Unravelling structural and mechanistic elements of catalytic subunits A and coupling subunit F and structural and kinetic studies of the A3B3D and A3B3DF-subcomplexes of the Methanosarcina mazei Gö1 provide insights into the single molecule dynamics of this engine

Singh, Dhirendra

2017

Singh, D. (2017). Unravelling structural and mechanistic elements of catalytic subunits A and coupling subunit F and structural and kinetic studies of the A3B3D and A3B3DF-subcomplexes of the Methanosarcina mazei Gö1 provide insights into the single molecule dynamics of this engine. Doctoral thesis, Nanyang Technological University, Singapore.

<http://hdl.handle.net/10356/70029>

<https://doi.org/10.32657/10356/70029>

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**Unravelling structural and mechanistic elements of
catalytic subunits A and coupling subunit F
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structural and kinetic studies of the A_3B_3D and A_3B_3DF -
subcomplexes of the *Methanosarcina mazei* Gö1 provide
insights into the single molecule dynamics of this engine**

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2016**

Acknowledgement

I wish to express my sincere gratitude to my advisor, Professor Gerhard Grüber, for his expert guidance and warm encouragement. This thesis has been possible only because of his help and support. I feel fortunate to be a part of his lab and shall remain ever grateful to him for providing a conducive environment.

I am very thankful to each and every present and past member of Grüber's lab for all the help and support. Special thanks to Dr. Asha Balakrishna for helping me with the protein purification and crystallography and also for her support and care. Truly appreciate Lavanya for being a good friend and for her constant support. Many thanks to Dr. Sandip, Dr. Phat, Dr. Lavanya and Saw for teaching me basic lab techniques and for all the love and care they bestowed on me. Special thanks to Dr. Goran, Dr. Yew kwang and Dr. Wilson for all the interesting discussions. I wish to express my gratitude to Dr. Neelu for accompanying me to synchrotron and helping me with crystallographic data collection and Dr. Sony and Dr. Ardina for their crystallographic inputs and. Special thanks to Dr. Hendrik for teaching me German phrases and introducing me to Thai food apart from the encouragement given in our scientific work. Thanks to Arvind, Ankita, Nesha, Ramya for being good juniors and listening to my suggestions and teaching me a lot in return. I am also grateful to the academic and technical staffs at the School of Biological Sciences, who have helped me in one way or the other in my research work.

I am thankful to all the support given by our collaborators, Prof. Shashi Bhusan, Prof. Wayne Frasch, Prof. Michael Börsch and Prof. Thorsten Wohland, which have been helpful in completing the projects. I gratefully acknowledge Prof. Volker Müller for kindly providing the clone for *MmA₃B₃D*. I acknowledge the financial support rendered by Ministry of Education as MOE Tier 2 grant and the Nanyang Research Scholarship. Thanks to the staff of beamline 13B1 at the National Synchrotron Radiation Research Centre (NSRRC), a national user facility supported by the National Science Council of Taiwan. The Synchrotron Radiation Protein Crystallography Facility at NSRRC is supported by the National Research Program for

Genomic Medicine. I wish to take this opportunity to extend my gratitude to my Thesis Advisory Committee members, Dr. Susana Geifman Shochat and Dr. Salil Bose.

I am also indebted to my innumerable friends worldwide, for their support and love. Last but not the least, my heartfelt gratitude to my parents and brother for their selfless support and love and also for understanding my absence.

Dedicated to

My Parents and My Brother

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Abbreviations

2D	Two-dimensional
3D	Three-dimensional
Å	Ångström
ADP	Adenosine diphosphate
AMP-PNP	Adenylyl imidodiphosphate
ATP	Adenosine triphosphate
APD	Avalanche photo diode
BSA	Bovine Serum Albumin
CHES	N-Cyclohexyl-2-aminoethanesulfonic acid
DTT	Dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
FCS	Fluorescence correlation spectroscopy
FPLC	Fast protein liquid chromatography
FRET	Fluorescence resonance energy transfer
HCl	Hydrochloric acid
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
IPTG	Isopropyl-β-D-thiogalactoside
ITC	Isothermal Titration Calorimetry
Kb	Kilo base pairs
kDa	kilo-Dalton
LB	Luria-Bertani
M	Molar
<i>MmA₃B₃D</i>	<i>Methanosarcina mazei</i> Gö1 A ₃ B ₃ D (WT)
<i>MmF</i>	<i>Methanosarcina mazei</i> Gö1 subunit F (WT)
<i>Mt</i>	<i>Mycobacterium tuberculosis</i>
<i>Mtε</i>	<i>Mt</i> F-ATP synthase subunit ε
NaCl	Sodium Chloride
Ni	Nickel
NMR	Nuclear magnetic resonance
nM	Nanomolar
nm	Nanometer
NTA	Nitrilotriacetic acid
PAGE	Polyacrylamide Gel Electrophoresis

PCR	Polymerase Chain Reaction
PDB	Protein data bank
PEG	Polyethylene glycol
Pefabloc ^{SC}	(4-(2-Aminoethyl)benzenesulfonylfluoride)
PMSF	Phenylmethylsulfonyl fluoride
RCSB	Research Collaboratory for Structural Bioinformatics
RMSD/r.m.s.d	Root mean square deviation
SAXS	Small Angle X-ray Scattering
SDS	Sodium dodecyl sulphate
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
ScF	<i>Saccharomyces cerevisiae</i> subunit F (WT)
TRIS	Tris-(hydroxymethyl) aminomethane

Abstract

ATP synthases and ATPases are membrane-bound enzymes that synthesize either ATP from ADP and phosphate by utilizing the electro-chemical potential of the membrane or generate electro-chemical potential by hydrolysing ATP, respectively. The ATP synthases found in archaeal cell membranes are mainly known as A-ATP synthases. The unique ability of archaea to grow on low energy substrates and thrive on harsh environmental conditions separates them from other domains of life and therefore A-ATP synthases are believed to be strongly related to the predecessor of the ATP synthases/ases [1]. Unravelling their structural and functional aspects will provide a deep insight into the evolutionary pathway of ATP synthases/ases.

A-ATP synthases (A_1A_O ATP synthases) are multi-subunit complexes with the subunit composition and stoichiometry of $A_3:B_3:D:F:(EG)_2:C:a:c_X$, whose functioning is determined by the coordination of these subunits [2]. Several studies have been carried out on A-ATP synthases addressing the question of ion-coupling in the membrane-embedded A_O -domain and ATP synthesis in the A_3B_3D -hexamer via the central stalk subunits C, D and F. However, details of the mechanistic coupling elements of subunit F, the nucleotide binding A-B interface, and the proposed rotating central stalk subunits (D and F) of this molecular engine are missing. In the present study, the role of subunit F as a stimulator of ATPase activity in the A_3B_3DF -complex of *Methanosarcina mazei* Gö1 (*Mm*) ATP-synthase is established with the combinatorial studies using electron microscopy and enzyme kinetics. Further, C-terminal deletions and mutations of subunit F revealed its importance in enhancing the ATPase activity. In another study, crystals of the A_3B_3D -complex of *M. mazei* Gö1 ATP-synthase were obtained and optimized for the purpose of retrieving information about the interface of subunits A and B of this enzyme complex. The mechanistic studies were carried out on the *Mm* A_3B_3DF complex, and its angular velocities were determined using gold-nanorod and compared to the distantly related eubacterial F-ATP synthases [3].

ATP synthases are described to have mechanisms which regulate the unnecessary depletion of ATP pool during an energy limited state of the cell. Mg-ADP inhibition is one of the regulatory features where Mg-ADP gets entrapped in the catalytic site, preventing the binding of ATP and further inhibiting ATP hydrolysis [4]. V-ATPases are ATP-driven proton pumps and localized in eukaryotic vacuoles, Golgi-derived vesicles, lysosomes, synaptic vesicles etc. They are mainly involved in membrane trafficking, cellular pH homeostasis, and neurotransmitter secretion [5, 6]. Currently, Mg-ADP inhibition is not observed in V-ATPases and to understand the involvement of their P-loop (phosphate binding loop) in shielding them from Mg-ADP inhibition, the P-loop of V-ATPases ($_{257}GAFGCGKTV_{265}$) was mimicked in

A-ATP synthases ($_{234}\text{G}\underline{\text{P}}\text{F}\text{G}\underline{\text{S}}\text{GKTV}_{242}$). The P-loop of V-ATPases and A-ATP synthases are mostly identical with differences in only two residues. These two residues of A-ATP synthases (proline and serine) were mutated to alanine and cysteine, respectively, in *MmA₃B₃D* and *MmA₃B₃DF*. The enzymatic assays revealed a decrease of ATP binding affinities for the mutated complexes (*MmA₃B₃D* and *MmA₃B₃DF*). In addition, the dismissal of Mg-ADP inhibition was observed in the mutated *MmA₃B₃DF* complex. The mutations were incorporated in the individual catalytic subunit A of A-ATP synthase and alterations in substrate-binding were studied by fluorescence correlation spectroscopy and X-ray crystallography.

1. Introduction

1.1. Introduction of ATP synthases and V-ATPases

Biological energy is required to maintain the highly ordered state of living cells, which can be supplied either by light in case of plants and photosynthetic bacteria (Rhodospirillaceae and Chlorobiaceae) or by chemical reactions in chemotropic archaea, bacteria and other organisms belonging to animalia [7, 8]. Primary energy source from light or chemical reaction, needs to be converted to an energy currency that can be used by the living cells. Adenosine triphosphate (ATP) and the electrochemical ion (H^+ or/and Na^+) gradient across a membrane are the two different circulatable energy currencies favoured by evolution [7]. The enzyme, which couples the free conversion of these currencies was referred to as the ‘coupling factor’ and later termed as ATP synthase [9-11]. As an integral part of the electron transport chain, the ATP synthases synthesize ATP by utilizing the electrochemical gradient or generate an electrochemical gradient by hydrolysing ATP with the latter function endowing them with the name ‘ATPases’.

The primal importance of ATP synthases and ATPases in the biological world is emphasized with their widespread prevalence across the phylogenetic tree that have survived natural selection. These enzymes are classified as V-ATPases and F- and A-ATP synthases on the basis of function and occurrence. F-ATP synthases were the first to be identified and are present in cell membranes of bacteria, thylakoid membranes of chloroplast and inner membranes of mitochondria and the letter ‘F’ stands for ‘Phosphorylation Factor’ [12, 13]. V-ATPases are present in the lysosomes, endosomes, synaptic vesicles, and eukaryotic vacuoles and capable of functioning as ATP-driven proton pump under physiological conditions [6, 14-18]. The third class of these rotary enzymes are A-ATP synthases present in archaea, which functions predominantly as an ATP synthase similar to F-ATP synthases [19, 20]. All the three rotary enzymes are divided into two domains; a soluble catalytic domain (F_1 , V_1 and A_1) and a membrane embedded ion-translocating domain (F_o , V_o and A_o) [5, 20, 21]. In the case of F_1 , V_1 and A_1 domains, the numerical ‘1’ stands for the first factor to be identified and in F_o , V_o and A_o , the letter ‘O’ represents the oligomycin binding site [22]. Though F_oF_1 , V_oV_1 and A_oA_1 -ATPases are classified differently, studies reveal that they share a common ancestral origin [20, 23].

1.2 Classification and subunit composition of ATP synthases and ATPases

As mentioned in the previous section, all the three rotary enzymes share structural features and they all function with the rotation of the central stalk subunits and the membrane embedded ring. The membrane embedded part (F_o , V_o and A_o) of the rotary ATPases is comprised of mainly hydrophobic amino acids. The soluble cytoplasmic part (F_1 , V_1 and A_1)

is divided into a hexameric head, a central stalk and peripheral stalk(s). Although these rotary enzymes share structural and functional features, they are highly diverse even in their classes. For example, F-ATP synthases of *Propiogenium modestum*, *Ilyobacter tartaricus* and *Acetobacterium woodii* are powered by Na⁺ ions, whereas majority of F-ATP synthases are dependent on H⁺-translocation [24-26]. Also, the regulation of wasteful ATP hydrolysis is different amongst F-ATP synthases of mitochondria, chloroplast and bacteria. Mitochondria possess an Inhibitory factor protein (IF₁), while bacteria rely on Mg-ADP inhibition and variable conformational states of ϵ -subunit. Chloroplasts circumvent the problem of wasteful ATP hydrolysis by combining the roles of Mg-ADP inhibition and ϵ -subunit, with the varying the oxidation/reduction states of a disulfide bond within the γ -subunit [27-37].

The widespread prevalence of F-ATP synthases and their major role as energy converter in prokaryotic and eukaryotic organisms have made them the most extensively studied enzyme amongst rotary enzymes. The eubacterial F-ATP synthases are simpler than the mitochondrial F-ATP synthases in composition, hence the model for eubacterial F-ATP synthases is discussed here along with those of V-ATPases and A-ATP synthases. The F-ATP synthases from eubacteria are comprised of eight subunits ($\alpha_3\beta_3\gamma\delta\epsilon:b_2:a:c_{9-14}$). The cytoplasmic catalytic F₁ domain, consists of $\alpha_3\beta_3$ hexamer responsible for the catalysis, a rotating subunit γ made up of coiled coil α -helices, a regulatory subunit ϵ , a peripheral stalk made of two b subunits and subunit δ capping the peripheral stalk. The membrane-embedded ion translocating F₀ part contains a c -ring made up of several helical c -subunits, each carrying a negatively charged glutamate residue, whose protonation and deprotonation results in the rotation of the c -ring, which is coupled to the rotation of the central stalk (subunit γ). The protonation of the carboxylate ion of this glutamate residue, present on the external surface of the c -ring, results in movement of glutamate towards the more hydrophobic environment, causing rotation of the c -ring. The stator subunit a is embedded in the membrane adjacent to the c -ring and a membrane embedded part of the peripheral stalk subunit b (Figure 1.1). Subunit a carries a positively charged residues which neutralises the carboxylate of c -subunits leading to the deprotonation of c -subunit glutamate and release of proton into the mitochondrial matrix or bacterial cytoplasm or stroma of chloroplast, depending of on the type of F-ATP synthase [38]. The number of carboxylates in the c -ring determines the number of translocated ions across the membrane.

Figure 1.1: An assembled model of bacterial F-ATP synthase generated using PyMOL [39]. The structural coordinates for the subunits in this model are drawn from the RCSB database [40]. The $\alpha_3\beta_3\gamma\epsilon$ -subcomplex (PDB: 3OAA), subunit δ (PDB: 2A7U), the tether domain (2KHK) and the membranous part of subunit b (PDB: 1B9U) are of *E. coli* F-ATP synthase, the peripheral stalk subunit b (PDB: 2CLY) is of bovine mitochondrial F-ATP synthase and the c -ring (PDB: 5DN6) is of *P. denitrificans* F-ATP synthase [41-46].

The A-ATP synthases containing nine subunits [$A_3:B_3:D:F:(EG)_2:C:a:c_X$], function predominantly in the direction of ATP synthesis, like its counterpart F-ATP synthase. The cytoplasmic A_1 domain is comprised of a hexameric head (A_3B_3) involved in catalysis, a central stalk (subunits C, D and F) responsible for coupling with A_0 domain and the two peripheral stalks $(EG)_2$ for the support and containment of elastic energy. The parts of membrane-embedded A_0 domain include a rotor (c -ring) and a stator (subunit a), which are responsible for the translocation of ions across the membrane [47, 48]. The stator subunit a (~95 kDa) of A-ATP synthases have more structural similarities to the V-ATPases subunit a (~95 kDa) than to the subunit a (~28 kDa) of F-ATP synthases (Figure 1.2) [1, 19].

The V-ATPases present in lysosomes, endosomes, synaptic vesicles and plant vacuoles are involved in acidification, membrane trafficking, neurotransmitter secretion, pH sensing etc [6, 14-18]. They are dedicated ATP-driven proton pumps and not capable of synthesizing ATP, unlike the counter parts (F-ATP synthase and A-ATP synthase) [49]. The most studied V-ATPase is that of *Saccharomyces cerevisiae*, with partial subunit composition similarity to A-ATP synthases. Variation arises from the presence of additional peripheral stalk, subunit H, subunit C and subunit e which are absent in A-ATP synthases [49-52]. All the structural

and functional differences along with 16S rRNA sequencing clearly suggest that A-ATP synthases and V-ATPases belong to separate branches of the phylogenetic tree warranting distinct classification [23, 53].

Figure 1.2: Surface representation view of an assembled model of A-ATP synthase generated using PyMOL [39]. The structures used for assembly are from the RCSB Protein Data Bank [40]. Subunit A (PDB: 3I4L) and subunit E (PDB: 4DT0) is of *P. horikoshii* A-ATP synthase, subunit B (PDB: 2C61) and subunit F (PDB: 2OV6) is of *M. mazei* Gö1 A-ATP synthase, subunit D (PDB: 3AON) and *c*-ring (PDB: 2BL2) is of *E. hirae* A-ATP synthase, subunit C (PDB: 1R5Z) and subunit G (PDB: 3K5B) of *T. thermophilus* A-ATP synthase and subunit *a* (PDB: 3RRK) of *M. ruber* A-ATP synthase [54-61].

The soluble V_1 domain has eight subunits [$A_3B_3C:D:F:(EG)_3:H$], where subunits A and B form the catalytic hexameric head, subunits D and F are part of central stalk required for the coupling process, subunits E and G are the peripheral stalks and are also involved in reversible disassembly along with subunit C and subunit H is required for the ATPase activity [5, 50, 62-64]. The V_O domain contains subunits $a:d:e:c_4-5:c':c''$, where subunit *a*, *c* and variants of *c* (*c'* and *c''*) are involved in translocation of protons [49, 65, 66]. The subunit *d*, located below the central stalk subunit is required for the ATPase activity [67]. The proposed function of subunit *e*, is in V-ATPase targeting and activation (Figure 1.3) [49, 68, 69]. A comparison of subunit composition of the three rotary enzymes is presented in table 1.1.

Figure 1.3: The assembled model of V-ATPase of *S. cerevisiae* generated using PyMOL [39]. The structure solved at 6.9 Å using electron microscopy was taken from the RCSB database and adapted for surface representation (PDB: 3J9T). High resolution structures of subunits D and F, solved at 3.1 Å of *S. cerevisiae* V-ATPase (PDB: 4RND) have been used to complement the assembled model [70, 71].

Table 1.1. Comparison of F-, A- and V-ATPases on the basis of subunit composition

Domain	V-ATPase	A-ATP synthase	F-ATP synthase	Function
V₁/A₁/F₁	A	A	β	Catalysis of ATP
	B	B	α	Nucleotide binding, as a regulatory subunit
	C	--	--	Assembly and reversible dissociation
	--	C*	--	Involved in ATPase activity
	D	D	γ	Coupling subunit, part of central stalk
	E	E	--	Peripheral stalk
	F	F	ε	Coupling subunit, part of central stalk
	G	G	b	Assembly, peripheral stalk
	H	--	--	Involved in ATPase activity
V₀/A₀/F₀	a	a	a	Involved in proton translocation
	d*	--	--	Involved in ATPase activity
	c	c	c	Proton translocation
	c'	c'	--	Proton translocation
	c''	c''	--	Proton translocation
	e	--	--	V-ATPase targeting and activation

*indicates homologous subunits.

1.3 Mechanism driving catalysis in ATP synthases

With the understanding of subunit composition of ATP synthases, the following section elaborates the mechanism behind the catalytic process in ATP synthases/ases. However, given that most studies have been performed using sub-complexes of the F_1 domain, much insight has been gained in the direction of ATP hydrolysis. The pioneering work by Boyer proposed as the binding change mechanism, shed light on the transitions occurring at the three α - β catalytic interfaces [72]. These interfaces comprise of residues mainly contributed by the catalytic subunit β and some from the non-catalytic subunit α . Although, there are a total of six α - β interfaces, only three are catalytically active.

The widely accepted mechanism for binding change accompanying catalysis is represented in figure 1.4 and shows the synchronised movements of $\alpha_3\beta_3$ and γ subunits in the sequence of catalysis that occurs at saturating concentration of ATP.

Figure 1.4: The mechanism of rotation of F-ATP synthases in the direction of ATP hydrolysis.

