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<td><strong>Citation</strong></td>
<td>Loganathan, N., Tsai, Y.-C. C., &amp; Mueller-Cajar, O. (2016). Characterization of the heterooligomeric red-type rubisco activase from red algae. Proceedings of the National Academy of Sciences of the United States of America, 113(49), 14019-14024.</td>
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Characterization of the hetero-oligomeric red-type rubisco activase from red algae

Short title: Hetero-oligomeric rubisco activase from red algae

Key words: Rubisco, activase, photosynthesis, AAA+ proteins, red algae

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ABSTRACT

The photosynthetic CO₂-fixing enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco) is inhibited by non-productive binding of its substrate ribulose-1,5-bisphosphate (RuBP) and other sugar phosphates. Reactivation requires ATP-hydrolysis powered remodeling of the inhibited complexes by diverse molecular chaperones known as rubisco activases (Rca). Eukaryotic phytoplankton of the red plastid lineage contain so-called red-type rubiscos, some of which have been shown to possess superior kinetic properties to green-type rubiscos found in higher plants. These organisms are known to encode multiple homologues of the α-proteobacterial red-type activase CbbX. Here we show that the gene products of two cbbX genes encoded by the nuclear and plastid genomes of the red algae Cyanidioschyzon merolae are non-functional in isolation, but together form a thermostable hetero-oligomeric rubisco activase that can use both α-proteobacterial and red algal inhibited rubisco complexes as a substrate. The mechanism of rubisco activation appears conserved between the bacterial and the algal systems and involves threading of the rubisco large subunit C-terminus. Whereas binding of the allosteric regulator RuBP induces oligomeric transitions to the bacterial activase, it merely enhances the kinetics of ATP hydrolysis in the algal enzyme. Mutational analysis of nuclear and plastid isoforms demonstrates strong co-ordination between the subunits and implicate the nuclear-encoded subunit as being functionally dominant. The plastid-encoded subunit may be catalytically inert. Efforts to enhance crop photosynthesis by transplanting red algal rubiscos with enhanced kinetics will need to take into account the requirement for a compatible rubisco activase.

SIGNIFICANCE STATEMENT

Eukaryotic phytoplankton of the red plastid lineage dominate the oceans and are responsible for a significant proportion of global photosynthetic CO₂ fixation. In contrast to their
ecological importance relatively little is known about the biochemical properties of their carbon dioxide fixation machinery. In plants, the carbon dioxide fixing enzyme rubisco forms inactive complexes with sugar phosphates, and needs to be constantly remodeled by the motor protein rubisco activase. Here we show that in red algae rubisco also forms inhibited complexes, which can be rescued by a convergently evolved rubisco activase similar to one described in photosynthetic bacteria. Some red algal rubiscos are a target for crop improvement strategies, since their kinetics are better suited to the current atmospheric gas composition.
INTRODUCTION

In all photosynthetic eukaryotes the enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco) catalyzes the incorporation of carbon dioxide into biomass during the Calvin-Benson-Bassham cycle (1). The majority of these organisms possess the Form I type enzyme, which forms an oligomer of large and small subunits in an L8S8 stoichiometry. Form I rubiscos are phylogenetically deeply divided between a green-type clade (Form IA and IB) derived from cyanobacteria and a red-type clade (Form IC and ID) of proteobacterial origin (2, 3). Eukaryotic phytoplankton of the red-plastid lineage all contain the red-type Form ID enzyme and dominate the modern oceans (4). The geochemical importance of these organisms is enormous, with diatoms alone believed to be responsible for ~20% of global net primary productivity (5).

