtration analysis at 280 nm with varying concentration of pure CmP isoform in the apo state was applied to Superdex 200 PC3.2/30 column and eluted using purification buffer A_CmP (Materials Table 2.4). (B, C) Oligomeric state of pure CmN isoform and CmNP. Gel filtration analysis with varying concentration of CmN and CmNP in the apo state were applied to Superdex 200 PC3.2/30 column and eluted using purification buffer A (Materials Table 2.4).

Figure 3.11| Negative stain electron microscopy analysis of CmNP.

(A) Electron micrograph of RsRca (0.1 mg ml^{-1}) in the presence of 1 mM Mg-ATP and 1 mM RuBP. (B-E) Electron micrograph of CmNP (0.1 mg ml^{-1}). (B) In the Apo state, (C) presence of 1 mM Mg-ATP and 1 mM RuBP, (D) presence of 1 mM Mg-ATP γ S and 1 mM RuBP and (E) CmNP Walker B (E138Q) double mutant, CmN^{BpB} (0.1 mg ml^{-1}) in the presence of 1 mM Mg-ATP. Imaged at 49000X magnification and 120 kV intensity. Scale bars, 100 nm.

3.6. Structural analysis of the algal activase by negative stain electron microscopy

With an aim to determine the arrangement of the subunits in CmNP and to verify its oligomeric state (apparently hexamer) independently, the complex was investigated by negative stain electron microscopy. We successfully observed ring shaped particles for RsRca in the presence of ATP and RuBP (Figure 3.11A), indicating that we were able to reproduce the previously reported results (Mueller-Cajar et al. 2011) and we presumed the established conditions would be adequate to evaluate the algal proteins. Unexpectedly, negative stain EM analysis of CmNP (Figure 3.11 B-E) with the similar conditions as RsRca (Figure 3.11A) showed no ring structures. The negative EM staining of Apo CmNP particles (with the same concentration as RsRca) displayed an amorphous nature (indefinite shape), precluding further structural analysis. (Figure 3.11B) This was unexpected, as it occupied an apparently stable hexameric state in solution. Even when CmNP was incubated with both Mg-ATP and RuBP (using the same concentrations as utilized for RsRca), no ring shaped particles were seen (Figure 3.11C). We also used the slowly hydrolysable ATP analogue, ATP γ S in an attempt to stabilize the CmNP complex. But we observed merely heterogeneous particles (no ring structures observable) of CmNP (Figure 3.11D). Next, we investigated if nucleotide (ATP) trapping in the subunits could facilitate ring structure. To confirm this, we employed a CmNP complex harbouring Walker B motif mutations (E138Q) in both isoform subunits (CmN^BP^B) (see Chapter 2 (Figure 4.6) for further details about this complex). This mutant also formed merely amorphous particles (no ring structures visible) in the presence of ATP in negative stain EM (Figure 3.11E). Thus the negative stain EM images obtained for CmNP with the indicated concentrations were not suitable for structural analysis to determine neither the arrangement nor the oligomeric state. In future, more conditions should be trialed to visualize the CmNP particles.

3.7. CmNP is a highly thermostable Rubisco activase

In plants, Rubisco activase is remarkably sensitive to heat inactivation, exhibiting a temperature optimum for activity below 40 °C (Salvucci and Crafts-Brandner 2004) and thermal inactivation of Rubisco activity is believed to arise primarily from temperature-dependent inactivation of Rubisco activase (Portis 2003, Salvucci and Crafts-Brandner 2004). Extensive studies have been carried out to confer thermostability to Rubisco activases. Kurek et al. were able to identify more thermostable *Arabidopsis* Rca variants but only up to 42 °C (Kurek et al. 2007). However, *C. merolae* is a thermophilic red algae, and it actively performs photosynthesis at a high temperature of 45 °C to 55 °C (Barbier et al. 2005). So we presumed that Rubisco activase from *C. merolae* is likely to be stable and functional at high temperatures. To understand the thermostability of the CmNP, we evaluated the temperature dependence of ATPase activity. The activity of CmNP was thus measured from 25-65 °C at regular intervals of 5 °C. In this approach, both the protein and the assay mixture were first heated to the target temperature for 10 min and then the ATPase activity was assayed at the same temperatures (Figure 3.12).

The results showed a linear increase in ATPase activity with increase in temperature. Even at 55 °C, the highest temperature at which ATPase activity could be measured using our experimental setup, the ATPase activity did not reach its maximum. The ATPase activity at 55 °C ($\sim 15 \text{ min}^{-1}$) was about 8 fold relative to 25 °C ($\sim 2 \text{ min}^{-1}$) (Figure 3.12A). Based on thermodynamic principles the rate of enzymatic reactions doubles with a 10 °C increase in temperature (Milo and Phillips 2015). This correlates with our observation of an 8-fold increase in rate with a 30 °C change.

We were further interested to study the temperature-ATPase relation of CmNP beyond 55 °C. Due to instrumental limitations, for ATPase measurements above 55 °C, we changed the experimental design for measuring activity at 60 °C and 65 °C. Here the protein was heated to the respective temperatures for 10 mins and then assayed at 45 °C. At these temperatures a white precipitate became apparent, and this was cleared by centrifugation prior to assaying. The activity was severely reduced and completely lost at 65 °C (Figure 3.12B). This indicates CmNP was stable up to at least 55 °C and further increases in temperature resulted in the loss of activity due to

denaturation. Similar to temperature dependent measurements for CmNP, we investigated if either of the isomers may gain ATPase function at its physiological temperature range. However, CmN and CmP showed no measurable ATPase activity at all studied temperatures (Figure 3.12A), suggesting these proteins were inactive at the temperature range from 25 °C to 55 °C. Interestingly, the ATPase activity of RsRca was at its highest at 35 °C. Further increases in temperature lead to decrease in ATPase activity which was completely lost at ~42 °C due to precipitation. These results indicated that the red algal activase is more thermostable than bacterial activase and any reported higher plant activases (Keown and Pearce 2014). This finding may present an interesting biotechnological opportunity if plant Rubisco could be modified to be compatible with red-type activases.

Figure 3.12| CmNP is highly thermostable.

CmNP complex proves to be most active and stable at its physiological temperature of 45 °C-55 °C. **(A)** To study the effect of temperature on CmNP, ATPase activity (5 μ M protomer) was measured at incremental temperatures up to 55 °C at 5 °C intervals. The enzymes were heated (10 mins) at the target temperature prior to measurement at the same temperature. **(B)** For 60 °C and 65 °C measurement, the CmNP was heated to that temperature, but the assay was performed at 45 °C. All ATPase assays were performed in the presence of 1 mM RuBP and ATP. Error bars shown are the standard deviation of three independent experiments.

3.8. RuBP stimulates ATP hydrolysis by enhancing enzyme kinetics

Although maximal ATPase activity was noted at 55 °C, we found that the coupling enzymes used in the spectrophotometric Rubisco assay reaction mix did not tolerate temperatures above 50 °C. Therefore, we chose 45 °C as a suitable temperature for more detailed measurements of ATPase and Rubisco activation (catalytic assays).

In RsRca, RuBP binding results in an oligomeric transition from an inactive fibril to the functional hexamer (Mueller-Cajar et al. 2011). This suggests that in the fibril, the residues involved in ATP hydrolysis are not positioned in an orientation that enables catalysis. RuBP binding, associated with hexamerization, allows the formation of the functional active site. In CmNP this was not the case. The oligomeric state was not affected by the presence of ATP or RuBP (Figure 3.9B, C) and the enzyme possessed a basal ATPase function in the absence of RuBP (Figure 3.7D). In order to understand the function of the regulator in this case we determined kinetic parameters at 45 °C with the ATP concentration varying between 10 µM to 2 mM while keeping CmNP concentration constant at 5 µM.

Curve-fitting showed that CmNP in the absence of RuBP was consistent with the Michaelis-Menten model while in the presence of the RuBP Michaelis-Menten kinetics resulted in a poor fit (Figure 3.13A). However, the RuBP bound CmNP data was readily fitted to a non-logarithmic Hill equation. The calculated parameters in the absence and presence of ligand namely maximum velocity ($V_{\max}$) were 6.4 ± 0.8 and 11.2 ± 0.27 ATP min⁻¹, the Michaelis-Menten constant (K_m) for apo CmNP was 0.49 ± 0.10 mM and in the presence of RuBP $K_{1/2}$ was 0.13 ± 0.01 mM with a Hill constant of 2.1 ± 0.1 (Figure 3.13A). Hence CmNP apo showed non-cooperativity whereas in the presence of the ligand RuBP it showed positive co-cooperativity. The concentration of ATP to reach half the maximum rate was reduced by 75% in the presence of the RuBP. Hence, binding of its allosteric regulator RuBP appears to trigger conformational changes essential for enhanced ATP binding and hydrolysis.

We compared the kinetic properties of bacterial red-type and plant green-type activase to learn if they display cooperativity and the difference in affinity for their substrate ATP. RsRca in the presence of RuBP fits to the Michaelis-Menten model indicating no co-operativity. The K_m (ATP) of the RsRca in the presence of RuBP and tobacco plant activase (NtRca) was found to be 0.015 ± 0.003 mM and 0.05 ± 0.006 mM respectively (Figure 3.13B).

Figure 3.13| RuBP induced kinetic changes in CmNP.

Dependence of the activase ATPase rates on ATP concentration. (A) The rate of ATP hydrolysis is plotted against ATP concentration for 5 μ M activase in the presence of RuBP (1 mM) (filled squares) or absence of RuBP (hollow red square). Kinetic analysis of CmNP ATPase activity measured at 45 °C in presence and absence of 1 mM RuBP. The Michaelis-Menten model (dashed lines) provided a good fit to the Apo data ($V_{max}=6.4 \pm 0.8$ min⁻¹, $K_m=0.49 \pm 0.10$ mM), but approximated the RuBP-bound form poorly. Fitting the Hill model (solid line) suggests positive co-operativity in presence of the ligand ($V_{max}=11.2 \pm 0.27$ min⁻¹, $K_{1/2}=0.13 \pm 0.01$ mM, Hill constant= 2.1 $\pm$ 0.1). (B) Kinetic analysis of RsRca and NtRca ATPase activity measured at 25 °C in the presence of RuBP (1 mM). The curve drawn through the points is calculated from the Michaelis-Menten equation. Kinetic constants of RsRca with RuBP: $V_{max} = 9.96 \pm 0.32$ min⁻¹, $K_m = 0.015 \pm 0.003$ mM and NtRca: $V_{max} = 42.65 \pm 0.73$ min⁻¹, $K_m = 0.055 \pm 0.006$ mM. Comparatively, RsRca has highest (15 μ M) and CmNP has lowest (130 μ M) affinity for ATP. Measurements of RsRca and NtRca were performed at 25 °C. All ATPase assays were measured in the presence of 1 mM ATP. Error bars indicate the mean and s.d. with n=3.

Comparatively, CmNP has 9 fold less affinity for ATP than RsRca (red activase) and 3 fold less affinity for ATP than plant activase (green activase). It is conceivable that these properties may reflect differences in physiological nucleotide concentrations.

Importantly the binding of the allosteric regulator RuBP to the algal activase results in conformational changes that lead to higher ATP hydrolysis efficiency and introduce co-operativity. However, the ligand-triggered oligomeric transitions is not seen for the bacterial homolog.

3.9. Purification of *C. merolae* Rubisco from source

Having established the identity of the red-algal activase, it was important to characterize its function in the context of its cognate Rubisco. This was especially pertinent, since earlier work had concluded that the inhibition properties of red-algal Rubisco were consistent with the absence of a Rubisco activase (Pearce 2006). In order to investigate if endogenous Rubisco of *C. merolae* can be activated by its activase, we decided to isolate the enzyme from its source, because no functional eukaryotic Rubisco has yet been successfully produced in *E.coli* (Cloney et al. 1993, Whitney and Sharwood 2007). *C. merolae* cells were cultivated at 45 °C with continuous aeration and light to isolate the native Rubisco enzyme (Minoda et al. 2004).

After several initial failed attempts to lyse the cells by sonication, homogenisation using a Glass/Teflon Potter Elvehjem homogenizer with cells suspended in lysis buffer (50 mM Tris-HCl pH=8.0, 50 mM NaCl) containing 0.1% v/v TritonX-100 and 1 mM PMSF was successful. The soluble fraction separated from debris by centrifugation was applied to an anion exchange column and eluted using a linear NaCl gradient. Rubisco enzyme was identified to be in fractions eluted at ~300 mM NaCl using SDS-PAGE (Figure 3.14A). These fractions were further purified using gel-filtration (Figure 3.14B). The purified enzyme was checked for carboxylation activity using the Rubisco assay by preparing active ECM and inhibited ER and ECM-C complexes as explained previously for bacterial Rubisco at room temperature.

Figure 3.14| Purification of endogenous Rubisco from *C. merolae* culture.

(A) Purification of Cm Rubisco using the linear gradient of NaCl (0-1 M) on anion exchange chromatography equilibrated with purification buffer A (Materials Table 2.4). Chromatogram representation of the eluted proteins (right) and 12.5% SDS gel for the respective fractions (right) (M: Molecular Weight Marker, F: Flow through, the numbers indicate the SDS samples from respective fractions of AIEC or size exclusion column). (B) The final step of purification using size exclusion chromatography (SuperDex 16/60) equilibrated with purification buffer A (Materials Table 2.4). Chromatogram representing the eluted proteins (left) and 12.5% SDS gel for the respective fractions (right) with a molecular marker.

3.10. *C. merolae* Rubisco complex is only partially inhibited at 25 °C and cannot be activated

Fully active CmECM complex showed maximum carboxylation activity at a rate of 0.43 s^{-1} at 25 °C (from Figure 3.15A data). Unexpectedly, RuBP and CABP inhibited complexes made at 25 °C, CmER²⁵ and CmECM-C²⁵ were not inactive but displayed only partial inhibition (Figure 3.15A). CmER²⁵ had 51% and CmECM-C²⁵ had 41% of ECM activity. One of the possible explanation for the observed Rubisco activity by CmER²⁵ and CmECM-C²⁵ could be due to the denatured or less active Rubisco could have been purified in addition to the pure Rubisco (as seen from the additional bands in the SDS-PAGE analysis of the final purification Figure 3.14B). This may be a manifestation of cooperative inhibitor binding as previously described for spinach Rubisco, where fast tight binding of sugar phosphates occur at first four active sites (from each RbcL dimer) and followed by weak binding in the next four low-affinity active sites (Vater and Salnikow 1979, Frank et al. 1996, Frank et al. 1998). Similar observation for CABP binding to Spinach Rubisco also showed negative co-operativity (Zhu and Jensen 1990). All these studies suggested that binding of ligands to the high-affinity sites changed the conformation of the other sites thus reducing the binding affinity of additional ligands. Studies by Pearce have also described the difficulties in obtaining the stable uncarbamylated Rubisco form of another thermophilic red algae (*Galdieria sulphuraria*) (Pearce 2006). The study also pointed out that red algal Rubisco is unlikely to get inhibited at low levels of inhibitor because of its very high inhibition constants.

The addition of its activase to CmER²⁵ could not increase the carboxylation rate of the partially inhibited complex, indicating CmNP activase was unable to release the bound metabolites or inhibitors and activating its endogenous Rubisco (Figure 3.15B). The addition of its isoforms independently and reconstituted activase (CmN+P) to CmER²⁵ was also unsuccessful in increasing the carboxylation rate of the enzyme complex (Figure 3.15B). Similarly, activation of the partially inhibited tight Rubisco complex, CmECM-C²⁵ by CmNP activase also displayed similar results as CmER²⁵ and did not result in more active Rubisco (Figure 3.15C). RsRca also failed to activate partially inhibited CmRubisco complexes (Figure 3.15B).

Figure 3.15| At 25 °C, native *C. merolae* Rubisco does not form an inactive complex in the presence of inhibitors and cannot be targeted by activase for activation.

(A) Rubisco activity assays were performed with activated CmECM²⁵ and inactivated CmER²⁵/ECM-C²⁵ Rubiscos (0.5 μM active sites and 10 mM NaHCO₃). (B, C) Partially inactive CmRbc cannot act a substrate for activases. Rubisco activity assays were performed with activated (CmECM²⁵) and inactivated (CmER²⁵/ECM-C²⁵) Rubiscos (0.5 μM active sites and 10 mM NaHCO₃) in the presence of pure red algal activase isoforms, reconstituted activase, CmNP and RsRca (5 μM protomer). (D) Partially inactive Rubisco fails to stimulate ATPase rate of activases. ATPase activity assays were performed with inactivated (CmER²⁵/ECM-C²⁵) Rubiscos (3 μM active sites) in the presence of bacterial and red algal activases (5 μM protomer). All *C. merolae* Rubiscos used in the assays were prepared at 25 °C. All ATPase and Rubisco assays were measured in the presence of 1 mM ATP and 1 mM RuBP at 25 °C. Error bars indicate the mean and s.d. with n=3.

Additionally, to check if there is any stimulation in ATPase rate of the activases by its partially inhibited substrate, we measured the activity with CmER²⁵ complex. No stimulation was observed in the presence of its substrate for all tested activases (Figure 3.15D), suggesting that partially inhibited Rubisco complex was not recognised or targeted by the activase to stimulate its ATPase.

These results were consistent with the Rubisco activity results. Hence this indicates that the conformation of the partially inhibited Rubisco complex is not suited for the activase interaction. We assume this would indeed not be the actual scenario *in vivo* because the presence of partially inhibited and non-activation of the Rubiscos by its activase only occur at its nonphysiological temperature condition. Also there is a possibility that by tweaking the experimental parameters (pH, crowding agent, increasing concentration of activase/Rubisco, etc.) we could make the activase to recognize the partially inhibited Rubiscos.

This implies CmER²⁵/ECM-C²⁵ which does not form a completely inhibited state is not amenable for CmNP targeting and activation. Different to completely inhibited ER or ECM-C, the partially inhibited state appears to present a surface that is not recognised by the activase. We also speculate that partial inhibition could result in non-equivalent active sites which may not be recognised by the activases. Nevertheless, properties of this partially inhibited red algal Rubisco species remain to be investigated in detail. In future to characterize the partially inhibited complexes it would be necessary to establish the identity of the species. The key property to test would be the degree of binding (tight or loose) to the partially inhibited Rubiscos. To address this, formed partially inhibited species can be buffer exchanged or dialyzed to remove unbound inhibitors and subsequently their carboxylation activity can be measured as a function of time to estimate the ECM formation. Additionally we can test if the degree of binding increases by incubating the free inhibitor removed species at 45 °C and measuring their catalytic rate. Kinetics of the partially inhibited Rubiscos (such as K_m (RuBP), K_{cat} , binding and dissociation constants) could provide a clue about the percentage of the active sites occupied by the inhibitors. By using a native mass spectrometry approach, Blayney MJ et al. 2011 (Blayney et al. 2011) had shown that inhibitors (CABP/RuBP) were bound to all eight active sites of the tobacco

Rubisco. We could also adopt the same approach to identify the number of active sites being blocked by the inhibitors in the partially inhibited form.

Complete inhibition of algal Rubisco can be achieved by incubation at physiological temperature

Previously, to overcome the problem of forming completely inhibited thermophilic red algal Rubisco complex, Pearce performed overnight dialysis of the enzyme against nitrogen-sparged buffer to drive the formation of the uncarbamylated E form (Pearce 2006). Since *C. merolae* being thermophilic algae, we hypothesized that heating the Rubisco to its physiological temperature could result in different biochemical properties. Incidentally Rubisco activity assays of CmER and CmECM-C heated to 45 °C for 15 mins and then assayed at 25 °C showed almost no or negligible 3PG production, confirming the formation of completely inhibited complexes (Figure 3.16A, B). Our alternative approach of heating the Rubisco of thermophilic algae to its near physiological temperature thus circumvented the necessity to achieve decarbamylated Rubisco through dialysis with CO₂ and O₂ free buffer. Conformational changes of E form (non-active) or ECM form (active) to the fully inhibited form thus apparently do not take place at 25 °C, but readily occur after heating at 45 °C. We assume only four of the eight active sites are occupied by the inhibitors under 25 °C condition, but heating facilitates the change in conformation leading the other four unoccupied sites to be bound with inhibitors resulting in a completely inhibited form. Another possibilities include that some unknown metabolite could be bound to the active sites of the Rubisco rendering it inactive and as a consequence of heating the unwanted metabolite inhibitor would be released from the active sites. Additionally heating could have removed the presence of partially denatured Rubisco during purification. The carboxylation rate of CmECM that was prepared at 45 °C (0.4 s⁻¹, measured at 25 °C) was noted to be almost similar as that of ECM prepared at 25 °C (from Figure 3.16A data).

To use these complexes for detailed ATPase and activation studies at 25 °C, we first wanted to know if the inhibition phenotype of the complexes achieved by heating could remain stable as a completely inhibited form at 25 °C, or whether it would return to the partially inhibited state over time. The heat inactivated red algal Rubisco incubated for hours at 25 °C showed no significant difference in Rubisco

activity over the incubation time (data not shown). Once a decarbamylated and RuBP inhibited Rubisco is formed by heating it continued to be stable and showed no spontaneous activation. A similar phenotype was also observed for inhibited complexes of *G.sulphuraria* at 25 °C by Pearce (Pearce 2006). In contrast, the bacterial counterpart, RsER does not exhibit the phenotype of the stable complex but rather shows spontaneous activation over time (Figure 3.16D). According to the argument of Pearce and Andrews 2003 (Pearce and Andrews 2003), the ability of the Rubisco enzyme to bind and release the sugar phosphates (substrate RuBP and inhibitors) is related to the flexibility of its active sites. The Rubiscos which releases the sugar phosphates rapidly are referred to as flexible and the others which releases slowly are referred to as less flexible or more rigid. Thus it suggests the flexibility of bacterial Rubisco is higher than the red algal enzyme. Along similar lines the observed phenotype of our stably inhibited algal Rubisco which does not demonstrate self-activation describes the algal Rubisco with less flexible or more rigid active site compared to the bacterial Rubisco.

This is consistent with the observation that rate of release of inhibitors for non-green algae was much slower compared to other forms of Form I and II Rubiscos (Schloss 1988, Pearce 2006, Parry et al. 2008). Overall these results suggest that heating together the Rubisco with its inhibitors at high temperature helps to reach the activation energy required for formation of the stable and tight complex with the inhibitors.

3.11. The stable ER and ECM-C Rubisoco complexes of the red algae can be activated by CmNP activase

To study whether the stable heated complex was amenable to activation, we tested the substrate remodelling capacity of CmNP by Rubisco assays at 25 °C. The addition of CmNP to the CmER complex resulted in a slow activation of inhibited Rubisco (Figure 3.16A). CmNP was also able to activate CmECM-C (Figure 3.16B). Activation of ECM-C by CmNP at these conditions was noted to be marginally faster than ER. After 5 min, ~32% of ECM-C active sites were activated while ~24% of ER active sites were activated by CmNP (Figure 3.16A, B) and this difference may be due to the time required for E complexes to form ECM (that is the carbamylation of the active site lysine, followed by Mg²⁺ binding). Under similar assay conditions, the

activation rate of inhibited Rubisco complexes of *C. merolae* by CmNP was observed to be significantly slower compared to activation of the *R. sphaeroides* counterpart. Red algal Rubisco, once inhibited has a very low release rate of inhibitors (low dissociation constant for inhibitors) compared to its bacterial counterpart (Pearce 2006) and Figure 3.16D supports that *C. merolae* Rubisco inhibited complex are very stable compared to the bacterial enzyme complexes. High stability of the red algal substrate may require a greater force application from the activase to remodel. Thus CmNP may not be able to generate enough force to readily activate the stable substrate complexes so observed rate of activation was slow. Another possible reason could be because of more unsuccessful activation attempts by CmNP. Possibly only a fraction of threading attempts by CmNP was successful for remodelling its Rubisco substrate compared to the bacterial substrates.