The 120° rotation of subunit γ in $\alpha_3\beta_3\gamma$ -complex of F-ATPases and conformational changes in the catalytic sites are shown in the figure. This is a representation of the membrane side view of the catalytic hexamer with the centrally located subunit γ . The hexameric catalytic head ($\alpha_3\beta_3$) is represented by hexagons, where orange hexagon is for catalytic subunit β and the subunit α is shown with green hexagon. The central stalk subunit γ (yellow) is divided into coiled-coil domain and the globular domain. The hexameric head has three potential sites for nucleotide binding and are represented as: β_E - empty, β_{TP} - ATP bound, β_{DP} -ADP bound. At saturated concentration of ATP, the progression of 120° rotation is shown by the small arrows. (A) The position of subunit γ at 0°, showing the catalytic dwell. The ATP hydrolysis occurs at β_{DP} and (B) phosphate releasing at β_E initiates the power stroke. (C) The power stroke continues till 120° with ATP binding at β_E and ADP release at β_{DP} . During this phase ATP binding to the β_E and ADP release from β_{DP} takes place. (D) After the 120° power stroke the β_{TP} site change conformation to become β_{DP} initiating ATP hydrolysis. The cycle of 120° rotation of γ subunit continues and one complete rotation (360°) of γ subunit results in the hydrolysis of 3 ATPs. This figure was adapted from *Sielaff et al.*, [3].

The hexagons depicted in the figure are representative of the three subunit α (green), three subunit β (orange). The coiled-coil and globular domains of the centrally located subunit γ are highlighted in yellow. In the event of ATP hydrolysis, at saturating concentrations of ATP, each catalytic site, denoted as β , sequentially progresses through three designations- β_E , β_{TP} and β_{DP} . β_{TP} and β_{DP} are abbreviations for the ATP and ADP occupied sites, respectively and β_E represents the empty site. Binding of ATP to one site leads to the other two sites

experiencing a knock-on effect on their conformations. Through its stepped rotation, the axle of subunit γ largely dictates the ATP molecule to bind differently at each of the catalytic sites and these events are strongly complemented by the adjoining subunit α .

1.3 Origin and evolution of the ATP synthases and ATPases

The origin and evolution of ATP synthases and ATPases are debatable topics considering their high efficiency and intelligent nanomotor design [73]. The understanding of evolution of these rotary enzymes will require the conjunctive studies of F- , A-ATP synthases and V-ATPases. As these rotary enzymes are divided into a membrane embedded (F_o , V_o and A_o) and a cytoplasmic domain (F_1 , V_1 and A_1), it is believed that they have evolved independently and assembled at a later stage, to function as a complete rotary enzyme.

The present day membrane embedded-part (F_o , V_o and A_o) of the rotary enzymes responsible for cation-translocation, could have originated as an ancestral membrane channel, functioning in translocation of RNA or/and proteins. Similarly, the catalytic hexameric ring of present day cytoplasmic part (F_1 , V_1 and A_1) resembles the RNA/DNA helicases involved in nucleic acid/ protein translocation suggesting that cytoplasmic part of these rotary enzymes are originated from RNA helicases (Figure 1.5) [74, 75]. Along with the structural and functional similarities (ATPase activity) of RNA helicases to the $F_1/V_1/A_1$ domain, they also demonstrated rotational movements during the course of unwinding. [76-78]. The similarity of the F_1 , V_1 and A_1 domains with the bacterial flagellar motor subunits, involved in ATP-driven transport of flagellin and the subunits of type III secretion system (T3SSs), indicates the evolutionary intersection of rotary enzymes and translocation system. Based on these evidences, it has been proposed that RNA helicases would have combined with the ancestral membrane channel and given rise to the ATP-driven RNA translocase, which then evolved into a protein translocase and subsequently into a cation-translocating ATPase [79]. In the course of the evolutionary stage of protein translocase, recruitment of membrane embedded peripheral stalks could have connected both the hexamer and the membrane channel (Figure 1.5). The central stalk of the present day rotary enzyme is possibly a result of a transported protein getting trapped due to hydrophobicity enhancing mutations in the inner surface of the membrane channel (c - ring). The hypothesis of recruitment of the central stalk preceding the peripheral stalk is supported by the experiment, where yeast mitochondrial F-ATP synthase assembled successfully without the central stalk. However, the enzyme was not functional, although ions passed the membrane freely [80]. The energy of ATP hydrolysis, which was earlier required for protein translocation, would now be used for rotation of entire central stalk with the membrane channel. This rotation might have coupled with the cation-

translocation across the membrane and would have been favoured by evolution, given that the energy from ATP hydrolysis will be stored in the form of an ion-gradient. The conversion of ion-translocating ATP-driven pump to ATP synthase possibly occurred alongside the evolution of other components of the respiratory chain [81]. The proposed scheme of evolution of rotary enzymes from RNA helicases and translocases is shown in figure 1.4.

Figure 1.5: The evolution of rotary ATPases based on the theory proposed by *Mulkidjanian et al.*, speculating the origin of ATP-driven pump from a membrane channel and RNA helicase fusion [79]. The fusion led to the development of RNA translocase, that later would have developed into protein translocase and subsequently given rise to the common ancestor of ATPase, from which all the rotary ATPases have evolved. This figure has been adapted from *Mulkidjanian et al.*, [79].

The speculation of events that occurred a billion years ago is a challenging task and therefore, the hypothesis on RNA translocase based evolution of rotary enzymes is disputable given the following questions; why the RNA translocases are not found in modern life forms and how an enzyme which synthesizes ATP is evolved from an enzyme which requires ATP

for its functioning? The plausible answers are, although RNA translocases would have held importance in the RNA world, but their ion-leaky nature would not have been an important evolutionary trait to carry forward. Also, the earlier ATPases would have functioned on ATP generated by fermentation or substrate level phosphorylation, which answers the possibility of ATP synthesizing enzyme evolving from an enzyme requiring ATP. The evolution of $F_1/A_1/V_1$ and $F_0/A_0/V_0$ part from RNA/DNA helicases ($F_1/A_1/V_1$) and a membrane channel ($F_0/A_0/V_0$), respectively, and then assembling into a single multi-subunit complex is a proposed hypothesis and future experiments might shed more light on the evolution of rotary enzymes.

After understanding the origin of rotary enzymes from RNA translocase, the immediate question is which of the rotary enzyme amongst F-, V- and A- type, originated first? The answer proposed on the basis of evidences states that all the three rotary enzymes evolved from a common progenitor [11, 82, 83]. As mentioned previously, A-ATP synthases are functionally similar to F-ATP synthases and share structural similarities with V-ATPases. This is also reflected in the phylogenetic tree of life where archaea and eukarya are shown to diverge lately from the common ancestor. The evolution amongst rotary enzymes is mainly studied on the basis of their functions as ATP synthase or ATPase. Therefore, the proposed hypothesis states that, the progenitor ATPase attained the function as an ATP synthase during evolution and then lost the ATP synthesising ability in some cases and became an ATPase [11]. This role reversal theory is dependent on the H^+/ATP coupling ratio denoted as ' n ' and defined as the ratio of number of protons translocated to the number of ATP consumed in one 360° rotation of the central stalk. The assumption states the large value of ' n ' will favour the functioning of enzyme as an ATP synthase with maintaining high phosphorylation potential. Whereas, a smaller ' n ' would be favourable to function exclusively as an ATPase, since large electrochemical gradient would be maintained, if less protons were pumped per ATP hydrolysed. The H^+/ATP coupling ratio is dependent on the number of proton translocating carboxylates in the c -ring and the number of the catalytic subunits. Given this, one carboxylate in the c -ring transport, one H^+ and one catalytic subunit hydrolyse or synthesise one ATP molecule per 360° rotation of the central stalk [11].

As described previously, the present day ATP synthases and ATPases are composed of subunits α and β in the hexameric headpiece in an alternate fashion. Of these, subunit β is catalytically active, while subunit α is also capable of nucleotide binding. This arrangement offers only three catalytic sites of the total six potential nucleotide binding sites in the hexamer. However, the proposed progenitor of these rotary enzymes, were suggested to house six catalytic subunits in the hexameric headpiece. It is believed that in this scenario a single

gene expressed all the 6 catalytic subunits (β). Duplication of this gene to a second copy would have helped the enzyme to achieve sufficient expression of the catalytic subunits. The H^+ /ATP coupling ratio in the progenitor ATPase was predicted to be 'two', given that it had six catalytic subunits and twelve functional *c*-subunit carboxylates. This low H^+ /ATP coupling ratio would have favoured it to function as a dedicated ATPase. The conversion of progenitor ATPase to ATP synthase [A-ATP synthase and F-ATP synthase (bacteria, mitochondria and chloroplast)] was proposed to be a result of point mutation of an important residue in one of the two catalytic genes (α or β) involved in catalysis. The mutation would have left the enzyme with only three catalytic sites increasing the H^+ /ATP coupling ratio to 'four', favouring ATP synthesis. The conversion of a catalytic subunit to non-catalytic by a single point mutation is supported by studies on the bovine mitochondrial F-ATP synthase. Here, E188 of catalytic β subunit is shown to be involved in hydrogen bonding with a water molecule to facilitate nucleophilic attack on the γ -phosphate of ATP is replaced by Q208 in subunit α , which cannot function similarly [84].

It is also proposed that another gene duplication would have preceded the second reversal of ATP synthase to ATP driven proton pump. It can be seen in sulfate-reducing archaeon, *Archaeoglobus fulgidus*, which contains two identical gene copies of functional *c*-subunit [85]. Also, in another species of archaea, *Methanothermobacter thermoautotrophicus*, a double sized *c*-subunit (*c-c*) with each containing a proton-translocating carboxylate was found [86]. This duplication could be a selective advantage for ensuring an optimal expression of *c*-subunits and also it could provide stability to the *c*-ring since half of the interfaces are merged. The transition to ATP-driven proton pump could be only a result of a single point mutation of the functional carboxylate from one of the *c-c* molecule. The mutation would have only left six functional carboxylates decreasing the H^+ /ATP coupling ratio to 'two', converting it to an exclusive ATPase. This can be seen in organisms growing on anaerobic fermentation as their primary ATP source such as *Enterococcus hirae*, or in V-ATPases of eukaryotes [87, 88].

So the role reversal theory (ATPase $\rightarrow$ ATP synthase $\rightarrow$ ATPase) postulates the evolution of rotary enzymes on the basis of gene duplication of catalytic subunit and the membrane embedded 'c' subunit. Further findings and structural studies will enhance the understanding of evolution of rotary enzymes.

1.4 Overview of archaea and methanogens

By the early 1900s, scientist believed the existence of only two kingdoms of life, comprising of prokaryotes and eukaryotes. In the 1970s, Carl Woese and co-workers

proposed the third kingdom of life named 'archaea' on the basis of 16S ribosomal RNA sequencing of methanogenic bacteria [89]. The development of molecular phylogenetics and whole genome sequencing revealed many such organisms differing from the prokaryotes and eukaryotes. The reasons archaea were neglected for years include their similar appearances to the bacteria under the microscope and the difficulties involved in culturing them in the laboratories due to their requirement to grow optimally in the extreme conditions.

Archaea was classified as prokaryotes differing from bacteria and were divided into halophiles growing at 3-5 M of NaCl (*Halobacterium salinarum*, *Halofaex japonica*) methanogens inhabiting the gastrointestinal tract of ruminants or humans (*Methanobrevibacter ruminantium*, *Methanobrevibacter smithii*, *Methanosphaera stadtmanae*) and hyperthermophiles thriving at 60- 113°C (*Pyrococcus furiosus*, *Pyrococcus woesei*, *Thermococcus profundus*) [7, 90-96]. Archaea have fascinated the scientific community with their remarkable ability to grow in extreme conditions and on low energy carbon sources. The occurrence of archaea in the hydrothermal-vents, hot springs, high salt and extreme pH conditions has resulted in increasing the stability of archaeal enzymes (biomolecules) and generation of other stable chemical compounds. These enzymes and other products are used in genetic engineering, food industries, bioremediation and many other biotechnological applications [97]. Along with the industrial applications of archaea, they are also a topic of interest in terms of studying evolution and existence of extra-terrestrial life, since they grow in conditions presumed to have existed billions of years ago on earth (No light, high CO₂, high temperatures) [98, 99].

As mentioned earlier, the origin of archaea is close to the origin of life. To combat the hostile environment of primitive earth, archaea had developed mechanisms for energy conservation which are diverse from bacteria or eukarya [47]. The ability of using low energy substrates for living, gives a unique identity to archaea and one such class comprises of methanogens that thrive on low energy carbon sources.

Methanogens, as the name suggests generate methane from CO₂ + H₂, acetate, formate, methanol, methylamines [7, 100, 101]. They grow in oxygen depleted environments including paddy fields, swamps and intestinal tracts of ruminants and generate about 1 giga ton of methane annually, thus playing an important role in carbon cycle [100]. The free energy change of these low energy substrates is not optimal for the synthesis of even 1 mol of ATP and hence, to thrive at the thermodynamic limit of life will require chemiosmotic mechanism of energy conservation [7]. To overcome the challenge of limited available energy, methanogens have coupled methanogenesis with ATP synthesis. They have also developed

membrane-bound electron transport system and an unusual ATP synthase to use Na^+ ions instead or along with H^+ .

The normal aerobic respiration of a glucose molecule provides ~30 ATP and the anaerobic fermentation of sugars to acetate provide around 4 mol of ATP, given that the free energy change during ATP to ADP hydrolysis is $-31.8 \text{ kJ mol}^{-1}$ under standard condition [102]. The methanogens do not survive on substrate level phosphorylation and also they cannot use aerobic respiration for their growth, instead they have integral membrane enzymes for the coupling of methanogenesis to the generation of ion gradients [103]. One of the important integral enzyme is methyltetrahydromethanopterin-coenzyme M methyltransferase (Mtr), which transfers the methyl group from methyltetrahydromethanopterin to coenzyme M, this is an exergonic reaction translocating sodium ion across the cytoplasmic membrane [104-107]. The Na^+ ion gradient is then used by the A-ATP synthases for the synthesis of ATP. The second important adaptation is to generate an additional proton gradient along with the sodium gradient, restricted to the methanogens containing cytochromes (*Methanosarcina mazei*, *Methanosarcina barkeri* and *Methanosarcina acetivorans*).

The pathway of methanogenesis coupled to ATP synthesis in methanogens (with cytochrome and without cytochromes) is described in figure 1.6. In methanogens containing cytochromes, the first endergonic reaction of reduction of ferredoxin is catalysed by the energy-converting hydrogenase (Ech) driven by the translocation of proton. The second reaction involves the Mtr enzyme, which transfers the methyl group to coenzyme M and translocates two sodium ions across the membrane. Then the Methyl-coM is reduced by coenzyme B with the release of methane (CH_4) and formation of heterodisulfide of CoM and CoB. This step is the same in methanogens with or without cytochromes. The additional step of proton gradient generation in cytochrome containing methanogens involves a membrane - embedded F_{420} non reducing hydrogenase (Vho) and a membrane bound heterodisulfide reductase (Hdr) along with an electron carrier methanophenazine (Figure 1.6). The Hdr enzyme reduces heterodisulfide of CoM and CoB with the translocation of protons across the membranes [103].

Figure 1.6: The pathway of ATP synthesis coupled to methanogenesis in methanogens with cytochromes (left) and without cytochromes (right). The energy-converting hydrogenase (Ech) reduces ferredoxin (Fd) and the methyltetrahydromethanopterin-coenzyme M-methyltransferase (Mtr) transfers the methyl group from methyltetrahydromethanopterin to coenzyme M and generates a primary Na^+ -gradient (common in both types of methanogens). Methane (CH_4) is released after the reduction of methyl Co-M with Co-B and a heterodisulfide is formed. The reduction of heterodisulfide of Co-M and Co-B is catalysed by the membrane embedded F_{420} non reducing hydrogenase and a membrane-bound heterodisulfide reductase (Vho-Hdr complex) in cytochrome containing methanogens with the translocation of protons across the membranes. This reduction is performed by a soluble complex of methylviologen-reducing hydrogenase and heterodisulfide reductase (Mvh-Hdr complex) in cytochrome free methanogens, without proton translocation. Therefore, the ATP synthase in cytochrome free methanogens uses only sodium ion gradient, whereas, in cytochrome containing methanogens both (sodium and proton) gradients are used. The figure is adapted from *Schlegel et al.*, [108].

These proton and sodium ion gradients are used by the A-ATP synthase for the generation of ATP. In case of cytochrome-free methanogens (*Methanothermobacter thermautotrophicus* and *Methanocaldococcus jannaschii*), Mvh-Hdr is a soluble complex of methyl viologen-reducing hydrogenase and heterodisulphide reductase that causes the heterosulphide reduction. The energy released is used to transfer electron from H₂ to ferredoxin. The reduced ferredoxin acts as the electron donor to the formyl methanofuran dehydrogenase reaction which is the Ist step of methanogenesis. Given that these species have only one coupling site from Na⁺, their ATP synthases are also Na⁺ selective. The use of both proton and sodium gradient for the generation of ATP is important for survival in energy limiting conditions and therefore, cytochrome containing methanogens are considered as evolutionarily advanced compared to their cytochrome lacking counterparts.

1.5 The unusual ATP synthase of archaea and its adaptation to harsh environmental conditions

The coupling of methanogenesis with translocation of Na⁺ and H⁺ ions as described in section 1.4 is an adaptive feature of methanogens to thrive in the hostile conditions existing on the primitive earth. The studies on A-ATP synthases and their comparison with the counterparts F-ATP synthase and V-ATPase have revealed the unusual characteristics unique to A-ATP synthase. The first adaptation is the use of Na⁺ ions for coupling as seen in cytochrome free methanogens and organisms growing at high temperatures (*P. furiosus*). The reason for selecting Na⁺ could be because of its high specificity as compared to H⁺ ions. As, these organisms are growing at the thermodynamic limit of life and any leakage of ions across the membrane could be detrimental for the biological system [109]. The sodium bioenergetics was probably an adaptation to survive on the primitive conditions on earth, but later on the cell membranes would have evolved to avoid the leakage of protons [110].

Adaptations in the membrane embedded rotor 'c'-ring has also been observed in archaea. The length of the *c* subunits differ from other rotary ATPases and also these *c* subunits are linked covalently providing the stability at higher temperatures. The mesophilic archaean, *M. mazei* Gö1 grows optimally at 35 °C and its *c* subunits have two transmembrane helices with one ion-binding site per *c* subunit. The other methanogenic species growing optimally at 65-70°C (*Methanothermobacter thermautotrophicus*), 85°C (*Methanocaldococcus jannaschii*) and 98°C (*Methanopyrus kandleri*) contain a *c*-ring with four, six and thirteen transmembrane helices, respectively. The *c*-ring has to rotate against the subunit *a* for ion-translocation and the interaction amongst the subunit *c* helices might decrease with the thermal fluctuations at high temperature. Therefore, covalently linking the subunit *c* is a way

to maintain stability at high temperatures and could be important for archaeal growth at high temperatures [20, 111].

Another adaptation is observed in the peripheral stalks made up of subunits E and G of the A₁-domain of A-ATP synthase. The asymmetrically mounted A₃B₃ sub-complex on the central stalk causes wobbling of the head during rotation of the central stalk. The crystal structure of subunit E of peripheral stalk of *P. horikoshii* revealed a S-shaped alpha helical structure and considered to facilitate the storage of elastic energy [54]. These EG heterodimers are required to stabilize the wobbling A₃B₃ head during catalysis by counteracting the torque generated by the central stalk [112].

1.6 Organisation of archaeal A₃B₃DF complex of A-ATP synthase

As mentioned in section 1.2, the A₁-domain is the cytoplasmic soluble part of A-ATP synthase divided into the A₃B₃ hexameric head, a central stalk of subunits C, D and F and the peripheral stalks of subunits E and G. The interface of subunits A and B provides the residues involved in catalysis of ATP, major residues are from subunit A. Therefore, subunit A is known as the catalytic subunit and subunit B has been proposed as regulatory subunit [61, 113-115]. Subunit A possesses a glycine rich phosphate binding loop (P-loop) with the sequence GXXGXGKTV, known as Walker A motif, and a conserved GER-loop, known as Walker B motif (Figure 1.7) [47, 116].