Rubisco has long been a target of crop improvement strategies (6) due to its low catalytic efficiency in addition to its tendency to catalyze abortive side reactions that result in damaged metabolites (7). This includes the oxygenation product 2-phosphoglycolate that needs to be repaired via photorespiration (8), and rubisco inhibitors such as xylulose 1,5-bisphosphate (XuBP) that are then dephosphorylated by specific phosphatases (9), (10). XuBP, other sugar phosphates and even rubisco’s bona fide substrate ribulose 1,5-bisphosphate (RuBP) can tightly bind to the active site (11), resulting in dead-end complexes that need to be reactivated for photosynthetic CO2 fixation to proceed. Conformational remodelling of dead-end complexes, which results in release of the inhibitor, is achieved in diverse organisms by a growing group of molecular chaperones known as the rubisco activases (Rca) (12). Three distantly related classes of Rca (green, red and CbbQO-type) have been identified so far (13-16). They all belong to the superfamily of AAA+ (ATPases associated with various cellular activities) proteins (17) and function as ring-shaped hexamers that couple the energy of ATP hydrolysis to rubisco remodeling. CbbQO requires one adaptor protein CbbO to associate
with the AAA hexamer CbbQ\textsubscript{6} in order to function (15). Common themes in the activation mechanism are emerging (such as manipulation of the large subunit C-terminus for red-type Rca and CbbQ\textsubscript{6}), although clear differences are also apparent (3, 12, 15).

Following the primary endosymbiotic event the green plastid lineage towards green algae and plants retained the green type Form IB rubisco from the cyanobacterial ancestor. In contrast the chloroplast genome of the red plastid lineage acquired a red-type Form I rubisco operon including the red-type Rca-encoding \textit{cbbX} gene from proteobacteria, probably via horizontal gene transfer (18, 19). All red-lineage phytoplankton for which data is available appear to encode an additional \textit{cbbX} gene in the nucleus (20).

Inhibition data on Form ID rubiscos from red lineage eukaryotic phytoplankton is limited. Rubisco from a number of species formed inhibited complexes of varying stability with RuBP (21), but in more detailed work the enzyme from the red algae \textit{Galdieria sulphuraria} was reported to exhibit high inhibition constants (22). Low rubisco activation states in rapidly extracted soluble lysates from various diatom species have been reported, suggesting the requirement for an activase (23, 24). Understanding and defining the activase requirement of eukaryotic red-type rubiscos is especially pertinent, since a number of these enzymes have been demonstrated to possess kinetic properties (such as high CO\textsubscript{2}/O\textsubscript{2} specificity factors) that would confer enhanced photosynthetic properties to land plants if successfully expressed (21, 25). Currently these efforts are hampered by an incomplete appreciation of their biogenesis requirements (3, 26).

Here we demonstrate that under physiological temperatures the red-algal rubisco from \textit{C. merolae} forms tightly inhibited complexes that can be activated by its cognate activase, which is a hetero-oligomeric complex consisting of the gene products of nuclear and plastid encoded \textit{cbbX}. By analyzing a series of mutant activases, we show that the two isoforms
function in highly co-ordinated manner, and that the nuclear-encoded subunit plays a more
critical role in activase function. Translational photosynthesis approaches that aim to take
advantage of red algal rubisco kinetics will need to take into account the requirement of a
compatible rubisco activase.