Figure 3.16| CmNP complex can only weakly activate its native inactive Rubisco at 25 °C.

(A, B) CmNP activates *C. merolae* Rubisco. Rubisco activity assays were performed with activated (CmECM) and inactivated CmER (A) and CmECM-C (B) Rubisco (0.5 μM active sites and 10mM NaHCO₃) in the absence and presence of red algal and bacterial activases (5 μM protomer). (C) CmNP shows weak stimulation by its endogenous Rubiscos. ATPase activity assays were performed with inactivated (CmER and CmECM-C) Rubiscos (3 μM active sites) in the presence of red algal and bacterial activases (5 μM protomer). (D) Rubisco activity assays were performed with activated (RsECM, CmECM) and inactivated (RsER, CmER) Rubiscos (0.5 μM active sites and 10 mM NaHCO₃) (dotted line indicated the ECM-C activation rate of the respective Rubisco and ER rate at 25 °C (hollow circles) is shown). The reaction mix for carbamylation and decarbamylation with Rubiscos were heated to the respective temperatures as indicated in the figure for about 10 min prior to the measurement at a temperature as indicated. Error bars shown are the standard deviation of three independent experiments.

Studies with ClpX has also shown that the AAA+ motor has the capacity to generate varied degree of force (more or less) depending on the stability of the substrate it remodels or unfolds (Sen et al. 2013). When individual subunits were tested if it could activate its cognate inhibited Rubisco complexes, as expected, they failed to activate (Figure 3.16A, B).

ATPase activity was determined to understand if there is any change in activity with the addition of stable algal Rubisco complex. ATPase rate of CmNP activase was increased ~2 fold in the presence of the tested substrates and the similar rate was observed for the reconstituted active complex (Figure 3.16C). No activity was noted for both isoforms individually (Figure 3.16C).

Next, we tested if the bacterial RsRca would be able to recognize and activate the highly stable Rubisco complexes with tightly bound inhibitors. Although a modest increase (~10-20 %) in ATPase activity of the bacterial activase with CmECM-C and CmER was observed (Figure 3.16C), no Rubisco activation was noted at 25°C. Hence although an interaction appeared to take place, any activation/remodelling attempts were unsuccessful.

The preceding results illustrate that CmNP can interact with and activate its cognate Rubisco substrate. Clearly, activation of CmRubisco does not fully correlate with the ATPase rate of the activase because RsRca with its much higher ATPase rate could not remodel the algal Rubiscos but the own algal CmNP activase with a much lower ATPase rate could achieve activation (albeit at a much lower rate). In the eukaryotic *C. merolae* Rubisco activation system, Rubisco remodelling success may be more dependent on species and thus structural complementarity of the activase. It appears to be more activase specific compared to the bacterial Rubisco which can be robustly activated by both eukaryotic and prokaryotic activases.

3.11.1. *C. merolae* Rubisco is readily activated at physiological temperature

In previous temperature dependence assays, we have demonstrated that CmNP has a faster ATPase rate at higher temperatures relative to 25 °C (Figure 3.12). Additionally, at a higher temperature, it is expected that the flexibility of the Rubisco increases (and the stability of inhibited complexes should decrease). Keeping these issues in mind, we decided to investigate further if enhanced ATP hydrolysis by CmNP can improve the remodelling rate of its Rubisco substrates, ER as well as ECM-C at its physiological temperature. This would also more closely resemble the *in vivo* condition of the red algae.

To test this hypothesis, we heated inactive complex and reaction mix independently to 45 °C for about 10 min, followed by performing the Rubisco activation assay with CmER and CmECM-C at the same temperature.

At 45 °C, fully active CmECM complex showed a pronounced increase in the carboxylation rate (0.8 s^{-1}) of about twofold compared to the 25 °C measurement (Figure 3.17A). CmER and CmECM-C remained completely inhibited as observed at 25 °C (Figure 3.17A, B). This observation complements the results by Pearce that the red algal Rubisco binds inhibitors very tightly and the release rate is very low (Pearce 2006). Interestingly, CmNP was noted to activate both inhibited algal Rubisco complexes with a much faster rate at 45 °C compared to 25 °C (Figure 3.17A, B). At the higher temperature, the conformational changes required to remodel the active site can be more easily achieved, enabling it to be activated by the cognate activase. The rate of activation of CmER and CmECM-C (red algal) at 45 °C was comparable to the rate of activation of RsER and RsECM-C (bacterial) at 25°C. This result is consistent with the idea that at the physiological temperature for organisms, the relative flexibility of Rubisco is similar (Pearce 2006). On the other hand, pure activase isoforms showed no detectable activity in the presence of CmER/ECM-C at 45 °C (Figure 3.17A, B).

Figure 3.17| CmNP complex can ready activate its cognate inactive Rubiscos at its physiological temperature.

(A, B) CmNP rapidly activates *C. merolae* Rubiscos at 45 °C. Rubisco activity assays were performed with activated (CmECM) and inactivated CmER (A), CmECM-C (B) Rubiscos (0.5 μM active sites and 10 mM NaHCO₃) in the absence and presence of red algal activases (5 μM protomer). (C) CmNP complex has a functional ATPase activity at 45 °C and can be stimulated by its endogenous Rubiscos. ATP hydrolysis rate comparison of pure CmN, CmP and CmNP. ATPase activity rate for 5 μM protomer was measured in the absence (Apo state) and presence of 1 mM RuBP. Also, ATPase activity rate was calculated with inactivated (CmER and CmECM-C) Rubiscos (3 μM active sites) in the presence of red algal activases (5 μM protomer). All ATPase and Rubisco assay were measured in the presence of 1 mM ATP and 1 mM RuBP. Only ATPase activity of Apo (C) was measured in the absence of RuBP. The activases were heated to 45 °C for 10 min prior to measurement at the same temperature. Error bars shown are the standard deviation of three independent experiments.

We were unable to test RsRca for activation of CmER/ECM-C at 45 °C because the activase aggregates upon heating to this temperature (Figure 3.12). ATPase data was consistent with the observed Rubisco activation. We noted a significant basal ATPase activity for CmNP $\sim 4 \text{ ATPmin}^{-1}$ which was increased by about twofold with the addition of RuBP $\sim 10 \text{ ATPmin}^{-1}$ when measured at 45 °C (Figure 3.17C). The ATPase rate was further stimulated ~ 1.5 fold with both inhibited Rubisco complexes and this was similarly observed for reconstituted (CmN+P) activase (Figure 3.17C, D). Once again the pure isoforms showed neither basal ATPase activity nor stimulation with Rubisco (Figure 3.17C, D) suggesting complete non-functionality even at their physiological temperature.

3.11.2. RsRca can activate CmER at 35 °C

Earlier we presumed that RsRca could not activate CmRubisco due to structural specificity issues (Figure 3.16). With our subsequent results indicating that successful activation of CmRubisco largely depends on the temperature (Figure 3.16 and Figure 3.17) and RsRca being ATPase functional at 35 °C (Figure 3.12A), we wanted to ask whether RsRca was incompatible with CmRubisco or whether activation was temperature dependent? Therefore activation of CmER and ECM-C by RsRca was measured at 35°C. Interestingly, RsRca was able to activate CmER at 35°C, but at a slower rate than CmNP (Figure 3.18A). ATPase stimulation was weak for both activases with CmER (Figure 3.18C). However, CmNP activated the Rubisco complex faster than RsRca in spite of displaying ~ 4 fold less ATPase activity (Figure 3.18C). Unexpectedly, at 35 °C CmECM-C was rapidly activated by CmNP, but RsRca displayed very low activity (Figure 3.18B), even though its ATPase activity was stimulated in the presence of the Rubisco complex (Figure 3.18C). Since the bacterial red-type activase can remodel CmRubisco, this confirms that successful remodelling of algal Rubiscos is temperature dependent and not limited to the specificity of its own activases. Evolutionarily, the activation mechanism as well as key residues required for activation are conserved across the two species.

Figure 3.18| Bacterial activase activates inactivated red algal Rubisco at 35 °C.

RsRca displays activation of *C. merolae* Rubisco at 35 °C and weak stimulation in ATPase rate was noted for the same. (**A**, **B**) Rubisco activity assays were performed with activated (CmECM) and inactivated CmER and CmECM-C Rubiscos (0.5 μM active sites and 10 mM NaHCO₃), and (**C**) ATPase activity assays were performed with CmER and CmECM-C Rubiscos (3 μM active sites), in the absence and presence of RsRca and CmNP (5 μM protomer). All measurements are performed in the presence of 1 mM ATP and 1 mM RuBP at 35 °C except ATPase activity of Apo (**C**), measured in the absence of RuBP. Prior to measurement, the activases were also heated to 35 °C. Error bars shown are the standard deviation of three independent experiments.

3.12. RsECM-C activation by CmNP is very rapid at increased temperature.

Since CmNP was able to perform better in activating CmRbc at 45 °C, we wanted to test whether the enzyme can activate bacterial Rubiscos efficiently with a higher rate at 45 °C. Preactivated and inactivated bacterial Rubisco complexes were prepared by heating to 45 °C for 15 mins along with the reaction mix prior to measurement. RsECM was seen to be very active, but notably, RsER complex was no longer inhibited by RuBP. RsER was seen to be spontaneously activated (Figure 3.16D). Our observation was consistent with previous studies which demonstrated that high temperature loosens the catalytic site and increases the rate of dissociation of RuBP (Schrader et al. 2006). At high temperature (45 °C) it was suggested that the interaction between the bound inhibitor and flexible domains of the binding site weakens and resulting in the increased rate of inhibitor release (Schrader et al. 2006). However, for the thermophilic *C. merolae* Rubisco the formed inhibited complex are stable indicating the active sites are not loosened due to heating.

In contrast to RsER, RsECM-C was very stable and remained completely in the inhibited state at 45 °C (Figure1.19A). This is additional evidence that compared to RuBP, CABP forms a more stable complex with the bacterial Rubisco. The addition of CmNP to the inhibited Rubisco enabled remodelling to the active form (Figure1.19A). The rate of activation was much faster than that observed at 25 °C. The rate of activation by the reconstituted complex was also identical to co-purified CmNP (Figure1.19A). Predictably, CmN and CmP isoforms were unsuccessful in activating RsECM-C (Figure1.19A) which is also consistent with the ATPase data (Figure1.19B). ATPase activity measurements of CmNP correlate to the observed Rubisco activation and showed a pronounced stimulation with bacterial ECM-C that was higher than stimulation by its cognate inhibited Rubiscos (Figure1.3A).

Figure 3.19| CmNP functions as a remodeller of RsECM-C at its physiological temperature and inhibited CABP complex stimulates its ATPase activity.

CmNP complex displays a rapid rate of activation with *R. sphaeroides* CABP-Rubisco at 45 °C and significant stimulation in ATPase rate was noted for CmNP. (A) Rubisco activity assays were performed with activated (RsECM) and inactivated RsECM-C Rubiscos (0.5 μM active sites and 10 mM NaHCO₃), and ATPase activity assays were performed with RsECM-C Rubisco (3 μM active sites) in the presence of red algal activases (5 μM protomer). All measurements are performed in the presence of 1 mM ATP and 1 mM RuBP at 45 °C. Prior to measurement, the activases were also heated to 45 °C. Error bars shown are the standard deviation of three independent experiments.

3.13. Summary

In this chapter, we have shown that individual isoforms (CmN and CmP) encoded by the nuclear and plastid encoded CbbX genes of *C. merolae* are functionally inert. The isoforms can, however, interact to form an active hetero-oligomeric complex (CmNP) with a stoichiometry of isoform subunits in 1:1 ratio. Our analytical gel filtration data showed that individual isoforms displayed a polydisperse nature, but CmNP complex appears to form hexameric complex in the apo state. The presence of ligands (ATP and RuBP) did not induce any oligomeric changes to the activase complex, in stark contrast to the earlier characterized bacterial activase. We found RuBP regulated CmNP by increasing the maximal ATP hydrolysis rate by twofold and increasing affinity for the nucleotide. Also, RuBP binding to the CmNP induces positive co-operativity for the binding of ATP ligands to the same. These properties differ for the regulation that is seen in RsRca.

We have shown here that CmNP is a functional activase by demonstrating that it can robustly activate RsER and RsECM-C complexes utilizing a mechanism similar to RsRca. Our data clearly illustrates that the proposed model of Rubisco activation by C-tail threading of the large subunit for RsRca is also conserved for CmNP.

We isolated the red algal Rubisco from the source and characterized the inhibition properties of this Rubisco. We found that at its non-physiological temperature, the Rubisco did not form a stable inhibited complex but heating to its physiological temperature (45 °C) made the complex stable with all the active sites bound with inhibitors. Its cognate activase could not generate enough force to activate the highly stable substrate complex robustly at its non-physiological temperature.

Temperature dependent ATPase assays had shown that CmNP is a thermostable activase. So when assayed at its physiological temperature, CmNP readily activated the stable inhibited Rubisco complexes (CmER, CmECM-C and RsECM-C). In conclusion, we have demonstrated that red algal's red-type Rubiscos is maintained by their heterocomplex activase (CmNP) effectively at its physiological temperature (45°C) which mimics the *in vivo* conditions.

Having characterized the basic biochemical properties of the CmNP so far, we were interested to identify the arrangement of the subunits in the ring and understand more about the regulatory role of each isoform in the activase complex. With an aim to make a framework of the mechanistic model, we mutated the key motifs in the individual isoforms to understand their roles in ATP hydrolysis, substrate targeting and activation and this work is described in the next chapter.

5. MECHANO CHEMICAL CHARACTERIZATION OF RED ALGAL ACTIVASE

The recently studied bacterial activase system functions as a homohexamer and a detailed mechanistic model was proposed (Mueller-Cajar et al. 2011). But the function of the activase in terms of the interaction between subunits was not studied. In a homohexamer, this can be done by subunit mixing or mutant doping experiments. In AAA proteins, a mutant doping strategy is often used to determine the relative contribution of individually mutated subunits towards ATPase and remodelling activity, and thus outline mechano chemical coupling mechanisms for the AAA+ hexamers. To investigate the mechanism, usually ATP hydrolysis or substrate binding impaired mutant subunits are mixed with wild-type subunits in different ratios, but keeping the total concentration of the protein constant, which permits generation of a heterogeneous population of hexamers. The distribution of the population is simulated with a binomial distribution that varies as a function of the molar ratio of each subunit (Werbeck et al. 2008). Therefore, for any specified percentage of mutant subunits, the probability distribution of hexamers containing 0, 1, 2, 3, 4, 5 or 6 mutant subunits can be derived. This approach was successfully adopted in other enzyme systems to obtain mechanical insights (Moreau et al. 2007, Martin et al. 2008b, Joly and Buck 2010, DeSantis et al. 2012, Yu et al. 2015). This distribution is then used to predict how activity could be inhibited at different mutant: wild-type ratios. As an alternative approach, two or more subunits can be covalently linked in a single-chain approach (Martin et al. 2005).

Our results demonstrated that the red algal activase functions as a hetero hexameric complex with 1:1 stoichiometry and could activate a range of Rubisco substrates. We decided to take advantage of this hetero-oligomeric system to investigate the relative contribution of each isoform to Rubisco activase function. To this end, we introduced a number of mutations in various key motifs of the CmN and CmP isoforms and characterized the resulting variant complexes. We aimed to propose a mechanistic model and compare this with already described models in the literature to illustrate the similarities and differences in the mechanism of AAA ATPases. Further, by understanding the role of each subunit in the complex, we hoped to understand the complexity introduced by nature into the Rubisco activation system of red-lineage phytoplankton.

Elucidating the subunit arrangement of the isoforms in the complex was key to interpreting the results generated by our approach. Two possible arrangements of the complex include a trimer of hetero dimers or dimers of homo trimers (Figure 4.4A). Based on the recalcitrant behaviour of the CmP isoform (see section 3.1) we considered a likely explanation to be poorly compatible subunit-subunit interfaces. Hence we hypothesized an alternating arrangement for CmNP. Negative stain electron microscopy was previously used to elucidate the subunit arrangement of hetero hexameric AAA+ proteins (Gardner et al. 2015), but as shown earlier CmNP was not amenable to this approach (Figure 3.11). Therefore we decided to follow a biochemical approach using mutants in the ATPase activase site and covalently linked complexes to address this issue.

4.1. CmNP was not readily amenable to a concatemerization approach

To test our hypothesis of an alternating subunit arrangement of CmN and CmP in the activase complex we initially considered to use a covalently linked subunit approach. We were inspired by the seminal work of Andreas Martin and colleagues, where ClpX Δ N subunits were covalently connected using a 20 amino acid residue linker (Martin et al. 2005). Successfully creating concatamers of CmN and CmP isoforms would also allow us to conduct similar studies in our system. This would allow us to precisely place amino acid substitutions in the context of the hexamer, allowing us to more thoroughly probe the mechanism and thus refine our working model. Such an approach has been used to mutate single subunits in the complex to probe the role of each motif in the functionality of the proteins (Martin et al. 2007, Martin et al. 2008a, Glynn et al. 2009, Joly and Buck 2011, Stinson et al. 2015). The concatamer approach provides the flexibility to arrange subunits in the complex i.e. it allows controlling the number of mutated subunits against the wild-type, to understand an issue such as the relative regulation in inter-subunit communication for ATPase cycle and substrate activation by this system. However, initially, we aimed to create a functionally active CmNP concatamer with alternating isoform subunits to support our hypothesized subunit arrangement.

To this end, we initially constructed concatemers with the same amino acid linker sequence used by Martin “L20” (Martin et al. 2005) owing to the structural similarities with ClpX (Hanson and Whiteheart 2005, Mueller-Cajar et al. 2011). According to a hexameric CbbX model, L20 should be able to connect the C-terminus of one subunit to the N-terminus of the clockwise subunit as the clockwise connection is expected to be similarly positioned in these proteins (Figure 4.1A). Two concatamers were made, N-P con (linker L20 connects the C-terminus of CmN to N-terminus of CmP) and P-N con (linker L20 connects the C-terminus of CmP to N-terminus of CmN) respectively (Figure 4.1A). Both were expressed, purified as explained in the methods and analysed using SDS-PAGE with Coomassie staining. A protein band of 70 kDa, which is the calculated size of the concatamers were spotted in the SDS-PAGE analysis of fractions from the Anion exchange chromatography of N-P and P-N con purifications (Figure 4.1B). Since the final concentration of purified P-N con was very less compared to the N-P con, the following biochemical analysis was performed only using N-P con protein. The presence of N-P concatamer was confirmed to be the desired concatamer by immunoblotting with an antibody raised against RsRca that recognises the plastid isoform CmP (Figure 4.1C). To find if the concatamer assembled to be a hexameric form, the protein was applied to an analytical gel filtration column. Surprisingly the concatamers were seen to be prominently eluted at three volumes 1.13 ml, 1.28 ml and 1.42 ml respectively. Stokes Radii of the concatamer peaks calculated from the calibration curve (Figure 3.9D) are 6.7 nm, 5.3 nm and 3.9 nm respectively. The elution profile of the concatamer suggests that it existed as a population of different oligomeric species, possibly as octamer ($R_s \sim 6.7$ nm), hexamer ($R_s \sim 5.3$ nm) and tetramer ($R_s \sim 3.9$ nm) (Figure 4.1D). When tested for its functionality, N-P con was ATPase active but appeared to have only a fraction of ATPase activity ($\sim 20\%$) relative to wild-type. On the other hand, P-N con showed no ATPase activity (Figure 4.1E). When assayed for Rubisco activation, both the concatamers were unable to activate the inhibited Rubisco (RsER) (Figure 4.1F).

Possibly, the observed non-productive oligomeric state complexes could have affected the activase function, which explains the low ATPase and the inactivity. This could have also resulted due to the inappropriate length of the linker, which may have perhaps constrained the N- and/or C-terminal movement to form the preferred active conformational state. In some AAA+ proteins, nucleotide binding to and hydrolysis by the AAA+ subunits induces N and C-terminal movement in the subunits that drive the conformational state changes. Any hinderance to these movement could affect the function of the protein. Hence, we increased the linker sequence by 5 amino acids. Since N-P con had better protein yield and activity compared to its counterpart, so it was further modified. The concatamer with extended linker N-P con (extended) (Figure 4.2A) was purified and the protein fractions with 75 kDa protomer was confirmed to be concatamer and assayed for its activity. Neither Rubisco activation activity (Figure 4.2B) or ATPase activity (Figure 4.2C) was detected for the complex with extended concatamer. We currently believe that the N-and C-terminus are important for sucessfully forming hexameric oligomer and function; restraining it by covalent linking could affect the functionality or the linker possibly interacts with surface residues of the subunits. In future work we will prioritize extending the linker further to achieve a functional concatamer. Our result clearly showed that concatamer is successfully expressed as a covalently linked CmN and CmP. But, currently, we remain uncertain as to why it does not display biochemical functionality.

Figure 4.1| Concatamer construction.

(A) Schematic representation of CmN connected to CmP via L20 linker with His₆ubiquitin tag (N-P concatamer and P-N concatamer) (right). Illustration of a concatamer model using the hexameric model of RsRca (PDB:3ZUH) with L20 linker (black) connecting N-terminal of CmN and C-terminal of CmP subunits (yellow and grey) (left). **(B)** SDS-PAGE analysis of the purification of concatamers. Successive purification by IMAC (Ni-NTA), followed by USP2 mediated cleavage of N-terminal tag protein was purified using anion exchange chromatography (AIEC). SourceQ 15 column equilibrated with purification buffer A and eluted with purification buffer B (Table 2.4) was used for the AIEC. SDS-PAGE (12.5%) gel represents purification profile of N-P concatamer (N-P con) (left) and P-N concatamer (P-N con) (right). The molecular weight marker bands and location of respective activase band are indicated. (M: Molecular Weight Marker, F: flowthrough of the SQ15 column, Ni-NTA: Fractions after immobilized metal affinity chromatography (IMAC), AC: Eluted protein after USP2 mediated cleavage of Ubiquitin. The numbers indicate the SDS samples from respective fractions of AIEC) **(C)** Presence of purified N-P con protein (69.8 kDa) detected by immunoblotting using α -RsRca antibody. Lane1: CmNP (WT) as a control, Lane2-4: purified N-P con protein loaded at increasing concentration and molecular weight marker indicated on the side. **(D)** Elution profiles of independent runs for N-P con and CmNP (WT) in apo form, loaded onto Superdex 200 PC3.2/30 column and eluted with purification buffer A (Table 2.4). The elution volume of N-P con suggests a population of different oligomers, though some fraction is similar to hexameric CmNP (WT). **(E)** ATPase activities of concatamers are negligible compared to CmNP (WT). ATPase activity was measured with concatamers (5 μ M protomer) at 45 °C. **(F)** Rubisco activity assays of activated (ECM) and inactivated (ER) Rubisco (0.5 μ M active sites, 10 mM NaHCO₃) in the presence and absence of concatamers (5 μ M protomer) measured at 25 °C. All ATPase and Rubisco assays were performed in the presence of 1 mM ATP and 1 mM RuBP.