The subunit A in A/V-ATPases possesses an 80-90 amino acid insertion at the N-terminus, which is not observed in subunit α or β of F-ATP synthase giving it a name as non-homologous region (NHR) (Figure 1.7) [117, 118]. The crystal structures (nucleotide bound and apo-form) of subunit A of A-ATP synthase from *P. horikoshii* revealed an arched P-loop (₂₃₄GPFGSGKTV₂₄₂), unique to the catalytic subunit of A-ATP synthases held by strong interactions with a *B*-factor of 27 Å². [55, 119]. The structure of subunit A when superimposed on the homologous catalytic β -subunit from F-ATP synthase, confirmed the differences in the P-loop of A-ATP synthases compared to the F-ATP synthases. Also, the superimposition of P-loop of A-ATP synthase on the members of superfamily of ATP/GTP binding proteins, reveals that the arched confirmation is a signature feature of A-ATP synthases (Figure 1.8).

Figure 1.7: The model of the A₃B₃DF subcomplex of A-ATP synthase generated by assembling subunits from different organisms. In centre is the assembled A₃B₃DF, where subunit A is from *P. horikoshii* (PDB: 1VDZ), subunits B and F are from *M. mazei* Gö1 (PDB: 2C61, 2OV6 respectively) and subunit D is from *E. hirae* (3AON). The crystal structure of subunit A (left) in orange showing the P-loop and GER-loop. Crystal structure of subunit B (right) in green showing the unique P-loop (SASGLPHN) and GIT-loop [55, 57, 59, 61].

Figure 1.8: The unique P-loop of A-ATP synthases. (A) The subunit A from *P. horikoshii* ATP synthase (PDB: 1VDZ, orange) is superimposed on homologous subunit β of bovine F-ATP synthase (PDB: 1BMF, cyan). The arched P-loop of subunit A is shown in red and flat P-loop of subunit β is shown in magenta. (B) The structural overlap of the P-loop of A-ATP synthase with members of the superfamily of ATP/GTP binding proteins. Kinesin-like protein KAR2 (PDB: 3KAR); N-Ethylmaleimide sensitive fusion protein (PDB: 1D2N); signal recognition protein FFH (PDB: 1FFH); Traffic ATPase (PDB: 1G6O); Kinesin motor domain (PDB: 1BG2); A_E (PDB: 3I72, orange) and S238A mutant (PDB: 3IKJ, green) of subunit A. The figure is modified according to Kumar *et al.*, [55].

The high resolution structures of empty, sulphate ion bound, ADP-bound and AMP-PNP-bound subunit A from *P. horikoshii* ATP synthase revealed a different manner of nucleotide binding in A-ATP synthases compared to F-ATP synthases [55, 119]. The role of G234, F236 and S238 in maintaining the arched conformation of P-loop was established through mutational and structural studies [55, 121, 122].

It is proposed that, in the unique arched conformation of P-loop, the nucleotides are more exposed to solvent and have weaker interactions with the P-loop residues [55]. Therefore, the P-loop of A-ATP synthase is capable of hydrolysing other nucleotides like GTP and UTP, a feature unique to A-ATP synthases [120].

Figure 1.9: Nucleotide bound P-loop of subunit A. (A) The AMP-PNP bound region of subunit A within 5 Å. The AMP-PNP is shown as ball-and-stick. The omit electron density map contoured at the 2.5 σ level for the AMP-PNP is given in *green*, and the residues surrounding it are shown in sticks. The P-loop residues are shown in *magenta*, and water molecules that interact with AMP-PNP are shown in *light-blue* sphere, with the hydrogen-bonding interactions shown by dotted lines. The S238 forms a bifurcated hydrogen bonding with γ -phosphate. The residue P235 also interacts with γ -phosphate of AMP-PNP via water molecule. (B) The ADP bound region of subunit A within 5 Å. The omit map contoured at the 2.5 σ level (*green*). The surrounding amino acids are represented in sticks, with water molecules represented by a sphere. The hydrogen bonds are given by *black* dotted lines. It can be seen that the S238 forms a hydrogen bond with the α and β -phosphates via a water molecule [55].

The S238A mutant resulted in a flattened P-loop resembling the P-loop of β subunit of F-ATP synthases, indicating the significance of the serine residue. The S238 forms a hydrogen bond with α and β -phosphate via a water molecule as revealed by the ADP-bound structure of subunit A. Also, in the AMP-PNP bound structure of subunit A, S238 forms a bifurcated hydrogen bonding with the γ -phosphate (Figure 1.9). These two nucleotide bound structures clearly suggests the importance of S238 in the P-loop of A-ATP synthases. Along with S238, the P-loop residue P235 also interacts with γ -phosphate of AMP-PNP via a water molecule, indicating the importance of P235 in catalysis (Figure 1.9).[55, 121].

The catalysis of ATP requires the involvement of another conserved loop known as the GER-loop or Walker B motif. The role of GER-loop in ATP catalysis is very well established in F-ATP synthases [84, 123]. The E188 residue of GER-loop of subunit β of F-ATP synthases is known as a mild base and is involved in the immobilization and polarization of a water molecule to facilitate the nucleophilic attack at the γ -phosphate of ATP [84, 123]. The residue E263 of subunit A of *P. horikoshii* A-ATP synthase is a homologue to residue E188 of subunit β of bovine F-ATP synthase. Earlier work in our laboratory proposed a different arrangement of the GER-loop in A-ATP synthases compared to that in F-ATP synthases. The superposition of the AMP-PNP bound structure of subunit A (A_{MP}) and subunit β (β_{MP}) revealed that the distance between E188 of β_{MP} and γ -phosphate of AMP-PNP is significantly closer (8.41 Å) than the distance between E263 of A_{MP} and γ -phosphate of AMP-PNP (14.31 Å) (Figure 1.10) [55].

The closer distances of the GER-loop and γ -phosphate in subunit β indicate tight binding of nucleotide, whereas, in the case of subunit A, the novel P-loop conformation is responsible for different arrangement of nucleotide inside catalytic site. The nucleotides in P-loop of subunit A faces weaker interactions, letting the nucleotide to be more solvent exposed and justifying the unique ability of subunit A of A-ATP synthases to hydrolyse other nucleotides like GTP and UTP with 86% and 54% activities, respectively. The decrease in selectivity of nucleotides could be an advantage for the archaea growing under limited energy and is considered as one of their adaptations for survival.

Figure 1.10: Superimposition of the structure of AMP (light orange) on β_{MP} (deep cyan). The P-loop of AMP is shown in red and β_{MP} is in magenta. The distances between the glutamate residue of GER-loop and the γ -phosphate of AMP-PNP measured as 8.4 Å and 14.3 Å for β_{MP} and AMP , respectively.

The mechanism of catalysis is not well understood in eukaryotic V-ATPases due to the lack of high resolution structures of the catalytic hexameric head (A_3B_3). Though there are structural similarities between A-ATP synthases and V-ATPases, the P-loop of V-ATPases (GA**F**GC**G**KTV) has a conserved cysteine in place of serine and an alanine residue replacing proline of A-ATP synthases. As mentioned earlier, the residues P235 and S238 play a role in maintaining the arched conformation of the P-loop and are also involved in catalysis. Therefore, it would be interesting to mimic the P-loop of the V-ATPase in the A-ATP synthase and to establish the role of C238 in subunit A.

The other subunit of the hexameric head is the non-catalytic subunit B, homologous to subunit α of F-ATP synthases. Subunit B was earlier considered as non-catalytic subunit but nucleotide binding and transition state studies revealed its regulatory role, given that it binds MgATP ($K_d = 22 \mu M$) with two-fold higher affinity than MgADP ($K_d = 50 \mu M$) [61, 113-115]. The crystal structure of subunit B from *M. mazei* Gö1 as shown in figure 1.6, is comprised of three domains: an N-terminal domain with six- β sheets (residues 13–76), a middle domain made up of α -helices and β sheets (residues 77–358), and a C-terminal domain with four helices (residues 359–460) [61]. Though subunit B has a compact structure with the shorter N- and C-terminal domains compared to the homologous non-catalytic α -subunit of bovine F-ATP synthase, the overall structure of both the subunits are comparable [61].

In F-ATP synthases the majority of residues involved in nucleotide binding and catalysis are from β -subunit with some contribution from α -subunit. The similar A-B dimeric interface is formed in A-ATP synthases and side-chain residues from subunit B contribute to nucleotide binding and catalysis. One of the important residue is R349 of subunit B, homologous to R373 of subunit α of bovine F-ATP synthase. The role of R373 in stabilisation of the negative charge developed on the terminal phosphate in a penta-coordinated transition state is well known and a similar function is proposed from R349 of subunit B [61, 84]. Another important residue of subunit B involved in nucleotide binding is L347, which is in vicinity of residue K240 of the catalytic subunit A (Figure 1.11) [124].

Figure 1.11: Structure of subunit B and its role in nucleotide binding. (A) The crystal structure of subunit B (green) of *M. mazei* Gö1 ATP synthase. The P-loop (SASGLPHN) is highlighted in red (PDB: 2C61) [61]. (B) The nucleotide binding interface of subunit A (yellow) and subunit B (green). The hexamer was modelled by fitting the X-ray coordinates of subunit A and B crystal structures on the A_1 -ATPase [117]. The AMP-PNP is shown in blue stick and the interacting residues are represented by yellow lines. The interface shows R349 from the subunit B to be in close proximity of the bound AMP-PNP, indicating the importance of B-R349 in nucleotide binding [55]. This figure has been adapted from Kumar *et al.*, [55].

Other than providing the interface for nucleotide binding, subunit B also has a unique P-loop (SASGLPHN) where the positively charged H156 residue is proposed to be involved in preferential binding of MgATP. It is also supported by mutational studies done on the P-loop of the related V-ATPase of *S. cerevisiae*, where 25% loss of hydrolytic activity and proton translocation were observed after mutating the histidine residue [125]. The preferential binding of ATP is unique for subunit B of A-ATP synthases and not observed in the homologous subunit α of F-ATP synthases. The preferential binding of ATP over ADP in

subunit B could be an attribute of A-ATP synthases to sense a steady state turnover in the catalytic A subunit allowing the unnecessary hydrolysis of ATP during energy limited scenario [113]. It is speculated that this kind of mechanism will allow archaea to adapt to their unique and harsh environments.

The binding of ATP to the P-loop of subunit B has been studied by crystallography, fluorescence spectroscopy and molecular dynamics simulations [115, 126, 127]. The studies revealed two intermediate states (Transient 1 and 2) of nucleotide binding in subunit B before reaching the final binding pocket (Final) (Figure 1.12). The nucleotide binds on transient 1 and its migration to transient 2 require the interaction of C-terminus of subunit B with the C-terminus of subunit F. Once the nucleotide occupies transient 2, the complex enters a transition state and the migration of ATP to the final site is driven by high stabilization of ATP on final site compared to transient 1 [127]. The role of subunit F in ATP migration is important given that the C-terminus of subunit F is flexible and it can attain an extended and a retracted state [128].

Figure 1.12: The transition states of ATP binding in subunit B of *M. mazei* Gö1 ATP synthase. The transition state structures are superposed on each other to show the position of nucleotide binding. The structure in *green* represents transition state I and the one in *yellow* shows transition state II. The ATP molecule for both the transition states are also shown in the same colours as the subunits. The putative final nucleotide is shown in *blue* spheres binding near the P-loop (SASGLPHN) [61, 115]. This figure has been adapted from Manimekalai *et al.*, [115].

The subunit F is unique to A-ATP synthases and V-ATPases, though always mistaken as homologous to subunit ϵ of F-ATP synthase, the uniqueness of subunit F is established in A-ATP synthases and V-ATPases [79, 129, 130]. The first low resolution structure of subunit F of *M. mazei* Gö1 A-ATP synthase was determined by SAXS. The structure demonstrated elongated C-terminus of subunit F and was supported by the high molecular weight determined by gel filtration chromatography [131]. The predicted length of subunit F was 7.5 nm determined by the low resolution structure. The cross-linking experiments revealed interaction of subunit F with regulatory subunit B and central stalk subunit D, hence rendering the name as “coupling subunit”. The solution structure of subunit F from *M. mazei* Gö1 ATP synthase exhibits a distinct two domain structure, where residues 1-78 comprise the N-terminal and residues 79-101 are the part of the flexible C-terminal domain (Figure 1.14) [57]. The well-ordered N-terminal globular domain is comprised of a four stranded parallel β sheet and three α helices.

Figure 1.13: Interaction between C-terminal of subunit F and subunit B is shown in an assembled model of A₃B₃DF. Subunit A (3I4L) is shown in *orange*, subunit B in *green* (2C61), subunit D (3AON) in *yellow* and subunit F (2OV6) in *magenta*. The residues identified to be involved in the cross-linking are highlighted in the box (PDB: 2OV6) [57, 61, 115, 126, 127, 131-133].

The elongated nature of subunit F, makes it a coupling subunit connecting the ion translocating part with the nucleotide binding subunit B. This coupling is supported by the

cross-linking of subunit F and subunit B in the presence of MgATP or MgAMP-PNP, where the zero length cross link occurred between $_{390}\text{EALSERDTK}_{399}$ of subunit B and $_{88}\text{REKIK}_{92}$ (Figure 1.13). The $_{390}\text{EALSERDTK}_{399}$ is homologous to the conserved DELSEED-region of the homologous F-ATP synthases. [132-134]. The interaction of subunit F and subunit B is also supported by tryptophan fluorescence spectroscopy which showed that residue W100 of subunit F was spatially close to residue W430 of subunit B and molecular dynamics studies revealing the intermolecular interaction between the side chains of V95 (subunit F) and K399 (subunit B), K101 (subunit F) and Q440 (subunit B) and backbone and side chains of K101 (subunit F) and G442 (subunit B) [57, 126].

Figure 1.14: NMR structure of subunit F from *M. mazei* Gö1 ATP synthase (magenta). The structure is divided into an N-terminal globular domain (1-78) and a C-terminal flexible domain (79-101) (PDB: 2OV6) [57].

Although structurally different, subunit F of A-ATP synthase was always considered as the homologue of subunit ϵ of F-ATP synthase on the basis of the globular N-terminal domain and the flexibility of C-terminal domain. The subunit ϵ of *E. coli* F-ATP synthase is proposed to undergo rearrangements during energy coupling due to its high flexibility and similar rearrangements will be possible with subunit F of A-ATP synthase [42]. The role of subunit ϵ of F-ATP synthase is established as an inhibitory subunit [135, 136]. It would be interesting to understand the role of subunit F in energy coupling and catalysis.

The central stalk subunit D of A-ATP synthases has structural and functional homology with the γ -subunit of F-ATP synthases. The coiled-coil region in both the subunits (subunit D or subunit- γ) resides in the central cavity of hexameric head (A_3B_3 or $\alpha_3\beta_3$). Along with the coiled-coil region, subunit γ is also made of a globular α/β fold. Though, this globular α/β

fold is lacking in subunit D of A-ATP synthases, the crystal structures of subunit F from *T. thermophilus* showed a similar globular α/β fold, indicating that γ -subunit of F-ATP synthases is a fusion of subunit D and F of A-ATP synthases [128, 137]. As mentioned in section 1.3, the ATP synthases and ATPases shares structural similarities with the flagellar protein or the protein translocation systems. The subunit D and homologue subunit- γ have a comparable coiled-coil domain that is comparable to FliJ, a bacterial flagellar protein (Figure 1.15) [138, 139]. I believe, studying the mechanistic role of subunit D in A_3B_3D and A_3B_3DF complex of *M. mazei* Gö1 ATP synthase will strengthen the understanding of the contributions of the central stalk in this rotary enzyme.

Figure 1.15: Structural relation of subunits DF to FliJ and subunit γ . The crystal structures shown are of flagellar protein (FliJ-blue) from *Salmonella typhimurium* (PDB: 3AJW), subunit γ (wheat) of bovine F-ATP synthase (PDB: 1E79) and subunits DF (D-yellow, F-magenta) from *E. hirae* ATPase (PDB: 3AON). The coiled coil domains of all the three protein share similarities and the globular domain of γ -subunit has structural similarities with the subunit F of A-ATP synthase [138, 139].

The ATP synthases considered for study in this thesis are the A- type ATP synthases of *Methanosarcina mazei* Gö1 (*Mm*) and *Pyrococcus horikoshii* (*Ph*). A-ATP synthases have carved a unique niche for themselves in terms of structure and bioenergetics, given that they have adapted to overwhelming environmental conditions that they have been subjected to. The findings so far have only opened up more scope for exploration to decode the intricacies of these biological systems. The structural and mechanistic understanding of individual

subunits and their functioning in A_3B_3D and A_3B_3DF complexes of ATP synthase will provide insights about the evolution and functioning of A-ATP synthases.

1.7 Objectives of the thesis

Archaea grow on limited energy and harsh environmental conditions, making them unique with their biochemical pathways and mechanism to conserve the energy. The study of their ATP synthases will unravel the functioning of primitive ATP synthases and will give insight about the evolution of rotary ATPases.

1) A-ATP synthases are the provider of ATP in archaea and made up of two domains: the soluble A_1 -domain for ATP hydrolysis or synthesis and the membrane embedded A_0 -domain for ion translocation. The central stalk made of subunits C, D and F is responsible for the coupling of these two domains. Although the solution structure of subunit F from *M. mazei* Gö1 A-ATP synthase is available, yet the role of subunit F in ATPase activity is not very well established. Therefore, the present study confirmed the assembly of A_3B_3D - and A_3B_3DF -complexes from *M. mazei* Gö1 by 2D classification and 3D reconstruction. Thereafter, focus was directed on enzyme kinetics of *Mm* A_3B_3D - and *Mm* A_3B_3DF -complexes to elucidate the role of subunit F in ATPase activity. To map the epitope of subunit F, responsible for playing a role in ATP hydrolysis, site directed mutagenesis, truncation and binding studies were planned and executed. The role of subunit F of related yeast V-ATPase in ATP hydrolysis is also studied. To understand the extended and retracted states of subunit F, mutants were generated and used in FRET (Fluorescence resonance energy transfer) experiments. To study the rotation of central stalk subunits (C and F), *Mm* A_3B_3DF mutants were generated and for the first time rotation of the A-ATP synthase was studied.

2) The A-ATP synthases and V-ATPases share structural similarities and both have a glycine rich P-loop in the catalytic subunit A. The P-loop of V-ATPases (GAFGCGKTV) have alanine and cysteine residues in place of proline and serine of A-ATP synthases (GPFSGGKTV). Since the V-ATPases are dedicated ATP driven ion-pumps, it would be interesting to study, if by mimicking these two residues of V-ATPases in the single subunit A from *P. horikoshii* OT3 alters the binding affinity of nucleotides or not. The crystal structure of the mutated subunit A was solved at 2.35 Å. After achieving interesting results from the single subunit, the aim was to incorporate P to A and S to C mutation in A_3B_3D - and A_3B_3DF -complexes from *M. mazei* Gö1 to study the effects in ATP hydrolysis.

3) The crystal structure of regulatory subunit B from *M. mazei* Gö1 is available and it gave insights about the nucleotide binding trail inside subunit B [61]. Now the aim was to understand the mechanism of nucleotide binding at the interface of subunits A and B and therefore, crystallisation of the *MmA*₃*B*₃*D*-complex was performed. In this work, efforts were directed towards screening and optimisation of the crystallisation conditions. The crystals for a 370 kDa *MmA*₃*B*₃*D*-complex were obtained and rigorous optimisation resulted in collection of diffraction data at low resolution. The data were processed to the best and information about the unit cell parameters and number of molecules in the asymmetric were determined.