RESULTS
The red algal rubisco activase functions as a heterooligomer
A sequence alignment of selected prokaryotic and nuclear and plastid encoded cbbX
sequences indicated that residues that have been demonstrated to be functionally important in
the bacterial activase from *R. sphaeroides* (RsRca) are in general highly conserved (Fig. S1).
Bioinformatic analyses have previously shown that *cbbX* sequences can be grouped in three
clusters: prokaryotic, plastid encoded and nuclear (or nucleomorph) encoded (Fig. S2) (27),
(20), (28). The prokaryotic sequences are more closely related to the plastid genes (~70%
amino acid identity), whereas the nuclear genes have diverged more extensively (~50 %
amino acid identity to the other groups).
In order to understand the significance of the distribution of rubisco activase genes observed
in red lineage phytoplankton, we decided to recombinantly produce in *Escherichia coli* and
biochemically characterize the gene products of both nuclear and plastid encoded *cbbX*
isoforms from the red algal model organism *Cyanidioschyzon merolae*(29). The construct
encoding the nuclear isoform (CmN) comprised residues 95-401, based on sequence
alignment to the functional bacterial activase RsRca and the plastid-encoded isoform CmP
(Fig. S1). These isoforms have been produced in *E. coli* previously, but were not tested for
activase function (30). Following overexpression in *E. coli*, CmN was easily obtained at high
purity by using a combination of affinity, anion-exchange and size exclusion chromatography
(Fig. 1A) (Fig. S3A). In contrast, in our hands purification of the plastid isoform (CmP) was
only possible at pH=10 while maintaining high salt concentrations (300 mM NaCl), to avoid protein aggregation (Fig. S3B). However, when cell pellets of *E. coli* producing CmN and CmP were mixed prior to lysis, the two proteins co-purified during anion exchange and gel-filtration chromatography (at pH=8) and aggregation of CmP was not observed. This suggested plastid and nuclear-encoded isoforms formed a hetero-oligomeric complex (CmNP) (Fig. 1A, S3C). As expected, co-expression of CmN and CmP in a strain harbouring a pETDuet plasmid encoding both isoforms also allowed purification of CmNP (Fig. S3D). In this case only CmP was fused to a N-terminal His6-Ubiquitin tag for affinity purification, providing additional evidence for complex formation. Densitometric analysis of the co-expressed CmNP complex was consistent with a 1:1 stoichiometry (Fig. S3E). Gel filtration analysis of the purified complexes indicated that both CmN and CmP formed polydisperse, concentration dependent oligomers (Fig. S4A,B). In contrast CmNP presented a concentration-independent monodisperse oligomeric state (albeit with a slight trailing shoulder), when applied at concentrations of 0.4 mg/ml (corresponding to 20 µg protein loaded) or higher. (Figs. 1B and S4C). The elution volume of CmNP corresponded to a Stokes radius of 5.8 nm (Fig. S4D), within 10% of the value measured for the hexameric AAA+ protein AfQ2 (5.4 nm) (15). In summary our data are consistent with the notion that at an equal loading concentration of 0.4 mg/ml the hetero-oligomeric CmNP is a hexamer consisting of three CmN and three CmP subunits, whereas the homo-oligomeric proteins occupy a mixture of smaller oligomeric states (Fig. 1B).

Next we tested whether the algal activase complexes were functional in activating the red-type rubisco from *Rhodobacter sphaeroides*. To carboxylate RuBP, rubisco active sites need to first bind two cofactors: a non-substrate CO2 and a Mg^{2+} ion (31, 32). This results in the activated holoenzyme (ECM). In the absence of the cofactors, active sites can bind RuBP instead to form the inhibited complex (ER)(33). Rubisco activases conformationally remodel
rubisco to convert ER to the apo form E (Fig. 1C). Here, using the spectrophotometric rubisco assay (34), we compare the carboxylation activities of ECM and ER complexes in the presence or absence of the activase.

Both CmN and CmP were non-functional, but the hetero-oligomer displayed robust activase activity (Fig. 1D), corresponding to ~50% of RsRca activase function when activase concentrations were limiting (Fig. S4E). Reconstituting CmNP from purified CmN and CmP also allowed formation of the functional activase (Fig. 1D). The bacterial red-type activase RsRca displays ATPase activity only in the presence of the allosteric regulator ribulose 1,5-bisphosphate (RuBP), and the activity is strongly stimulated by its substrate rubisco (Fig. 1E) (14). No ATPase activity could be detected for CmN and CmP in isolation, whereas both co-purified and reconstituted CmNP displayed a low basal ATPase activity that could be stimulated by RuBP and inhibited rubisco. Strikingly the observed catalytic rate for CmNP was only a fraction (10-20%) of that measured for the bacterial activase RsRca under equivalent conditions (Fig. 1E). Finally we tested the ability of RsRca and CmNP to remove the non-physiological, tight binding transition state analog 2-carboxy-arabinitol 1,5bisphosphate (CABP) (35) from the ECM holoenzyme (Fig. S4F). Both activases were able to rapidly activate ECMC complexes (Fig. S4G). In comparison green-type Rca from spinach is unable to dissociate CABP from its cognate rubisco (36), whereas the CbbQO activases from chemoautotrophic bacteria are able to do so (15).