Figure 4.2| Concatamer with the extended linker.

(A) Schematic representation of concatamer with L25 linker (N-L25-P). **(B)** Rubisco activity assays with activated (ECM) and inactivated (ER) RsRbcLS (0.5 μM active sites, 10 mM NaHCO_3) in the presence and absence of concatamer (5 μM protomer) measured at 25 °C. The assay shows no activation by the concatamer with the extended linker. **(C)** ATPase activity of new concatamer was negligible. ATPase activity was measured with concatamer (5 μM protomer) at both 25 °C and 45 °C. All ATPase and Rubisco assays were measured in the presence of 1 mM RuBP and ATP.

4.2. Purification of the CmNP variants

To characterize the relative contribution of each isoform to the ATPase and Rubisco activation activity of the heterocomplex, we mutated some of the key residues of the functional motifs and elements. Site-directed mutagenesis (Methods section 2.2.1.6) were performed using the designed primers (Table 2.5) to produce mutants of CmN and CmP expressed in pHue vectors (Table 2.11) as described in materials and methods (Methods section 2.2.1.8.6).

For the desired mutant heterocomplex, the respective CmN and CmP plasmids (Table 2.11) in *E.coli* BL21 (DE3) cells overexpressing chaperonins GroEL and GroES by pBADESL was grown in 1 l LB medium independently induced by IPTG and L-Arabinose at 23 °C overnight. Harvested cells of isoforms (mutant or wild type) were mixed in equal ratio prior to lysis step as explained in methods (Figure 3.5C, D). This mixing of lysates enabled reconstitution of CmN isoform with its counterpart CmP to form the CmNP variant complex during the opening of the cells. The representative SDS-PAGE gel shows the purified variants used in this chapter (Figure 4.3).

Figure 4.3| SDS-PAGE analysis of pure CmNP variants.

12.5% SDS-PAGE analysis. 3 μ g protein is loaded per lane. Superscripts indicate the mutated element of the respective isoform. (WT: wild-type, A: Walker A (K80A), B: Walker B (E138Q), R: arginine finger (R194A), S: RuBP binding site (R239A), Y: Pore loop (Y114A), K123A: surface-exposed lysine).

4.3. Biochemical evidence in support of an alternating arrangement of the isoform subunits

In the study of the heteromeric Yta10/12 system, Augustin et.al assigned the arrangement of subunits using a biochemical approach (Augustin et al. 2009). The principle of this approach is to make a complex with arginine finger mutation (R) in one isoform and Walker B (B) mutation in the other isoform subunits and study the ATPase activity. Mutation in arginine finger of one subunit affects the ATP hydrolysis at the catalytic site of the neighbouring subunit and certainly the subunits with Walker B mutation are defective for ATP hydrolysis. Hence, the ATPase rate of the variant complex provides an indication regarding the arrangement of subunits. If the arrangement is trimer of homodimers (alternating), three functional active sites can be formed (Figure 4.4A), and the resulting ATPase rate should be 50% or more compared to the wild-type enzyme. In a dimer of homotrimer arrangement, only one functional active site can be formed (Figure 4.4A) and the ATPase rate would thus be expected to be 1/6 or less compared to wild-type. We adopted this approach for our CmNP hetero hexameric system and introduced Ala mutation at Arg194 (arginine finger) and Gln mutation at Glu138 (conserved glutamate residue at Walker B) substitutions into the subunits. Variant complexes N^{RPB} and N^{BPR} were prepared to study the ATPase activity (see section 4.6 for a more detailed characterization). The ATPase rate of the variant harbouring mutations in arginine finger of CmN and Walker B motif of CmP (N^{RPB}) was relatively similar to CmNP, although lesser than variant complex with mutated Walker B motif of CmP and wild-type CmN subunits (an interesting phenotype, which will be discussed in depth later) (Figure 4.4B). Consistent with an alternating subunit arrangement, ATPase of the variant was more than half of wild-type, so theoretically this suggests that formation of a dimer of homotrimers complex is highly unlikely and correlates with the formation of a trimer of heterodimer complex (alternating arrangement) with an alternating arrangement of isoform subunits. Despite the fact that ATPase rate of the variant (N^{RPB}) favors the conclusion that more than one functional active site could exist, this approach cannot formally exclude the possibility of other heterogeneous subunit arrangements (NNPNPP, NPNNPP, and NNNPPP). Indeed N^{RPB} is a very interesting variant with atypical phenotypes (such as gain of function of ATP hydrolysis function relative to its non-functional CmN^{RP} parent), we studied this in detail (in the current section 4.6)

to gain more insights into the intersubunit signaling mechanism by understanding its ATPase activities at different conditions and its Rubisco activation activity with different substrates.

Figure 4.4| Alternating arrangement of isoform subunit in the CmNP complex.

(A) Representation of possible arrangement of subunits in the CmNP complex. Surface (top) and ribbon representation (bottom) of red-type Rca with arginine finger (R194) and Walker B (E138) are shown in stick form. The stick representation of residues with colour (E138-pink and R194-blue) indicates mutated and black sticks represent non-mutated residues. The black circle indicates functional active site. If the CmNP would be a “dimer of homotrimers”, the combination of arginine finger (R194) and Walker B (E138) mutations (*cis*- and *trans*-element) would inactivate five out of six active sites and thus reduce its ATPase activity drastically. If the CmNP would be a “trimer of homodimers” this mutation combination would result in three of six active sites and theoretically, should have half or more ATPase compared to WT. **(B)** ATPase assay with wild-type and variants (5 μ M protomer) in the presence of RuBP (1 μ M) measured at 45 °C. Thus the formation of a “dimer of homotrimer” is highly unlikely. Error bars indicate the mean and s.d. with n=3.

4.4. Active site mutants reveal that the subunits are highly coordinated

In most cases mutating the Walker A motif of an AAA+ protein results in a nucleotide-binding deficient enzyme (Wendler et al. 2012). In the bacterial activase RsRca, a mutation in the Walker A motif was consistent with an ATP binding defect, rendering it inactive for both ATPase and Rubisco activation functions (Mueller-Cajar et al. 2011).

The conserved lysine residue (K80) of the Walker A motif (red sticks) in nucleotide (ATP) binding site is shown in all subunits (alternating gold and grey surfaces represent CmN and CmP respectively) and a closer view of the active site containing the K80 is also shown (Figure 4.5A). The CmNP model is based on the atomic model of the RsRca hexamer (PDB:3ZUH). Both CmN and CmP isoforms contain a highly conserved Walker A motif (GXXXXGK) in their AAA+ module (Hanson and Whiteheart 2005) (Figure 3.1). So, we investigated the relative role of Walker A motif by each isoform subunits in the process of ATP hydrolysis and activation of Rubisco by substituting the conserved lysine residue (K80) with alanine. The three variants with mutations in CmN or CmP or both were cloned and purified (CmN^{AP}, CmNP^A and CmN^{AP}A). These proteins were first checked for proper assembly using analytical gel filtration. The elution profile for all variants was similar compared to the wild-type activase, indicating the mutation did not result in an oligomerization defect as all three variants stably assembled as hexameric oligomers (Figure 4.5B).

Next, for all variants, the rate of ATP hydrolysis was measured at 25 °C and 45 °C, in the presence or absence of RuBP (1 mM), and also in the presence of its substrate. All Walker A variants failed to show any basal ATPase activity (in the absence of RuBP) or stimulation of ATPase in the presence of RuBP at both measured temperatures (Figure 4.5C, D). Although there appears to be a weak ATPase activity for the mutants at 25 °C measurement we consider this negligible since measurements of $\sim 0.3 \text{ min}^{-1}$ are close to the instrumental detection limit. Overall dysfunctional Walker A motif in CmN or CmP isoform rendered the complex (with other functional isoform subunits) inactive for ATPase function and predictably the variant with all subunits mutated for Walker A also resulted in no ATPase function (Figure 4.5C, D). This shows that the subunits are highly coordinated for ATP hydrolysis and one

possible explanation would involve obligate binding of a nucleotide in the subunit adjacent to the one involved in ATP hydrolysis. We plan to investigate this issue further in the future.

In the CmNP^A and CmN^AP variants, although presumably ATP binding in mutated active sites of one isoform is likely impaired, nucleotide could theoretically bind to the active sites of the other isoform and display comparable ATPase activity, but in fact, the complex becomes inactive for ATP hydrolysis. So, in spite of three alternative catalytically active subunits with intact *cis* (Walker A and B) and *trans* (arginine finger) elements capable of ATP binding and hydrolysis, no activity was detected. This shows that the isoforms are highly functionally coupled. In contrast m-AAA YTA10/12 (Arlt et al. 1996, Augustin et al. 2009) and peroxisomal PEX1/PEX6 (Blok et al. 2015, Gardner et al. 2015) heterocomplexes behaved differently. Walker A motif defects in either isoform subunits of both the complex did not completely abolish the ATPase activity of CmNP complex but only displayed a partial inhibition (Yta10/12 ~40%, Pex1/6 ~30% active) indicating that the isoforms of either complexes are independently active and not as tightly coupled as the isoforms of CmNP complex for ATPase. Nevertheless, similar phenotype as observed for CmNP Walker A variants was reported recently for an admittedly artificial situation of a covalently linked dimer of ClpB D1 AAA+ module (Zeymer et al. 2014). Here a Walker A mutation resulted in complete loss of ATPase i.e. no catalytic activity from the neighbouring intact active site. Since our results also indicate that ATPase activity at any catalytic site is dependent on the nucleotide-bound state of its neighbour, hence both CmN and CmP active sites must be nucleotide binding competent for ATPase activity. This suggests the nucleotide bound status of a subunit is being allosterically communicated to the neighbouring subunits influencing its activity (inter-subunit communication). Further, no ATPase activity was seen even in the presence of its protein substrates (Figure 4.5C, D) and consequently no activation of its substrates was detected at all measured conditions (Table 4.1).

Figure 4.5| Impaired nucleotide binding reveal strong co-operativity between subunits.

Characterization of Walker A motif defective variants (K80A) of CmNP to understand the role of CmN and CmP in ATP hydrolysis and Rubisco activation. **(A)** Ribbon representation of activase model (PDB:3ZUH) with Walker A residue (K80) shown in the sticks (Red). CmN depicted in gold and CmP subunits depicted in grey (Left side). Closer view of the K80 (red sticks) in the active site (Right side). Variants with alternate and all K80 residues mutated to A was made and characterized. **(B)** Size exclusion chromatography profiles for the Walker A variants compared to WT (20 μ g each) eluted on a Superdex 200 PC3.2/30 column equilibrated with purification buffer A. **(C, D)** Walker A mutation (K80A) in either subunit abolishes the ATPase activity. ATPase activity of the CmNP Walker A variants (K80A) (5 μ M protomer) in the absence (apo) or presence of RuBP (1 mM) and ER/ECM-C of Rs and Cm Rubiscos (3 μ M active sites) measured at 25 °C **(C)** and 45 °C **(D)**. ATPase inactive variants failed to target and activate inhibited Rubiscos at 25 °C and 45 °C. All ATPase were performed in the presence of 1 mM RuBP and ATP, except Apo (absence of RuBP). Error bars indicate the mean and s.d. of at least three independent experiments.

Table 4.1 A: Rubisco activity of Walker A mutants (25 °C)

CmNP Mutants	Activase Activity (25 °C)			
	RsER	RsECM-C	CmER	CmECM-C
CmNP ^A	No Activity (<1% of WT)			
CmN ^A P	No Activity (<1% of WT)			
CmN ^A P ^A	No Activity (<1% of WT)			

Table 4.1 B: Rubisco activity of Walker A mutants (45 °C)

CmNP Mutants	Activase Activity (45 °C)		
	CmER	CmECM-C	RsECM-C
CmNP ^A	No Activity (<1% of WT)		
CmN ^A P	No Activity (<1% of WT)		
CmN ^A P ^A	No Activity (<1% of WT)		

Activase activity of Walker A (K80A) variants assayed with 5 μ M activase protomer in the presence of inactivated Rubiscos of Rs and Cm (0.5 μ M active sites, 10 mM NaHCO₃) at 25 °C and 45 °C showed no activity (Table 4.1). All Rubisco assays were performed in the presence of 1 mM RuBP and ATP, except Apo (absence of RuBP).

The arginine finger mediates allosteric communication between subunits and senses the nucleotide binding state of the adjacent subunit (Wendler et al. 2012). From the RsRca model, Arg 194 residue, close to the nucleotide-binding site of the subsequent subunit was identified as the putative arginine finger. Mutation of Arg 194 (arginine finger) in RsRca affected the oligomerisation of the enzyme (Mueller-Cajar et al. 2011). The arginine finger variant formed fibrillar structures in negative stain EM in the presence of ATP, indicating the activase binds to ATP. But the same variant did not facilitate the formation of hexamer in the presence of ATP and RuBP, suggesting the arginine finger mutated subunits were unable to respond to RuBP binding by forming functionally active hexameric ring and consequently due to the absence of the active oligomeric state no ATPase and activase function was displayed by the variant (Mueller-Cajar et al. 2011).

Figure 4.6| Arginine finger mutants.

Characterization of R-finger defective variants (R194A) of CmNP (**A**) Ribbon representation of activase model (PDB:3ZUH) with alternating subunits of CmN (gold) and CmP (grey) is shown with arginine finger (R-finger) residue (R194) in the sticks (blue) (Left side). Closer view of the R194 (blue sticks) from the supplied subunit participating in the active site of its neighbouring subunit (Right side). Variants with alternate and all R194 residue mutated to A was made and characterized. (**B**) Size exclusion chromatography profiles for the arginine finger variants compared to WT (20 μ g each) eluted on a Superdex 200 PC3.2/30 column equilibrated with purification buffer A. (**C**, **D**) Defective R-finger in either alternating isoform abolished the ATPase function. ATPase activity of the R-finger variants (5 μ M protomer) in the absence (apo) or presence of RuBP (1 mM) and ER/ECM-C Rubiscos of Rs and Cm (3 μ M active sites) measured at 25 °C (**C**) and 45 °C (**D**). R-finger variants failed to target and activate inhibited Rubiscos measured at 25 °C and 45 °C. Rubisco activation activity of R-finger (194A) variants assayed with 5 μ M activase protomer in the presence of Rs and Cm inactivated Rubiscos (0.5 μ M active sites, 10 mM NaHCO_3) at 25 °C and 45 °C (**Table 4.2**). All ATPase assays were performed in the presence of 1 mM RuBP and ATP, except Apo (absence of RuBP). Error bars indicate the mean and s.d of at least three independent experiments.

Table 4.2 A: Rubisco activity of arginine finger mutants (25 °C)

CmNP Mutants	Activase Activity (25 °C)			
	RsER	RsECM-C	CmER	CmECM-C
CmNP ^R	No Activity (<1% of WT)			
CmN ^R P	No Activity (<1% of WT)			
CmN ^R P ^R	No Activity (<1% of WT)			

Table 4.2 B: Rubisco activity of arginine finger mutants (45 °C)

CmNP Mutants	Activase Activity (45 °C)		
	CmER	CmECM-C	RsECM-C
CmNP ^R	No Activity (<1% of WT)		
CmN ^R P	No Activity (<1% of WT)		
CmN ^R P ^R	No Activity (<1% of WT)		

R-finger variants failed to target and activate inhibited Rubiscos measured at 25 °C and 45 °C. Rubisco activation activity of R-finger (194A) variants assayed with 5 µM activase protomer in the presence of Rs and Cm inactivated Rubiscos (0.5 µM active sites, 10 mM NaHCO₃) at 25 °C and 45 °C (Table 4.2). All Rubisco assays were performed in the presence of 1 mM RuBP and ATP, except Apo (absence of RuBP).

Through sequence alignment, we found that the arginine finger appears fully conserved in bacterial and algal isoforms of red-type activase (Chapter 3, Figure 3.1). Figure 4.6A shows the arginine finger (R194) as blue sticks in the ribbon representation of the CmNP model. Close view showing the arginine finger of CmN (gold ribbon) forming the active site of the neighbouring CmP (grey ribbon). To understand the relative influence of arginine fingers of CmN and CmP in ATPase function and intersubunit communication, we decided to replace conserved arginine residues by alanine in CmN (CmN^RP) or CmP (CmNP^R) or in both (CmN^RP^R). All three variants with arginine finger mutation resulted in the loss of ATPase activity and consequently, Rubisco activase function at both 25 °C and 45 °C (Figure 4.6C, D)(Table 4.2), as observed with RsRca (Mueller-Cajar et al. 2011). These phenotypes were thus very similar to the Walker A mutants (Figure 4.5). Apparently in CmNP^R, CmN subunits catalytic active sites are compromised because of the mutated trans-acting arginine finger from the neighbouring CmP subunit. Similarly, in CmN^RP, catalytic sites of CmP subunits are inactive owing to mutated arginine finger from CmN subunits. However in this arrangement the CmN subunits possess intact catalytic sites that could be expected to be functional. But, the variant was ATPase inactive and non-functional, emphasizing allosteric regulation of ATPase by arginine fingers. Also, it was important to characterize the oligomerization of the variants to

confirm the loss of activity was due to the allosteric effect of the arginine fingers and not by mutation induced oligomerization changes. We performed analytical gel filtration runs with wild-type activase and variants with arginine finger mutation. All three variants showed no oligomeric transitions and had same elution profile as wild-type, representing the hexameric species even in the apo state (Figure 4.6B). Arginine finger mutation nevertheless did not impact the oligomerisation of the red algal activase, in contrast to the bacterial activase.

Complete loss of ATPase activity (even basal activity) in the Arginine mutants with intact active sites in hexameric conformation (Figure 4.6C,D), strongly suggests that arginine finger not only influences the catalytic site of the subunit it contacts (trans) but also influences the subunit that supplied (cis) the arginine finger as observed for covalently linked ClpB dimer by Zeymer et.al (Zeymer et al. 2014). As observed for Walker A mutants these results suggest strong cooperativity within subunits for ATPase hydrolysis. Hence disrupting either, CmN or CmP active sites by Walker A or arginine finger mutations result in complete inactivity of the CmNP complex (Figure 4.5 and 4.6).

4.5. ATP-hydrolysis by the Nuclear encoded isoform is sufficient for activase activity

The Walker B mutant (E138Q) of the bacterial activase was reported to be ATPase inactive and unable to activate its Rubisco substrate (Mueller-Cajar et al. 2011). Regarding the oligomeric transitions observed for RsRca, this mutant behaves like the wild-type (Mueller-Cajar, unpublished data), suggesting the Walker B mutant is nucleotide-binding competent, but cannot hydrolyze ATP. This is the phenotype observed for Glu-Gln substitutions of the catalytic Walker B glutamate (Hanson and Whiteheart 2005, Hersch et al. 2005). In the YTA10/12 heteromeric complex, variants with Walker B mutation in different isoforms were reported to maintain significant fractional functionality (Augustin et al. 2009). Figure 4.7A shows the glutamic acid residue (E138) as red sticks in the ribbon representation of the CmNP model. Close view showing the position of the E138 residue of CmN (gold ribbon) in its nucleotide binding pocket. To probe the role of nucleotide binding to the CmNP isoforms for their functionality, we introduced single mutation E138Q in both isoforms (Figure 4.7A). Three variants (NP^B, N^BP and N^BP^B) were made and tested for oligomerization

defects. All three variants stably formed hexameric species (Figure 4.7B). We anticipated the complex with only three catalytic sites of the CmN or CmP would have at least half or less ATPase activity compared to wild-type. Unexpectedly, NP^B was able to hydrolyse ATP twice as fast as wild-type which harbours six functional catalytic sites. The ATPase activity of the complex with mutated CmP subunits showed a 2 fold increase in basal activity and stimulation with RuBP compared to wild-type (Figure 4.7C). Hence, ATP binding to all CmP subunits appears to unleash the ATPase activity at the CmN catalytic site. ATP bound CmP subunits in the complex thus appear to trigger a favourable conformational state for ATP hydrolysis at CmN active sites. However, this mutant did not exhibit significant ATPase stimulation with the protein substrates at both temperatures (Figure 4.7C, E). The substrate processing in a number of AAA+ (ClpX, ClpB, DNA threading proteins) proteins is shown to couple the ATP hydrolysis energy to the conformation changes of the central pore loop (Martin et al. 2008a). It has been demonstrated that impairing the active sites of the subunits affects the substrate processing capability. Since the active sites of CmP have been impaired by Walker B mutation we expected that the ATP powered conformational changes in the pore loops of CmP would also be impaired and as a consequence we expected a reduced activation rate for different substrates. But on the contrary to our expectation the CmNP^B variant activated all Rubiscos at almost the same rate as the wild-type at all measured conditions except for CmER (86% at 25 °C and 72% at 45 °C) (Figure 4.7D, F)(Table 4.3). Contrary to the published data in other AAA+ systems, our data suggests that ATPase activity of CmP in the hetero complex might not be coupled to the conformational changes for the substrate processing by its pore. Unexpectedly, It was interesting to note that although the working ATPase was higher than wild-type, the variant exhibited only a very weak ATPase stimulation with the substrates compared to 1.5-2 fold increase for the wild-type. This suggests a substrate-triggered switch is no longer functional. One such switch in AAA ATPases has been described as the “glutamate switch” (Zhang and Wigley 2008). This allows us to assume that the loss of the switch may have resulted in the highest gear with maximum ATP hydrolysis in the presence of RuBP and so no further stimulation could be achieved by the substrates. With only three ATP hydrolysis-competent subunits of CmN (CmNP^B), almost wild-type activation rates were achieved (Table 4.3). Assuming that the hydrolysis generates mechanical energy for the pore loops in its own subunit, then CmN pore loops alone, are

competent for substrate remodelling. This suggests that pore loops of CmP subunits are of minor importance to translocate the substrate for activation. But they might be essential for grasping/ holding the substrate during translocation cycle until the next CmN subunit pulls the substrate (until the next power stroke by CmN occurs). Assuming full nucleotide occupancy at Walker B mutated subunits, it appears that ATP binding at CmP allosterically regulates the ATPase activity of the complex by affecting the ATP binding and hydrolysis at its neighbouring site. A similar result was also seen in a ClpB-based system (Zeymer et al. 2014).

Table 4.3 A: Rubisco activity of Walker B mutants (25 °C)

CmNP WT/Mutants	Relative Activity (%WT)			
	RsER	RsECM-C	CmER	CmECM-C
CmNP	100 ± 10.94	100 ± 6.71	99.57 ± 10.3	100
CmNP^B	101.02 ± 6.19	n.m	85.9 ± 5.44	97.14 ± 6.86
CmN^BP	No Activity (<1% of WT)			
CmN^BP^B	No Activity (<1% of WT)			

Table 4.3 B: Rubisco activity of Walker B mutants (45 °C)

CmNP WT/Mutants	Relative Activity (%WT)		
	CmER	CmECM-C	RsECM-C
CmNP	100 ± 7.25	100.9 ± 8.53	100
CmNP^B	71.9 ± 2.34	100.65 ± 3.09	n.m
CmN^BP	No Activity (<1% of WT)		
CmN^BP^B	No Activity (<1% of WT)		

Figure 4.7| ATP binding to CmP unleashes ATPase activity of CmN.