2. Materials and Methods

2. 1 Materials

2.1 Materials

2.1.1 Chemicals

All the chemicals used in the current study were of at least analytical grade. Chemicals were purchased from the following companies:

2.1.1.1 Buffers and chemicals

	Sigma (St. Louis, MO, USA)
	USB (Sampscott, MA)
	Calbiochem (Darmstadt, Germany),
	Fluka (Sigma, Buchs Germany)
	Roth (Karlsruhe, Germany)
	Serva (Heidelberg, Germany)
PMSF	Sigma (St. Louis, MO, USA)
DTT	Hoefer (San Francisco, CA, USA)
Ni ²⁺ -NTA	QIAGEN (Hilden)
LB Media	BD (Sparks, MD, USA)
Agarose, SDS, Glycine, APS etc.	Bio-Rad (Hercules, CA, USA)
Antibiotics	Calbiochem, Sigma and Gibco (Invitrogen)
IPTG	Fermentas
BSA	GERBU (Heidelberg, Germany)
ATP / ADP / AMPPNP	Sigma (St. Louis, MO, USA)

2.1.2 Molecular biology materials

Primers	IDT and 1 st Base (Singapore)
<i>Pfu</i> DNA Polymerase	Fermentas (Glen Burnie, MD, USA)
KAPA HiFi DNA Polymerase	Kapa Biosystems
<i>Nco</i> I, <i>Xba</i> I, <i>Kpn</i> I	Fermentas and New England Biolabs
T4 DNA Ligase	Fermentas and NEB
T4 Quick Ligase	Promega
CIAP	Fermentas (Glen Burnie, MD, USA)
Miniprep Plasmid Kit	Qiagen (Hilden, Germany)
Nucleobond AX mediprep Kit	MN & Co (Düren, Germany)

<i>Escherichia coli</i> expression strains	DH5 α , DK8 cells were kindly provided by Prof. M. Futai (Japan), BL21 (DE3), BL21 (RIL)
Plasmid vectors	pGEM-4Z (Kindly provided by Prof. V Müller (Germany), pET-9d1 [140], pET-32b, pET24b

2.1.3 Chromatography

2.1.3.1 Ion exchange

RESOURCE TM Q (6 ml)	Amersham Biosciences (New York City, USA)
Ni ²⁺ -NTA resin	Qiagen (Hilden, Germany)

2.1.3.2 Gel filtration

Superdex 200 HR (10/30)	GE Healthcare (Uppsala, Sweden)
Superdex 75 HR (10/30)	GE Healthcare (Uppsala, Sweden)

2.1.3.3 Instruments and accessories

Äkta FPLC, Äkta Prime	GE Healthcare (Uppsala, Sweden)
Millex Filters (0.45 μ M)	Millipore (Bradford, USA)
Syringe, needles and accessories	BD Biosciences

2.1.3.4 Protein concentration devices and estimation

Centriprep YM10	Millipore (Co-cork, Ireland)
Amicon ultra (3, 50 and 100 kDa)	Millipore (Co-cork, Ireland)
BCA Assay Kit	Pierce (Rockford, IL, USA)

2.1.4 Other instrumentation

PCR Thermocycler:	
Biometra T personal	Biometra
Biometra T gradient	Biometra
Sonoplus Sonicator	Bendelin
Micropulser Electroporator	Bio-Rad

Ultraspec 2100 Pro Spectrophotometer	Amersham Biosciences
BioSpec-Nano	Shimadzu, Tokyo, Japan

2.1.5 Computer software

Vector NTI 10.3.0	Invitrogen
PyMOL v0.99	DeLano Scientific LLC, USA [39]
HKL2000 package	Zbyszek and Wladek, 1997 [141]
Refmac5 (CCP4 package)	Murshudov <i>et al.</i> , 2011 [142]
MOLREP	Vagin and Taplyakov, 2010 [143]
PHASER	McCoy <i>et al.</i> , 2007 [144]
Coot	Emsley and Cowtan, 2010 [145]
Ramachandran plot	Ramachandran <i>et al.</i> , 1963 [146]
PROCHECK	Laskowski <i>et al.</i> , 1993 [147]
Quantity One	Bio-Rad (Hercules, CA, USA)
CCP4	Winn <i>et al.</i> , 1994 [148]
EMAN2	Tang <i>et al.</i> , 2007 [149]
UCSF Chimera	Pettersen <i>et al.</i> , 2004 [150]

2. 2 Methods

2.2.1 Cloning of A₃B₃D and A₃B₃DF-subcomplexes of A-ATP synthase from *M. mazei* Gö1

The construct pMG3 (pGEM-4Z + subunit ABD) was kindly provided by Prof. Müller from Germany (Johann Wolfgang Goethe-Universität Frankfurt) [151]. In this construct, genes for *ahaA*, *ahaB*, and *ahaD* (encoding the subunits A, B and D) from *M. mazei* Gö1 were cloned between *Xba*I and *Kpn*I (restriction sites) in vector pGEM-4Z (Figure 2.1). The His₈-tag was introduced at the N-terminus of subunit A, to enable the purification using Ni²⁺-NTA affinity chromatography. *E. coli* DK8 cells (kindly provided by Prof. M. Futai) used for further expression, since *E. coli* DK8 cells do not have their internal ATP synthase gene, the expressed protein will be the desired recombinant protein (Figure 2.1).

Figure 2.1: Plasmid carrying ABD and ABFD genes of *M. mazei* Gö1 A-ATP synthase. Vector map showing the ABD (A) and ABFD (B) genes in pGEM-4Z (restriction sites *Xba*I and *Kpn*I used for cloning) [151].

Similarly, for the cloning of ABDF genes, the plasmid containing genes *ahaA*, *ahaB*, *ahaF* and *ahaD* of A-ATP synthase from *M. mazei* Gö1 were obtained from DNA 2.0 and provided in the shuttle vector pJ367 (~5 kbp). Electroporation was done to achieve the transformation of plasmid (pJ367 + ABFD) into electro-competent cells (*E. coli* DH5 α). Electro-competent cells were prepared as per the protocol from Current Protocols in Molecular Biology (Wiley InterScience) manual. 2.5 μ l (concentration: 100 ng/ μ l) of plasmid was added to 100 μ l of competent cells and incubated on ice for 5–10 min. Electroporation was done at a constant voltage of 2500 V (Micropulser Electroporator, Bio-Rad). The cuvette was then immediately placed back on ice for 1 min, following addition of Luria Bertani (LB) media to a total volume of 1 ml. The mixture was incubated at 37 °C for 1 h at 180 rpm. Appropriate dilutions were prepared before plating the cells on antibiotic (kanamycin) selection plates. Single colonies were picked the next day and grown for a large scale culture of 50 ml, which later was used to extract the plasmid containing genes ABFD by using midiprep kit (QIAGEN).

The plasmid (pJ367 + ABFD) was finally eluted in 200 μ l of elution buffer (EB- buffer; 10 mM Tris-HCl, pH 8.5, QIAGEN). Vector pJ367, containing the genes ABFD was double digested overnight at 37 °C by taking an appropriate amount of the restriction enzymes, *Xba*I and *Kpn*I. In order to check the digested product after overnight digestion, 5 μ l of the samples were applied onto an analytical agarose gel (0.8 %). The gel was kept in ethylene dibromide solution for 20 minutes and observed under UV light. The size of the PCR product was confirmed with the appropriate DNA marker. The remaining reaction mixture was applied onto a preparative agarose gel. The portion corresponding to the insert (ABFD-4098 bp) was cut out immediately and purified by gel extraction kit (QIAGEN) as per the manufacturer's protocol. Plasmid pGEM-4Z was also digested overnight with appropriate amounts of *Xba*I and *Kpn*I at 37 °C. The reaction mixture was applied onto an analytical agarose gel (0.8 %). The desired band was cut out and purified by gel extraction kit (QIAGEN) and further used as a vector for ligation. The reagents used were as follows:

Ligase buffer (10x)	1 μ l
Vector (V)	50 ng
Insert (I)	Same as vector
T4 Ligase	1 μ l
MilliQ water	3.9 μ l
Total	10 μl

The ligation mixture was kept at room temperature for 2 h and the reaction was terminated by adding 90 μ l of Milli Q water to make a final volume of 100 μ l. 1 ml of butanol was added into the mixture and mixed thoroughly. Afterwards, the mixture was centrifuged at 13,000 x g (using an Eppendorf Mini-centrifuge) for 10 min. and the supernatant was discarded gently. The pellet was resuspended in 500 μ l of 70% ethanol and centrifuged again for 10 min. Supernatant was again removed and the pellet was re-dissolved in 7 μ l of Milli Q water. 7 μ l of ligation mixture was used for the transformation into *E. coli* DH5 α cells. Single colonies were picked from the transformation plate on the next day and were incubated at 37 °C.

Figure 2.2: Confirmatory gel for ABFD clone. Clone check by digesting with the restriction enzymes, *Xba*I and *Kpn*I. *Lane 1*, Marker, representing the corresponding bands. *Lane 2*, Digested bands of insert (4100 bp) and vector (2746 bp).

The plasmid DNA was isolated using standard protocol (QIAGEN mini-prep kit) and subsequently double digested and applied onto a 1.2% agarose gel, in order to confirm the presence of ligated insert. The size of the insert and vector were compared with appropriate controls and markers (Figure 2.2). Verified plasmid was finally transformed into *E. coli* DK8 cells for protein production and they were grown on 100 µg/ml ampicillin-containing LB agar plates.

2.2.2 Growth, induction and production of the A₃B₃D- and A₃B₃DF complexes of

M. mazei Gö1 A-ATP synthase

Reagents used: *Luria Bertoni media*: 10 g/l Tryptone

5 g/l Yeast Extract

5 g/l NaCl

Glycerol stocks of *E. coli* DK8 cells containing pGEM-4Z plus subunit ABD and pGEM-4Z plus subunit ABFD were used for overnight small scale inoculation in 500 ml of ampicillin positive (100 µg/ml) LB medium. Later upscale secondary culture was grown to OD₆₀₀ of 0.6-0.7 at 37 °C with shaking of 180 rpm (infors HT Minitron shaker). Protein induction was initiated with addition of 1 mM of isopropyl (thio)-β-D-galactopyranoside (IPTG) and kept at 37 °C for 4 h (as per the established protocol). Cells were harvested using a centrifuge at 8500 x g for 13 min, 6 °C, flash frozen with liquid nitrogen and later stored at -80 °C.

2.2.3 Purification of complexes A₃B₃D and A₃B₃DF of *M. mazei* Gö1 A-ATP synthase

To isolate His₈-*MmA*₃B₃D and *MmA*₃B₃DF, the cells were lysed on ice by sonication for 3 x 1 min in buffer A (50 mM Tris/HCl, pH 7.5, 350 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mM Pefabloc^{SC} (BIOMOL). Cell debris was separated by centrifugation at 10 000 x g for 35 min. Supernatant was filtered (0.45 µm; Millipore) and incubated with 2.5 ml of Ni²⁺-NTA resin for 1.5 h at 4 °C to allow binding of the His-tagged protein to the matrix.

The recombinant proteins were eluted with an imidazole-gradient (0-600 mM) in buffer A and applied on an SDS-PAGE [152]. Fractions containing the His₈-tagged *MmA*₃B₃D were pooled and applied on an ion-exchange chromatography column (ResourceTM Q column GE Healthcare). The buffers used were B: 50 mM Tris/HCl (pH 7.5), 0.8 mM DTT and C: 50 mM Tris/HCl (pH 7.5), 1 M NaCl and 0.8 mM DTT. A step gradient was performed, starting from 50 mM of NaCl to 1 M of NaCl. Collected fractions were applied on a SDS-gel, and the peak appearing at 200 mM NaCl included the desired protein. The eluted samples were concentrated using a 100 kDa centricon (100 kDa cut off) to the final volume of 1 ml and applied on a Superdex 200 column in buffer D (50 mM Tris/HCl (pH 7.5), 200 mM NaCl) and the desired peak eluted at approximately 12 ml volume. The purity and homogeneity of all the samples were analysed by SDS-PAGE [152] stained with Coomassie Brilliant Blue G250.

2.2.4 Quantification of purified proteins using bicinchoninic acid (BCA) assay and nanodrop

BSA is used as a standard for protein quantification by BCA method. BCA reagent available in kit form from Pierce (Rockford, IL, USA.) was used for measuring protein concentration. In general, two dilutions of protein samples were taken and each of them was measured in triplicates. Optical density was measured at 562 nm against a blank. A standard curve with 50 µg/ml, 125 µg/ml, 200 µg/ml and 250 µg/ml of BSA was then drawn from the OD₅₆₂ and fitting equation was derived. The same equation was used to calculate the protein concentration. The protein concentration was also measured with the help of a nanodrop (BioSpec-Nano, Shimadzu) by measuring the absorption of aromatic amino acids at 280 nm. Molar extinction coefficients were determined as 319960 M⁻¹cm⁻¹ for the *MmA*₃B₃D and 326950 M⁻¹cm⁻¹ for the *MmA*₃B₃DF, using ExpASy ProtParam [153]. The values obtained were reliable and reproducible, so this technique was accepted for its feasibility.

2.2.5 Electron microscopy and 2D image analysis of the A₃B₃D- and A₃B₃DF complexes of *M. mazei* Gö1 A-ATP synthase

The *Mm*A₃B₃D- and *Mm*A₃B₃DF complexes were diluted to a final concentration of 36 µg/ml in buffer B. A volume of 4 µl of protein sample was applied to a glow discharged carbon coated copper TEM grid and stained with 2% (v/v) uranyl acetate. Electron micrographs were recorded on a Tecnai spirit T12 transmission electron microscope (FEI) equipped with a 4K CCD camera (FEI) operated at a voltage of 120 kV at a calibrated magnification of 66,350x under low dose conditions. Thirty micrographs were recorded at 0° angle for both *Mm*A₃B₃D and *Mm*A₃B₃DF complexes, respectively. A total of 3,619 particles for recombinant *Mm*A₃B₃D and 1,360 particles for *Mm*A₃B₃DF were selected. The selection criteria were clear visibility of single molecules and separation from neighboring particles. The particle selection and processing was performed using EMAN2 [149].

2.2.6 ATP hydrolysis assay and Kinetic studies of complexes A₃B₃D and A₃B₃DF of *M. mazei* Gö1 A-ATP synthase

A continuous ATP hydrolysis assay was used to measure the specific ATP hydrolysis activity of all A-ATP synthase constructs. In this assay, ATP was constantly regenerated by an enzymatic reaction, while the consumption of NADH was measured spectroscopically at 340 nm (Figure 2.3). The change in absorbance was measured for 250 s in 2 s intervals at 37 °C after adding 50 µg ATPase to 1 ml reaction solution (25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.5, 25 mM KCl, 5 mM MgCl₂, 5 mM KCN, 2 mM phosphoenolpyruvate, 2 mM ATP, 0.5 mM NADH, 30 units L-lactic acid dehydrogenase, 30 units pyruvate kinase), and its activity was derived by fitting the initial linear section of the slope.

Figure 2.3: Flow diagram of the ATP hydrolysis assay. This is coupled to the oxidation of NADH. The decrease of NADH is measured at 340 nm.

The Michaelis constant (K_m) and the maximum rate (V_{max}) of MmA_3B_3D and MmA_3B_3DF were determined by substrate-dependent ATP-hydrolysis experiments with varying concentrations of 0.025 – 4 mM ATP (Figure 3.9).

2.2.7 Cloning of *M. mazei* Gö1 A-ATP synthase subunit F (*MmF*) and its mutant forms

To amplify subunit F (*MmF*) and its mutant forms of A-ATP synthase from *M. mazei* Gö1 the following primers incorporating *NcoI* and *SacI* restriction sites were used.

Construct		Oligonucleotide sequence 5'-3'
<i>MmF</i> (R88L)	Forward	GCTCATG <u>CCATGG</u> AGTTAGCAGTGATCGGAAAGAGCGAATT C
	Reverse	GCCCGAGCTCTTACTTCCACAGATCAACACCTACCGCTTGTT TTATTTTTTCAAGTAAACTCGTTGAGCC
<i>MmF</i> (K92L)	Forward	GCTCATG <u>CCATGG</u> AGTTAGCAGTGATCGGAAAGACGAATTC
	Reverse	GCCCGAGCTCTTACTTCCACAGATCAACACCTACCGCTTGAA GTATTTTTTCTCTTAAACTCGTTGAGCC
<i>MmF</i> (S84A)	Forward (a)	GCTCATG <u>CCATGG</u> AGTTAG CAGTGATCGGAA
	Internal reverse	TCTTAAACTCGTCGCGCCTGATCCGCT
	(b)	
	Internal forward	AGCGGATCAGGCGCGACGAGTTTAAGA
	(c)	
<i>MmF</i> (L87C)	Forward	GCTCATG <u>CCATGG</u> AGTTAGCAGTGATCGGAAAGAGCGAATT C
	Reverse	GCCCGAGCTCTTACTTCCACAGATCAACACCTACCGCTTGTT TTATTTTTTCTCTACAACCTCGTTGAGCC
<i>MmF</i> (K92C)	Forward	GCTCATG <u>CCATGG</u> AGTTAGCAGTGATCGGAAAGAGCGAATT C
	Reverse	GCCCGAGCTCTTACTTCCACAGATCAACACCTACCGCTTGAC ATATTTTTTCTCTTAA
<i>MmF</i> (A94C)	Forward	GCTCATG <u>CCATGG</u> AGTTAGCAGTGATCGGAAAGAGCGAATT C
	Reverse	GCCCGAGCTCTTACTTCCACAGATCAACACCTACACATTGTT TTATTTTTTC
<i>MmF</i>₁₋₈₇	Forward	GCTCATG <u>CCATGG</u> AGTTAGCAGTGATCGGAA
	Reverse	TTACGAGCTCTTATAAACTCGTTGAGCCTGATCCGCT
<i>MmF</i>₁₋₉₁	Forward	GCTCATG <u>CCATGG</u> AGTTAGCAGTGATCGGAA
	Reverse	CTACGAGCTCTTATATTTTTTCTCTTAAACTCGTTGAGCCTG ATCCGC

MmF₁₋₉₅	Forward	GCTCATGCCATGGAGTTAGCAGTGATCGGAA
	Reverse	ACAGGAGCTCTTATACCGCTTGTTTTATTTTTCTCTTAAACTCG
MmF₅₋₁₀₁	Forward	GAGTCCATGGTGATCGGAAAGAGCGAATTC
	Reverse	GCCAGAGCTCTTACTTCCACAGATCAACACCTACC
MmF_{1-87C}	Forward	CATCACCCCATGGAGTTAGCAGTGATCGGAAAGAGCGAA
	Reverse	TTATGAGCTCTTAACAACCTCGTTGAGCCTGATCCGCT

The Plasmid pET-9d1 [140] with the cloned subunit *MmF* (WT) was used as template for polymerase chain reaction (PCR) [134]. The total volume for PCR set up was 50 μ l. The reaction was setup on ice as mentioned below with the appropriate concentration of constituents as:

Reagents	Amount
<i>Pfu</i> buffer (10x)	5 μ l
dNTP's (2 mM)	1.5 μ l
Primers (100 μ M)	2 x 0.5 μ l
Template [pET9d1+Subunit F(WT)]	1 μ l
MilliQ water	40.5 μ l
<i>Pfu</i> DNA polymerase	1 μ l

All reagents were pipetted into a PCR tube kept on ice. The reagents were mixed by spinning it shortly in the centrifuge. The lid of the PCR thermocycler (Biometra T personal) was preheated to 99 °C before reaction tubes were placed inside the machine. The following PCR thermocycler program was used for amplification:

Cycle steps	Temperature	Time
Lid	99 °C	
Initial denaturation	96 °C	3 min
Cycle denaturation	96 °C	30 sec
Annealing	Varied with the T _m	45 sec
Extension	72 °C	45 sec
Final extension	72 °C	5 min
End	4 °C	

The PCR strategy used for the amplification was same for all the subunit F constructs except for the S84A mutant, which was amplified by overlap extension PCR (Figure 2.4). Here, two separate fragments were amplified using primers *a* and *b* and primers *c* and *d*, respectively. Both the fragments contained the mutation, since they overlap each other. The two fragments were then fused together via a second PCR using the primers *a* and *d*, as mentioned in figure 2.4. All the PCR amplified products were applied onto the analytic agarose gel (1.5%) to check the amplification and purity. Depending on the purity of the products, the remaining reaction mixture was applied onto a preparative agarose gel and purified by gel extraction kit (QIAGEN) according to the manufacturer's protocol. The purified PCR product was finally eluted in 50 μ l volume of elution buffer (Qiagen).