**Characterization of the red algal rubisco activation system**

Having established the identity of the functional algal rubisco activase, we aimed to reproduce the entire algal activation system. As eukaryotic rubiscos have still not been successfully produced recombinantly, we purified the *C. merolae* rubisco from algal culture to homogeneity using anion exchange chromatography and gel filtration (Fig. S5A).
Consistent with earlier work using the rubisco from another red algal thermophile *Galdieria sulphuraria* (22), we found that *C. merolae* rubisco did not form stably inhibited complexes with RuBP or CABP at 25 °C (Fig. S5B). We therefore decided to investigate the properties of this enzyme at the more physiologically relevant temperature of 45 °C (29). ECM formation was efficient at both temperatures with the holoenzyme displaying linear activity consistent with a carboxylation velocity of 0.4 s⁻¹ at 25 °C and 0.7 s⁻¹ at 45 °C. At the higher temperature, incubation of rubisco with either inhibitor readily led to the formation of stably inhibited complexes that did not spontaneously reactivate at 25 °C (Fig. S5C, D). At 45 °C the CmNP activase was able to remove both inhibitors from the inhibited red-algal rubisco complexes with complete activation of CmER achieved after 2 minutes. (Fig. 2A, B). At 25 °C activation of inhibited algal rubisco by CmNP was marginal (Fig. S5C, D), in stark contrast to its robust ability to activate the bacterial enzyme at this temperature (Fig. 1D). At 35 °C (but not at 25 °C) the simpler homo-oligomeric RsRca was able to activate inhibited algal CmER, but displayed low activity when exposed to CmECMC (Fig. S5E, F). At 45 °C approximately one CmNP hexamer per inhibited active site was required to achieve full activation of CmER (Fig. S6A, B). At subsaturating CmNP concentrations rubisco activity became linear corresponding to a fractional activity of ECM, indicating that activation and inhibition by RuBP was proceeding at equivalent rates (Fig. S6A, B). When CmNP concentrations were saturating, full activation could be achieved even when rubisco active site concentrations were as low as 0.05 µM (Fig. S6C).

When comparing the relative ability of different rubisco complexes to stimulate ATPase activity, we noticed that inhibited algal complexes did not stimulate RsRca ATPase at 25 °C, whereas both algal and bacterial complexes resulted in modest, but concentration dependent stimulation of CmNP at both 25 °C and 45 °C (Fig. 2C, S6D,E). Intrigued by the temperature dependence of the algal rubisco activation system, we determined the optimal temperature of
CmNP for ATP hydrolysis to be $\geq 55$ °C (Fig. 2D). CmNP lost activity upon incubation at 60 °C and was completely denatured at 65 °C (Fig. S6F). The bacterial RsRca exhibited a thermal optimum of 35 °C for its ATPase function, and the protein aggregated at higher temperatures (Fig. 2D). Given the thermolabile nature of crop plant rubisco activase (37), thermostable activases are of potential biotechnological interest (38).