Characterization of Walker B defective variants (E138Q) of CmNP. **(A)** Ribbon representation of activase model (PDB:3ZUH) with alternating subunits of CmN (gold) and CmP (grey) is shown with putative Walker B residue (E138) in the sticks (pink) (Left side). Closer view of the E138 (pink sticks) in the active site (Right side). Variants with alternate and all E138 residues mutated to Q was made and characterized. **(B)** Size exclusion chromatography profiles for the Walker B variants compared to WT (20 µg each) eluted on a Superdex 200 PC3.2/30 column equilibrated with purification buffer A. **(C, E)** ATP binding to Walker B defective CmP unleashes ATPase activity of CmN while CmN Walker B defective variants is non-functional. ATPase activity of the CmNP Walker B variants (5 µM protomer) in the absence (apo) or presence of RuBP (1 mM) and ER/ECM-C Rubiscos of Rs and Cm (3 µM active sites) measured at 25 °C **(C)** and 45 °C **(E)**. **(D)** (Table 4.3A) Rubisco activation activity of Walker B variants measured with 1 µM activase protomer for RsER (0.3 µM active sites, 10 mM NaHCO₃) or 2 µM activase for CmER and CmECM-C (0.3 µM active sites, 10 mM NaHCO₃), measured at 25 °C. **(F)** (Table 4.3B) Rubisco activity of Walker B variants measured with 1µM activase for CmER and CmECM-C (0.3 µM active sites, 10 mM NaHCO₃), at 45 °C. All ATPase and Rubisco assays were performed in the presence of 1 mM RuBP and ATP, except ATPase activity of Apo form (absence of only RuBP). Error bars indicate the mean and s.d of at least three independent experiments. (n.m in the table means activity is observed but not measured).

Interestingly, when only CmN contained the Walker B mutation (CmN^{BP}), the complex was completely ATPase-deficient despite the presence of three wild-type ATPase pockets in CmP (Figure 4.7C, E). As expected, in the CmN and CmP Walker B double mutant, no ATPase activity was observed (CmN^{BP}P^B) (Figure 4.7C, E).

ATPase activity $< 0.3 \text{ min}^{-1}$ at 45 °C was considered within instrumental uncertainty. Thus we speculated that CmP is completely inhibited when CmN is trapped in a permanently ATP-bound state or CmP has no ATPase activity. In any case when CmN is mutated the complex becomes inactive. No Rubisco activation activity was observed with both the variants (Figure 4.7D, F). We also observed that independent of CmP's neighbouring subunit (CmN) in ATP-bound or unbound state, variant with non-mutated CmP (CmN^{AP}, CmN^{BP}) was functionally inactive (Figure 4.5 and 4.7). These results strongly imply that CmP isoform does not contribute to the ATPase activity of the complex and it may be catalytically inert. This evidence collectively suggests that ATP hydrolysis may occur only at the CmN active site while CmP regulates the hydrolysis rate by just binding ATP molecules. From the kinetic analysis of the CmNP^B, K_m for ATP was reduced by 31% in the presence of RuBP (1 mM). This showed increased binding affinity for the ATP molecules by this variant resulted in increased V_{max} in the presence of RuBP (Figure 4.8). Interestingly the co-operativity regarding ATP binding earlier observed for the wild-type (Figure 3.13) was not observed for CmNP^B, and this result warrants further investigation.

Figure 4.8| RuBP induced kinetic changes in the CmNP^B variant.

Dependence of the activase ATPase rates on ATP concentration. The rate of ATP hydrolysis is plotted against ATP concentration for 5 μ M activase in the presence of RuBP (1 mM) (filled triangles) or absence of RuBP (hollow triangles). Kinetic analysis of CmNP^B ATPase activity measured at 45 °C in presence and absence of 1 mM RuBP. The Michaelis-Menten model (dashed lines) provided a good fit to both the Apo data ($V_{\max}$ =16 $\pm$ 1.15 min⁻¹, K_m =0.32 $\pm$ 0.1 mM) and the RuBP-bound form. ($V_{\max}$ =34.1 $\pm$ 2 min⁻¹, K_m =0.23 $\pm$ 0.05 mM).

In summary ATP-bound state of CmP subunits appears to influence ATP binding to and hydrolysis by the CmN subunits and hence ATP-bound CmP conformation is favourable for increased ATPase rate at CmN site. Since CmP will be unable to hydrolyse ATP in CmNP^B, we propose its pore loop tyrosines will remain stationary during activase function. In this context, the CmP tyrosines may perform a holdase role, whereas the CmN tyrosines will be moving and allow threading to occur. The energy of the increased ATPase hydrolysis rate may compensate for the assumed immobility of the CmP pore loops.

When the catalytic site of the CmN isoform was made defective for hydrolysis ATPase was abolished. Eventually, ATP binding competent CmP failed to have any hydrolysis at its active site and there is no substrate activation. The ATPase phenotype of CmNP (E138Q) variants was partially similar to the other heterocomplexes studied (Augustin et al. 2009, Ciniawsky et al. 2015, Gardner et al. 2015) except no increased ATPase was observed in these systems due to altered inter-subunit signaling in activase. Consistent with those results, one isoform is functionally dominant (in our case CmN) over the other.

The next question we considered concerned the nature of the ATP binding signal from one subunit to the other. How do the subunits communicate with each other regarding ATP hydrolysis, substrate targeting and/or subsequent activation? The literature suggests that in AAA+ proteins communication between subunits is mediated by the R-finger. ATP binding could be signalled to the next subunit through its arginine fingers. So, we next investigated the role of arginine fingers in inter-subunit communication.

4.6. Gain of ATPase function by CmN^RP when Walker B motif of CmP is mutated

As discussed earlier (section 4.2), we found N^RP^B, showed ATPase activity similar to the wild-type. On the other hand, the inverse variant was ATPase inactive.

Figure 4.9A shows the glutamic acid residue (E138) and arginine finger (R194) as sticks in the ribbon representation of the CmNP model. E138 of one subunit and R194 of the neighbour subunit form an active site. Three active sites are formed by non-mutated residues (black sticks; coloured sticks are mutated residues) in CmN^BP^R (left) and CmN^RP^B (right).

Intrigued by this surprising result, we characterized N^RP^B further. We found no oligomerisation defect compared to the wild-type (Figure 4.9B). We noticed that its basal ATPase activity was similar to N^PB variant, which is twice that of a basal activity of wild-type CmNP (Figure 4.9C, E). However, no stimulation by RuBP was observed, thus resulting in similar activity to CmNP in the presence of the ligand. This trend was observed at both temperatures (Figure 4.9C, E). In this variant the active site of CmN is intact, but both the *cis* and *trans* acting elements of CmP active sites are mutated. Consequently, CmP will be catalytically inert. Notably, N^RP^B variant achieving the same ATPase rate as the wild-type with only active sites of CmN isoform subunits once more suggests that the CmP subunits are unlikely to contribute to the ATPase activity of the wild-type CmNP. Further, N^RP^B showed negligible stimulation of ATPase with its substrates at 25 °C and 45 °C (Figure 4.9C, E). Accordingly, activase function was severely crippled in spite of full ATP-hydrolysing capacity (Table 4.4). The inverse mutant N^BP^R showed no ATPase activity in the presence and absence of RuBP, no ATPase or Rubisco activation activity with substrates was recorded for N^BP^R at 25 °C (Figure 4.9C, D) and 45 °C (Figure 4.9E, F) (Table 4.4).

Table 4.4 A: Rubisco activity of Walker B/arginine finger mutants (25 °C)

CmNP Mutants	Activase Activity (25 °C)			
	RsER	RsECM-C	CmER	CmECM-C
CmN ^R P ^B	No Activity (<1% of WT)			
CmN ^B P ^R	No Activity (<1% of WT)			

Table 4.4 B: Rubisco activity of Walker B/arginine finger mutants (45 °C)

CmNP Mutants	Activase Activity (45 °C)		
	CmER	CmECM-C	RsECM-C
CmN ^R P ^B	No Activity (<1% of WT)		
CmN ^B P ^R	No Activity (<1% of WT)		

Figure 4.9| The ATPase activity of a CmN arginine finger mutant can be restored by a concomitant Walker B mutation in CmP.

(A) Ribbon representation of activase model (PDB:3ZUH) with alternating subunits of CmN (gold) and CmP (grey) showing arginine finger (R194) and Walker B residue (E138) in the sticks. Right side shows CmN^BP^R variant with CmN mutated for E138 (pink) and CmP mutated for R194 (blue) and other variant CmN^RP^B with mutations reversed (left side). The residues represented as black sticks are non-mutated. (B) Size exclusion chromatography profiles for the variants compared to WT (20 µg each) eluted on a Superdex 200 PC3.2/30 column equilibrated with purification buffer A. (C, E) ATPase activities of CmNP variants harbouring mutations both in the R-finger of CmN (R194A) and Walker B motif of CmP (E138Q) hydrolyse ATP comparably well as opposed to variant with CmN harbouring E138Q and CmP with the R-finger mutation (R194A). ATPase activities with CmNP variants (5 µM protomer) in the absence (apo) or presence of RuBP (1 mM) and with ER/ECM-C Rubiscos (3 µM active sites) measured at 25 °C (C) and 45 °C (E). (D, F) (Table 4.4) Both variants cannot activate their Rubisco substrates. Rubisco activity assays of activated (ECM) and inactivated (ER) RsRbcLS (0.5 µM active sites, 10 mM NaHCO₃) in the presence and absence of CmNP variants (5 µM protomer) measured at 25 °C (D). Rubisco activity assays of activated (ECM) and inactivated (ER) CmRbcLS (0.5 µM active sites, 10 mM NaHCO₃) in the presence and absence of CmNP variants (5 µM protomer) measured at 45 °C (F). (Table 4.4) Summary of activase activity of CmN^RP^B and CmN^BP^R (5 µM protomer) with Rs and Cm Rubiscos (0.5 µM) at 25 °C and 45 °C. All ATPase and Rubisco assays were performed in the presence of 1 mM RuBP and ATP, except ATPase activity of Apo form (absence of only RuBP). Error bars indicate the mean and s.d of at least three independent experiments.

Given that arginine finger mutation in any isoform subunits made the complex non-functional, surprisingly introducing an additional Walker B mutation into CmP isoform of the N^{RP} inactive complex resulted in a gain-of-function, with the resulting variant N^{RPB} being ATPase active. Since ATPase and activase activity are completely uncoupled in CmN^{RPB}, the arginine finger of the nuclear subunit is thus implicated in coupling the energy of ATP hydrolysis to conformational changes in Rubisco.

Thus this atypical phenotype observed in N^{RPB} variant can be argued as both gain-of-function (in ATPase) to inactive arginine variant (CmN^{RP}) or a loss of function (in Rubisco activation) to an active Walker B variant (CmNP^B). A conceivable explanation is that locking CmP into a nucleotide trapped state as a result of the Walker B mutation overcomes the R finger defect by favouring a hydrolysis-competent conformation for the CmP arginine finger. Hence this variant gave a better insight to understand inter- and intra- subunit communication pathway.

4.7. Variants at the RuBP binding site are severely functionally impaired

A unique feature of the RsRca enzyme is the existence of a conserved allosteric site for the binding of its effector RuBP located in the C-terminal subdomain that regulates its oligomeric state and activity (Mueller-Cajar et al. 2011). The other Rubisco activases classes CbbQO (Tsai et al. 2015) and plant activases (Stotz et al. 2011) do not respond to RuBP. Binding of RuBP to RsRca promotes hexamer ring formation and stimulates ATPase rate. From the crystal structure, RuBP is predicted to bind to a positively charged pocket formed by highly conserved residues R239, S250, N253, R257 and R261 at the α -helical subdomain, adjacent to the inter-subunit interface of RsRca. Mutation of the RuBP binding site severely affects oligomerization as mutants of these residues form fibrils rather than ring-shaped species in the presence of ATP and RuBP. Biochemically they are ATPase inactive in the presence of RuBP (Mueller-Cajar et al. 2011).

Measured at 25°C

Measured at 45°C

Table 4.5 A: Relative Rubisco activity of RuBP binding defective mutants (25°C)

CmNP WT/Mutants	Relative Activity (%WT)			
	RsER	RsECM-C	CmER	CmECM-C
CmNP	100 ± 10.94	100 ± 6.71	99.57 ± 10.3	100
CmNP^S	10.68 ± 1.57	n.m	9.6 ± 0.9	8.16
CmN^SP	No Activity (<1% of WT)			
CmN^SP^S	No Activity (<1% of WT)			

Table 4.5 A: Relative Rubisco activity of RuBP binding defective mutants (45°C)

CmNP WT/Mutants	Relative Activity (%WT)		
	CmER	CmECM-C	RsECM-C
CmNP	100 ± 4.4	100.9 ± 8.53	100
CmNP^S	13.02 ± 0.67	13.07 ± 5.6	n.m
CmN^SP	No Activity (<1% of WT)		
CmN^SP^S	No Activity (<1% of WT)		

Figure 4.10| Characterization of variants mutated at the RuBP binding pocket.

(A) Surface representation of activase model (PDB:3ZUH) with alternating subunits of CmN (gold) and CmP (grey) showing RuBP binding pocket with interacting residue R239 highlighted in green. Closer view of the RuBP pocket is shown in ribbon representation with residue R239 in stick representation (green). (B) Size exclusion chromatography profiles for the RuBP binding defective variants compared to WT (20 µg each) eluted on a Superdex 200 PC3.2/30 column equilibrated with purification buffer A. (C, E) CmP mutated variant showed no ATPase stimulation while CmN mutated variants were completely ATPase inactive. ATPase activity of the CmNP RuBP binding variants (5 µM protomer) in the absence (apo) or presence of RuBP (1 mM) and ER/ECM-C Rubiscos (3 µM active sites) measured at 25 °C (C) and 45 °C (E). (D, F) (Table 4.5) Weak activase activity by CmP mutated variant but none for CmN mutated variants relative to WT. (D) Rubisco activation activity of RuBP variants measured with 1 µM activase protomer for RsER (0.3 µM active sites, 10 mM NaHCO₃) or 2 µM activase protomer for CmER and CmECM-C (0.3 µM active sites, 10 mM NaHCO₃), measured at 25 °C. (F) Rubisco activity of RuBP variants measured with 2 µM activase protomer for CmER and CmECM-C (0.3 µM active sites, 10 mM NaHCO₃), at 45 °C. All ATPase and Rubisco assays were performed in the presence of 1 mM RuBP and ATP, except ATPase activity of Apo form (absence of only RuBP). Error bars indicate the mean and s.d of at least three independent experiments. (n.m in the table means activity is observed but not measured).

The residues involved in RuBP binding are conserved in both red algal activase isoforms (Figure 3.1). However, RuBP does not influence the oligomeric transition of red algal activase and CmNP has a basal ATPase activity (Figure 3.9, 3.17). Indeed, CmNP acts under the allosteric regulation of RuBP (Figure 3.7) with stimulation of ATP hydrolysis in the presence of RuBP. It is not clear in our heteromeric system how important RuBP binding is to activase function and whether it has to bind to both subunit types. Clarifying this issue would improve our understanding how the RuBP is involved in coupling ATP binding and hydrolysis to Rubisco remodelling and would further help to advance our understanding in developing a working model of the hetero-oligomeric complex activase complex.

The RuBP binding pocket and the conserved interacting residue R239 highlighted in green is shown in Figure 4.10A using the surface representation of CmNP model. Close view shows the position of R239 residue (green sticks) in the RuBP binding pocket. The conserved RuBP binding residue in CmN and CmP, R239 was mutated to alanine to probe the role of RuBP binding to the isoform subunits in regulating the ATPase activity and Rubisco activation. Three CmNP variants (CmNP^S, CmN^SP and CmN^SP^S, where S stands for sugar binding) were purified and analysed using analytical gel chromatography for any oligomeric state transitions as a result of mutation. All variants eluted at 1.23 ml, similar to the wild-type indicating the hexameric nature of the mutants (Figure 4.10B). When CmNP^S was analysed at its physiological temperature, the basal ATPase rate was only half of wild-type (2 min⁻¹) and was not stimulated by RuBP or its Rubisco substrates (Figure 4.10E). However, it very weakly activated all the Rubisco substrates tested (Figure 4.10D, F). Though the measured activity was only 10-15% relative to wild-type at limiting concentrations of activase and Rubisco (Table 4.5), the activation rate increased with the increase in activase concentration for RsER and CmER (Figure 4.11A, B). The ATPase and the activase activity phenotype of this variant when assayed at 25 °C was similar to that of 45 °C (Figure 4.10C, D). However, it remains uncertain why the R239 mutation results in reduced basal ATPase activity at both temperatures. We suspect that the mutation at this residue in some way affected interaction with a nucleotide in the subunit. Unexpectedly the other two variants, with CmN mutated (CmN^SP and CmN^SP^S) were completely ATPase inactive and consequently did not mediate the activation of inhibited Rubiscos at all measured conditions (Figure 4.10C-F).

Increasing the concentration of the variants did not result in measurable activities (Figure 4.11A, B). The observed results are likely due to a significant structural perturbation in the R239A mutants that impair an ATPase hydrolysis-competent conformation to be achieved and thus complicates interpretation of these variants. To answer questions regarding RuBP binding to CmN additional mutants (of RuBP interacting residues) in the binding sites should be generated which would maintain the wild-type basal ATPase activity.

To probe in detail the RuBP binding mediated signaling by CmP in the hetero-oligomer, we mutated Walker B (E138Q) and arginine finger (R194A) in addition to the RuBP interacting mutation (R239A) in both subunits. The CmNP variants (CmNP^{BS}, CmNP^{RS}, CmN^{BS}P and CmN^{RS}P) were purified and analysed using analytical gel chromatography for any oligomeric state transitions as a result of mutation. All variants eluted similar to the wild-type indicating the hexameric nature of the mutants (Figure 4.12A). When the variants were analysed, except CmNP^{BS} all the variants were completely inactive and the ATPase phenotype of all the variants at 25 °C was similar to that of 45 °C (Figure 4.12B-D). The inactive phenotype of CmN^{BS}P and CmN^{RS}P could be due to the speculated structural perturbation occurring due to the mutation of R239 in CmN (CmN^SP), as discussed above. Although CmNP^S had a basal ATPase activity, additionally mutating the arginine finger (CmNP^{RS}) made the complex to ATPase inactive mutant (Figure 4.12C, D). The loss of ATPase activity of CmNP^{RS} corresponds to the loss of Rubisco activation activity (Figure 4.12B) confirming the role of arginine finger in ATPase activity. The CmNP^{BS} variant was particularly interesting because the increased basal ATPase activity of this variant was similar to that of CmNP^B, indicating a gain of function of CmNP^S (Figure 4.12C, D). However, there was no ATPase stimulation by RuBP, indicating that conformational transition for the ATPase stimulation at the CmN active sites is likely mediated by the CmP RuBP binding site. However, the activation rate was still low despite increased basal ATPase activity (Figure 4.12B). Hence RuBP binding by CmP is crucial for regulating the ATPase activity and Rubisco remodelling.

Figure 4.11| (A, B) Dependence of the rate of Rubisco activation on the concentration of the CmNP^S variant.

Rubisco assays were performed with activated (CmECM) and inactivated (CmER) Rubiscos (0.5 μM active sites and 10 mM NaHCO_3) measured at 45 °C (**A**), and with activated (RsECM) and inactivated (RsER) Rubiscos (0.5 μM active sites and 10 mM NaHCO_3) measured at 25 °C (**B**) in the absence and presence of activases. The concentration of the activase used in (**A**, **B**) is 5 μM protomer if otherwise indicated for CmNP^S. All ATPase and Rubisco assays were performed in the presence of 1 mM RuBP and ATP, except ATPase activity of Apo form (absence of only RuBP). Error bars indicate the mean and s.d of at least three independent experiments.

Figure 4.12| RuBP-dependent stimulation of CmNP^B requires the CmP RuBP binding site.

(A) Size exclusion chromatography profiles for the double mutant variants compared to WT (20 μ g each) eluted on a Superdex 200 PC3.2/30 column equilibrated with purification buffer A. (B) Weak activase activity by CmNP^{BS} while other double mutants were inactive. Rubisco activity assays performed with activated (RsECM) and inactivated (RsER) Rubisco (0.5 μ M active sites) in the presence and absence of the activases (5 μ M protomer) measured at 25 °C. (C,D) CmNP^{BS} showed ATPase activity similar to CmNP^B but no stimulation by RuBP and the other double mutant variants were completely ATPase inactive. ATPase activity of the RuBP binding variants (5 μ M protomer) in the absence (apo) or presence of RuBP (1 mM) measured at 25 °C (C) and 45 °C (D) was compared with WT, CmNP^S and CmNP^B ATPase activity. All ATPase and Rubisco assays were performed in the presence of 1 mM RuBP and ATP, except ATPase activity of Apo form (absence of just RuBP). Error bars indicate the mean and s.d of at least three independent experiments. The absence of error bars indicates only a single experiment was performed.

In summary, the phenotype of the CmNP^{BS} variant provided insights about the importance of CmP's interaction with RuBP in the regulation of hetero-oligomeric function. In future, we plan to characterize these variants further to understand more about its phenotypes in activating its cognate Rubiscos.

4.8. The pore loop tyrosines of both isoforms contribute to CmNP function

In the best characterized AAA+ protein remodelers, ATPase activity is coupled to protein remodelling via its axial pore loop, which engages the protein substrate for remodelling. Likewise in CbbX, engagement of the Rubisco C-terminal tail by the central pore loops of CbbX is part of the current mechanistic model. It was shown that mutating the conserved aromatic tyrosine residue in pore motif of CbbX (Mueller-Cajar et al. 2011) and ClpX (Siddiqui et al. 2004) homo hexamer proteins eliminated or severely diminished the enzymes function, demonstrating the importance of the aromatic residue of the motif in substrate targeting and remodelling. The conserved tyrosine residues (Y114) of the pore loop (red stick) in all subunits are depicted in the ribbon representation of the CmNP model. A closer view shows how the Y114 lines the central pore of the CmNP activase (Figure 4.13A). To determine the relative contribution of CmN and CmP in substrate remodelling we used the pore loop variants (Y114A) of CmNP to examine pore loop 1 function for activation of bacterial and algal Rubisco substrates at 25 °C and 45 °C. These experiments are especially interesting since CmNP^B suggests that the CmP subunit appears to have a low or non-existent ATP hydrolysis rate (Figure 4.7C, D).

The pore loop variants did not show any oligomeric changes compared to the wild-type (Figure 4.13B). The variants with just CmN mutated (N^YP) and both isoforms mutated (N^YP^Y) showed an increased ATPase rate of ~1.5 and 1.75 fold respectively (Figure 4.13C, E). The phenotype of increased ATPase of pore variants was also observed with bacterial activase, as the mutation in all the subunits of the homohexameric complex (Rs^{Y114A}) showed double the ATPase rate of the wild-type (Mueller-Cajar et al. 2011). Similar observations in ClpX have been explained by reasoning that the mutations with the decreased side chain volumes (Y→A) tend to increase ATPase rates, with tighter packing of residues in the pore resulting in slower conformational transitions (Martin et al. 2008a) by removing the restrictive contacts of Y sidechains (Iosefson et al. 2015). The same effect was also seen in FtsH

(Yamada-Inagawa et al. 2003) and HsIU (Park et al. 2005). Unlike the other two variants, when only the pore loops of CmP were mutated (NP^Y), the basal ATPase rate and stimulation by RuBP were similar to the wild-type (Figure 4.13C, E).