Figure 2.4: Flow chart of overlap extension PCR. A flow diagram showing the procedures involved in site directed mutagenesis using overlap extension polymerase chain reaction method [154].

The purified PCR product was double digested for 2 h at 37 °C (SW22 Shaking Water Bath, Julabo, Germany) by taking appropriate amounts of the restriction enzyme *NcoI* and *SacI*. After the digestion, the reaction mixture was again purified with an enzyme reaction purification kit from QIAGEN. Subsequently, a ligation reaction between vector (Figure 2.5)

and insert (amplified PCR product) was setup at room temperature for 2 hr. The reagents used were as follows:

Ligase buffer (10x)	1 μ l
Vector (V)	50-100 ng
Insert (I)	Variable (1-5 times of vector)
T4 Ligase	1 μ l
MilliQ water	variable
Total	10 μ l

The Ligation reaction was terminated by adding 90 μ l of Milli Q water to make a final volume of 100 μ l. 1 ml of butanol was added into the mixture and mixed thoroughly to precipitate the DNA from rest of the reaction mixture. Afterwards, the mixture was centrifuged at 13 000 x g (using Eppendorf Mini-centrifuge) for 10 min and the supernatant was discarded gently. The pellet was resuspended in 500 μ l of 70% ethanol and centrifuged again for 10 min. Supernatant was again removed and the pellet was re-dissolved in 7 μ l of elution buffer (10 mM Tris-HCl, pH 8.5; EB buffer, QIAGEN) or Milli Q water.

The ligation mixture was then transformed into *E. coli* DH5 α cells. Single colonies were picked from the transformation plate on the next day and were incubated at 37 °C. The plasmid DNA was isolated using standard protocol (QIAGEN mini-prep kit) and subsequently double digested and applied onto a 1.5% agarose gel, in order to confirm the presence of the ligated insert. The size of insert and vector were compared with appropriate controls and markers. The verified plasmid was finally transformed into *E. coli* BL21 (DE3) cells for protein production

and they were grown on 30 μ g/ml kanamycin-containing LB agar plates.

For generating a clone with the cleavable thioredoxin-Tag, the plasmid pET-9d1 containing the gene of subunit *MmF* (WT) was digested with *Nco*I and *Sac*I enzymes and the subunit F gene was ligated in the pET32b vector via the same restriction sites.

Figure 2.5: Vector map of modified pET-9d (+). The pET-9d (+) vector showing full map and unique multiple cloning sites (MCS) that has been used to clone various genes [140].

2.2.8 Electroporation transformation

Electroporation was done for the transformation of plasmid into electro-competent cells. Electro-competent cells were prepared as per the protocol from Current Protocols in Molecular Biology (Wiley InterScience) manual. 1 – 50 ng of plasmid was added to 80 – 100 μ l of competent cells and incubated on ice for 5 – 10 min. Electroporation was done at constant voltage of 2500 V (Micropulser Electroporator, Bio-Rad). The cuvette was then immediately placed back on ice for 1 min., following addition of LB media to a total volume of 1 ml. The mixture was incubated at 37 °C for 1 h. Appropriate dilutions were prepared before plating the cells on antibiotic selection plates.

2.2.9 Production of *M. mazei* Gö1 A-ATP synthase subunit F (*MmF*) and mutants

Induction test

Reagents for 4 x lysis buffer: 250 mM Tris/HCl, pH 6.8
9.2% SDS
40% Glycerol
0.2% Bromophenol Blue
1 mM DTT

At least 5 single colonies were randomly picked and grown to OD₆₀₀ of 0.6 - 0.7 at 37 °C with shaking of 180 rpm (Infors HT Minitron shaker). A final concentration of 0.5-1 mM IPTG was then added to start the induction. The mixture was kept at 30 – 37 °C for 2 – 3 h. The concentration of IPTG depended on the characteristics of protein being produced and the conditions have been optimised for the respective proteins. Respective controls were left uninduced under identical conditions. Cells were pelleted down and resuspended with 60 μ l of 1x lysis buffer in the presence of 1 mM DTT. Resuspended cell mass was heated at 95 °C for 5 min and 15 – 20 μ l of each sample were loaded onto a 17% SDS-PAGE gel. The expression was more in the induced cultures ((+) supplemented with IPTG) than uninduced cultures ((-) not supplemented with IPTG).

To produce His₆-*MmF* mutants at large scales, liquid cultures were incubated in kanamycin-positive (30mg/ml) LB medium at 37 °C with shaking at 180 rpm, until an optical density OD₆₀₀ of 0.6 - 0.7 was achieved. The His₆-subunit F mutants were expressed by inducing the cells with IPTG to a final concentration of 1 mM. Following incubation for another 4 h at 30 °C, the cells were harvested at 8 500 x g for 12 min, 4 °C, frozen in liquid nitrogen, and stored at –80 °C until further use.

2.2.10 Purification of *M. mazei* Gö1 A-ATP synthase subunit F (*MmF*) and mutants

For purification of His₆-*MmF* mutants, cells were lysed on ice by sonication for 3 x 1 min in buffer E (50 mM Tris/HCl, pH 8.5, 100 mM NaCl, and 4 mM Pefabloc^{SC} (BIOMOL)). DTT was added to a final concentration of 0.8 mM, for the purification of the subunit F mutants, where cysteine was incorporated. Precipitated material was separated by centrifugation at 10 000 x g for 30 min. Supernatant was filtered (0.45 µm; Millipore) and incubated with 2 ml Ni²⁺-NTA resin column for 1.5 h at 4 °C and eluted with an imidazole-gradient (0 - 250 mM) in buffer E. Fractions containing His₆-tagged subunit F mutants were identified by SDS-PAGE, pooled and concentrated upto 1 ml of volume using Centricon YM-3 (3 kDa molecular mass cut off) spin concentrators (Millipore) and injected on a Superdex 75 HR 10/30 column (GE Healthcare) using a buffer of 50 mM Tris/HCl (pH 8.5), 100 mM NaCl.

For purification of *MmF* with the cleavable thioredoxin tag, a similar protocol was used until the Ni²⁺-NTA binding step. This was followed by incubation of the Ni²⁺-NTA beads bound to the protein with 20 units of thrombin and the cleavage was performed overnight at 4 °C. The cleaved subunit F was eluted with 1 ml of buffer containing 50 mM Tris/HCl, pH 8.5. The eluted protein was applied to a size exclusion (Superdex 200 HR 10/30, GE Healthcare) column with buffer containing 50 mM Tris/HCl (pH 8.5), 100 mM NaCl.

Purified protein for both subunit F (*ScF*) of the *Saccharomyces cerevisiae* V-ATPase and its C-terminal truncated form (*ScF*₁₋₉₄) were used from the earlier purifications as per the published protocol [155]. Subunit epsilon (*Mtε*) from *M. tuberculosis* H37Rv was purified according to the published protocol [156].

2.2.11 Reconstitution of *Mm*A₃B₃D with *MmF*, *MmF* mutants, *ScF*, *ScF* mutants and *Mtε*

For reconstitution of the *Mm*A₃B₃D with *MmF*, recombinant *Mm*A₃B₃D was purified as mentioned in section 2.2.3 and 2.2.10, respectively and incubated with a 3-fold molar excess of freshly purified *MmF* and its mutants at 4 °C for 4 h in 50 mM Tris/HCl, pH 7.5, 100 mM NaCl and 0.4 mM DTT). 5-fold molar excess of *ScF*, *ScF*₁₋₉₄ and *Mtε* was used when reconstituted with *Mm*A₃B₃D. The reconstitution was followed by size exclusion chromatography (Superdex 200 HR 10/30, GE-Healthcare), where the bound fractions were eluted in single peak and the binding was confirmed by the SDS-PAGE. The fractions were concentrated with 100 kDa centricons and used subsequently for ATP hydrolysis activity measurements as per the protocol mentioned in section 2.2.6.

2.2.12 Binding studies of *MmA₃B₃D* with *MmF* (WT) and *MmF_{1-87C}* using Fluorescence correlation spectroscopy

Fluorescence correlation spectroscopy (FCS) was performed at 23 °C on a ConfoCor 3 microscope (Carl Zeiss, Jena, Germany) with a water immersion objective (40x/1.2 W Corr UVVIS-IR, Zeiss) and the 633 nm line of a 5 mW HeNe633 laser to study the binding of *MmF* (WT) and *MmF₁₋₈₇* to *MmA₃B₃D*. The two subunit F proteins were genetically modified to contain a cysteine at F87C that was labeled with Atto647N-maleimide. After labelling, the free dye was separated from the labeled protein by size exclusion chromatography (Superdex 200 HR 10/30, GE Healthcare). 15 µl of samples containing subunit F-Atto647N and varying concentrations of *MmA₃B₃D* in buffer D (pH 7.5) were pipetted into Nunc 8-well chambers.

Before usage, the cover glasses were treated with 3 % of gelatin, in order to prevent unspecific binding of the fluorophors, later removed by H₂O [157]. Fluorescence autocorrelation functions were measured for 15 sec each with 30 repetitions. Solutions of Cy5 in pure water (*Fluka*) were used as references for the calibration of the confocal microscope. The measurements of autocorrelations curves of the fluorescent *MmF* (WT), *MmA₃B₃D*+*MmF*, *MmF₁₋₈₇* and *MmA₃B₃D*+*MmF₁₋₈₇* yielded the ratio of complex formation, as plotted in figure 3.16. The calculations of the bound fractions and the dissociation constants were done using the ConfoCor 3-software 4.2 and Origin 7.5.

2.2.13 Densitometric analysis of the wild-type and reconstituted *A₃B₃DF* complexes

The reconstitution efficiency of *M. mazei* Gö1 *A₃B₃D* with wild-type *MmF*, *MmF* (R88L), *MmF₁₋₈₇*, and *MmF₅₋₁₀₁* was analyzed by measuring the band intensities of the subunits from the SDS-gels. Here, the reconstituted complexes were collected after the elution from the size exclusion column (Superdex 200 HR 10/30, GE Healthcare) and were applied on a 17% SDS-gel. The intensity of the bands of subunits D and F were analyzed and compared with the ones of *A₃B₃DF*. The band intensities of subunits D and F were determined by calculating integrals of the respective peaks with ImageJ [158]. The intensity of subunit F is given relative to subunit D, which in case of the wild-type *A₃B₃DF* complex is 0.3:1.

2.2.14 Cloning of *A_(P235A,S238C)B₃D* and *A_(P235A,S238C)B₃DF* complexes containing the double mutation (P235A, S238C) in subunit A

Full plasmid amplification method was used to incorporate the mutation (P235A, S238C) in subunit A of *MmA₃B₃D* and *MmA₃B₃DF*. These double mutants will be referred as *A_(P235A,S238C)B₃D* and *A_(P235A,S238C)B₃DF*. Since the same mutation had to be generated in

the P-loop of subunit A, the following set of primers used were the same for *MmA₃B₃D* and *MmA₃B₃DF*.

Primer	Primer sequence (5' – 3')
Forward primer	CTGCTGCAATTCCAGGAGCTTTCGGGTGTGGCAAAACCGTTACT CAGCAGC
Reverse primer	GCTGCTGAGTAACGGTTTTGCCACACCCGAAAGCTCCTGGAATT GCAGCAG

The amplification was done using PCR and the reaction was the same as mentioned in section 2.2.15, though the template used here were pGEM-4z + ABD (WT) for *A_(P235A,S238C)B₃D* and pGEM-4z+ ABDF (WT) for *A_(P235A,S238C)B₃DF* respectively [2, 151]. KAPA HiFi polymerase was used for the amplification and the PCR setup was similar to the protocol mentioned in section 2.2.15, with only the annealing temperature changed to 65 °C.

The PCR product was analyzed on a 1.2 % agarose gel and was purified using a gel extraction kit. *DpnI* digestion was performed as mentioned in section 2.2.15, followed by transformation in *E. coli* DH5 α cells and later to *E. coli* DK8 cells for the production of the recombinant protein. Protein expression and purification was performed as mentioned in section 2.2.3 following the same protocol except for skipping the step of ion-exchange chromatography. ATP hydrolysis assay was performed as per section 2.2.6.

2.2.15 Cloning of subunit A mutant (S238C) and double mutant (P235A, S238C) of A-ATP synthase from *P. horikoshii* OT3

Overlap extension PCR was used for the cloning of double mutant (P235A, S238C), and inverse PCR with tail to tail primers was used for the cloning of the S238C mutant. In both the cases, the desired gene encoding subunit A of the A₁A₀ ATP synthase from *Pyrococcus horikoshii* OT3 (*Ph*) was amplified by PCR using the mutagenic primers. The template of subunit A used here was an insert of subunit A (WT) in pET22b (+) and is devoid of the intein region. In case of the double mutant (P235A, S238C), mutations were incorporated by site directed mutagenesis using overlap extension method (Figure 2.6). In this case, two separate PCR reactions were setup, one with a flanking primer (forward primer *a*) and an internal primer (internal reverse primer *b*) and the second PCR with the internal primer (internal forward primer *c*) and flanking primer (forward primer *d*), as mentioned below.

Primer	Primer sequence (5' – 3')
Forward primer <i>a</i>	TATACATATGGTGGCGAAGGGGAGGATAATTAGGGTTACTGGA CC
Internal Reverse primer <i>b</i>	CACCGTCTTACCACAGCCGAAAGCCCCAGGAATTGC
Internal Forward primer <i>c</i>	GCAATTCCTGGGGCTTTCGGCTGTGGTAAGACGGTG
Reverse primer <i>d</i>	GCTTGTCGACTCACGCCCCATACTTCTTGAAGAGCTCC

The PCR reactions were setup in a total volume of 50 µl as follows:

Reagents	Amount
<i>Pfu</i> buffer (10x)	5 µl
dNTP's (10 mM)	1.5 µl
Primers (100 µM)	2 x 0.5 µl
Template [pET922b+Subunit A(WT)]	1 µl (7 ng)
MilliQ water	40.5 µl
<i>Pfu</i> DNA polymerase	1 µl

The flanking primers (*a* and *d*) hybridized at each end of the subunit A sequence, thereby incorporating *NdeI* and *Sall* restriction sites (underlined), and an internal primer (forward primer *b*) and (Reverse primer *c*), that hybridized at the internal site. The PCR reaction was set up on ice and the lid of the PCR thermocycler (Biometra T personal) was preheated to 99 °C before reaction tubes were placed inside the machine. The following PCR thermocycler program was used for the amplification:

Cycle steps	Temperature	Time
Lid	99 °C	
Initial denaturation	96 °C	3 min
Cycle denaturation	96 °C	30 sec
Annealing	61 °C	1 min
Extension	72 °C	3 min
Final extension	72 °C	5 min
End	4 °C	

In separate PCRs, two fragments of the subunit A gene were amplified, using primers *a* and *b* and primers *c* and *d*, respectively. The amplified fragments were fused together via a second PCR using the primers *a* and *d*. The overlap allowed one strand from each fragment to act as a primer on the other, and extension of this overlap by flanking primers (*a* and *d*) in a second PCR reaction resulted in the final product. To check the amplification and purity of

the desired product, 5 μ l of PCR amplified product were applied onto the analytical agarose gel (1%). On verifying product purity, the remaining reaction mixture was applied onto a preparative agarose gel and purified using gel extraction kit (QIAGEN) as per the manufacturer's protocol. The purified PCR product was finally eluted in 50 μ l volume of elution buffer (Qiagen). The purity of the gel extracted PCR product was ascertained by running 1 μ l sample on a 1% analytical agarose gel.

Figure 2.6: A flow chart showing the procedure involved in site directed mutagenesis using overlap extension-PCR method [154]. The same strategy was used to generate the mutant *PhA*_{P235A S238C}.

The purified PCR product (insert) was double digested with restriction enzymes *NdeI* and *Sall* for 2 h at 37 °C and the pET-22b(+) vector was digested with *NdeI* and subsequently with *Sall* for 3 h at 37 °C. The digested vector and inserts were applied onto a preparative agarose gel and purified by gel extraction kit (QIAGEN) as per the manufacturer's protocol. Analytical agarose gel (1%) was run to ensure complete digestion. The double digested PCR

product and the pET-22b (+) vector were then ligated. The ligation reaction mixture of the vector and amplified product was setup as mentioned in section 2.2.7. The ligation mixture was incubated overnight at 4°C and butanol precipitation and transformation in *E. coli* DH5 α cells was carried out as mentioned in sections 2.2.7 and 2.2.8. To ascertain, successful ligation of subunit A inserts into the vector, the purified plasmids were double digested with restriction enzymes *NdeI* and *Sall*. The sizes of vector and insert were then confirmed with appropriate markers and positive controls. The mutations were verified by DNA sequencing. The verified plasmids were finally transformed into *E. coli* (BL21–CodonPlus (DE3)–RIL) cells (Stratagene). The selection plates contained ampicillin and chloramphenicol.

To clone subunit A mutant (S238C), inverse PCR was used and the following tail to tail mutagenic primers were generated for amplification.

Primer	Primer sequence (5' – 3')
Forward primer	TGTGGTAAGACGGTGACTCAGCATCAGCTAGCCAAGTGGAG
Reverse primer	GCCGAAAGGCCAGGAATTGCAGCTGTTCCACCTTTAGC TTGCGG

KAPA HiFi polymerase was used for the amplification of the plasmid pET22b (+) with subunit A mutant (S238C). PCR reaction was setup in a total volume of 50 μ l as follows with the template containing insert of subunit A (WT) in pET22b (+). The PCR reaction used the following setup:

Reagents	Amount
KAPA HiFi buffer (5x)	10 μ l
dNTP's (10 mM)	1.5 μ l
Primers (100 μ M)	2 x 0.3 μ l
Template [pET922b+Subunit A(WT)]	1 μ l (25 ng)
MilliQ water	35.9 μ l
KAPA HiFi DNA polymerase	1 μ l

The amplified product was then digested with *DpnI* enzyme to get rid of the parental methylated plasmid and the product was then transformed into *E. coli* DH5 α cells and later to *E. coli* (BL21–CodonPlus (DE3)–RIL) cells (Stratagene) for protein expression.

Cycle steps	Temperature	Time
Lid	99 °C	
Initial denaturation	95 °C	2 min
Cycle denaturation	98 °C	20 sec
Annealing	63.5 °C	15 sec
Extension	72 °C	5 min
Final extension	72 °C	7 min
End	4 °C	

2.2.16 Purification of subunit A mutants (*PhA*_{P235A, S238C} and *PhA*_{S238C})

To produce subunit A mutant *PhA*_{P235A, S238C} and *PhA*_{S238C}, liquid cultures were shaken in LB medium containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol. At an OD₆₀₀ of 0.6, the cells were induced by the addition of 1 mM IPTG and growth continued at 30 °C for 4 h. The cultured cells were pelleted down by centrifugation at 9 000 x g (Biofuge *Primo* R, Heraeus) for 10 min. The cell pellet was then resuspended in Tris-HCl buffer F (50 mM Tris-HCl, pH 8.5, 50 mM NaCl, 1 mM DTT and 1 mM PMSF) and disrupted using sonication method. The cell lysate was incubated at 70 °C for 30 min, kept on ice and then centrifuged at 40 000 x g for 30 min. Ammonium sulfate was added to the supernatant to 60% saturation. The solution was mixed thoroughly till ammonium sulphate dissolved completely, followed by centrifugation at 20 000 x g for 20 min. The protein pellet thus obtained was resuspended in buffer G (50 mM Tris-HCl, pH 8.5, 50 mM NaCl, 1 mM DTT). The resuspended protein solution was applied on a PD-10 (GE Healthcare) desalting column (equilibrated with 50 mM Tris-HCl, pH 8.5, 1 mM DTT) to remove excess salt. The protein eluted in buffer H (50 mM Tris-HCl, pH 8.5, 1 mM DTT), which was then loaded onto a Resource Q column (GE Healthcare) equilibrated with buffer H. The bound protein eluted by a linear NaCl gradient (0.05 - 1.0 M). Subunit A mutants eluted at a concentration of 0.15 M NaCl. The peak fractions were pooled together and concentrated using 50 kDa concentrators (Amicon) to a volume of 500 µl. The fractions containing subunit A mutants were applied onto a column of 26/60 Superdex200 (GE Healthcare) equilibrated with buffer G (50 mM Tris-HCl, pH 8.5, 1 mM DTT, 50 mM NaCl). The subunit A mutant eluted at 15 ml volume.