The role of the allosteric regulator RuBP

The nucleotide-bound bacterial RsRca undergoes an oligomeric transition from an ATPase inactive fibril to the functional hexamer, which is triggered by the binding of the allosteric regulator RuBP and provides a regulatory mechanism to respond to increases in Calvin-Benson cycle flux (14). These transitions were earlier demonstrated using negative-stain electron microscopy, but we found they can be recapitulated using gel filtration (Fig. 3A). In the Apo form, RsRca behaved as a large ~400 kDa polydisperse oligomer. In buffer containing Mg-ATP RsRca eluted close to the void volume, consistent with previously observed fibrils. In contrast in the presence of both RuBP and MgATP, the condition under which hexamers were previously observed, a monodisperse symmetrical peak at 1.23 ml was obtained. In contrast, under equivalent conditions CmNP eluted as a species resembling the RsRca hexamer (although a shoulder was observed, indicating some level of subunit dissociation) (Fig. 3B). Hence, although the allosteric activation of ATPase activity by RuBP is conserved in the algal activase, the associated oligomeric transition from fibril to hexamer has apparently not been retained during evolution. Kinetic analysis of CmNP ATP hydrolysis was consistent with a non-cooperative model in the absence of RuBP. In the presence of the ligand the substrate concentration required for half-maximal activity was reduced by 75%, and the maximal catalytic rate almost doubled (Fig. S6G). Importantly, the data was not consistent with the Michaelis-Menten model, but indicated positive co-operativity with a Hill constant of 2.1. Hence for the algal activase binding of the allosteric regulator RuBP results
in conformational changes that lead to higher ATP hydrolysis efficiency and introduce co-
operativity, but the ligand does not trigger oligomeric transitions as seen for the bacterial
homolog. Positive co-operativity has recently also been described for the green-type Rca
from tobacco, where it was induced both by the presence of the competitive inhibitor ADP or
excess Mg$^{2+}$ (39).

CmN subunits are more critical than CmP for ATPase and activase function

Given the strict requirement for both nuclear and plastid encoded subunits in the functional
red algal activase, we decided to ask whether the subunits had evolved unique roles, or
whether both contributed equally to their function of ATP hydrolysis and rubisco activation.
Towards this end we constructed a series of amino-acid exchanges in both subunits and, by
mixing cell pellets expressing the desired CmN and CmP variants, purified CmNP complexes
harbouring different combinations of mutations. All variant enzymes examined here could be
purified as the assembled hetero-hexamer (Fig. S7A, B).

Initially we considered the rubisco activase and ATPase activity of mutants containing
substitutions in catalytic residues of the Walker motifs and arginine fingers of their
nucleotide binding domains (NBD). The Walker motifs interact with the nucleotide bound to
its NBD, and the arginine finger interacts with the γ-phosphate of the nucleotide bound to the
adjacent active site (40) (Fig. 4A). Both single and double Arginine finger substitutions
(R194A – residue numbering follows RsRca, Fig. S1) abolished ATPase and activase activity,
irrespective of subunit (Fig. 4B). Similarly, a Lys to Ala (K80A) substitution in the Walker A
motif that generally abolishes nucleotide binding (17) led to completely inactive ATPase and
activase complexes in all instances (Fig. S7C). These results indicated that the subunits do
not operate independently as proposed for ClpX (41), but are instead stringently functionally
coupled. In contrast to the complete loss of functionality observed for Walker A and arginine
finger substitutions, we found that a mutation of the Walker B glutamate (E138Q) was largely tolerated by CmP alone (maintaining 72% of activase activity), but abolished ATPase and activase activity when present in either CmN or both subunits (Fig. 4B, S7D). This result is reminiscent to those observed for two other hetero-oligomeric AAA+ proteins, Pex1/6 (42, 43) and Yta10/12 (44) where a Walker B mutation in one subunit, but not the other abolished functionality of the complex. In contrast to those systems, CmNP\textsuperscript{B} complexes hydrolyzed ATP at around twice the rate of CmNP in both the absence and presence of the regulator RuBP. The Walker B substitution used here generally leads to an active site that binds but does not hydrolyze ATP (17). Nucleotide-bound CmP thus appears to strongly stimulate CmN ATPase activity, a phenomenon seen previously in ClpB (45).