Inhibited Rubisco did not stimulate the ATPase activity of the pore loop mutant of bacterial activase (RsRca^{Y114A}) (Mueller-Cajar et al. 2011) consistent with a lack of substrate engagement. Therefore we also examined the effects of substrates on the stimulation of the ATPase activity of CmNP pore loop variants. No stimulation in ATPase was noted for NP^Y with all substrates at 25 °C and 45 °C. Although some stimulation with substrates was noted for NP^Y at 25 °C, this was not observed at 45 °C and further analysis (such as upwards titration of Rubisco concentrations) would be necessary to establish significance (Figure 4.13C, E). The ATPase rate of NP^Y was no longer stimulated by *R. sphaeroides* Rubisco complexes at both measured temperatures. But interestingly *C. merolae* Rubisco complexes weakly stimulated the ATPase activity of the NP^Y variant suggesting successful cognate substrate engagement by the variant with intact CmN pore loops. Additionally, the ATPase activity with CmECM-C was recorded to be maximum for NP^Y variant (Figure 4.13C, E). Having observed that the mutants had varied ATPase rate, we subsequently tested if these variants could use this increased motor activity to remodel the substrate at both temperatures.

Table 4.6 A: Relative Rubisco activity of Pore loop mutants (25 °C)

CmNP WT/Mutants	Relative Activity (%WT)			
	RsER	RsECM-C	CmER	CmECM-C
CmNP	100 ± 10.94	100 ± 6.71	99.57 ± 10.3	100
CmNP^Y	12.3 ± 0.6	No Activity (<1% of WT)	5.12 ± 3.3	No Activity (<1% of WT)
CmN^YP		No Activity (<1% of WT)		
CmN^YP^Y		No Activity (<1% of WT)		

Table 4.6 B: Relative Rubisco activity of Pore loop mutants (45 °C)

CmNP WT/Mutants	Relative Activity (%WT)		
	CmER	CmECM-C	RsECM-C
CmNP	100 ± 4.4	100.9 ± 8.53	100
CmNP ^Y	18.9 ± 1.67	No Activity (<1% of WT)	
CmN ^Y P	No Activity (<1% of WT)		
CmN ^Y P ^Y	No Activity (<1% of WT)		

Figure 4.13| Pore loop mutation uncouples ATPase and activase activity.

(A) Ribbon representation of activase model (PDB:3ZUH) with alternating subunits of CmN (gold) and CmP (grey) highlighting Y144 residue in red. Closer view of the top surface of the activase model, showing conserved Y114 residue in the sticks (red) lining the central pore (left side). (B) Size exclusion chromatography profiles for the pore loop variants compared to WT (20 µg each) eluted on a Superdex 200 PC3.2/30 column equilibrated with purification buffer A. (C,E) Rates of ATP hydrolysis measured for each pore loop CmNP variant (5 µM protomer), Apo in the absence or in the presence of RuBP (1 mM) or in the presence of RuBP and CABP inactivated Rubiscos (ER/ECM-C) of Rs and Cm (3 µM active sites) at 25 °C (C) and 45 °C (E). (D, F) Weak activity by mutated pore loops of CmP (CmNP^Y) but none when pore loops of CmN mutated (CmN^YP) relative to WT. (D)(Table 4.6A) Relative Rubisco activation activities measured for pore loop variants (1 µM protomer) with RsER (0.3 µM active sites, 10 mM NaHCO₃) or CmER (0.3 µM active sites, 10 mM NaHCO₃) with 2 µM protomer at 25 °C. (F) (Table 4.6B) Relative Rubisco activities measured for pore loop variants (2 µM) with CmER (0.3 µM active sites, 10 mM NaHCO₃) at 45 °C. All ATPase and Rubisco assays were performed in the presence of 1 mM RuBP and ATP, except ATPase activity of Apo form (absence of only RuBP). Error bars indicate the mean and s.d of at least three independent experiments.

Despite possessing the three functional pore loops of CmN subunits, the CmNP^Y variant merely activated RsER with a rate of ~ 10% and CmER with <10% compared to wild-type CmNP at 25 °C (Figure 4.13D) (Table 4.6A). But at the higher temperature, this variant activated CmER at a rate of ~20% relative to wild-type (Figure 4.13F)(Table 4.6B). Most interestingly when this variant was challenged with CABP-Rubisco complexes, it failed to activate (Table 4.6). This is likely due to the tight binding nature of CABP conferring higher stability to the ECM-C complex than the ER (Pearce 2006). Thus to activate very stable substrates like the ECM-C complex, either more or all pore loops are required to be intact. CmN^YP and CmN^YP^Y were unable to activate any substrate at both 25 °C and 45 °C (Table 4.6 and Figure 4.13D, F). Hence, CmNP pore-loop variants were most deleterious when the mutation was in the CmN subunit of the complex. Although there is a high ATPase activity and possibly exhibiting some stimulation in the presence of some substrates (Figure 4.13C, E), these variants failed to activate their substrate. Clearly, as expected mutation in pore loops uncouples ATPase activity and activase function. Overall, the conserved pore residues appear to be important for regulated ATP hydrolysis by CmNP. Pore loop mutations may reduce activase ability by failing to tightly engage the C-tail of Rubisco into the central pore of the activase (or by allowing the Rubisco to continuously slip from the central pore) before the activase is successful in helping the release of inhibitor from the Rubisco. It indicates that substrate is being targeted to the pore and only activated if the complex has functional CmN pore loops. Interestingly, though ECM-C substrates stimulated the ATPase activity of the NP^Y mutants but could not be activated. Consequently, the aromatic side chain of CmN pore loop seems to have a special role in substrate gripping and remodelling during a power stroke than CmP, but is not exclusively responsible for its substrate remodelling. The defective pore loops in CmNP^Y reduced the activity by 80% (Table 4.6), showing that the CmP subunits also contribute to engagement of the substrate during a power stroke. Although less indispensable than the CmN subunit it appears both CmN and CmP have to work together to successfully remodel the inhibited Rubisco. Future work measuring the relative efficiency of such mutants in activation will be insightful. We hypothesize that similar to ClpX much more ATP will be hydrolysed for remodelling an active site when the pore is perturbed.

In a recent substrate gripping study of ClpX, it was shown that at least two adjacent wild-type pore loops (Y-Y pair) are necessary for effective substrate threading. Alternatively, if mutated pore loop (A) was placed next to wild-type in the ring, it resulted in a severely defective substrate processing. The mutated pore loop is denoted by A and wild-type pore loop by Y for ClpX. Utilizing the previously discussed system by Martin et. al (Martin et al. 2005) Dimer of trimer of mutated and a wild-type pore loops arrangement in a ring (A-A-A-Y-Y-Y) was reported to be quite active as opposed to the variant with alternating mutated and wild-type arrangement (Y-A-Y-A-Y-A) in the hexameric ring, which was completely inactive (Iosefson et al. 2015). By analogy our results are also consistent with our model of an alternating arrangement of the CmN and CmP isoforms Presence of functional pair of CmN and CmP adjacent to each other could be vital for the proper engagement, gripping and remodelling of the Rubisco C-tail and this explains the observed decrease in the rate of activation by CmNP^Y which could be attributed to the absence of functional CmP pore loop adjacent to the CmN. This suggests that engagement of Rubisco also involves CmP subunit pore loops for the activation.

4.9. The role of the conserved surface lysine 123

Although the presented results confirm that the protein-processing pore of CmNP plays a role in the recognition and engagement of Rubisco substrates, it is certain that there will be more than one site of interaction of CmNP for the different Rubisco substrates. At present with the absence of Rubisco-activase complex structure, we do not know exactly the different sites of interaction. But from the structure of RsRca, it was predicted that the protruding lysine (K 123) residue on the highly conserved top surface of the hexamer could interact with Rubisco. On mutating this conserved lysine residue, the activation rate of RsRca was dramatically reduced to about 20% (Mueller-Cajar et al. 2011), but ATPase was increased (similar to the pore loop mutant), indicating the possibility of interaction with Rubisco.

Table 4.7 A. Relative Rubisco activity of surface exposed lysine mutants (25 °C)

CmNP WT/Mutants	Relative Activity (%WT)			
	RsER	RsECM-C	CmER	CmECM-C
CmNP	100 ± 10.94	100 ± 6.71	99.57 ± 10.3	100
CmNP^{K123A}	21.7 ± 2.6	22.9 ± 0.9	35.9	n.m
CmN^{K123A}P	No Activity (<1% of WT)			
CmN^{K123A}P^{K123A}	No Activity (<1% of WT)			

Table 4.7 B. Relative Rubisco activity of surface exposed lysine mutants (45 °C)

CmNP WT/Mutants	Relative Activity (%WT)		
	CmER	CmECM-C	RsECM-C
CmNP	100 ± 4.4	100.9 ± 8.53	100
CmNP^S	25.76 ± 2.6	25.8 ± 0.5	31.03
CmN^SP	No Activity (<1% of WT)		
CmN^SP^S	No Activity (<1% of WT)		

Figure 4.14| Surface lysine 123 of CmNP is critical for Rubisco engagement.

(a) Surface representation of activase model (PDB:3ZUH) with alternating subunits of CmN (gold) and CmP (grey) highlighting the surface K123 residue in blue. Closer view of the top surface of the hexamer model with K123 located close to the central pore (blue). (B) Size exclusion chromatography profiles for the surface lysine variants compared to WT (20 µg each) eluted on a Superdex 200 PC3.2/30 column equilibrated with purification buffer A. (C, E) Weak ATPase stimulation by only CmP mutated variant in the presence RuBP but in the presence of substrates while CmN mutated variants were completely inactive. ATPase activity of the CmNP variants (K123A) (5 µM protomer) in the absence (apo) or presence of RuBP (1 mM) and ER/ECM-C Rubiscos (3 µM active sites) measured at 25 °C (C) and 45 °C (E). (D, F) (Table 4.7) K123 mutated at CmP (CmNP^{K123A}) showed weak activase activity relative to WT. Rubisco activity of surface lysine (K123A) variants measured with 1 µM activase for RsER (0.3 µM active sites, 10 mM NaHCO₃) or 2 µM activase protomer for CmER and RsECM-C (0.3 µM active sites, 10 mM NaHCO₃), measured at 25 °C (D). Rubisco activity of surface lysine (K123A) variants measured with 2 µM activase protomer for CmER, CmECM-C and RsECM-C (0.3 µM active sites, 10 mM NaHCO₃) at 45 °C (F). (G) Dependence of the rate of Rubisco activation on the concentration of the CmNP^{K123A} variant. Rubisco assays were performed with activated (CmECM) and inactivated (CmER) Rubiscos (0.5 µM active sites and 10 mM NaHCO₃) in the absence and presence of activases (5 µM) measured at 45 °C. All ATPase and Rubisco assays were performed in the presence of 1 mM RuBP and ATP, except ATPase activity of Apo form (absence of only RuBP). Error bars indicate the mean and s.d of at least three independent experiments. The absence of error bars indicates only a single experiment was performed. (n.m in the table means activity is observed but not measured).

Figure 4.14A shows the lysine (K123) highlighted in blue on the surface of all subunits of the CmNP model. A closer view shows the surface protrusion of the K123 and its proximity to the pore loop tyrosine residue. To investigate the possible roles of this residue in CmNP and relative function of the isoforms we purified three variants with the substitution of alanine at lysine 123 in only CmN or CmP and in both isoforms of the wild-type complex. As predicted these variants showed no oligomerization defects (Figure 4.14B).

Based on the demonstrated non-equivalency of subunits, we hypothesized that CmNP might bind to Rubisco in a specific orientation. Hence CmN and CmP may encounter different surfaces. The variant with Ala substitution for Lys 123 in CmP subunits (CmNP^{K123A}) showed basal ATPase rate and stimulation by RuBP similar to wild-type (Figure 4.14C, E). Nevertheless, no Rubisco substrates stimulated the variants ATPase activity despite activation of Rubisco substrates (Figure 4.14C-F) (Table 4.7). Conversely mutation of K 123 in CmN subunits either independently or together with CmP (CmN^{K123A}P and CmN^{K123A}P^{K123A}) completely abolished the ATPase activity and thus substrate activation (Figure 4.14C-F) (Table 4.7). This phenotype is unexpected because K 123 residue does not directly contribute to the active site nor involved in ATP binding. Unfortunately, the severe defect of this mutation precludes any interpretation regarding the function of this residue in the nuclear subunit and limits analysis to CmNP^{K123A}.

The measured rate of activation of CmER with limiting concentration of CmNP^{K123A} (1 μ M) was ~20% of the wild-type at 45 °C. But at higher activase concentration (5 μ M) of the variant, the rate of activation was increased (Figure 4.14G). These results are consistent with the idea that the K 123 of the plastid isoform being involved in substrate binding. However, its reduced essentiality at higher concentrations indicates that the CmN lysine alone is sufficient for successful Rubisco engagement.

The equivalent mutation in RsRca resulted in an almost doubled ATPase rate in the presence of RuBP and retained ~20% activation rate compared to wild-type RsRca (Mueller-Cajar et al. 2011). In conclusion mutation in all subunits of RsRca was tolerated but not in the heteromeric CmNP complex. Mutation in alternating CmP subunits of the complex is tolerated but not vice versa. This suggests the positively

charged K123 residues are involved in protein-protein interactions by functioning as anchoring sites at negatively charged pocket the Rubisco large subunit surface. The functionality of CmNP^{K123A} indicates three anchoring sites are sufficient.

Subsequently, we tried to predict the possible binding sites on the Rubisco for the red-type activase. Earlier studies have shown that certain surface residues of Rubisco are involved in the activase interaction. The surface exposed P89 residue in the large subunit of the higher plants (non-Solanaceae) was shown to be essential for plant activase interaction (Larson et al. 1997). A recent study from our lab showed that mutating the corresponding surface exposed residues in *Acidithiobacillus ferrooxidans* (Af) Rubisco, D82 of the of the AfL and the E75 in AfM eliminated or severely affected the activation by CbbQO activase (Tsai et al. 2015). Based on these we hypothesized that the positively charged K123 could likely interact with the negatively charged E90 or the adjacent P91 on the surface of the large subunit. Coincidentally the distance between the alternative K123 residues on the RsRca hexamer model and the distance between the negatively charged E90 in the large subunit dimer (RbcL2) is approximately the same, ~41 Å (Figure 4.15A). Our hypothetical model is schematically illustrated in Figure 4.15A. The corresponding residues E90A and P91 were mutated to alanine. The Rubisco variants (RsE90A and RsP91A) were purified. The Rubisco activity of fully activated ECM and inhibited ER of the respective Rubisco variants were similar to the wild-type (80-90%), indicating that the mutations did not largely affect the catalytic performance (Figure 4.15B, C). According to our hypothesis, the activation of inhibited Rubiscos by the activase should be impaired. Alternatively, the activation of inhibited RsP91A by the activases was only weakly reduced compared to the activation of inhibited wild-type Rubisco (Figure 4.15C). However, the activation of inhibited RsE90A by the activase was significantly reduced compared to the wild-type (Figure 4.15B), even though not severely impaired. Although these preliminary results suggest that E90 and P91 of RsRbc are not the key target residues for the activase, but the observed reduction in the activation rate of E90 variant gives some hope. Possibly other structural interactions of the Rubisco- activase in addition to the pore loop are necessary for Rubisco activation. So in future, activation assays with careful titrations of activases and characterization of more surface residue variants of the RsRbc will need to be performed.

A

B

C

Figure 4.15| E90 and P91 are not essential for activation by red-type activases.

(A) Schematic illustration using activase and Rubisco model to show the hypothetical interaction of K123 residue with Rubisco during the remodelling. Surface representation of activase model (PDB:3ZUH) highlighting the pore loop tyrosine residue (Y114) (purple) and lysine123 on the surface (blue) with the distance between the K123 residue in the alternating CmN (gold surface) subunit is indicated (dotted line) and the nucleotide is shown in the sticks (Left). Y114 and K123 are shown to be crucial for substrate activation. Y114 is likely involved in the C-tail threading, but the role of K123 is unknown. The distance of the E90/P91 on RbcL dimer is similar to the distance of alternating K123 residue on the activase (41 Å). We predicted that K123 could interact with the E90 or P91 (blue on the Rubisco) residues and this binding will act as an anchor to hold the Rubisco close to the activase while the pore loop partially threads the C-tail (yellow tail on the Rubisco) during the remodelling process (Right). (B) The rate of activation of RsE90A Rubisco variant by the RsRca and CmNP was reduced compared to the results RsER which indicate E90 residue could be involved in the remodelling process. (C) RsRca and CmNP showed an almost similar rate of activation of RsP91A Rubisco variant, indicating that P91 residue not likely to be involved in the remodelling process. Rubisco assays were performed with activated (RsE90A/RsP91AECM) and inactivated (RsE90A/RsP91AER) Rubiscos (0.5 µM active sites and 10 mM NaHCO₃) the absence and presence of RsRca and CmNP (5 µM protomer) measured at 25 °C. The RsER WT data are shown in dotted lines for comparison with the variants data. All Rubisco assays were performed in the presence of 1 mM RuBP and ATP. Error bars indicate the mean and s.d of at least three independent experiments.

4.10. 3D structure of the CmNP predicted by homology modeling

From the mutational studies of the CmNP we observed interesting results and atypical phenotypes which helped us to propose a mechanistic model for its ATPase activity and Rubisco activation activity. The variants in our study were generated based on the conserved motifs and elements between RsRca, CmN and CmP (Figure 1.16A). These variants helped only to understand the basic working mechanism of the CmNP activase system. However, towards understanding mechanism in detail a structure guided functional characterization approach has to be adopted (i.e. with the aid of the CmNP structure the role of the residues involved in subunit-subunit interaction, Rubisco interaction etc.). The limitation for this approach is that no experimentally determined structure of the CmNP activase is currently available. So we constructed a homology model of the CmNP activase using I-TASSER server (Roy et al. 2010).

The protein structures of individual isoform CmN and CmP were computationally predicted with RsRca (PDB:3ZUH) as a reference structure template (Figure 4.16B). Structural alignment of CmN and CmP homology models (with 54% sequence similarity) in PyMol showed very minor differences as indicated with arrows in the figure (Figure 4.16B). Similarly aligning homology models of CmN and CmP with RsRca showed very minor differences for CmN and Rca (Figure 1.16C), while the structures of CmP and RsRca was almost identical (Figure 4.16D). Subsequently, CmNP complex with alternating arrangement of isoform subunits (N-P-N-P-N-P) and same isoform subunits arrangement (N-N-N-P-P-P) models were constructed using PyMol (Figure 4.17A,B). Because of high sequence similarity (Figure 4.16A) (~60%) and structural similarity between the template and target proteins, the homology model of CmNP was similar to the RsRca. To computationally predict the subunit arrangement in CmNP complex, the generated 3D protein models of the CmN and CmP was docked against each other using PatchDock (Schneidman-Duhovny et al. 2005). The docking score for heterodimer of CmN and CmP (which was similar to homodimer of RsRca) was more than homodimer of CmN or CmP. Although this suggests the possibility of alternating subunit arrangement further validation has to be done in future. However, the 3D homology model of CmNP can

be used in future for structure-guided mutations for further understand the role of individual isoform.

Figure 4.16| Sequence and structural alignments of CmN and CmP homology models.

(A) Amino acid sequences of RsRca, CmN and CmP were aligned using Multalin (Corpet 1988) and sequence similarity is shown in table (right). Functional motifs and key residues as determined previously (Mueller-Cajar et al. 2011) are indicated using coloured bars and triangles. Secondary structure elements of RsRca (PDB: 3SYL) are shown above the sequences. Uniprot accession codes of the CbbX sequences are: *Rhodobacter sphaeroides* (RsRca-P95648), *Cyanidioschyzon merolae* (nuclear: M1UXG8, residues 95-401; plastid: O22025). The figure was prepared using Esprict (Gouet et al. 1999). (B-D) RsRca (blue), homology models of CmN (red) and CmP (green) (from I-TASSER) were structurally aligned using PyMol. (B) Structural alignment of CmN and CmP. (C) Structural alignment of CmN and RsRca. (D) Structural alignment of CmP and RsRca. Homology models of CmN and CmP and RsRca are shown as cartoon representation in red, green and blue respectively. The arrow indicates an extra turn in the $\alpha 11$ of CmN model.

Figure 4.17| Subunit arrangement model with homology models of CmN and CmP with RsRca.

(A) CmNP complex with alternating arrangement of isoforms (trimer of heterodimer). (B) CmNP complex with same subunit arrangement (dimer of homotrimer). Top panel shows cartoon representation and bottom panel shows surface representation of the CmNP arrangement. These CmNP models were made using Pymol with homology models of CmN (red) and CmP (green).

4.11. Summary

Following the characterization of the CmNP hexameric complex in Chapter Three, we initially wanted to learn the arrangement of the complex. Our biochemical results supported alternating arrangement of CmN and CmP partners. We went on to comprehensively dissect the relative role of the CmN and CmP partners in ATPase activity and activase function. Simultaneously our goals included understanding the regulation and mechanism of ATPase and Rubisco activation. We found that mutating key residues of the nucleotide binding site (Walker A and arginine finger) of either CmN or CmP resulted in complete inactivity of the activase. Subunits thus showed strong cooperativity for ATP hydrolysis. Nucleotide bound state of the CmP (Walker B mutation) counterintuitively increased the ATPase rate of the complex and the Rubisco activation rate was largely equivalent to wild-type. Disrupting pore loop function affects Rubisco engagement, thus affecting ATPase stimulation by Rubisco and its activation. Mutating pore loops of CmP was tolerated but for CmN it abolished the Rubisco activation. Conserved surface lysine residue may play a role in substrate engagement. Unfortunately, regarding the RuBP binding residue and the conserved surface lysine residue, mutation resulted in reduced basal ATPase activity, and this impaired the strength of the conclusions we could draw. However, in summary, we found that mutating any key residues in CmN was in general not tolerated and resulted in a completely inactive complex. Hence, CmN emerged as a dominant partner in CmNP, and ATP hydrolysis in its active site is sufficient to generate enough force to remodel Rubisco. CmP, on the other hand, appears to play more of a structural and regulatory role in activase activity. Importantly, our results suggest that when CmP active sites are nucleotide-bound, they signal to an adjacent nuclear subunit and stimulate its ATPase activity. We envisage that further development and refinement of the results presented in this Chapter will provide useful information to a wide range of workers interested in the mechanisms of molecular chaperones of the AAA+ class.

5. CHARACTERIZATION OF AN α - CYANOBACTERIAL CbbX PROTEIN

The work presented thus far has supported the biochemical function of CbbX proteins as Rubisco activases that have co-evolved with red-type Rubiscos which evolutionarily have a proteobacterial origin (Delwiche and Palmer 1996). Since all red-type Rubiscos found encoded in genomes so far appear to be associated with *cbbX* homologues, CbbX proteins have been designated as so-called red-type activases (Hauser et al. 2015). However, it has been known for some time, and this has been underlined by recent large scale genome sequencing efforts (Zarzycki et al. 2012, Shih et al. 2013), that *cbbX* genes are also found distributed in both α - and β -cyanobacterial genomes. This is not consistent with a red-type Rubisco activase function since all cyanobacteria encode green-type Rubiscos (either FormIA or FormIB) (Badger and Bek 2008).