Buffer exchange was performed for the eluted protein during the concentration process using 50 kDa concentrators (Amicon) in a stepwise manner such, that the pH of the buffer (50 mM Tris-HCl, pH 8.5) was reduced gradually from 8.2 to 7.8 and finally, the protein was

obtained in the buffer (50 mM Tris-HCl, pH 7.5). The purity of the protein samples were analyzed by SDS-PAGE [152].

2.2.17 Nucleotide-binding studies of *PhA*_{P235A, S238C} using Fluorescence correlation spectroscopy (FCS)

In order to determine the binding affinity of nucleotides with *PhA*_{P235A, S238C}, FCS was performed at 25 °C on a ConfoCor 3 (Zeiss, Jena, Germany) using the AMP-PNP- and ADP-analogues EDA-ATP ATTO-647N and EDA-ADP ATTO-647N (*ATTO-TEC*, Siegen, Germany). The 488 nm laser line of an HeNe633 laser were attenuated to 5 mW and focused into the aqueous solution by a water immersion objective (40 x / 1,2 W Korr UL-VIS-IR, Zeiss). FCS was measured in 15 µl droplets of the diluted fluorescent derivatives of ATP and ADP, which were placed on Nunc 8 well chambered cover glass. The following filter sets were used: MBS: HFT UV/488/543/633, EF1: LP 655, EF: None, DBS: Plate, DBS1: Plate, DBS3: Mirror. Out-of-focus fluorescence was rejected by a 90 µm pinhole in the detection pathway, resulting in a confocal detection volume approximately 0.25 fl. Fluorescence autocorrelation functions were measured for 30 sec each with 30 repetitions. Solutions of Cy5 in pure water (*Fluka*) were used as references for the calibration of the confocal microscope. To analyze the autocorrelation functions of fluorescent nucleotides bound, in part, both the subunit A and its mutant *PhA*_{P235A, S238C} respectively, models with the diffusion time and the triplet state were used for fitting (FCS-LSM software, ConfoCor 3, Zeiss). The diffusion times of fluorescent nucleotides and fluorescently labeled subunits were measured independently, and kept fixed during the fitting of the FCS data. Therefore, the determination of the binding constants required only the calculation of the relative amounts of free nucleotides with the short diffusion time and of the bound nucleotides with the diffusion time of subunit A and its mutant *PhA*_{P235A, S238C}. The calculations of the bound fractions and the dissociation constants were done by the ConfoCor 3-software 4.2 and Origin 7.5.

2.2.18 Crystallization of the subunit *PhA*_{P235A, S238C}

Larger crystals for *PhA*_{P235A, S238C}, suitable for X-ray diffraction measurements were obtained by the hanging-drop vapor diffusion method at 18 °C from a solution containing 50% (v/v) MPD and 0.1 M acetate pH 4.5 with paraffin oil and silicone oil (v/v 1:1). 2 µl protein solution was mixed with an equal volume of reservoir solution and equilibrated against 500 µl reservoir solution containing paraffin oil and silicone oil. This overlaying of oils prolonged the nucleation time and hence large sized crystals could be obtained.

2.2.19 Co-crystallization of subunit A mutant *PhA*_{P235A, S238C} with nucleotides

For co-crystallization with nucleotides, the purified subunit A mutant *PhA*_{P235A, S238C} was divided into three batches and incubated separately with 2 mM MgATP, 2 mM MgADP, 2 mM MgAMP-PNP and 2 mM MgAMP at 4 °C for 30 min on a sample rotator and this mixture was then concentrated to 10 - 12 mg/ml using 50 kDa centricon concentrators (Amicon). During this concentration process, buffer exchange was performed in a stepwise manner to finally obtain the protein in the crystallization buffer (50 mM Tris-HCl, pH 7.5). The protein-ligand complexes were then set up for crystallization in the same optimized conditions in hanging drop plates and incubated at 18 °C. The presence of 50% (v/v) MPD in the crystallization buffer acted as a good cryo-protectant. Therefore, the crystals thus obtained were directly flash-cooled in liquid nitrogen.

2.2.20 Data collection

With the help of Dr. Neelagandan Kamariah from our laboratory, single-wavelength datasets for *PhA*_{P235A, S238C} crystals were collected at 140 K on beamline 13B1 at the National Synchrotron Radiation Research Center (NSRRC, Hsinchu, Taiwan) using the ADSC Quantum 315 CCD detector. Data sets were collected as a series of 0.5° oscillation images with 20 s exposure time with a detector distance of 350 mm. Staff at beamline 13B1 (NSRRC) kindly provided technical assistance. All the diffraction data were indexed, integrated and scaled using the HKL2000 suite program [141]. All the crystals belong to tetragonal space group P4₃2₁2 and have similar unit cell parameters as wild type subunit A. The Matthews's coefficient [159] was determined, which revealed one molecule in the asymmetric unit.

2.2.21 Crystal structure determination of subunit A mutant *PhA*_{P235A, S238C}

The structure of subunit A mutant (P235A; PDB: 3M4Y) [121] from *P. horikoshii* OT3 (*Ph*) was used as model for structure determination by molecular replacement method using the programs PHASER [144] and MOLREP [143]. Rigid body refinement was carried out followed by difference Fourier syntheses calculations and iterative cycles of model building and refinement were carried out using the programs COOT [145] and REFMAC5 [142] of the CCP4 suite (1994). The template of wild type subunit A used for cloning had a mutation at position 79 from glycine to arginine, which has been reported in earlier structures [55] and this was clearly visible in the electron density map. The initial 50 residues of the N-terminal are disordered in the structure because of the high flexibility of the N-terminus. The geometry of the final model was checked with PROCHECK [147] and MOLPROBITY [160]

and the figures were drawn using the PyMOL [39]. Structural comparison analysis was carried out using the SUPERPOSE program as included in the CCP4 suite [161].

2.2.22 Crystallization of the A₃B₃D complex of the *M. mazei* Gö1 A-ATP synthase

Crystallization of complex *MmA*₃B₃D was attempted using vapour diffusion method [162]. Sparse matrix screens from Hampton research and Emerald biosystems were used for the initial screening. Hanging drops were set up by mixing 1 µl of the purified complex *MmA*₃B₃D (3-5 mg/ml) in buffer D (50 mM Tris/HCl, pH 7.5, 200 mM NaCl, 0.8 mM DTT) with 1 µl of the precipitant solution and incubated at 18 °C. Initial crystals were observed with Emerald biosystems wizard screen 1 and wizard screen 2. On confirming the crystals as protein crystals, further optimization of the crystallization condition was done in order to decrease the nucleation and to promote further crystal growth. Sitting drop plates were used for indirect vapour diffusion and better looking crystals were obtained. Further optimization of protein concentration (10 mg/ml) and drop size (2 µl of purified complex *MmA*₃B₃D and 2 µl of precipitant solution) resulted in bigger and better looking crystals, which later diffracted upto 10 Å with the addition of 0.3 M Glycyl-glycyl-glycine. The final crystallization solution was comprised of 10% PEG-8000, 0.1 M CHES (pH 9.5), 0.2 M NaCl and 0.3 M Glycyl-glycine-glycine. Crystals with 2 mM MgADP, 2 mM MgATP and 2 mM MgAMP-PNP were also obtained.

2.2.23 Crystal analysis and data collection

Crystals were analyzed in-house at 100 K on a Rigaku RAXIS IV image plate detector with a Rigaku RA-Micro & HFM rotating copper anode generator (Rigaku/MS). Low resolution data were collected by Dr. Asha Balakrishna and Dr. M.M.S. Sony from our laboratory at 140 K at (NSRRC, Hsinchu, Taiwan) using the ADSC Quantum 315 CCD detector. The diffraction data were indexed, integrated and scaled using the CCP4 suite of programs. The crystal belongs to cubic space group I2₁3. Molecular replacement was carried out using CCP4 suite.

2.2.24 Genetic engineering of the *M. mazei* Gö1 mutants A₃B₃DF-M1 and-M2

The purpose of generating *MmA*₃B₃DF mutants (*MmA*₃B₃DF-M1 and *MmA*₃B₃DF-M2) was to study the rotation of subunit D and F in the A-ATP synthase. The *MmA*₃B₃DF-M1 mutant protein included the amino acid substitutions C36V, C72V, C181A and C380V in subunit A as well as the substitutions C67S and A71C in subunit B and D, respectively.

The fragment containing genes *ahaA*, *ahaB*, *ahaF*, and *ahaD* of the *MmA₃B₃DF-M1* mutant were synthesized and provided by *DNA 2.0* in the vector pJ367 (in-house cloning vector). The fragment was further subcloned into pGEM-4Z. The second mutant protein *MmA₃B₃DF-M2* contained the single substitution A71C in subunit D, which was generated by replacing the gene encoding subunit D of *MmA₃B₃DF-WT* with the gene encoding subunit D mutation A71C of *MmA₃B₃DF-M1*-pJ367. The replacement of subunit D was possible because of the presence of an endogenous *NcoI* site at the N-terminal of subunit D, which was then used along with *SacI* (followed by the stop codon of subunit D). The constructs were transformed into *E. coli* DH5 α cells and later into *E. coli* DK8 cells. All constructs contained a His₈-tag at the N-terminus of subunit A and the respective proteins were produced and purified as mentioned in section 2.2.3. ATPase hydrolytic activity was tested as per section 2.2.6.

2.2.25 Labeling and rotation assay of *M. mazei* Gö1 mutant A₃B₃DF-M2

The following labeling and rotation experiments were performed by Dr. Hendrik Sielaff at Arizona State University under the guidance of Prof. Wayne Fräsch using the following protocol. The cysteine in the *M. mazei* Gö1 subunit D (A71C) of *MmA₃B₃DF-M2* was labeled with biotin-maleimide for the attachment of a neutravidin-coated gold nanorod. 12.7 μ M of *MmA₃B₃DF-M2* in 25 mM HEPES, pH 7.5/ HCl, 25 mM KCl, 5 mM MgCl₂ were incubated for 1 min at 4 °C with a 1.5-fold molar excess of biotin-PEAC₅-maleimide (Dojindo, Japan).. The reaction was stopped by adding 2 mM acetyl-cysteine, and unbound biotin-maleimide was removed by adding buffer E, followed by filtration in a centrifuge, using Centricon YM 100. Samples were frozen in liquid N₂ and kept at -80 °C until further use.

For rotation assay, samples were pipetted into flow cells consisting of two coverslips, separated by double-adhesive tape. Prior to use, the bottom coverslip was cleaned for 30 min by sonication in 1% Hellmanex III, followed by rigorous washing with H₂O. Gold nanorods (40x76 nm, Nanopartz, USA) were prepared mixing 9 μ l of nanorods with 1 μ l Neutravidin (5 mg/ml in H₂O, Molecular Probes, USA) for 1 h, before adding 1 μ l BSA (10% solution, Aurion, The Netherlands) for 1 min and 89 μ l of buffer RB (50 mM Tris pH 8.0, 10 mM KCl). Solutions were infused in a flow cell (20 μ l, 5 min incubation) in the following order, with washing steps of 60 μ l buffer RB in between: (1) Ni²⁺-NTA-horseradish peroxidase conjugate (Qiagen, Germany) in deionized H₂O; (2) 1 nM biotinylated protein in buffer RB; (3) gold nanorods in buffer RB; (4) 2 mM ATP, 1 mM MgCl₂ in buffer RB.

A gold nanorod was attached to the protein as a visible probe of rotation. Samples were illuminated by a mercury lamp through a dark field condenser in a confocal microscope (Axiovert, Zeiss, Germany). When observed through a band pass filter and a polarizer, the intensity of red light scattered from the nanorod, detected by an avalanche photo diode (APD), changed sinusoidal from a maximum to a minimum when the long axis of the nanorod was parallel and perpendicular, respectively, to the axis of polarization. Data were collected for 10 s and 30 s at 1 kHz and 200 kHz, respectively. The obtained signals were evaluated with custom software (Matlab, MathWorks) to obtain intensities and velocities at each angular position of the rotating protein-nanorod complex [163, 164]. From the light intensity fluctuations recorded by the APD, power strokes were identified by intensity changes from a minimum taken from the lowest 10 percentile of peaks to a maximum taken from the highest 5 percentile of peak, for which a linear least square fit gave an R-value of at least 0.9.

2.2.26 Genetic engineering of the *M. mazei* Gö1 mutant A₃B₃D-M1 mutant

The *Mm*A₃B₃D-M1 mutant included the amino acid substitutions C36V, C72V, C181A and C380V in subunit A as well as the substitutions C67S and A71C in subunit B and D, respectively similar to mutant *Mm*A₃B₃DF-M1. For easy understanding, this mutant is referred to as A_{(ΔCys)3}B_{(ΔCys)3}D_{A71C} in the results and discussion sections. The generation of this mutant was essential to perform FRET experiments along with the subunit F cysteine mutants [*Mm*F (L87C), *Mm*F (K92C) and *Mm*F (A94C)]. *Mm*A₃B₃D-M1 was generated from *Mm*A₃B₃DF-M1 by deleting subunit F from the operon. The order of genes in the operon was *ahaA*, *ahaB*, *ahaF* and *ahaD* corresponding to subunit A, B, F and D respectively. I took advantage of the intrinsic *Xho*I site present in the subunit B and designed four primers which are mentioned below.

Primer	Primer sequence (5' – 3')
Forward primer <i>a</i>	AGTTCCTCGAGTTTGCCGACCTGTTTGAG
Internal Reverse primer <i>b</i>	CCATGGCAATCACTTAGCCTTTCTGTG
Internal Forward primer <i>c</i>	GCTAAGTGATTGCCATGGCTCAGCAGG
Reverse primer <i>d</i>	TTCGAGCTCGGTACCTTAATCTCTCATTCTTG

Two PCR reactions were setup, one using the forward primer *a* and the internal reverse primer *b* and the other using internal forward primer *c* and reverse primer *d* as

described in figure 2.7. In both the PCR reactions, *MmA₃B₃DF-M1* was used as the template.

Figure 2.7: Flow chart explaining the cloning of *MmA₃B₃D-M1*. The operon containing *ahaA*, *ahaB*, *ahaF* and *ahaD* genes are shown with green arrows. (□) indicates overlapping region from subunit D and (Δ) indicates overlapping region from subunit B.

The PCR with primers *a* and *b* resulted in amplification of a part of subunit B carrying *XhoI* site (product I). The second PCR with primers *c* and *d* resulted in amplification of subunit D with the *SacI* site (product II). The part of subunit B and subunit D were ligated by a third PCR using equimolar ratio of product I and II as the template and primers *a* and *d* as forward and reverse primer, respectively. This ligated product of subunit B and D was termed as product III. The product III was double digested with *XhoI* and *SacI* enzymes for 2 h. at 37 °C and ligated to the already double digested *MmA₃B₃DF-M1*, where part of subunit B and entire subunit F and D were deleted as shown in figure 2.7. The ligation of product III with *MmA₃B₃DF-M1* (without subunit B, F and D) resulted in generation of *MmA₃B₃D-M1*, where subunit F has been deleted.

The construct was transformed into *E. coli* DH5α cells and later into *E. coli* DK8 cells. The construct contained a His₈-tag at the N-terminus of subunit A and the respective proteins

were produced and purified as mentioned in section 2.2.3. The ATPase hydrolytic activity was tested as per section 2.2.6.

2.2.27 Cloning of the *M. mazei* Gö1 mutants A₃B₃D-M2 and A₃B₃DF-M3

Both mutants *MmA₃B₃D-M2* and *MmA₃B₃DF-M3* were designed for the purpose of rotation studies and they carry an additional cysteine in subunit D at position 131 (E131C), along with the A71C mutation. Subunits A, B and F were devoid of any mutation in this case. The rationale for the mutation was that two cysteines at the tip of subunit D will enhance the binding of the streptavidin beads and hence the probability of recording the rotating molecule. The mutagenic primers used here are stated below.

Primer	Primer sequence (5' – 3')
Forward primer <i>a</i>	ATTGCCATGGCTCAGCAGGACGTAAAACCAAC
Internal Reverse primer <i>b</i>	GGTCCTCGTAGGCATCAGCAGTACAATCAATATATGAATTTGTTC CG
Internal Forward primer <i>c</i>	CGGAACAAATTCATATATTGATTGTACTGCTGATGCCTACGAGG ACC
Reverse primer <i>d</i>	ATTCGAGCTCGGTACCTTAATCTCTCATTCTTGCTTGACTC TCT

The template used for the mutation was *MmA₃B₃DF-M1*, which already has an A71C mutation in subunit D. The two PCR products were generated with primers *a* and *b* and with primers *c* and *d* and then mixed in equimolar ratios and used as a template for the third PCR. The third PCR was performed with primers *a* and *d*, resulting in the mutated subunit D, which was extracted from the gel, purified and used for the double digestion performed with *NcoI* and *SacI* restriction enzymes. The mutated subunit D (A71C and E131C) was then replaced with the subunit D (WT) of the *MmA₃B₃D* (WT) and *MmA₃B₃DF* (WT) which resulted in *MmA₃B₃D-M2* and *MmA₃B₃DF-M3*, respectively. The ligation reaction was setup with the following components:

Ligase buffer (10x)	1 µl
Vector (V)	50-100 ng
Insert (I)	Variable (1:3 and 1:6 of vector: insert)
T4 Ligase	1 µl
MilliQ water	variable
Total	10 µl

The ligation reaction was kept overnight at 4 °C and followed by the transformation in *E. coli* DH5 α cells and mutation was confirmed with sequencing. The construct was then transformed into *E. coli* DK8 cells for the expression of protein. The process of cloning of these two mutants has been pictorially explained in figure 2.8.

Figure 2.8: Flow chart for cloning *MmA₃B₃D*-M2 and *MmA₃B₃DF*-M3.

3. Results

3.1 Production, purification and characterization of complexes A_3B_3D and A_3B_3DF of the A-ATP synthase from *M. mazei* Gö1 and establishing the role of subunit F as an ATPase stimulating subunit

Production, purification and characterization of sub-complexes A_3B_3D and A_3B_3DF of the A-ATP synthase from *M. mazei* Gö1 and establishing the role of subunit F as an ATPase stimulating subunit

3.1.1 Production and purification of A₃B₃D of the A-ATP synthase from *M. mazei* Gö1

The construct pMG3 (pGEM-4Z + subunit ABD) was kindly provided by Prof. Müller from Germany (Johann Wolfgang Goethe-Universität Frankfurt)[151]. Earlier attempts to purify the A₃B₃D-complex from *M. mazei* Gö1 did not achieve desirable yield and homogeneity of the protein [165]. As described in details below, optimization steps were developed including a three step protein purification protocol, comprised of Nickel-nitrilotriacetic acid (Ni²⁺-NTA) affinity chromatography, followed by anion exchange - and gel filtration chromatography. The outcome of this purification strategy was satisfactory given the better yield and highly purified recombinant *MmA*₃B₃D-complex protein. The yield of recombinant protein was further enhanced by upscaling the purification.

Figure 3.1: Purification of *M. mazei* Gö1 A₃B₃D (A) Ni²⁺-NTA purification profile showing the desired bands of subunits A, B and D corresponding to the sizes of 62 kDa, 52 kDa and 27 kDa, respectively. Fractions from 125 mM to 400 mM imidazole were pooled and applied onto (B) an anion exchange column (Resource Q, 6 ml) at a constant flow rate of 1 ml/min with a linear NaCl gradient. The gray area of the peak refers to the pooled fractions. (C) Protein obtained after the anion exchanger was applied onto a gel filtration column (Superdex 200). *MmA*₃B₃D eluted at 12.5 ml column volume and its purity was confirmed by an 17 % SDS-gel (inset).