Unexpectedly, ATPase (but not activase) activity of a complex containing a Arginine finger substitution in CmN could be restored by a concomitant Walker B mutation in CmP. The resultant complex CmN\textsuperscript{R}\textsuperscript{P}\textsuperscript{B} had a high basal ATPase rate equivalent to that of CmNP\textsuperscript{B}, which was however not stimulated further by RuBP or inhibited rubisco (Fig. 4B). In contrast the inverse arrangement CmN\textsuperscript{B}\textsuperscript{P}\textsuperscript{R} was non-functional (Fig. 4B). We conclude that subunits in the heterohexamer are arranged in an alternating sequence, which in complex CmN\textsuperscript{R}\textsuperscript{P}\textsuperscript{B} permits the formation of three functional ATPase sites harbouried by the nuclear encoded subunit. These are formed by the Walker motifs of CmN and the arginine finger of CmP (Fig. 4A, B). An alternative dimer of trimer arrangement would lead to only a single functional active site per hexamer (44), and is not consistent with this result. The other described heterohexameric AAA+ systems Yta10/12 and Pex1/6 also follow a strictly alternating sequence (42-44).

**Both subunit types are involved in rubisco remodeling**

Similar to other AAA+ ATPases (17, 46), the mechanism of bacterial RsRca uses an axial pore loop 1 tyrosine to transiently thread the C-terminus of the rubisco large subunit to
trigger the release of bound inhibitors. We tested the ability of CmNP to activate R. sphaeroides rubisco mutants with two or four amino acid deletions at the C-terminus of the large subunit (Fig. 5A). As observed for RsRca earlier (14), both activation (Fig. 5B) and ATPase stimulation of CmNP was impaired (Fig. 5C), supporting a conserved mechanism.

We therefore decided to probe the relative importance of the key pore loop tyrosine in the two isoforms. Pore loop tyrosine (Y114A) substitutions led to complete abolishment of activase activity, when present in the nuclear or both subunits (CmN^Y and CmN^YP^Y) (Fig. 5D). These complexes displayed increased basal and RuBP stimulated ATPase activity, likely due to decreased steric constraints of pore loop movement as observed for ClpX(46). The CmNP^Y variant displayed wild-type ATPase activity (although stimulation by inhibited rubisco was reduced) and retained 19% of activase activity. These results confirm the importance of the axial pores in the remodeling mechanism of the algal activase and similar to the catalytic variants suggest that the nuclear subunit plays a more critical (but not exclusive) role in remodeling inhibited rubisco complexes.

To further understand the requirement for binding of the allosteric regulator, we characterized variants mutated at the RuBP binding site. All residues shown to be involved in RuBP binding in RsRca(14) are conserved in both CmN and CmP (Fig. S1), so we decided to analyze the effect of substituting Arg-239 with alanine. This residue is predicted to coordinate a phosphate moiety of RuBP. When present in either the nuclear (CmN^S^P) or both isoforms (CmN^S^PS^S), both ATPase and rubisco activase activity were completely abolished (Fig. 5D). CmNP^S retained ~50% basal ATPase activity, which was no longer stimulated by RuBP or rubisco. Nevertheless this complex had 13% of the wild-type rubisco activase activity (Fig. 5D). The phenotype of these variants is consistent with the notion that allosteric RuBP binding to the main catalytic subunit CmN of the algal Rca is essential for activase function.
It has been unclear whether the propensity of rubisco to form dead-end inhibited complexes and the requirement for an activase to remodel these extends into the red-lineage eukaryotic phytoplankton (22). Here we provide unambiguous biochemical evidence that red algal Form ID rubisco forms stably inhibited complexes at physiological temperatures that can be rescued by its cognate activase CmNP. The activase is a 1:1 hetero-hexamer of plastid and nuclear-encoded subunits. In accordance with observed gene distributions we expect this observation to hold widely for organisms of the red-plastid lineage, which includes diatoms, brown algae and cryptophytes. It is noteworthy that the algal activase overall was highly functional in remodeling bacterial rubisco even at suboptimal temperatures (Figs. 1D and S4E), whereas RsRca exhibited relatively less activity (and a lack of ATPase stimulation) with algal rubisco as the substrate (Fig. S5C-F and Fig. 2C). It remains to be established whether these results are caused by differences in relative rubisco complex stability, activase efficiency, protein-protein interaction compatibility or a combination of these factors.