Cyanobacterial *cbbX* is generally found to be positioned in the neighbourhood of genes encoding carboxysomal proteins downstream of the Rubisco subunit encoding genes (*rbcL* and *rbcS*) indicating that the cyanobacterial CbbX could likely function as a carboxysomal activase (Figure 5.1B).

Alignment of red-type activases encoded by proteobacteria with similar amino acid sequences from cyanobacteria containing green-type Rubiscos indicated that the key motifs and residues highlighted in Figure 5.1A (under the sequences) are highly conserved in all α -type and with a few exception in β type cyanobacterias (Figure 5.1A). The pore loop1 which was shown to be critical for the activation of Rubiscos by the red-type activase (this study) was not conserved in some β -cyanobacterial sequences (Figure 5.1A), indicating that a different mechanism of activation could have evolved for CbbX in these organisms.

In Figure 5.1C sequences of few red and green-type Rubisco large subunits are aligned to show that in general the large subunits of green-type Rubiscos from cyanobacteria or plants lack the extended C-terminus region (boxed in blue) which is shown to be important for the activity by red-type activases. This indicates the cyanobacterial CbbX should have evolved to identify an alternative structural element on the surface of the green-type Rubisco in a somewhat similar way as green-type activase to recognise the inhibited Rubisco forms. (Figure 5.1C).

[illegible]

Figure 5.1| Sequence alignment of cyanobacterial red-type Rubisco activase sequences.

(A) Amino acid sequences of CbbX proteins from α -cyanobacteria and β -cyanobacteria were aligned using T-Coffee (Notredame et al. 2000). Functional motifs and key residues as determined previously (Mueller-Cajar et al. 2011) are indicated using coloured bars and triangles. Secondary structure elements of RsRca (PDB: 3SYL) are shown above the sequences. (B) Gene neighborhood of α -carboxysome-associated *cbbX*. The representative α -carboxysome locus encoded in the genome of in *Syn. WH8102* (top), *MIT9313* (middle) and *MIT9303* (bottom). (C) Alignment of Rubisco Large subunit C-termini of representative four red and five green Rubisco proteins using T-Coffee (Notredame et al. 2000). Similar residues are shown in red on a yellow background and identical residues in white on red background. Framed sequences on coloured background indicate homologous regions. Blue background highlights C-tail extension differences between red-type and green-type large subunit sequences. UniProt or NCBI accession codes are shown adjacent (left side) to the respective CbbX or RbcL sequences. The figures were prepared using Esript (Gouet et al. 1999).

Figure 5.2| Phylogenetic tree of Cyanobacterial CbbX protein sequences.

The sequence alignment shown in Figure 5.1 was used to produce a phylogenetic tree by Neighbor-Joining in the MEGA6 software suite (Tamura, Stecher et al. 2013) using the p-distance model and pairwise gap deletion. Bootstrap values are the percentage from 1000 replicates where a given node was present.

Phylogenetic analysis of the aligned CbbX sequences indicated that both α - and β -cyanobacterial activases form separate branches. The phylogenetic relationship of β -cyanobacterial encoded activases appears to be closer to proteobacterial than the α -cyanobacterial encoded counterparts (Figure 5.2). In spite of the non-conserved pore loop motif critical for the activation function, β -cyanobacterial activases are in close evolutionary terms with the respective proteins of proteobacteria while α -cyanobacterial activases seem to have diverged during evolution. Both types of cyanobacterial sequences are more closely related to the red algal plastid encoded activase than the widely branched nuclear encoded activase (CmN). Overall our analysis appears to be consistent with the suggestion of Hovde et al. (Hovde et al. 2015) that plastid genome encoded (CmP) and cyanobacterial activases could have been derived from a proteobacterial ancestor.

We chose *Synechococcus* sp. WH8102 CbbX (boxed in green in Figure 5.2) which is relatively diverged (68% sequence identity) from the proteobacterial activase to confirm its activase function and to study if there is a new mechanism evolved in this species to maintain the function of its green-type Form IA carboxysomal Rubisco. To characterize this activation system, we produced the activase and Rubisco protein (in vitro).

5.1. Purification of cyanobacterial activase and Rubisco proteins

In order to characterize the Rubisco activation system of WH8102, the activase and the Rubisco genes were cloned from the genomic DNA into the respective plasmids as described in the methods. The genomic DNA was a kind gift from Dr. Dean Price (Australian National University).

WH8102 activase (8102Rca) (Uniprot: Q7TTT9) was produced in BL21 (DE3) cells harbouring pHue8102Rca and the pBADESL plasmid encoding the GroEL/ES chaperonin system. The protein was purified as described in methods. The pure protein used in the following studies after the polishing step (size exclusion chromatography) is shown in Figure 5.3A.

The wild-type WH8102 Rubisco and its variant WH8102RsC with an additional C-terminal extension (TASVAM), and wild-type MIT9313 Rubisco expressed by

pET30WH8102RbcLS, pET30WH8102RsC and pET24bMIT9313RbcLS respectively in BL21 (DE3) cells were purified as described in the methods. The pET30WH8102RsC construct was made by my colleague Dr. Tsai Yi-Chin. The pure Rubisco proteins after the final purification step, which are to be used in the following assays are shown in the Figure 5.3B.

5.2. Regulation of the oligomeric states by the ligands

Considering the quite diverse behaviour exhibited by proteobacterial and red algal activases as described in Section 3.5 of this thesis, we were interested to see whether the cyanobacterial protein resembled either of these systems. Hence we performed analytical gel filtration analysis of the cyanobacterial activase. The shift in the elution peak towards a higher oligomeric state with an increase in the concentration of 8102Rca implies a polydisperse nature of the apo enzyme (Figure 5.4). In the presence of the ligand ATP, 8102Rca elutes at a void volume which indicates a very large oligomeric form. This suggests a fibrillar structure formation with ATP similar to RsRca (Figure 5.4). 8102Rca with both ATP and RuBP shows a shift in the elution peak (1.14 ml) (Figure 5.4). The corresponding Stokes radius (R_s) calculated for the elution volume of the activase in the presence of ATP and RuBP using standard curve was 6.61 nm, which would be consistent with a ~ 130 Å diameter. This estimation is very close to the measured diameter of the RsRca ring (~ 140 Å) (Mueller-Cajar et al. 2011). Thus it strongly indicates ATP and RuBP induce the hexameric ring formation of the 8102Rca enzyme. Overall these experiments indicate that 8102Rca undergoes similar oligomeric transitions as the proteobacterium RsRca when exposed to the ligands Mg-ATP and RuBP.

Since the regulation by these ligands is conserved in 8102Rca, we subsequently wanted to understand if the activation function 8102Rca with red-type Rubiscos is also conserved.

Figure 5.3| SDS-PAGE analysis of the purified activase and the Rubisco proteins.

(A) 12.5% SDS-PAGE gel. The molecular weight of marker bands and location of the pure 8102Rca after the final size exclusion purification process are indicated. (B) 16% SDS-PAGE gel with 3 μ g protein loaded per lane. Molecular weight markers as well as the location of the Rubisco large and small subunit are indicated. Lane1-WH8102Rbc (WT), Lane2-WH8102Rbc with RsRbc C-tail and Lane3-MIT9313Rbc (WT).

Figure 5.4| Analysis of the oligomeric state of WH8102 activase.

Elution profiles for the 8102Rca in Apo form, activases in the presence 1 mM ATP and 5 mM MgCl_2 and in the presence of 0.1 mM RuBP, 1 mM ATP and 5 mM MgCl_2 measured at 280 nM. Independent run in Superdex 200 PC3.2/30 column was loaded with 20 µg of protein unless otherwise indicated and eluted using purification buffer A at pH=8

5.3. WH8102Rca is a functional Rubisco activase

8102Rca is a red-type activase found with a green-type Rubisco (Form IA). This gave rise to a hypothesis that 8102Rca could have evolved or adopted a new mechanism to activate the green-type Rubisco without a carboxy terminal extension (C-tail) of the large subunit (RbcL) is required according to the transient threading model (Figure 5.1C). In chapter three we showed that CmNP (red algal activase complex), although found with Form ID red-type Rubisco can activate *R. sphaeroides* red-type Rubiscos (Form IC) and at 35 °C RsRca activated red algal Rubiscos (Form ID). This indicates that red-type activases found with red-type Rubiscos can interchangeably activate their Rubiscos. However, to extend the interpretation to cyanobacterial red-type activases, we wanted to know whether it has retained the ability to activate red-type Rubiscos.

So we used the RuBP (RsER) and CABP (RsECM-C) inhibited Rubisco complex of *R. sphaeroides* to test the functionality of 8102Rca as a red-type activase. These inhibited complexes along with the activated complex (RsECM) were prepared as explained in Methods (Section 2.2.3.1) to be used in Rubisco and ATPase activity assays. On comparing the carboxylation activities of RsECM and RsER in the presence or absence of activases, 8102Rca activated RsER implying that 8102Rca is a functional red-type activase (Figure 5.5A). Comparatively, the activation by 8102Rca was observed to be faster than CmNP but similar to RsRca (Figure 5.5A). 8102Rca displayed ATPase activity only in the presence of RuBP ($\sim 10 \text{ min}^{-1}$) (Figure 5.5B). The ATP hydrolysis rate was further stimulated in the presence of RsER in a concentration dependent manner up to threefold and the approximate concentration of RsER to reach half maximum rate was $1.3 \text{ }\mu\text{M}$ (Figure 5.5C). The magnitude of stimulation by RsER varied significantly between the activases and at saturating concentrations the ATPase activity was increased up to 8-fold for RsRca and 3-fold for 8102Rca and CmNP (Figure 5.5C).

Next to check the thermostability of the 8102Rca, the enzyme was incubated at various temperatures for 10 min followed by ATPase measurement at the same temperature. Although ATPase peaked at 30 °C, it dropped sharply thereafter and was lost completely around 40 °C (Figure 5.4D). 8102Rca appear to be the most thermolabile red-type activase studied so far. The reason could be that *Synechococcus*

species inhabits the ocean where the temperature is relatively stable and does not reach extremes. So the activase enzyme of these organisms did not need to evolve thermostable properties.

Surprisingly, carboxylation activity assay with RsECM-C in the presence of activases revealed that 8102Rca was unable to remove CABP from the RsECM-C complex to make it active (Figure 5.5E). This phenotype is in contrast to the other two red-type activases tested (RsRca and CmNP) but happens to be similar to plant activases, which activate their green-type Rubiscos (Form IB) (Robinson and Portis 1988). It thus appears that 8102Rca cannot generate enough force (unlike RsRca and CmNP) to remove the tightly bound inhibitor from the active sites of the carbamylated Rubisco. Since no activation was observed, we expected to see no stimulation of ATPase activity of 8102Rca by RsECM-C, but in contrary the ATPase rate was significantly stimulated by RsECM-C in a concentration-dependent manner, comparable to the stimulation by RsER (Figure 5.5F, C). The accelerated ATP hydrolysis suggests that 8102Rca was able to recognize and make a productive interaction with ECM-C but could not achieve in remodelling it.

Figure 5.5| WH8102 activase can activate bacterial red-type Rubisco.

8102Rca activates RuBP inhibited but not CABP inhibited Rubisco complex of *R. sphaeroides*. Rubisco activity assays were performed with activated (RsECM) and inactivated Rubiscos (0.5 μ M Rubisco complexes and 10 mM NaHCO₃), RsER (**A**) and RsECM-C (**E**) in the absence and presence of cyanobacterial, bacterial and red algal activases (5 μ M). (**B**) ATPase activity rate of 5 μ M 8102Rca enzyme (protomer) was measured in the absence (Apo state) and in the presence of 1 mM RuBP together with inactivated (RsER and RsECM-C) Rubisco complex (3 μ M). Stimulation of ATPase activity of 8102Rca by different concentrations of RsER (**C**) and RsECM-C (**F**) compared with ATPase stimulation of RsRca and CmNP. ATPase rate was measured with the concentration of inactivated (RsER and RsECM-C) Rubiscos as indicated in the figure in the presence of 1 mM RuBP and with respective activase (5 μ M). (**D**) To study the effect of temperature on 8102Rca, ATPase rate of the activase (5 μ M) was measured at incremental temperatures from 25 °C up to 55 °C with 5 °C intervals. The enzyme was heated (10 mins) at respective temperature prior to the measurement at the same in the presence of 1 mM RuBP. All measurements were made at 25°C except for the thermostability study (**D**). All ATPase and Rubisco assays were performed in the presence of 1 mM ATP. Error bars shown are the standard deviation of three independent experiments.

5.4. Mechanism of activation of RsER by 8102Rca is consistent with the C-tail threading model

We had previously shown that both CmNP and RsRca activated RsER in a manner consistent with transient threading of the C-tail of RbcL through the central pore (Section 3.4.3). If 8102Rca is the activase of its cognate green-type cyanobacterial Rubisco, the C-tail would not be available, thus an alternative mechanism of activation may exist. We used the C-tail deletion mutants (Rs Δ 2 and Rs Δ 4) test whether 8102Rca uses the same mechanism for activation or adopted a different mechanism.

The ECM and ER complexes of both Rubisco mutants were prepared. Activation of Rs Δ 2ER by 8102Rca was severely impaired (Figure 5.6A). The relative activation rate Rs Δ 2ER by 8102Rca compared to RsRca was ~14% (Figure 5.6C). 8102Rca was unable to activate Rs Δ 4ER (Figure 5.6B). This relative rate was similar to that of RsRca. This was unexpected since it implies the mechanism is conserved in 8102Rca. Moreover, 8102Rca was only weakly stimulated by inactive Rs Δ 2ER and Rs Δ 4ER complexes which is in agreement with activation data (Figure 5.6D). These stimulated ATPase rates by Rs Δ 2ER for 8102Rca and RsRca seem to be similar, but ATPase stimulation by Rs Δ 4ER was seen only for 8102Rca whereas CmNP showed no stimulation by ER complexes of both the deletion Rubisco mutants.

Overall, ATPase stimulation by Rubisco substrates showed that 8102Rca could target the inactive complex of red-type Form IC Rubiscos and activate them by the C-tail dependent mechanism, consistent with the transient threading mechanism model proposed for RsRca and CmNP. Thus above results convince that 8102Rca is a functional red-type activase. Red-type activase from different phylogeny have conserved the same mechanism of activation regardless of being associated with green or red-type Rubiscos.

Figure 5.6| Activation of RsER by 8102Rca is via the conserved C-tail dependent mechanism.

(A)(B) Activation of *R. sphaeroides* inhibited complex by 8102Rca is impeded by the deletion of C-tail residues. (C) The rate of activation of Rs Δ 2ER by all three activases is compared with the WT Rubisco. Rubisco activity assays were performed with activated (Rs Δ 2/Rs Δ 4 ECM) and inactivated (Rs Δ 2/ Rs Δ 4 ER) Rubisco complexes ($0.5 \mu\text{M}$ active sites and 10 mM NaHCO_3) in the absence and presence of cyanobacterial, bacterial and red algal activases ($5 \mu\text{M}$). (D) ATPase activity of 8102Rca was weakly stimulated by Rs Δ 2 but not by Rs Δ 4 ER complexes. ATPase activity was determined in the presence or absence of RuBP inhibited (Rs Δ 2/ Δ 4 ER) Rubiscos ($3 \mu\text{M}$) and cyanobacterial, bacterial and red algal activases ($5 \mu\text{M}$). All ATPase and Rubisco assays were performed in the presence of 1 mM ATP and 1 mM RuBP. All measurements were performed at 25°C . Error bars shown are the standard deviation of three independent experiments. The absence of error bars indicates only a single experiment was performed.

5.5. 8102Rca activates inactive *C. merolae* Rubisco complexes poorly

Next, we tested whether 8102Rca can target and activate the inhibited Rubisco complexes (Form ID) of the red algae, *C. merolae*. Activated (CmECM) and stable inactivated Rubisco complexes by RuBP (CmER) and CABP (CmECM-C) were made as described in Chapter three (at 45 °C) to be tested for activation and ATPase stimulation. Carboxylation activity of CmER in the presence of 8102Rca showed a very slow activation rate (Figure 5.7A). This phenotype was different to RsRca which could not activate CmER at 25 °C (Figure 5.7A). The weak ATPase stimulation of 8102Rca by CmER complements the slow activation of CmER (Figure 5.7B).

When the CmECM-C complex was challenged with 8102Rca, it failed to activate the Rubisco complex (Figure 5.7C). Nevertheless, a weak but genuine concentration dependent ATPase stimulation by the inhibited Rubisco complex was observed indicating some interaction with the algal Rubisco enzyme (Figure 5.7D). A similar ATPase phenotype was observed for RsRca although it cannot activate the CmER nor CmECM-C at the measured conditions (25 °C) (Figure 5.7B, D), indicating that it could interact but cannot activate the complex.

Overall 8102Rca resembled RsRca in its ability to activate and interact with the red-algal Rubisco.

5.6. 8102Rca does not function as activase on its cognate Rubisco

To find whether 8102Rca is functional with the green-type Rubisco encoded in the same organism, we purified the WH8102 Rubisco by overexpressing the construct pET30WH8102RbcLS in *E.coli* cells as described in methods. Using pure Rubisco, activated complex (8102ECM) and inhibited complex with RuBP (8102ER) and CABP (8102ECM-C) was prepared.

From (Figure 5.8A), fully activated ECM-complex had a carboxylation rate of 1.8 s^{-1} at the subsaturating CO_2 concentration used (10 mM NaHCO_3). This low CO_2 concentration was used to favour the decarbamylated (E) form. As described for other cyanobacterial Form IB Rubiscos (Andrews and Abel 1981, Lee et al. 1991), WH8102 Rubisco did not form a stable inactive complex with RuBP and the ER complex showed only partial inhibition, whereas the Rubisco formed a stable inactive

complex with its transition analog CABP. With a brief initial inhibition in activity, ~25% of the ER complex was spontaneously activated after only 4 min exposed to CO₂, Mg²⁺ and RuBP of the reaction mix (Figure 5.8A). This shows that RuBP fails to bind tightly to the decarbamylated sites for making it inactive.

Recently it has been shown by our group that a Form II Rubisco, which also does not form stable ER complexes, can be targeted by its CbbQO-type activase (Tsai et al. 2015). Hence it is conceivable that the cyanobacterial activase could favour ECM formation, even when ER is not a stable entity. However, counter to this hypothesis, we found that the carboxylation activity of 8102ER in the presence of 8102Rca was unchanged (Figure 5.8B). This indicates 8102Rca could neither target nor activate its cognate ER complex. Subsequently testing other red-type activases (RsRca and CmNP) with 8102ER complex also failed to activate (Figure 5.8B).

Consistent with the previous results, 8102Rca was also unable to activate even the completely inhibited 8102ECM-C complex (Figure 5.8C). This is consistent with the observation that 8102Rca was unable to remodel the extremely stable ECM-C complexes formed by proteobacterial and red-algal Rubiscos. However, RsRca and CmNP which were successful in activating red-type Form I Rubisco's ECM-C complex also failed to activate the 8102ECM-C complex (Figure 5.8C). Hence 8102Rca, although biochemically a functional Rubisco activase, may not perform this function *in vivo*.

Formation of CmECM/ER/ECM-C at 45 °C → Assayed at 25 °C

Figure 5.7| Red algal Form ID Rubisco was weakly activated by 8102Rca and its ATPase was only weakly stimulated by the Rubisco.

(A, C) 8102Rca weakly activates *C. merolae* ER complex but not ECM-C complex. Rubisco activity assays were performed with activated (CmECM) and inactivated CmER (A) and CmECM-C (C) Rubisco complexes (0.5 μM and 10 mM NaHCO₃) in the absence and presence of 8102Rca, RsRca and CmNP activases (5 μM). (B, D) 8102Rca shows weak stimulation by red algal inhibited complexes. ATPase activity assays were performed with inhibited (CmER and CmECM-C) Rubisco complex at the concentrations indicated in the figure with respective activase (5 μM). All measurements were performed at 25 °C in the presence of 1 mM RuBP and 1 mM ATP. Error bars shown are the standard deviation of three independent experiments. The absence of error bars indicates only a single experiment was performed.

5.7. α and β -cyanobacterial Rubiscos were not targeted nor activated by 8102Rca

To further investigate the incompatibility of red-type activase with green-type Rubiscos (Form IA), we tested the activation of Rubisco complex from two more cyanobacterial species. As an α -cyanobacterial species, we chose Rubisco of *Prochlorococcus MIT9313* because it's a Form IA Rubisco (as found in *Synechococcus PCC8102*) and importantly this organism also encodes a CbbX protein. As a β -cyanobacterial model we chose *Synechococcus elongatus PCC 6301*, which encodes a well-studied Form IB Rubisco and no known activase in the genome (Zarzycki et al. 2012). The synthesized gene of MIT9313 Rubisco by Genscript (USA) was cloned into a pET24b vector (pET24bMIT9313RbcLS) before expressing it in *E. coli* cells. The purification of MIT9313 Rubisco was similar to WH8102 Rubisco. 6301 Rubisco was encoded on pTrcSynLS (Mueller-Cajar and Whitney 2008), expressed in *E. coli* cells and purified by Ms. Guo Zhijun.

Fully activated 9313ECM and 6301ECM complex displayed a carboxylation rate of 1.2 s^{-1} at 10 mM NaHCO_3 . The 9313ER and 6301ER were not stable (Figure 5.8E, F). These catalytic properties were similar to the counterparts of WH8102 which reflect that all cyanobacterial Rubiscos behave the similarly. 9313ER complex did not stimulate the ATPase of 8102Rca, suggesting the complex was not recognized by the activase (Figure 5.8D). Subsequently, the 8102Rca did not enhance the Rubisco activity of the ER complexes.

Figure 5.8| 8102Rca cannot activate both Form IA and IB Green Rubiscos.

α - and β -Cyanobacterial Rubiscos does not form an inactive complex with RuBP and cannot be targeted by activase for activation. (A) Rubisco activity assays were performed with activated WH8102ECM and inactivated WH8102ER/ECM-C Rubisco complexes (0.5 μ M and 10 mM NaHCO₃). (B, C) Partially inactive WH8102ER and completely inactive ECM-C cannot act a substrate for 8102Rca. Rubisco activity assays were performed with activated (WH8102ECM) and inactivated (WH8102ER/ECM-C) Rubisco complexes (0.5 μ M and 10 mM NaHCO₃) in the presence of 8102Rca, CmNP and RsRca (5 μ M). (D) Partially inactive Rubisco weakly stimulates ATPase rate of 8102Rca. ATPase activity assays were performed with 8102Rca (5 μ M) in the presence and absence of inactivated (WH8102ER/MIT9313ER) Rubisco complex (3 μ M). (E, F) 8102Rca failed to activate inhibited Form IA Rubisco from *Syn. MIT9313* and Form IB Rubisco from *Syn. PCC6301*. Rubisco activity assays were performed with activated (9313/6301ECM) and inactivated (9313/6301ER) Rubisco complexes (0.5 μ M and 10 mM NaHCO₃) in the presence of the three red-type activases (5 μ M). All measurements were performed at 25 °C in the presence of 1 mM RuBP and 1 mM ATP. Error bars indicate the mean and standard deviation of at least three independent experiments. The absence of error bars indicates only a single experiment was performed.

Likewise, other red-type activases were also not able to accelerate the spontaneous activation of the ER complexes (Figure 5.8E, F). Overall we found red-type activases cannot activate ER complexes of Form IA and IB Rubiscos. This suggests that one of the reasons for the inability of the red-type activases to activate green-type Rubisco could be the absence of the C-terminal extension at the large subunit, which is deemed to be important for the recognition of inhibited Rubiscos by the red-type activases. Alternatively, our experimental condition could not mimic the carboxysomal environment. So we then decided to attempt and mimic the *in vivo* environment.