Ni²⁺-affinity chromatography was performed to selectively isolate *MmA₃B₃D* from the entire cell extract, using the His₈-tag at the N-terminus of subunit A. Prominent bands of subunit A (62 kDa), subunit B (52 kDa) and subunit D (27 kDa) were identified in the fractions of 125-400 mM imidazole on a 17 % SDS-gel (Figure 3.1A). The fractions were applied onto an anion exchange chromatography column (Resource Q, 6 ml) to separate remaining contaminants by their different charge. The *MmA₃B₃D* eluted at 200 mM NaCl and the fractions were concentrated to 500 µl, and applied on a gel filtration column (Superdex 200 column, GE Healthcare), equilibrated in a buffer comprised of 50 mM Tris/HCl (pH 7.5), 200 mM NaCl, 0.8 mM DTT. The peak obtained at 12.5 ml elution volume was confirmed as *MmA₃B₃D* by SDS-PAGE (shaded area in figure 3.1C) and by negative stain electron microscopy that will be described in section 3.1.3.

3.1.2 Cloning, production and purification of the *M. mazei* Gö1 A₃B₃DF sub-complex

Plasmid containing the genes ABFD (4098 bp) of the A-ATP synthase from *M. mazei* Gö1 was synthesized by the company DNA 2.0 and provided in the shuttle vector pJ367 (~5 kbp). I cloned these ABFD genes into pGEM-4Z vector and transformed them into *E. coli* DK8 cells for the production of the recombinant *MmA₃B₃DF* as explained in section 2.2.1 and 2.2.3. Purification of *MmA₃B₃DF* was performed as per the established three step purification protocol of *MmA₃B₃D* mentioned in section 3.1.1. The complex eluting from the Ni²⁺-NTA affinity column was analysed on a 17 % SDS-gel, showing that subunits A, B, and subunit D were obtained. The band for subunit F was not visible after Ni²⁺-NTA affinity chromatography (Figure 3.2A), because of the low concentration of the entire complex in the eluted and loaded samples. Similar to *MmA₃B₃D*, fractions containing 125-400 mM of imidazole were pooled and applied on an ion-exchange column (Resource Q, 6ml) and the desired protein was obtained at 200 mM of NaCl (Buffer B and C were used) (Figure 3.2B). Subsequently, the protein obtained from the ion-exchange chromatography was applied onto a gel filtration column (Superdex 200), resulting in the elution of highly homogeneous *MmA₃B₃DF* (Figure 3.2C), which was evident from the SDS-gel (Figure 3.2C inset).

Figure 3.2: Purification of *M. mazei* Göl A₃B₃DF (A) Ni²⁺-NTA purification profile showing the desired bands of subunits A, B and D corresponding to the sizes of 62 kDa, 52 kDa and 27 kDa, respectively. Fractions from 125 mM to 400 mM imidazole were pooled and applied onto an (B) anion exchange column (Resource Q, 6 ml) at a constant flow rate of 1 ml/min with a NaCl gradient. The gray area of the peak refers to the pooled fractions. (C) Protein obtained after the anion exchanger was applied onto a gel filtration column (Superdex 200). *Mm*A₃B₃DF eluted at 12 ml column volume and its purity was confirmed by a 17 % SDS-PAGE gel (inset).

3.1.3 Electron microscopy and 2D reconstruction of *Mm*A₃B₃D

To observe single molecules under the electron microscope, samples were diluted with 50 mM Tris/HCl, pH 7.5, and 200 mM NaCl to an optimal concentration of 36 µg/ml and stained with uranyl acetate (negative stain) to improve the visibility of the molecules by providing better contrast. Class averages are represented in figure 3.4. As observed in class *11*, a pseudo-hexagonal arrangement of the particles with the presence of a seventh mass proposed to be subunit D (Figure 3.4 and class *11* focused as image *II*) can be distinguished. This so-called bottom view reveals an alteration of a bigger and smaller mass, reflecting the alteration of the nucleotide-binding subunit A and B, respectively. The so-called top view is

represented in class 15, showing a ‘crown’ like arrangement. The centre of this crown has a cavity, which in the bottom view is filled by subunit D (Figure 3.4 class 15 focused as image I).

Figure 3.3: Negative staining electron microscopy of MmA_3B_3D . (A) Electron micrograph of the negatively stained complex MmA_3B_3D shows the widely spread MmA_3B_3D particles. (B) Image of the self-organizing map of MmA_3B_3D particles. Altogether 3619 particles were used for obtaining the class averages.

Figure 3.4: Class averages of the negatively stained complex MmA_3B_3D . The bottom view is represented in class 11 and is also focused as image II. A pseudo-hexagonal arrangement of particles and also a seventh mass proposed to be subunit D can be seen in class 11. The so-called top view as shown in class 15 and also focused as image I, reveals an alteration of a bigger and smaller mass representing nucleotide-binding subunit A and B. Subunit A is represented by the bigger globular mass indicating the presence of the Non-Homologous Region (NHR).

A homogeneous distribution of the MmA_3B_3D complex is shown in the EM images of Figure 3.3A, where the boxes represent the particles picked for processing. A total of 32 images were recorded from which 3619 particles were selected by EMAN2 (image processing suite) and 31 classes were obtained.

3.1.4 Electron microscopy and 2D reconstruction of MmA_3B_3DF

Figure 3.5: Negative stain electron microscopy data of MmA_3B_3DF . (A) Electron micrograph of negatively stained MmA_3B_3DF shows the widely spread MmA_3B_3DF particles. (B) Image of the self-organizing map of the MmA_3B_3DF particles which were picked using EMAN2. 1360 particles were used to compute 26 class averages. (C) Classes showing the alternating A-B hexamer (3:3) and the 7th mass are seen in the bottom view (class 6 and 22 are represented as I and III, respectively), which could be subunit D and subunit F (subunit F is just 11 kDa). The top view shows the central cavity and also the crown made up of the A_3B_3 hexamer (class 11 represented as II in the enlarged view).

Similar to MmA_3B_3D , the MmA_3B_3DF complex was also analysed by negative stain electron microscopy. The protein samples were diluted to 29 $\mu\text{g/ml}$ to obtain a better spread of the molecules on the carbon grid. A total of 24 images were recorded and 1360 particles were picked and processed by the software EMAN 2. These 1360 particles were classified in 26 classes and 2D reconstruction was performed. The hexameric head made of alternating subunits A and B and the central cavity were similar to the MmA_3B_3D complex (Figure 3.5). The position of the seventh mass including subunits D and F in class 22 seems to be more centered compared to the position in class 6 (Figure 3.5C). This variation of the central mass is assumed to be caused by the preferred orientation of the molecule or could it be caused by the displacement of the central mass with respect to the tilt. Class 11 shows the central cavity and the hexameric globular masses (Figure 3.5C)

The surface view of the three-dimensional representation of MmA_3B_3DF (Figure 3.6) also confirms the presence of the hexamer and the central cavity when looking from the top. The bottom view represents the additional seventh mass, which should be representing subunits D and F. The 3D models were generated from the 2D classes by using EMAN2 and the reconstruction was performed. The 3D structure was resolved upto 30 Å and complex formation was confirmed.

Figure 3.6: Surface representation of the three-dimensional reconstruction of MmA_3B_3DF . The top view of MmA_3B_3DF represents the pseudo-hexagonal arrangement of the MmA_3B_3 subunits, with the presence of a central cavity (*left*). The bottom view of the complex MmA_3B_3DF shows the additional seventh mass representing subunits D and F (*right*).

3.1.5 ATP hydrolysis and comparison of the kinetic properties of the MmA_3B_3D and MmA_3B_3DF complexes

To understand the enzymatic activity of the MmA_3B_3D and MmA_3B_3DF , an NADH-coupled ATPase assay was performed using 2 mM ATP in the enzymatic assay. The assay

Figure 3.7: Specific activity of the *MmA₃B₃D* and *MmA₃B₃DF* using 2 mM ATP in the enzymatic assay.

revealed an ATPase activity of about 1.2 $\mu\text{mol}/\text{min}/\text{mg}$ protein for *MmA₃B₃D* and 4.6 $\mu\text{mol}/\text{min}/\text{mg}$ protein for *MmA₃B₃DF* (Figure 3.7). The higher activity in case of the *MmA₃B₃DF* complex does signify the importance of subunit F as an activator for ATP hydrolysis. To determine the role of subunit F, substrate-dependent ATP-hydrolysis experiments with varying concentrations of ATP (0.025 – 4 mM) were performed (Figure 3.8). The Michaelis constant (K_m) and the maximum rate (V_{max}) of *MmA₃B₃D* and *MmA₃B₃DF* were determined (Figure 3.9).

Figure 3.8: ATP hydrolysis of *MmA₃B₃D* and *MmA₃B₃DF* at varying concentrations of MgATP. Continuous ATPase activity of *MmA₃B₃D* (A) and *MmA₃B₃DF* (B) measured at different concentrations of MgATP (0.05 to 4 mM). Decrease in NADH absorption at 340 nm is plotted against the progressing time in seconds.

The calculated V_{max} values of *MmA₃B₃D* and *MmA₃B₃DF* are $7.9 \pm 0.3 \text{ s}^{-1}$ and $30.4 \pm 0.9 \text{ s}^{-1}$, respectively, while the K_m values are nearly the same for both ($0.19 \pm 0.04 \text{ mM}$).

and 0.15 ± 0.02 mM, respectively) (Figure 3.9). We calculated the second order binding rate constant V_{max}/K_m for both complexes and found that MmA_3B_3DF ($2.0 \cdot 10^5 \text{ M}^{-1}\text{s}^{-1}$) is catalytically 5 times more effective than MmA_3B_3D ($0.4 \cdot 10^5 \text{ M}^{-1}\text{s}^{-1}$). The kinetic data clearly state that the presence of subunit F has no effect on the K_m and therefore substrate-binding is the same for both MmA_3B_3D and MmA_3B_3DF . However, the V_{max} is 4 times slower in MmA_3B_3D compared to MmA_3B_3DF , indicating that subunit F affects another step of the ATP hydrolysis reaction, namely the cleavage of ATP to ADP and P_i , and/or the release of the products.

Figure 3.9: Michaelis-Menten plot of MmA_3B_3DF and MmA_3B_3D . The measurements for MmA_3B_3D (○) and MmA_3B_3DF (□) were performed at varying concentrations of MgATP (0.05 mM to 4 mM) performed at 37 °C.

In the related F-ATP synthases the cleavage of ATP in the nucleotide-binding site is independent of rotation, while the release of phosphate from the active site is coupled to the rotation of the central stalk, which is necessary for the overall hydrolysis reaction to continue with the binding of an ATP to an empty site. A strong coupling between the reaction site and the central rotating stalk is needed for the enzyme to be effective [2]. As subunit F can interact with both subunit D and subunit B [126, 127, 134], it seems to act as a mediator element for rotation. By increasing the tightness of the coupling between rotor and stator subunits it may increase the maximal velocity of the reaction [2].

3.1.6 Purification of subunit F (WT) of *M. mazei* Gö1 and reconstitution with *MmA₃B₃D*

To understand the role of subunit F as a stimulator of hydrolytic activity in *MmA₃B₃DF*, it was necessary to purify recombinant subunit F of *M. mazei* Gö1 (*MmF*) for the reconstitution with the *MmA₃B₃D*. *MmF* was purified using Ni²⁺-NTA chromatography following the protocol described in [134] and mentioned in section 2.2.10.

Imidazole fractions containing subunit F were pooled, concentrated (spin concentrator, molecular cut-off: 3 kDa, Millipore) and applied to a size exclusion column (Superdex 200 HR 10/30, GE Healthcare). The protein was eluted with a buffer containing 50 mM Tris/HCl, pH 8.5, and 100 mM NaCl (Figure 3.10).

Figure 3.10: Purification of *MmF* by gel filtration (Superdex 75). Protein obtained after Ni²⁺-NTA chromatography was applied onto a gel filtration column (Superdex 75) and the desired protein eluted at 13 ml volume. The purity was confirmed by running an 17 % SDS-PAGE. Lane 1 represents the protein marker and lane 2 shows the purified subunit F.

For reconstitution of the *MmF* with the *MmA₃B₃D* complex, freshly purified *MmA₃B₃D* was incubated with a 3-fold molar excess of freshly purified *MmF* at 4 °C for 4 h in 50 mM Tris/HCl, pH 7.5, 100 mM NaCl and 0.4 mM DTT. The reconstituted mixture was then passed over a gel filtration column (Superdex 200 HR 10/30, GE-Healthcare) (Figure 3.11). The protein complex eluted at 12 ml and was concentrated using a 100 kDa centricon to remove the excess of *MmF*. The concentrated sample, when analyzed on a SDS-gel, confirmed that individual subunit F (*Mm*) has bound to the *MmA₃B₃D* complex (Figure 3.11A, inset). Subsequently ATP hydrolysis activity measurements with the reconstituted *MmA₃B₃D* + F (WT) complex showed an activity of 3.3 $\mu\text{mol}/\text{min}/\text{mg}$ protein, which is 3 times higher than the activity of *MmA₃B₃D*, and reached 72% of the *MmA₃B₃DF* (WT) activity. These results clearly indicate subunit F plays an important role in ATPase activity.

Figure 3.11: Reconstitution of *M. mazei* Gö1 A₃B₃D and subunit F. (A) Purified *MmA3B3D* and *MmF* were reconstituted and passed through a Superdex S200 column (A) and the peak containing *MmA3B3D*+*MmF* was run on a 17 % SDS-gel (inset). (B) Bar diagram showing the specific activities of *MmA3B3DF*, *MmA3B3D* and *MmA3B3D*+*MmF*, indicating the increase in activity of *MmA3B3D* after addition of subunit F.

3.1.8 Mapping of the critical residues of subunit F involved in the stimulation

As shown above, subunit F of A-ATP synthases stimulates ATP hydrolysis in the recombinant *MmA3B3DF* by a factor of four (Figure 3.7) and in the reconstituted complex *MmA3B3D* + *MmF* by a factor of three when compared to the *MmA3B3D* complex. The structural information available about subunit F indicates the flexibility of the C-terminus, which might enable the coupling subunit F to switch between an elongated and retracted state. To investigate the role of the C-terminus of subunit F and also to identify critical residues of the C-terminal region of *MmF*, truncations and point mutations were genetically engineered in *MmF*, based on the available structural information [57, 126, 127].

The C-terminal truncated forms of subunit F (*MmF*₁₋₈₇, *MmF*₁₋₉₁ and *MmF*₁₋₉₅) were generated by PCR as mentioned in section 2.2.7. The protein was expressed in *E. coli* BL21 (DE3) cells (Stratagene) and were purified similar to *MmF* (see above) and as shown in figure 3.12A. The truncated *MmF*₁₋₈₇, *MmF*₁₋₉₁ and *MmF*₁₋₉₅ were then incubated with *MmA3B3D* to generate the reconstituted complexes, followed by gel filtration chromatography (Superdex 200). The reconstituted complex eluted around 13 ml and the peak fractions were concentrated and applied on a SDS-gel (Figure 3.12B). It was concluded from the SDS-gel that all the C-terminal truncated forms bound to the *MmA3B3D* complex, indicating that the globular N-terminal domain is responsible for the binding of *MmF* with the *MmA3B3D* complex. Importantly, the reconstituted *MmA3B3D* + truncated subunit F (*MmF*₁₋₈₇, *MmF*₁₋₉₁ and *MmF*₁₋₉₅) complexes did not reveal increased ATPase activity as observed for the

reconstituted $MmA_3B_3D + MmF$ complex, indicating the stimulating role played by the C-terminus of subunit F. The activities of the reconstituted $MmA_3B_3D + MmF_{1-87}$, $MmA_3B_3D + MmF_{1-91}$ and $MmA_3B_3D + MmF_{1-95}$ were 0.6, 1.0, and 1.6 $\mu\text{mol}/\text{min}/\text{mg}$ protein, respectively (Figure 3.12C).

Figure 3.12: Activities of reconstituted MmA_3B_3D and C-terminal truncated subunit F. (A) SDS-gel showing the purified truncated forms of subunit F. Lanes 2 and 5 are the protein ladder, lane 1 is MmF_{1-87} , lanes 3 and 4 represent MmF_{1-91} and MmF_{1-95} , respectively. (B) Gel filtration profile representing the elution of reconstituted $MmA_3B_3D + MmF_{1-91}$ and $MmA_3B_3D + MmF_{1-95}$. The inset shows the SDS-gel of reconstituted $MmA_3B_3D + MmF_{1-91}$ (Lane 2) and $MmA_3B_3D + MmF_{1-95}$ (Lane 3). (C) Specific activities of the reconstituted complexes of MmA_3B_3D with the truncated forms of subunit F.

To get more insight about the critical C-terminus residues of subunit F involved in enhancing ATPase activity five mutants containing the point mutations $MmFS84A$, $MmFR88L$, $MmFK92L$, $MmFW100I$ and $MmFW100Y$ were generated. The importance of

residues R88 and K92 can be inferred from their presence in the sequence ⁸⁶SLREKIKQA⁹⁴ (Figure 3.13A-C), which has a propensity to form a helical structure [127] (Figure 3.13A). Also, in the extended state of *MmF*, R88 has been shown to interact with E391 of the so-called DELSEED region of subunit B [131]. S84 was found to interact with the phosphate moiety of ATP and residue W100 has been identified to interact with the adenine-ring of ATP as shown in figure 3.13B [127]. These single mutations were incorporated by site directed mutagenesis as described in section 2.2.7. Purification of the *MmF* mutants was done using the protocol described in section 2.2.10 and the purity of the mutants was confirmed by the SDS-gel shown in figure 3.14D.

Reconstitutions of *MmA₃B₃D* with *MmF* mutants were carried out in a molar ratio of 1:3 respectively. The reconstituted mixture was incubated at 4 °C for 4 h in 50 mM Tris/HCl, pH 7.5, 100 mM NaCl and 0.4 mM DTT and applied onto a Superdex 200 column. The purified reconstituted complexes were used for ATP hydrolysis experiments and the specific ATP hydrolysis activities were calculated accordingly.

The reconstituted *MmA₃B₃D* + *MmF*(K92L), *MmA₃B₃D* + *MmF*(W100I) and *MmA₃B₃D* + *MmF*(W100Y) mutant forms (Figure 3.14) showed ATPase activities of 3.4, 3.1, and 3.2 µmol/min/mg protein respectively, which are similar to the reconstituted wild-type *MmA₃B₃DF* complex. In comparison, the reconstituted complex including *MmF* mutant R88 showed a 20 % drop in ATPase activity (2.6 µmol/min/mg protein; Figure 3.14). Previous studies showed that R88 of *MmF* is part of a peptide sequence, which forms a zero-length cross-link with the nucleotide-binding subunit B in the related entire *M. jannaschii* A-ATP synthase in the presence of MgAMP-PNP or MgATP, respectively [134]. Substitution of serine to alanine in the subunit F mutant S84A caused a slight increase in activity (3.6 µmol/min/mg protein) of the reconstituted *MmA₃B₃D*+ *MmF*(S84A) mutant complex in all the repeated experiments (Figure 3.14).

Figure 3.13: Structural model of *MmF* and interactions of the C-terminus of *MmF* with the C-terminus of subunit B (*MmB*). (A) Two states of subunit F are shown in *orange* (extended) and *wheat* (retracted) [2]. Similarly, structure for the two transition states of subunit B are T1 (*gray*) and T2 (*green*). (B) A 21-amino acid peptide of subunit F (F₈₁₋₁₀₁) showing the helical domain and the critical residues interacting with ATP [127]. (C) NMR structure of *MmF* critical residues, which were mutated, are represented in *yellow* accordingly. (D) SDS-PAGE gel showing the purity of the subunit F mutants. Lane 1 indicates the protein ladder and lanes 2-6 represent *MmF*-R88A, -K92L, -W100I, -W100Y and -S84A, respectively. Figures taken from Singh *et al.*, and Devanathan *et al.*, [2] [127].

Figure 3.14: ATP hydrolysis activity of *MmA₃B₃D*, *MmA₃B₃D* with *MmF* and *MmA₃B₃D* with *MmF* single amino acid mutants.