Although the algal activase has conserved its dependence on pore loop 1 tyrosines and the rubisco large subunit C-terminus for rubisco remodeling activity (Fig. 5B,D), and thus utilizes the same mechanism as the bacterial activase, its regulatory properties differ markedly (Fig. S8). Whereas RsRca forms ATPase inactive fibrils in the absence of the allosteric regulator RuBP, the algal enzyme has a basal constitutive ATPase rate and forms the hexamer under the same conditions. In RsRca RuBP triggers an oligomeric transition to the ATPase active hexamer, whereas the catalytic efficiency for ATP hydrolysis increases for CmNP. Once the inhibited rubisco complex is engaged, ATPase activity of RsRca increases several fold and in comparison is only modestly stimulated in the algal enzyme (Fig. S8). We predict that characterization of more red-type activases will uncover more diverse regulatory
behavior that will guide the choice of activases to be used in biotechnological ventures aiming to engineer photosynthetic CO₂ fixation.

Our characterization of activase variants mutated in key functional residues suggests an alternating arrangement of subunits with a catalytically dominant CmN (Fig. S8). We interpret the high activase functionality of CmNP[^B] (Fig. 4B) to indicate that in the wild-type the CmP subunit is either catalytically inert, or its ATP hydrolysis function does not significantly contribute to the energy utilized during rubisco remodeling. In spite of its relegated role in ATP hydrolysis, the phenotype of Walker A and arginine finger variants suggests CmP plays a critical role in tightly co-ordinating ATP hydrolysis.

The availability of sensitive and rapid biochemical assays for both ATPase and activase function makes rubisco activation a highly accessible system to study structure-function relationships of evolutionarily diverse AAA+ proteins. At the same time, efforts to engineer photosynthesis, in particular the rate-limiting CO₂-fixing reactions, are gaining momentum (47). In order to effectively and efficiently harness the potential of synthetic biology it is essential to carefully characterize the diversity of CO₂-fixation modules that are found in the vast treasure troves of metagenomic studies (48).

**MATERIALS AND METHODS**

Detailed experimental procedures are described in *SI Text*. All activase proteins and *R. sphaeroides* rubisco were produced recombinantly in *E. coli*. *C. merolae* rubisco was obtained from algal culture. Proteins were purified as described in *SI Text*. Rubisco and ATPase activity was measured using coupled spectrophotometric assays (34, 49). Inhibited rubisco complexes were prepared as described in *SI Text*. 
ACKNOWLEDGMENTS

CPBP was a gift of Dr. Spencer Whitney (Australian National University). We thank Melvin Chua, Maria Claribel Lapina and Lynette Liew for technical assistance. The research was funded by a Nanyang Technological University startup grant and a Ministry of Education (MOE) of Singapore AcRF Tier2 grant (MOE2013-T2-2-089) to O. M.-C.