5.8. Crowding approach with PEG did not improve Rubisco-activase interaction

In vivo, the cyanobacterial Rubisco and presumably the activase are localized in a crowded environment inside the carboxysome (Rae et al. 2013). We speculated that due to a tight packing environment, the activase could have been placed in close proximity to the Rubisco *in vivo* to facilitate activation.

The characteristic of the intracellular environment is that the macromolecules are present in a crowded environment. This has a positive effect on the affinity and interaction between proteins. Thus crowding affects the macromolecular function *in vivo* (Ellis 2001). So we attempted to partially mimic the macromolecular crowding of

the carboxysomal cellular environment. We hoped this would increase the affinity between the activase and Rubisco and would also enhance its ability to activate the cyanobacterial Rubisco. A macromolecular crowding approach was shown to enhance activase activity for both the plant activation system (Salvucci 1992) and the chemoautotrophic CbbQO system (Tsai et al. 2015). Both studies used 5% polyethylene glycol 3350 (PEG) and demonstrated increased activation rate of Rubisco by its activase.

Since carboxysomes are presumably more tightly packed with Rubisco than the chloroplast stroma, we used 10% v/v PEG in our assays to cause crowding. In the presence of PEG, the 8102ECM had CO₂ fixation activity of 0.75 s⁻¹ at the subsaturating CO₂ concentration used (10 mM NaHCO₃) and the inhibition property of the ER complex was not changed. The addition of 8102Rca to ER complex did not result in enhanced Rubisco activity even in the presence of PEG (Figure 5.9). Similarly, RsRca and CmNP were not successful in activating the ER complex in the mimicked crowded condition. Possibly 10% PEG was not enough to favour a productive activase-Rubisco interaction. Thus the crowding approach was not successful to achieve activation of 8102ER complex by red-type activases.

Figure 5.9| Molecular Crowding using PEG did not confer the ability to activate Rubisco to red-type activases.

Rubisco activity assays were performed with activated (WH8102ECM) and inactivated (WH8102ER) Rubisco complexes (0.5 μ M and 10 mM NaHCO_3) in the presence of 8102Rca, CmNP and RsRca (5 μ M). All assays were performed in the presence of 10% PEG 3350, 1 mM ATP and 1 mM RuBP at 25 $^{\circ}\text{C}$.

5.9. Attempt to engineer activase compatible WH8102 Rubisco by extending the C-terminus

As mentioned earlier, green-type Rubiscos lack the C-terminal extensions (Figure 5.1C). Since we have demonstrated the importance of the C-tail dependent mechanism for all activases, we hypothesized this C-tail could hold the answer for the targeting and activation of green Rubisco by red activases. We attempted to change the incompatibility of *WH8102* Rubisco for 8102Rca and other red-type activases by extending RbcL with the last 6 amino acids of the *R. sphaeroides* large subunit's C-tail (TASVAM) onto the counterpart of the *WH8102* Rubisco. We hypothesized that this chimeric Rubisco would be targeted and activated by the red activase via a C-tail dependent mechanism if the key missing link for activation of green Rubisco is the C-tail. If the Rubisco with C-tail fails to be activated by red activase, it would suggest that targeting and activation requires additional structural elements.

WH8102 Rubisco with the 6 amino acid residue extension (WH8102RsC) was purified using the same procedure as the wild-type enzyme. The chimeric Rubisco was functional and behaved like wild-type under all tested conditions. Like the wild-type enzyme, it did not form stable inhibited complex with RuBP (Figure 5.10A, B).

None of the red-type activases, when added to ER of the chimeric Rubisco, were able to increase the carboxylation rate (Figure .10A). Although we have shown 8102Rca cannot activate ECM-C complexes, the *R. sphaeroides* ECMC complexes can be remodelled by RsRca and CmNP (Chapter One). So we hypothesized that the completely inhibited 8102 ECM-C complex with Rs C-tail could possibly be activated by RsRca and/or CmNP by the C-tail dependent mechanism. However, neither activase was able to do so (Figure 5.10B).

We showed that 8102Rca activation of RsER is via the conserved C-tail dependent mechanism. Surprisingly even RsRca which could be assumed to have a higher affinity for its cognate Rubisco's C-tail than CmNP or 8102Rca also failed to activate the ER and ECM-C complexes. These results strongly suggest that elements on Rubisco's surface in addition to the large subunit C-tail are required for productive engagement and remodelling by red-type activases. These elements appear not to be present on 8102 Rubisco.

Figure 5.10| WH8102 Rubisco engineered with the C-tail extension of the *R. sphaeroides* large subunit of did not facilitate activation by 8102Rca.

(A, B) Inhibited forms of chimeric Rubisco was not activated by 8102Rca as well as RsRca or CmNP. Rubisco activity assays were performed with activated (WH8102RsC ECM) and inactivated WH8102RsC ER (A) and WH8102RsC ECM-C (B) Rubisco complexes (0.5 μ M and 10 mM NaHCO_3) in the presence of 8102Rca, CmNP and RsRca (5 μ M). All assays were performed in the presence of 1 mM ATP and 1 mM RuBP at 25 $^{\circ}\text{C}$. Error bars indicate the mean and standard deviation of at least three independent experiments. The absence of error bars indicates only a single experiment was performed.

5.10. Investigating the possibility of inhibitor specific complexes

Next, we hypothesized that 8102Rca could be involved in maintaining the optimal catalytic activity of its Rubisco by triggering the release of specific other inhibitors that are formed as a consequence of misfire catalytic reactions of RuBP, which bind to the catalytic sites (Pearce 2003,2006). Although from structural considerations, one would expect inhibited Rubisco to display similar features to an activase (defined by a closed active site (Duff et al. 2000)), it is not impossible that specific inhibitors may result in slightly different conformations that could then be targeted by an activase.

Following this train of thought, we decided to use the inhibitor and Rubisco misfire product Xylulose 1,5 biphosphate (XuBP) ((Zhu and Jensen 1991). XuBP is a stereoisomer of the substrate RuBP and therefore, we wanted to test whether the red-type activase can remove the XuBP from 8102 Rubisco active sites and increase the rate of carboxylation.

XuBP was synthesized by Ms. Maria Claribel Lapina following the protocol described by Bracher et al. (Bracher et al. 2015), and this was used to prepare inhibited Rubisco complex of WH8102 (8102EX) as described in methods (Section 2.2.3.1). As a control to test whether red activases trigger removal of XuBP, *R. sphaeroides* Rubisco inhibited with XuBP (RsEX) was prepared and tested in Rubisco activity assays. The spontaneous activation of RsEX was slower than RsER. After 6 min ~10% of ER was spontaneously activated, whereas only ~ 5% of EX was spontaneously activated to ECM. This indicates that XuBP forms a more tightly bound complex in comparison to RuBP with decarbamylated proteobacterial Rubisco (Figure 5.11A). Testing the activation of RsEX by activases, resulted in all three red activases (RsRca, CmNP and 8102Rca) activating the inhibited complex (Figure 5.11A). As expected and similar to RsER activation, RsRca showed a maximum rate of activation (almost spontaneous activation of RsEX) followed by 8102Rca and CmNPRca. This clearly shows that 8102Rca is capable of triggering the release of XuBP from inactive Rubisco complexes.

Having demonstrated the ability of all red-type activases to remove XuBP from the proteobacterial Rubisco, we set out to assess whether XuBP would inhibit 8102 Rubisco. The assayed Rubisco activity of 8102EX was similar to 8102ER (Figure 5.11B), did not form a stably inhibited complex and showed rapid spontaneous activation, indicating XuBP does not form tight a complex with decarbamylated sites of cyanobacterial Rubisco. Testing the activation of 8102EX by its activase did not result in an increase of activity. Subsequently addition of neither RsRca nor CmNP activated 8102EX. In spite of these negative results it is quite possible that a positive result could be obtained using one or more of a large number of other reported (or yet unreported) Rubisco inhibitors such as PDBP and KABP (Parry et al. 2008).

Figure 5.11| 8102Rca was not successful in activating XuBP inhibited WH8102 Rubisco complex.

8102Rca was able to activate XuBP inhibited red-type Rubisco but not of the green-type Rubisco. **(A)** Rubisco activity assays were performed with activated (RsECM) and XuBP inactivated (RsEX) Rubisco complex (0.5 μM and 10 mM NaHCO_3) in the presence of 8102Rca, CmNP and RsRca (5 μM). **(B)** Rubisco activity assays were performed with activated (WH8102ECM) and XuBP inactivated (WH8102EX) Rubisco complex (0.5 μM and 10 mM NaHCO_3) in the presence of 8102Rca, CmNP and RsRca (5 μM). All assays were performed in the presence of 1 mM ATP and 1 mM RuBP at 25 $^{\circ}\text{C}$. Error bars indicate the mean and standard deviation of at least three independent experiments. The absence of error bars indicates only a single experiment was performed.

5.11. Summary

Since phylogenetically related red-type activase to proteobacterial and red algal activase was found in cyanobacteria associated with green-type Rubisco with no C-tail extension we hoped to discover and understand a novel mechanism of Rubisco activation. This would be of particular interest since the insights gleaned would potentially allow us to engineer compatibility between the green plant Rubiscos and the red-type activases. In this chapter, we have characterized ATPase and Rubisco (red and green) activating activity of WH8102 α -cyanobacterial activase.

The oligomeric transitions and thus regulatory properties were very similar to the RsRca (and differed from the algal CmNP enzyme). The 8102Rca apo protein displayed polydispersity and with ATP it formed fibrillar while with RuBP addition it shifted to hexameric form. Similar to other red activases the ATPase activity of 8102Rca was regulated by RuBP and inhibited Rubisco complexes. When challenged with RuBP inhibited *R. sphaeroides* Rubisco (red Rubisco), 8102Rca readily activated the complex at a rate similar to RsRca but the ATPase stimulation by RsER was less than RsRca. We have also shown that 8102Rca appears to activate RuBP inhibited *C. merolae* Rubisco (red Rubisco) albeit at a very low rate. Subsequently, by studying the mechanism of red-type Rubisco activation by 8102Rca, we found that it appears to activate red-type Rubiscos via the same conserved mechanism of transient C-tail threading through the central pore of the activase ring.

Our results with green-type Rubiscos of WH8102, MIT9313 and *Synechococcus* PCC6301 showed that the ER complex was not stable and was spontaneously activated, as observed previously for Rubiscos of cyanobacterial species. These partially inhibited complexes were not activated by 8102Rca. Furthermore, our approach to provide an environment conducive for carboxysomal proteins by increasing the molecular crowding with PEG also failed to improve the ability of the activase to activate the cyanobacterial Rubiscos. Activation of RsER by 8102Rca implied that C-tail extension of Rubisco is a critical substrate recognition element. Following this idea despite engineering the WH8102 Rubisco with the recognition element, 8102Rca still failed to activate the inhibited complex. Finally

with the idea that 8102Rca could recognize Rubisco inhibited by different inhibitors, we tested if XuBP inhibited Rubisco can be activated by 8102Rca. Although the activase successfully remodelled the red-type XuBP inhibited Rubisco complex, it failed to remodel the partially XuBP inhibited WH8102 complex.

An interesting property of 8102Rca was that it was not able to activate the ECM-C complex of both red and green-type Rubiscos. Although observed ATPase stimulation with these complexes implied that 8102Rca could recognize the Rubisco, but it probably cannot generate enough force to remodel the highly stable complex formed with the transition state analog CABP.

Our results have also shown that neither RsRca nor CmNP was able to remodel any green-type Rubisco inhibited complexes. Overall, with the brief characterization of the WH8102 activation system, we were unable to biochemically assign 8102Rca as a carboxysomal activase in our study, but we have demonstrated that 8102Rca is a functional activase with conserved regulation and activation mechanism. Many of its properties closely resemble the proteobacterial activase RsRca.

6.DISCUSSION

In this thesis, I set out to biochemically confirm the existence of Rubisco activation systems in non-green algae and cyanobacteria that were implied by the discovery of a Rubisco activase system in α -proteobacteria. We were able to provide evidence for a hetero-oligomeric red-algal activation system and perform a detailed functional dissection of the two activase subunits. In contrast, although a homomeric α -cyanobacterial red-type activase was found to be functional in the context of heterologous red-type Rubisco, activity towards its cognate Rubisco could not be confirmed *in vitro*. In this chapter, we summarize differences and similarities in the properties of the red activases that have been studied. We also propose a mechanistic framework consistent with the behaviour of algal activase subunits mutated in key functional residues.

6.1. A comparison of red-type activases from diverse systems

Our key finding regarding the red algal Rubisco activase was that individual isoforms are non-functional, but when combined they form a functional complex at a stoichiometry of 1:1. In contrast to the proteobacterial RsRca, which undergoes ligand-triggered oligomeric transitions, CmNP appears to occupy a stable hexameric oligomeric state in the absence and presence of either or both ligands (Figure 6.1). Consistent with its hexameric state, CmNP displayed a basal ATPase activity in the absence of RuBP, which was further stimulated in the presence of RuBP and its substrate Rubisco. In contrast, the cyanobacterial 8102Rca underwent oligomeric transitions as observed earlier for RsRca. The difference in the oligomeric state and the rate of stimulation of ATPase by RuBP shows the difference in the regulation for CmNP activase. In contrast to RsRca and 8102Rca, RuBP reduced the affinity for ATP and increased the $V_{\max}$ of CmNP. Since the oligomeric transitions and ATPase activity of 8102Rca are fairly similar to RsRca (Figure 6.1), we assume the regulation in homo hexameric red-type activases will be similar in other models as well. This regulation of ATPase by the Calvin cycle metabolite RuBP appears unique to the red-type activase system as it has not been reported for plant activase and the CbbQO systems. Regulation of ATPase by inhibited substrate is absent in plant activase systems (Robinson and Portis 1989) but is observed for the chemoautotrophic CbbQO activases (Tsai et al. 2015).

Figure 6.1| Comparison of the properties of proteobacterial, red algal and cyanobacterial red-type activases in the presence and absence of ligands.

CmN and CmP likely originated as a result of an ancient duplication of an ancestral proteobacterial *cbbX* gene that had inserted into the plastid genome as part of a red *rbcL-rbcS-cbbX* operon. (Delwiche and Palmer 1996, Maier et al. 2000, Fujita et al. 2008). Given that the proteobacterial *cbbX* encoded protein forms a productive homo-hexameric activase, the increased complexity of the eukaryotic system may be a consequence of an evolutionary ratchet rather than a functional specialization (Gray et al. 2010, Finnigan et al. 2012). Soon after duplication, the two isoforms were functionally identical. They could have formed productive activase in a homo-hexameric or heterohexameric arrangement in any combination. But during evolution, this plasticity was lost. Over time the subunit-subunit interface was likely modified so it can no longer interact with the same subunit to form the productive hexameric complex. Subsequent mutations in the individual isoforms during evolution could have made them lose some and modify certain functions of the motor (Finnigan

et al. 2012). As a consequence, CmN and CmP became obligate partners and had to interact with each other in a strict stoichiometry (1:1) to complement their losses occurred during evolution. Thus the complexity increased. Since evolution has functionally coupled them to be obligate partners, theoretically the production of the nuclear-encoded CmN is expected to be coupled to that of CmP, which would occur by the process known as retrograde signalling (Nott et al. 2006). In other words, *in vivo* mechanisms should be in place to strictly control the CmN production, similar to other components of the photosynthetic machinery that are encoded by the nuclear genome in eukaryotic photosynthetic organisms (Allen et al. 2011).

6.2. The mechanism of activation appears conserved

Since all activases were able to activate RsER we hypothesized that these follow the same Rubisco remodelling mechanism as RsRca, which is partial threading of the C-tail of the large subunit terminus through its central pore. Our results for inhibited Rubiscos with shorter large subunits (deletion constructs of 2 and 4 residues from the large subunit) with CmNP and 8102Rca supports a C-tail dependent mechanism of activation similar to RsRca. Further, the participation of the central pore in the remodelling process was confirmed for CmNP by mutating the conserved tyrosine lining the pore in all subunits. This mutant eventually failed to engage its Rubisco substrate. These results together indicated that CmNP also follows the proposed partial threading model. Our study suggests a conserved mechanism of activation for red-type Rubisco remodelling by the red algal, cyanobacterial and proteobacterial activases. We hypothesize this model can be extrapolated to all red-type activases.

6.3. The α -cyanobacterial Rubisco activation system

Whether carboxysomal Rubiscos require an activase is an unresolved question. Normally the organisms that have carboxysomes do not encode activases (at least belonging to the three identified classes), but a number of cyanobacteria and proteobacteria with carboxysomes do encode an activase (Badger and Bek 2008). Certain proteobacteria having carboxysomes encode the CbbQO activase while some β -cyanobacteria encode a green-type activase like protein or CbbX and some α -cyanobacterias encode CbbX (Zarzycki et al. 2012, Long et al. 2016). On the basis of the observation that in some cases activase proteins are encoded but are absent in

others, does this physiologically mean some require activation by activase and others are not regulated by the activase? Or does this imply the majority of cyanobacteria are a potential source of undiscovered activases? It is, of course, conceivable that in some organisms a general molecular chaperone (AAA+ type or other) can perform an activase function, but clearly the carboxysomal localization would need to be fulfilled.

The other puzzling property regarding cyanobacterial Rubiscos is that they do not form stable ER complexes *in vitro* (Andrews and Abel 1981, Lee et al. 1991, Li et al. 1999). The work by Pearce has also demonstrated that although cyanobacterial Rubisco and higher Plant Rubisco share high sequence identity, the binding and release of inhibitors is considerably different (Pearce 2006). In the cyanobacteria, the binding of the inhibitor is loose and thus released faster compared to higher plant Rubiscos. Consistent with Pearce's work we also observed that three different cyanobacterial Rubiscos were only partially inhibited by RuBP, suggesting that the inhibitors do not bind tightly to the active sites of the decarbamylated enzyme and were rapidly released.

The role of CbbX as an activase *in vivo* for cyanobacteria is thus debatable. So far only experimental evidence for required role of activase (green-type Rca) in the maintenance of the cyanobacterial Rubisco has been obtained in β -cyanobacteria (Li et al. 1999). However, no *in vitro* activity could be established in that work and the reported physiological effect of a Rca mutant was not strong and apparently quite conditional. So we characterized the α -cyanobacterial activase with an aim to understand the mechanism model for activation of green-type Rubisco. We were able to show that CbbX can function as activase *in vitro* with red-type Rubiscos. Strong evidence for its role in the Calvin cycle is provided by our data that regulation by RuBP is conserved (formation of productive hexamer and ATPase activity is induced by RuBP). Although no direct evidence for interactions of CbbX with carboxysomal proteins is available, since CbbX is seen in the neighbourhood of the carboxysomal assembly genes suggests that it could possibly be assembled into the carboxysome to regulate the Rubisco. However, unfortunately, we could not demonstrate its role as activase for its cognate Rubisco *in vitro*. Furthermore, the observed C-tail dependency

to activate Rubisco is problematic because the cognate green-type Rubisco does not have this C-terminal extension.

Due to the significance of the extended C-tail in the remodelling mechanism, we engineered C-terminus of the *WH8102* to be similar to *R. sphaeroides*. The chimeric cyanobacterial Rubisco with extended C-tail showed no difference in phenotype. This approach clearly demonstrated that interaction between activases and Rubisco is not restricted just to the C-tail in the green-type Rubisco.

There could be other inhibitors *in vivo* that bind to the active site. Also in carboxysomes, Rubisco is believed to be incorporated into larger-order structures with other proteins such as carbonic anhydrase (CA), CsoS family of proteins (in α -carboxysomes), or Ccm family of proteins (in β -carboxysomes) and other proteins in the carboxysomes. Some of these may be required by the Rubisco *in vitro* to successfully characterize its regulation by the activase.

Finally, the requirement of a “carboxysome-specific” adaptor protein by activase to recognize its substrate Rubisco analogous to ClpX-ssrA or ssrB systems (Dougan et al. 2002) could be a possibility. Such an adaptor could possibly compensate for the missing C-tail.

In summary, we believe our data are strongly supportive of a genuine Rubisco activase function for carboxysomal Rubiscos, given the biochemical activity and RuBP dependency of the 8102CbbX protein. We anticipate that future work will uncover the specific assay conditions that will reproduce the correct environment for carboxysomal Rubisco activases to function. We feel that evolution of an alternative function is unlikely, and should have resulted in significant signatures in the primary sequence, as well as loss of the *in vitro* Rubisco activase function.

6.4. Activation of relatively stable substrate complex depends on the strength of the activase

From the outcome of our results, we observed a difference in stability among the inhibited red-type Rubiscos of proteobacteria (RsER and RsECM-C) and algae (CmER and CmECM-C). In general, our study shows that remodelling the CABP complexes by the activases is more challenging than RuBP complexes. At 25 °C, RsER shows weak spontaneous activation, but at 40 °C, the RsER was spontaneously activated. All the three activases were able to robustly activate the RsER (Table 6.1). This suggests that the binding of RuBP is loose and this makes RsER the least stable red-type Rubisco complex studied. At 25 °C and 45 °C the RsECM-C was completely inhibited. This inhibited complex was not activated by all the activases (Table 6.1), the activation rate was also lower than the RsER, suggesting that this inhibited Rubisco is relatively stable than RsER. Earlier studies have also shown that ECM-C complexes of various Rubiscos are more stable than their ER complexes (Schloss 1988, Servaites 1990, Pearce 2006). The Rubisco of the thermophile algae formed a partially inhibited complex at sub-optimal temperature but when heated to its physiological temperature it formed stable inhibited complex. At 25 °C and 45 °C inhibited CmER and CmEM-C were very stable and showed no spontaneous activation. The outcome of the activation data for our algal complexes suggested that CmER and CmECM-C were only weakly activated by CmNP activase and other activases were completely unable to activate the complex at 25 °C even though the inhibited algal Rubiscos stimulated their ATPase activity (Table 6.1). This indicates that algal inhibited Rubiscos are relatively more stable than bacterial counterparts, but as expected, present a surface to the activases that is recognized. The rigid active sites of red-type Rubisco (especially algal) are hypothesized to be a trade-off required for their high CO₂ specificity (Pearce 2006). As a consequence, the ligands tend to bind strongly to the active sites which restrict catalytic rate and facilitates the formation of a very stable tightly inhibited complex. Since bacterial activase can activate CmER faster than CmECM-C at 35 °C this suggested that CmECM-C complex is relatively more difficult to activate than ER complex (Table 6.1). Also with the lower activation rate of CmECM-C compared to CmER by CmNP wild-type at 45 °C and additionally the ability of CmNP^Y pore loop variant to activate CmER and not CmECM-C suggests that ECM-C complex of algal Rubisco is relatively the most stable inhibited

complex. Based on these observations, we have arranged the inhibited Rubiscos in their order of relative stability (Figure 6.2). The different degree of the stability of the complex indicated that the activases are required to be powerful enough to remodel the complex. The ability of the activase to activate the increasingly stable complex is a measure of their relative ability to generate sufficient force required to remodel the target complex. From all the outcomes of the activation rate data for the activases with different Rubiscos (Table 6.1) prompts us to hypothesize a difference in relative strength of the activases. Figure 6.2 illustrates the predicted stability of the Rubisco complexes used in our study and the potential of the activase to activate these complexes. On the basis of our hypothesis, 8102Rca is a relatively weak activase because it can only activate RsER (relatively least stable complex). However, it can recognize other inhibited forms (ATPase stimulation) but could not generate sufficient force to remodel the stable Rubisco complexes. RsRca can readily remodel RsER and RsECM-C and CmER (only at 35 °C) but CmECM-C (relatively most stable) at a very low rate. This makes it a relatively stronger activase. However, CmNP can activate all the Rubisco complexes (Table 6.1) which were difficult for the other activases to remodel. This makes the red algal CmNP activase the strongest that can generate force to effectively remodel the stable Rubisco complexes (Figure 6.2). Another observation is that CmNP achieved a robust activation of RsER and RsECM-C with very weak stimulation of ATPase activity $\sim 12\%$ compared to RsRca. Although CmNP prompts us to assume that high ATPase is not essential for effective remodelling and force generation, the efficiency of the activases (ATP used per active site remodelling) needs to be determined to make conclusive statements.