Author Contributions

N. L. and O. M.-C designed experiments. N. L. performed most experiments with help from Y.-C.T. O. M.-C wrote the manuscript.
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**Fig. 1.** The red algal rubisco activase functions as a heterooligomer. (A), SDS-PAGE analysis of purified activase proteins. 3 µg protein loaded per lane. (B) Gel filtration analysis of the algal activase proteins. At identical loading concentration (20 µg) the hetero-oligomer CmNP elutes as a larger complex than the individual isoforms. Buffer conditions: CmN and CmNP, buffer A; CmP, buffer A adjusted to pH=10, 300 mM NaCl (C) Rubisco activases catalyze the removal of inhibitory RuBP from inhibited active sites (ER) to form the inactive apo-enzyme E. E spontaneously binds the cofactors CO₂ and Mg²⁺ to form the functional holoenzyme ECM. (D) The hetero-oligomer, but not individual isoforms, can activate *R. sphaeroides* rubisco in the inhibited form. Rubisco activity assay of active (RsECM) and inhibited (RsER) rubisco complexes (0.5 µM active sites) in the presence and absence of *C. merolae* activase complexes (5 µM activase protomer). Assay temperature, 25 °C. (E) The ATPase activity of the hetero-oligomer, but not individual isoforms, is stimulated by RuBP and inhibited *R. sphaeroides* rubisco. ATPase assays of the activases (5 µM protomer) performed at 25 °C in the presence and absence of RuBP (1 mM) and *R. sphaeroides* ER (3 µM active sites). Error bars indicate the mean and s.d. of at least three independent experiments. “CmN + CmP” indicates the hetero-oligomeric complex reconstituted *in vitro* from purified CmN and CmP whereas CmNP is the co-purified complex.
Fig. 2. Biochemical characterization of the red algal rubisco activation system. (A,B) The algal CmNP activase can efficiently activate inhibited algal ER (A) and ECMC (B) complexes at physiological temperature. Rubisco activity assay conditions: 45 °C, 0.5 µM rubisco active sites, 5 µM activase protomer. (C) Regulation of the ATPase activity of bacterial and algal activases (5 µM protomer) by RuBP (1 mM) and inhibited bacterial and algal rubisco complexes (3 µM active sites). *, not measured due to RsER instability at 45 °C. (D) The algal activase is thermostable. ATPase assays of bacterial and algal activases (5 µM protomer) were performed at temperatures up to 55 °C in the presence of 1 mM RuBP. Error bars indicate the mean and s.d. of at least three independent experiments.
Fig. 3. The bacterial, but not the red-algal, activases undergo RuBP-triggered oligomeric transitions. Gel filtration analysis of 20 µg of bacterial (A) and algal (B) activase protein in buffer A supplemented with 0.1 mM RuBP, 1 mM ATP and 5 mM MgCl₂ as indicated.
Fig. 4. The subunits are highly co-ordinated and arranged alternately. (A) Active site architecture based on the atomic model of the RsRca hexamer (PDB:3ZUH). (B) Biochemical characterization of CmNP active site variants. Superscripts indicate the following amino acid substitutions: B- E138Q (Walker B), R- R194A (Arginine finger). The inset schematic illustrates the alternative arrangement of subunits consistent with formation of three functional ATPase active sites (black letters- motifs intact, red letters- mutated) for the N^{R}B^{B} variant. ATPase assays were performed at 45 °C using equivalent conditions to those in Fig 2C. Rubisco activity assays: red numbers indicate no detectable activity under conditions as in Fig. 2A. Green numbers: relative initial rate of ECM formation was quantified in assays containing 0.3 µM active sites (CmER) and 1 µM activase protomer at 45 °C (Fig. S7D). Error bars indicate the mean and s.d. of at least three independent experiments.
Fig. 5. The mechanism of the algal activase is conserved. (A) Alignment of the rubisco large subunit C-termini of *R. sphaeroides* and *C. merolae*. (B) Impaired activation of *R. sphaeroides* Rubisco with two or four amino acids truncated from the large subunit. Assay conditions were as described in Fig. 1D and the relative rate of ECM formation was quantified. (C) Truncated *R. sphaeroides* rubisco does not stimulate the ATPase activity of the activases. Assays were performed as in Fig. 1E in the presence of 1 mM RuBP. (D) Biochemical characterization of CmNP variants mutated at the putative RuBP binding site and pore loop 1. Superscripts indicate the following amino acid substitutions: S- R239A (Sugar binding), Y- Y114A (Pore loop tyrosine). Experiments were performed as in Fig. 4B but using 2 µM activase protomer to quantify activase activity (green numbers). Error bars indicate the mean and s.d. of at least three independent experiments.