This model is only based on our observations. To test the stability of the complexes used in our studies one has to study the properties of substrate binding sites of the Rubisco by carefully analysing the inhibitor binding kinetics such as inhibition constant (K_i), binding constant (K_{on}), dissociation constant (K_d or K_{off}) at 25 °C and 45 °C to characterize the degree of inhibition similar to what has been presented by Pearce (Pearce 2006). Activase strength obviously depends on the substrate stability (Siddiqui et al. 2004, Sen et al. 2013). By using efficiency assays for the activase we could measure the number of ATP molecules used /active site activated). However, this would not give a measure of the force generated by the activases (Sen et al. 2013) It is thus difficult to test our hypothesis rigorously using

only biochemical assays. Similar questions are currently being addressed by other groups using single molecule experiments (Martin et al. 2008b, Sen et al. 2013, Iosefson et al. 2015). Although it is considerably difficult to establish this set up to test every activase-Rubisco complex, it could be a worthwhile challenge in the future.

Table 6.1| Overview of the ATPase stimulation and activation profile of the wild-type red-type activases.

Activase Rubisco	RsRca		CmNP		8102Rca	
Assayed at 25 °C						
RsER (Assayed 25 °C)	✓	+++	✓	+	✓	+++
CmER (Assayed 25 °C)	✗	+	✓	+	✗	+
RsECM-C (Assayed 25 °C)	✓	+++	✓	+	✗	+
CmECM-C (Assayed 25 °C)	✗	+	✓	+	✗	+
WH8102ER (Assayed 25 °C)	✗	○	✗	○	✗	+
WH8102ECM-C (Assayed 25 °C)	✗	○	✗	○	✗	○
WH8102RsC ER (Assayed 25 °C)	✗	○	✗	○	✗	○
WH8102RsC ECM-C (Assayed 25 °C)	✗	○	✗	○	✗	○
9313ER (Assayed 25 °C)	✗	○	✗	○	✗	○
6301ER (Assayed 25 °C)	✗	○	✗	○	✗	○
Assayed at 35 °C						
CmER (Assayed 35 °C)	✓	+	✓	+		○
CmECM-C (Assayed 35 °C)	✓	+	✓	+		○
Assayed at 45 °C						
CmER (Assayed 45 °C)		○	✓	+		○
CmECM-C (Assayed 45 °C)		○	✓	+		○
RsECM-C (Assayed 45 °C)		○	✓	+		○

Rubisco Activation	
✓	Ribust
✓	Weak
✗	No
ATPase Stimulation	
+	Strong
+	Weak (< onefold)
○	Not measured

Rubisco Activation	
✓	Ribust
✓	Weak
✗	No
ATPase Stimulation	
+	Strong
+	Weak (< onefold)
○	Not measured

*All CmER/CmECM-C showed were heated to 45 °C to form a stable complex before assaying

Figure 6.2| Stable Rubisco complex requires strong activase to remodel their sites.

The top panel shows the inhibited Rubisco complexes arranged in the order of increasing relative stability and the bottom panel shows the activase characterized. The arrows indicate the activation of the Rubisco by the respective activase (Green-8102Rca, Violet-RsRca and Red-CmNP). The arrows with the dotted lines indicate activation only possible at 35 °C.

6.5. Arrangement and of role of the CmNP subunits

After failed attempts to identify the arrangements of the isoform in the complex by electron microscopy, biochemical characterization of the CmNP variant harbouring Walker B mutation in CmP and arginine finger mutation in CmN suggested an alternative arrangement of subunits (trimer of heterodimer). This arrangement is consistent with other well-characterised hetero-oligomeric AAA+ proteins such as YTA10/12 and PEX1/6 (Augustin et al. 2009, Ciniawsky et al. 2015).

In the heterocomplex, CmN seems to be a dominant isoform while CmP acts as a regulatory isoform. The variants listed in the table (Table 6.2) support that CmN is a dominant partner of the complex. Mutating any residues of the key motifs (Table 6.2) in the CmN renders the complex inactive for both ATPase and Rubisco activation except pore loop motif in which only Rubisco recognition and activation is lost. But on the other hand mutating residues of Walker B, RuBP binding, pore loop or surface

lysine (K123) of CmP is tolerated although the mostly the ATPase and the Rubisco activation is reduced. This shows that the CmP likely acts as a regulatory partner.

Table 6.2| Overview of the ATPase stimulation and activation profile of the red-activase variants mutated in the key residues.

CmNP Mutants Rubisco	WalkerB Mutant (E138Q)	Arg Finger/ WalkerB	Poreloop Mutant (Y114A)			RuBP Mutant (R239A)	Surface Lysine Mutant (K123A)
	CmN ^{NPB}	CmN ^{RpB}	CmN ^{NPY}	CmN ^{VP}	CmN ^{VPY}	CmN ^{PS}	CmN ^{Pk123A}
RsER (Assayed 25 °C)	+	-	-	-	+	-	-
CmER (Assayed 25 °C)	+	-	+	-	-	-	-
RsECM-C (Assayed 25 °C)	+	-	-	-	+	-	+
CmECM-C (Assayed 25 °C)	+	-	+	-	-	-	-
CmER (Assayed 45 °C)	+	-	-	-	-	-	-
CmECM-C (Assayed 45 °C)	+	-	+	+	-	-	-
RsECM-C (Assayed 45 °C)	+	-	-	-	-	-	-

* Only the ATPase functional mutants are displayed.

ATPase Inactive Mutants		
WalkerAMutant (K80A)		
CmN ^{AP}	CmN ^{PA}	CmN ^{APA}
Arginine finger Mutant (R194A)		
CmN ^{RP}	CmN ^{PR}	CmN ^{RPR}
Arg Finger/ WalkerB Mutant		
CmN ^{BPR}		
RuBP Mutant (R239A)		
CmN ^{SP}	CmN ^{SPS}	
Surface Lysine Mutant (K123A)		
CmN ^{K123AP}	CmN ^{K123APK123A}	

Rubisco Activation			ATPase Stimulation

Ribust	Weak	No	Strong	Weak (< onefold)	No

6.6. A first mechanistic model for CmNP function

In our study, we aimed to understand the relative contribution and arrangement of the two CmNP isoforms, by producing a series of variant complexes. Some of these had interesting biochemical phenotypes, and here we attempt to place our observations into a model that is amenable to future experimental verification. Assuming that Walker A motif mutation prevents binding of ATP to the active sites of CmNP, our results with mutation of Walker A motif (K80A; Figure 4.4) had shown that impairing the nucleotide binding in alternative subunits makes the complex completely inactive. This suggests a strong coordination for ATP binding and hydrolysis between the subunits. Similarly mutating the arginine finger (R194; Figure 4.5) in any isoform completely abolished the activity of the complex. This strongly suggests inter-subunit communication for ATP binding and hydrolysis because the arginine finger of *cis* subunit constitutes the nucleotide binding site of the *trans*-subunit (Figure 6.3A). In summary, a Walker A or arginine finger mutation in either isoform is sufficient to totally eliminate the activity of the subunit harbouring the mutation and the nearest neighbour intact active site, thus causing the complex to be completely inactive.

Mutating the Walker B glutamate to glutamine usually prevents ATP hydrolysis but does not impair ATP binding to the active sites (Erzberger and Berger 2006), so we assume the same for the CmNP system (E138; Figure 4.6). Mutating Walker B of CmN (CmN^BP) abolished the ATPase activity of the complex implying CmN active sites are critical for the complex. The equivalent mutation in CmP (CmNP^B) displayed an interesting phenotype. The conformation of the nucleotide locked CmP (CmNP^B) apparently unleashed the ATPase activity of CmN. A similar phenotype was observed in the covalently linked ClpB dimers (Zeymer et al. 2014) but not in other heteromeric complexes YTA10/12 or PEX1/6 (Augustin et al. 2009, Ciniawsky et al. 2015, Gardner et al. 2015). The increased ATPase rate of this variant, coupled with robust activase activity, suggests that the CmP subunit in the wild-type activase is not required to hydrolyse nucleotide. In fact, it may be catalytically inert (Figure 6.3A).

The mutated catalytic site of either subunit must somehow communicate with the neighbouring nucleotide binding-competent subunit and confer a conformation that makes it inactive or active for ATP hydrolysis. The phenotypes of both Walker A and B variants gave us a clue that the conserved trans-acting arginine finger (R194) could be a very crucial player in regulating the ATPase of the overall heteromeric complex. Herein we present a model, which predicts the arginine finger (R194) in the CmNP complex as an integral element for intersubunit communication for catalysing ATP hydrolysis in the *trans*-subunit (Figure 6.3B). Initially, we would have assumed that the arginine finger of CmN senses the presence of bound nucleotide at the CmP harboured active site, and communicates this state back to the *cis* CmN for inducing ATP hydrolysis based on the phenotype of CmNP^B. However, the CmN^{RPB} phenotype implicated a possible role of the CmP arginine finger in the nucleotide signaling instead. The ATPase activity of CmN^{RPB} indicates that the presence of bound nucleotide to CmP is clearly not communicated to the CmN active site via the arginine finger of the CmN subunit. But rather the arginine finger of CmP (*cis*) (counter clockwise) signals the bound nucleotide at CmP to the *trans* CmN active site, thus making CmN highly active for ATP hydrolysis (Figure 6.3B (black dotted lines)). Based on our model we believe CmP, in the nucleotide-bound state, signals to CmN in the heterocomplex, strongly stimulating its ATPase activity.

Our results with mutating RuBP binding sites (R239; Figure 4.8) in CmN (CmN^{SP}) and in CmP (CmNP^S) suggested that binding of RuBP to both isoforms simultaneously was important for the ATPase stimulation. An important caveat to this interpretation, however, is that the chosen R239A mutation in CmN led to the abolishment of basal ATPase activity. Thus a different mutation should be chosen to support this interpretation in the future. An ideal RuBP binding defect would lead to a variant with identical basal ATPase activity to the wild-type, and selective loss of RuBP-induced ATPase stimulation.

Keeping in mind the limitations of the chosen mutation, preventing RuBP binding to CmP while CmN is RuBP binding competent (CmNP^S) resulted in the activase to have only basal ATPase activity and no stimulation with RuBP was observed. Moreover, mutating the RuBP site in a CmP subunit harbouring the Walker B mutation (CmNP^{BS}) resulted in the same phenotype and had no RuBP stimulation

(Figure 4.12). Thus it indicates that RuBP binding to CmP is necessary for the stimulation of ATPase in CmN subunits to take place (Figure 6.3). However mutating RuBP site at CmN (CmN^{SP}) abolished the basal ATPase activity as well, which is inconsistent with the model. In future, for better understanding the effects of RuBP binding to CmNP the mutant with defective RuBP binding phenotype but maintaining full basal activity need to be identified. Other possible good candidates could be alanine substitutions of S250, N253, R257 and R261 based on the crystal structure of RsRca (Mueller-Cajar et al. 2011). Our observation of RuBP signaling for ATPase stimulation on comparing the phenotype of CmN^{RPB} variant, which shows no RuBP stimulation, with CmNP^B, which retains RuBP signaling implied that mutating arginine finger of the CmN (CmN^{RPB}) crippled the RuBP signaling effect for the stimulation of ATPase (Figure 6.3). This suggests that RuBP binding to CmP (clockwise) is signaled to the CmN via the arginine finger of the CmN which signals to the active site for the stimulation of ATPase in CmN (Figure 6.3). Understanding of the role of arginine finger in ATP hydrolysis from these variants gave more insights into the working principles of the system, but further characterization needs to be established to appropriately illustrate its role in inter- and intra-subunit communication.

Moreover, since the Rubisco remodelling capacity of CmNP^B with defective CmP catalytic site was similar to the wild-type, this suggested that pore loops of CmP could be non-essential (Here we assume that ATP hydrolysis in the CmN active site will power movement of the CmN pore loop1, and not that of CmP). However, when we tested this hypothesis by mutating tyrosine residue (Y114) of the pore loop of CmP (CmNP^Y), the Rubisco remodelling was severely affected (~80% reduction in activase activity) (Figure 4.9). This variant, contrary to our hypothesis showed that intact pore loops of CmP are important for substrate remodelling, but not as indispensable as CmN (Figure 6.3A). These results together indicate that in CmP the function of coupling ATP hydrolysis energy to their pore loops during substrate remodelling seems to be nonessential. However, CmP is absolutely required to bind nucleotide and their pore loops must remain intact for the proper functioning of the hetero-oligomeric CmNP complex. During Rubisco remodelling by CmNP, probably CmN couples ATP hydrolysis energy to the conformational changes that accompanies the movement of their pore loops (only CmN can actively hydrolyse ATP) while CmP

pore loops might be required to engage the substrate by performing a holdase function during remodelling to prevent slippage. Preventing RuBP binding to CmP while CmN is RuBP binding competent in either CmNP^S or CmNP^{BS} crippled the Rubisco remodelling activity by ~90% (Figure 4.8, 4.12). This suggested that RuBP binding to CmP is essential for activase activity of the complex. Importantly, the loss of RuBP signaling in CmN^{R^PB} correlates with a loss in Rubisco activation (Figure 6.3). This variant thus failed to couple the ATPase energy for the conformational changes that lead to substrate remodelling (similar to pore-loop mutants). Probably the best explanation for this phenotype is that although this variant is able to rapidly hydrolyse ATP, the conformational changes required to remodel Rubisco can no longer take place. This implicates the CmN arginine finger in transferring movements that are required for force transmission from the motor to the substrate. Alternatively, the CmN^{R^PB} variant may simply prefer to occupy a conformation that can no longer recognize the protein substrate, and this would explain the lack of ATPase stimulation when exposed to inhibited Rubisco.

Figure 6.3| Proposed model for ATPase activity and its regulation in the CmNP complex.

(A) ATP hydrolysis highly is co-ordinated not stochastic in CmNP. Schematic representation of the CmNP activase complex (top view) with alternating CmN and CmP subunits. The active sites are at the interface of two subunits constituted by the nucleotide binding domain and an arginine finger from the neighbour (counterclockwise). The figure highlights that CmN active sites (green circle) are involved in ATP hydrolysis and, CmP sites are required to bind nucleotide but are likely catalytically inert (red circle). The pore loops of CmN are more important than those of CmP. (B) Signaling for ATPase activity at the CmN active site is presumed to be from CmP subunit (clockwise). This model is supported by the phenotype of CmN^{RPB} and CmNP^{BS}. ATPase activity of CmN^{RPB} suggests that the bound nucleotide at CmP active site is signaled via its arginine finger to the CmN active site in a clockwise direction. (dotted black lines indicate the bound Nucleotide signaling). The loss of ATPase stimulation in CmNP^{BS} suggests the RuBP binding to CmP regulates the stimulation

of ATPase at CmN active site (Purple arrows indicate possible RuBP signaling to the CmN active site).

In summary, our model predicts that the CmNP complex works in a strongly cooperative fashion and signals the presence of bound ATP and RuBP at one subunit to its adjacent subunit's active site via trans-acting arginine fingers. Local allosteric conformational changes are triggered that allow substrate binding and hydrolysis to occur. The outcomes of our work have thus established a framework for analysing additional variants and provided a rough sketch for the mechanism. We have generated a tool box to understand the working of a heterocomplex AAA+ motor. To increase understanding in our mechanistic interpretation, this study should further be supplemented with other methods and techniques such as nucleotide binding assays (for example using fluorescent nucleotide analogues) for validating our assumptions on nucleotide binding for Walker A and Walker B variants. These analyses would also indicate the ratio of bound nucleotides to the subunits of the wild-type and variant complexes. Biophysical approaches such as Surface plasmon resonance (SPR) and Isothermal titration calorimetry (ITC) can be used to assess RuBP and nucleotide binding to the wild-type or mutant subunits. Such experiments would lay the foundation to understand the detailed mechanism of CmNP in future. A critical and powerful future tool would be the isolation of functional covalently linked concatameric complexes, as was attempted during our study. We also anticipate utilizing Rubisco-dependent *in vivo* evolution systems (Mueller-Cajar and Whitney 2008) to evolve second site suppressors of interesting impaired variants that will highlight functionally important residues that cannot be identified by rational means. (Augustin et al. 2009) have already beautifully illustrated this possibility for the Yta10/Yta12 complex by using evolution in yeast.

In summary, the availability of rapid biochemical assays for ATPase and Rubisco function make the CmNP an eminently accessible model for dissecting structure-function relationships for AAA+ ATPases. Future work in the laboratory hopes to take advantage of this situation and improve the admittedly still highly speculative nature of our model.

Phylogenetic analysis indicated that the CbbX encoded by the plastid and nuclear genomes diverged significantly during evolution. However, from sequence alignments, there were no clear signature sequences for plastid and nuclear encoded

activases, which suggests that their biochemical properties may vary across species (Hovde et al. 2015). Hence it is highly probable that characterization of activase pairs from different algal species would form a 1:1 stoichiometry complex of alternating subunits, as a result of the evolutionary ratchet (Finnigan et al. 2012), but the relative biochemical properties could be diverse. For instance, in some cases, the role of the plastid encoded activase could be dominant. From a biotechnological point of view, which is aiming to improve photosynthetic performance, it is necessary to use systems with suitable properties. Thus it may prove useful to characterize other activase complexes to understand the diverse solutions that may exist.

6.7. Towards understanding structure-function relationship

Determining the crystal structure of CmNP would be highly informative to further understand the arrangement and subunit interactions. Understand the structure-function relationship could give more insights about the inter-subunit signaling. More importantly, crystal structures with bound ligands (ATP, ADP and RuBP) would provide a wealth of knowledge about the arrangement of the nucleotide binding pockets, a conformational change associated with the binding and hydrolysis of ATP. Obtaining various structural snapshots could provide a better insight to understand the observed coordination among the subunits during ATP hydrolysis and substrate engagement. Overall structural information of the complex would greatly help refinement of our mechanistic model.

6.8. Biotechnological outlook

To meet the global food demand of the future the CO₂ fixation, the field is currently engaging in an intense rush towards engineering crop plant photosynthesis and yield potential (Zhu et al. 2004, Lin et al. 2014). Rubisco is the key enzyme in photosynthesis. So one of the efforts to engineer the photosynthesis in crop plants would be to add a red-algal Rubisco with high specificity for CO₂ (Read and Tabita 1994, Andrews and Whitney 2003, Shih et al. 2016) which would result in higher photosynthetic efficiencies in plants. Although at the moment we are not successful in folding the algal Rubisco in plants (Whitney et al. 2001), but these issues are likely to be resolved soon with as a result of our rapidly increasing understanding of Rubisco biogenesis (Liu et al. 2010, Hauser et al. 2015, Joshi et al. 2015). In the future, even when we were successful in engineering the algal Rubisco in plants, it would likely

not be useful because we have shown it is prone to be inhibited and requires its activase for maintaining its function. My work has extensively characterized the red algal activase and shown that Rubisco can be rescued by RsRca or heterocomplex CmNP but not by just CmP or CmN. So we must incorporate either RsRca or CmNP together with the algal Rubisco enzyme in plants for maintaining its photosynthesis. Thus the work in this thesis has provided additional information required for a biotechnological venture aiming to engineer photosynthetic CO₂ fixation in plants.

Another central challenge in photosynthesis of plants is the thermal intolerance of photosynthesis caused by denaturation of the green-type Rubisco activase (Salvucci and Crafts-Brandner 2004). Our CmNP activase displays some superior biochemical properties compared to green-type Rca. CmNP has the desired thermostability property, and it can potentially remodel the highly stable inhibited Rubisco complexes that green Rca fails to perform. This thermostability property of the activase could thus be used in plants to increase the heat tolerance and avoid the shutdown of photosynthesis due to heat stress (Salvucci and Crafts-Brandner 2004). Since the red and green Rubiscos are structurally quite similar and with the understanding of the mechanism of activation, it should be fairly straightforward to introduce the factors to the green Rubisco which are responsible for the activation by red-type activases. As an initiative step, we engineered the conserved substrate target, C-terminal extension, onto the green Rubisco of cyanobacteria. Although we were unsuccessful, we believe it should be feasible in future to engineer Rubisco to be compatible with other activases if we build a better understanding of these proteins and their working model.

The stramenopile algae, *Nannochloropsis gaditana* is of great interest for the biofuel industry (Radakovits et al. 2012) and in fact has red-type Rubiscos and CbbX. They use the Rubisco activation system similar to what we have characterized and not the well-studied models. Detailed biochemical information of the activation system could possibly be useful for strain improvement.

6.9. Physiological and geological implications

In plants, activases are crucial checkpoints in Calvin cycle regulation. The activity of the plant activase is regulated by light intensity through the ferredoxin-thioredoxin system (Zhang and Portis 1999), in dark the activase activity is low. In red algae, the regulation of the photosynthetic dark reactions is relatively poorly characterized. Based on our model we hypothesize activase activity could be regulated by the RuBP; if RuBP levels are low in the dark then activase becomes less functional. The role of activase in algae will not be merely limited to rescue the Rubisco function but may play other roles in the regulation of CO₂ photosynthetic assimilation.

In plants, there are specific inhibitors like the night-time inhibitor carboxyarabinitol 1-phosphate (CA1P) (Andralojc et al. 2002, Andralojc et al. 2012) and catalytic misfire products. In red algae, due to lack of detailed physiological studies, we have no knowledge about other inhibitors. There could be other naturally occurring sugar phosphates that are formed during catalysis might inhibit the Rubisco (Lorimer et al. 1977, Edmondson et al. 1990, Parry et al. 2003, Parry et al. 2008). So my work here highlights a facet of the red algal photosynthetic system that has been ignored in the past. Since we are certain that there is an activase for red algae, subsequent future studies should aim to answer if there are very specific ways to regulate Rubisco in these algae similar to what is found in plants which had been studied in detail over the years.

Finally, the activase protein we characterized is found in organisms that are responsible for a significant portion (~25%) of global CO₂ fixation (Nelson et al. 1995) (Falkowski et al. 2004). Thus red-type eukaryotic Rubisco activase systems can be considered to be major regulators and thus gatekeepers of CO₂ uptake into the biosphere, and our work represents the first glimpse into their biochemical properties. We are anticipating an upcoming era of ever-increasing anthropogenic CO₂ emissions and the associated global climate change. It is, therefore, to be welcomed if humanity chooses to invest a small amount of the capital gained from burning fossil fuels into trying to obtain an initial understanding of the biochemical processes that may in future be essential to counteract these grave issues.

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