3.1.9 Binding studies of *MmA₃B₃D* with subunit *MmF* and the C-terminal truncated *MmF₁₋₈₇* and densitometric analysis of reconstituted complexes

From the above activity assays, it is clear that the C-terminus of subunit F plays a role in increasing the ATPase activity of *MmA₃B₃DF*. To understand the effect of the C-terminus of subunit F on its binding to *MmA₃B₃D*, FCS experiments were performed with the subunit F truncation mutants as described in section 2.2.12. In FCS, the diffusion time is an indicator for the size of a molecule and it increases when two molecules bind to each other due to the increased size of the complex. Here, *MmF* (11 kDa), *MmF₁₋₈₇* (9 kDa) and the *MmA₃B₃D*+*MmF*-complex (380 kDa) showed diffusion times of 177 μs, 166 μs and ~570 μs, respectively, in good agreement with their molecular masses. In both complexes, *MmA₃B₃D* + *MmF* and *MmA₃B₃D* + *MmF₁₋₈₇*-complex, the saturation point of complex formation was reached at very low *MmA₃B₃D* concentrations (Figure 3.15). Because of the tight binding it was not possible to measure the complex formation at very low concentrations (<15 nM). The calculated K_d -values, derived from a fit of the data points with the Hill equation, were 2 nM and 32 nM for *MmA₃B₃D* + *MmF* and *MmA₃B₃D* + *MmF₁₋₈₇*, respectively. These data indicate that the C-terminus of subunit F is not playing a significant role in binding to *MmA₃B₃D* complex, rather is only important for ATP hydrolysis stimulation.

Figure 3.15: FCS of *MmA3B3D* with *MmF* and *MmF*₁₋₈₇. (A) Plot revealing the ratio of complex formation, as plotted for *MmA3B3D*+*MmF* (red) and *MmA3B3D*+*MmF*₁₋₈₇ (blue). The autocorrelations curves (23 °C, 7.5 min with 15 s repetitions) of - (B) *MmA3B3D*+*MmF* and - (C) *MmA3B3D*+*MmF*₁₋₈₇

The role of the N-terminus was also established by truncating the initial four residues ¹MELA⁴ of the subunit F of *M. mazei* Gö1. These residues are in close proximity of the ⁸⁸VSIS⁹¹ peptide of the β -hairpin of subunit D (Figure 4.3) and have the conformational mobility which might affect the movement of the β -hairpin of subunit D. To study a possible effect of the ⁸⁸VSIS⁹¹ peptide, the N-terminal truncated *MmF*₅₋₁₀₁ mutant was engineered and reconstituted with purified *MmA3B3D* (Figure 3.16).

Figure 3.16: Gel filtration profile of the reconstituted *Mm*A₃B₃D + *Mm*F₅₋₁₀₁. The protein eluted at around 12 ml and the shaded gray area of the peak indicate the fractions which were pooled and applied on the SDS-gel, lane 2. Lane 1 represents the protein ladder.

The complex of *Mm*A₃B₃D reconstituted with *Mm*F₅₋₁₀₁ revealed a decrease in ATPase activity (1.5 $\mu\text{mol}/\text{min}/\text{mg}$ protein) (Figure 3.16), indicating the importance of the very N-terminus of subunit F in ATPase activity.

Densitometric analysis was carried out to confirm the nature of stoichiometric binding of subunit F and its mutants to *Mm*A₃B₃D. This was needed to understand the effect of the N and C-terminal truncations or point mutations in *Mm*F on the formation of reconstituted *Mm*A₃B₃D and *Mm*F complexes (Figure 3.17).

The densitometric analysis showed that the ratio of subunit D: F remains constant (1: 0.3) in case of the reconstituted *Mm*A₃B₃D+ *Mm*F (R88L) complex and *Mm*A₃B₃D+ *Mm*F₅₋₁₀₁ complex when compared to the wild type *Mm*A₃B₃DF complex reflecting that the drop in hydrolysis is due to the mutation or truncation and not because of the alteration in binding of subunit F with subunit D (Figure 3.17). In case of the reconstituted *Mm*A₃B₃D+ *Mm*F₁₋₈₇ complex, we observed that the ratio of subunits D: F altered 1: 0.1. This could be caused by the overall lower mass of *Mm*F₁₋₈₇ and the loss of its highly rich positively charged C-terminal amino acids, which have a higher affinity towards the negatively charged Coomassie Brilliant Blue G-250 dye molecule used for staining (Figure 3.17).

Figure 3.17: Densitometric analysis of the *MmA3B3DF*-complex and the reconstituted *MmA3B3D* with mutant F forms. (A) The densitometric plot represents band intensities of subunits D (left peak) and F (right peak) in the *MmA3B3D+MmF*₅₋₁₀₁ complex derived from the 17% SDS-gel (inset). Integrals of the peaks were used to determine the ratio of subunit D to F. (B–D) The plots represent the densitometric analysis of the complexes *MmA3B3D+MmF* (R88L), *MmA3B3D+F*₁₋₈₇, and *MmA3B3DF*, respectively. Similar to the wild-type *MmA3B3DF*-complex, the integrals of the respective peaks of subunit D and F in *MmA3B3D+MmF* (R88L) and *MmA3B3D+MmF*₅₋₁₀₁ show a ratio of 1:0.3, indicating complete complex formation. The band intensities of reconstituted *MmA3B3D+MmF*₁₋₈₇-complex revealed a ratio of the integral peaks of 1:0.1 for subunits D:F.

3.1.10 Interaction of *MmA3B3D* with the related coupling subunits *ScF* from *S. cerevisiae*

V-ATPase and *Mtε* from *M. tuberculosis* F-ATP synthase

From the previous experiments it was established that subunit F from *M. mazei* Gö1 does stimulate ATPase activity of *MmA3B3D* complex. To understand whether the function is conserved throughout other species and ATPases, subunit F (*ScF*) from *Saccharomyces cerevisiae* was reconstituted with *MmA3B3D* by incubating 5:1 molar ratio (*ScF*: *MmA3B3D*) at 4 °C for 4 h in 50 mM Tris/HCl, pH 7.5, 100 mM NaCl and 0.4 mM DTT. The reconstituted mixture was applied onto the gel filtration column (Superdex 200) and the complex was obtained at about 12 ml, which was applied on the SDS-gel to visualize the binding of *ScF* to the *MmA3B3D* complex. It was interpreted from the SDS-gel that *ScF* was bound successful to *MmA3B3D* (Figure 3.18A). ATPase activity was measured for this reconstituted complex and was calculated to be 1.6 μmol/min/mg protein, depicting a slight increase in activity as compared to *MmA3B3D* (1.2 μmol/min/mg protein) (Figure 3.19A).

Figure 3.18 Gel filtration profile for the reconstituted *MmA3B3D* + *ScF*. (A) The protein eluted around 12 ml and the shaded gray area of the peak indicated the fractionated which were pooled and applied on the SDS-gel lane 2. (B) Gel filtration profile for the reconstituted *MmA3B3D* plus C-terminal truncated *ScF* (*ScF*₁₋₉₄). Lane 1 is the protein ladder and lane 2 represents the peak fraction applied on the gel. *ScF*₁₋₉₄ did not bind to *MmA3B3D*.

The successful hybrid formation demonstrates the likeness of the archaeal ATP synthases and vacuolar ATPases. Even though *MmF* and *ScF* does not have much sequence similarity (<23 %), structural similarity can be seen in their N-terminal globular domain (Figure 3.19B) which might be a reason for the formation of a reconstituted complex (*MmA3B3D* + *ScF*). Similar to the C-terminal truncated *MmF*₁₋₈₇, the homologue C-terminal truncated *ScF*₁₋₉₄, was incubated with *MmA3B3D* complex (Figure 3.18B), which did not result in the formation of a reconstituted *MmA3B3D* + *ScF*₁₋₉₄ complex and no change in the ATPase activity was observed (1.2 $\mu\text{mol}/\text{min}/\text{mg}$ protein) (Figure 3.18B and 3.19A). The data indicate that the C-terminus of *ScF* is required for the binding with *MmA3B3D*, which is in contrast to *MmF* as shown in figure 3.12B. Although structurally different [57], subunit epsilon (ϵ) of the related *M. tuberculosis* F-ATP synthase has been describe to be functionally homologue to subunit F of ATP synthases and V-ATPases. Here, subunit ϵ of the *Mycobacterium tuberculosis* (*Mt* ϵ) F-ATP synthase has been isolated (Figure 3.20) as a representative subunit ϵ . Reconstitution of *Mt* ϵ and *MmA3B3D* even at a ratio of 5: 1 did not lead to an *MmA3B3D* + *Mt* ϵ complex as demonstrated in figure 3.20, and was also inferred from the ATPase activity of *MmA3B3D* + *Mt* ϵ , which was the same as for *MmA3B3D* (3.20B) [2].

Figure 3.19: ATPase activity of reconstituted *MmA₃B₃D*+*ScF* and *MmA₃B₃D*+*ScF*₁₋₉₄. (A) The bars indicate the specific ATPase activity of *MmA₃B₃D*, reconstituted *MmA₃B₃D* + *MmF*, *MmA₃B₃D* + *ScF* (1.6 μmol/min/mg protein) and *MmA₃B₃D* + *ScF*₁₋₉₄ (1.2 μmol/min/mg protein). (B) Structural alignment of *MmF* and *ScF* depicting the similarity of the globular domain. NMR structure of *MmF* is represented in magenta and crystal structure of *ScF* is shown in dark blue [71].

Figure 3.20: Gel filtration profile and activity assay for the reconstituted *MmA₃B₃D*+*Mte* complex. (A) The gel shows that only *MmA₃B₃D* (lane 2) is present in the major peak I (large grey area) and unbound *Mte* (lane 3) eluted as smaller peak II (small grey area). The lane 1 shows the protein marker (kDa) and (B) ATPase activity of reconstituted protein of peak I (*MmA₃B₃D* + *Mte*) was calculated to be (1.1 μmol/min/mg protein).

3.1.11 Insights into the extended and retracted states of subunit F with respect to *Mm*A₃B₃D complex

It was established that the C-terminus of subunit F is stimulating the ATPase activity. In order to communicate structural alterations of the subunit F with the catalytic A-B interfaces inside *Mm*A₃B₃DF, subunit F has to come in close proximity to the catalytic center via its C-terminus. To prove whether such movements occur, fluorescence resonance energy transfer (FRET) experiments were designed. With a FRET experiment it is possible to measure the distance between a donor and an acceptor fluorescent dye. If the dyes are attached to moving domains within a protein, different states of the protein can be distinguished. Maleimide modified fluorescent dyes can be attached to cysteines in the protein. Therefore, a mutant A_(ΔCys)₃B_(ΔCys)₃D_{A71C} was generated, where all the cysteines in subunit A and B were mutated (Except conserved C261 in subunit A). In addition, a cysteine was introduced in subunit D by replacing Alanine 71 to Cysteine. Subunit F mutants were generated by incorporating cysteines at crucial positions. The substitutions include *Mm*F (L87C), *Mm*F (K92C) and *Mm*F (A94C). These mutations were designed based on analysing a structural model of *Mm*A₃B₃DF (Figure 3.21A) and choosing residues with resonance transfer distances in the range of 2 nm to 10 nm to perform FRET experiments effectively. The A_(ΔCys)₃B_(ΔCys)₃D_{A71C} complex was purified as per the protocol developed for *Mm*A₃B₃D and subunit F mutants (L87C, K92C and A94C) were purified as described in section 3.1.6. The purified A_(ΔCys)₃B_(ΔCys)₃D_{A71C} complex was labelled with the donor dye Atto488 maleimide and subunit F mutants were labelled with the acceptor dye Atto647N maleimide. The excess dyes were removed by injecting the labelled protein onto a gel filtration column and the labelling was confirmed by a multi-wavelength scanner (Figure 3.21C and D).

The single molecule FRET (smFRET) experiments were carried out in the laboratory of our collaborator, Prof. Michael Bösch at Jena University Hospital (Germany). The A_(ΔCys)₃B_(ΔCys)₃D_{A71C}- Atto488 maleimide (donor) was combined with *Mm*F-L87C- Atto647N maleimide (acceptor) in 1:1 ratio (~1 μM). The mixture was diluted to get a concentration less than 1 nM, which is optimal for performing smFRET. The FRET efficiency was measured and compared in presence of different nucleotides (MgATP, MgAMP-PNP and MgADP). The preliminary smFRET data reveal nucleotide-dependent conformational changes of subunit F (Data not shown). However, these changes are in contrast to the regulatory movements found in subunit ε of F-ATP synthases, implicating a different regulatory mechanism of A-ATP synthase. Thus, adding another unique feature to the A-ATP synthases.

Figure 3.21: Protein production for FRET experiment. (A) A model describing the arrangement of subunits A, B, D and F in a complex with respect to the FRET experiment. The model was generated using PyMOL [39], by assembling the three dimensional structures. Subunit A is highlighted in *orange*, B in *green*, D in *yellow* and F is in *magenta*. The substituted residues are labeled and shown as a sphere. The distance between subunit D-A71C and subunit F-A94C is shown by dashed line and calculated to be of 7 nm, which is in the permissible range for a FRET experiment. (B) The gel filtration profile of A $_{(\Delta Cys)3}$ B $_{(\Delta Cys)3}$ D $_{A71C}$. Peak I represents the oligomeric state of A $_{(\Delta Cys)3}$ B $_{(\Delta Cys)3}$ D $_{A71C}$ and the peak II is the eluted monomeric A $_{(\Delta Cys)3}$ B $_{(\Delta Cys)3}$ D $_{A71C}$. The shaded area of peak II indicate the purified protein used for the labelling. (C) The SDS-gel shows A $_{(\Delta Cys)3}$ B $_{(\Delta Cys)3}$ D $_{A71C}$ -Atto488 in lane 2, lane 1 is the protein ladder and lane 3 represents fluorescence scan (488 nm) of lane 2. Lane 3 shows a dark band for labelled D $_{A71C}$ and few light bands above D $_{A71C}$ for some impurities. The dark band is labeled subunit D. (D) SDS-gel of a Atto647N maleimide labeled subunit F cysteine mutants (L87C, K92C and A94C) after scanning (wavelength of 647 nm). The dark bands represent the monomeric subunit F mutants.

3.2 Role of P-loop mutations (P235A, S238C) in recombinant subunit A and in *MmA*₃B₃D and *MmA*₃B₃DF complexes

Role of P-loop mutations (P235A, S238C) in individual subunit A and in complexes *MmA*₃B₃D and *MmA*₃B₃DF

3.2.1 Production and purification of $A_{(P235A, S238C)3}B_3D$ and $A_{(P235A, S238C)3}B_3DF$ complexes

The glycine rich phosphate binding loop (P-loop) is involved in the catalysis of ATP synthesis and hydrolysis, and is conserved amongst the A-ATP synthases (GPFGSGKTV) and V-ATPases (GAFGC $\underline{C}$ GKTV). The substitution of Proline to Alanine and Serine to Cysteine is unique to the P-loop of V-ATPases. Here, we have tried to mimic the P-loop of V-ATPase in the A-ATP synthase and to address the question whether this alters the ATPase activity of the A_3B_3D and A_3B_3DF complexes of *M. mazei* Gö1 A-ATP synthase. Therefore, the mutant complexes $A_{(P235A, S238C)3}B_3D$ and $A_{(P235A, S238C)3}B_3DF$ were generated and expressed in the ATP synthase deficient strain *E. coli* DK8. The Ni^{2+} -affinity chromatography was performed as mentioned in section 3.1.1 and the imidazole fractions (100 mM -400 mM) were pooled and concentrated up to 1 ml using a 100 kDa centricon. The sample was injected into a Superdex 200 gel filtration column and the purified mutants eluted at 12.5 ml in buffer comprised of 50 mM Tris/HCl (pH 7.5), 200 mM NaCl, 0.8 mM DTT. The eluted $A_{(P235A, S238C)3}B_3D$ and $A_{(P235A, S238C)3}B_3DF$ was then concentrated and further used for the experiments.

Figure 3.22: Purification of $A_{(P235A, S238C)3}B_3D$ and $A_{(P235A, S238C)3}B_3DF$ complexes. (A) Elution profile of complex $A_{(P235A, S238C)3}B_3D$ when run on a gel filtration (Superdex 200) column. The protein eluted at 12.5 ml and the purity was checked by running it on a 17% SDS-gel (shown in inset). (B) Gel filtration chromatogram of $A_{(P235A, S238C)3}B_3DF$ complex. The elution profile was similar to the one of $A_{(P235A, S238C)3}B_3D$. The purity of the mutant was analysed on a 17% SDS-gel (shown in inset).

3.2.2 ATP hydrolysis and kinetic studies of $A_{(P235A, S238C)3}B_3D$

To understand the effect of the P235A and S238C substitutions in the *Mm* A_3B_3D complex, an NADH-coupled ATPase assay was performed with $A_{(P235A, S238C)3}B_3D$ and ATPase activity of 2.4 $\mu\text{mol}/\text{min}/\text{mg}$ protein was determined. This was comparatively two times higher than the activity of WT *Mm* A_3B_3D (1.0 $\mu\text{mol}/\text{min}/\text{mg}$ protein), indicating that the double mutation in the P-loop played an important role in increasing the ATPase activity (Figure 3.23). Further kinetic studies with $A_{(P235A, S238C)3}B_3D$ revealed that the V_{max} for $A_{(P235A,$

$s_{238C}B_3D$ is $3.7 \mu\text{mol/min/mg protein}$ or $22.8 \pm 0.5 \text{ s}^{-1}$, which is three times higher than the one of MmA_3B_3D ($1.3 \mu\text{mol/min/mg protein}$ or $7.9 \pm 0.3 \text{ s}^{-1}$). Surprisingly, the K_m of $A_{(P235A, S238C)}B_3D$ ($1.07 \pm 0.08 \text{ mM}$) was much higher than the K_m of MmA_3B_3D ($0.19 \pm 0.04 \text{ mM}$), indicating that the binding affinity for MgATP has been decreased by the incorporation of the P-loop mutations (Table 3.1).

Figure 3.23: Enzyme kinetics of MmA_3B_3D and $A_{(P235A, S238C)}B_3D$. (A) ATP hydrolysis of MmA_3B_3D and $A_{(P235A, S238C)}B_3D$ at 2 mM of ATP. Decrease in NADH absorption at 340 nm is plotted against the progressing time in seconds. The blue line indicates MmA_3B_3D and the magenta line represents $A_{(P235A, S238C)}B_3D$. (B) Comparison of ATPase activity of MmA_3B_3D and $A_{(P235A, S238C)}B_3D$. $A_{(P235A, S238C)}B_3D$ shows an ATPase activity of $2.4 \mu\text{mol/min/mg protein}$ which is much higher than the ATPase activity of MmA_3B_3D ($1.0 \mu\text{mol/min/mg protein}$). (C) Comparison of the Michaelis-Menten plot of $A_{(P235A, S238C)}B_3D$ ($\square$) with MmA_3B_3D ($\circ$) at varying concentrations of MgATP (0.1 mM to 6 mM) performed at 37°C .

The enzymatic reaction is only complete after the formation of the product, therefore the low binding affinity might result in faster release of the products and hence increase the

V_{max} in case of the $A_{(P235A, S238C)}B_3D$ complex. The decrease in binding affinity of the $A_{(P235A, S238C)}B_3D$ complex is in line with the K_D values of subunit $A_{(P235A, S238C)}$ which were obtained by the FCS experiment and shown in section (3.3.6).

3.2.3 ATP hydrolysis and kinetic studies of $A_{(P235A, S238C)}B_3DF$

Similar to the $A_{(P235A, S238C)}B_3D$ complex, the NADH-coupled ATPase assay was also performed with the $A_{(P235A, S238C)}B_3DF$ complex and the ATPase activity was 3.9 $\mu\text{mol}/\text{min}/\text{mg}$ protein, nearly similar to the of MmA_3B_3DF -WT (4.2 $\mu\text{mol}/\text{min}/\text{mg}$ protein).

It was observed that the enzyme complex $A_{(P235A, S238C)}B_3DF$ was active for longer duration and suspected to relieve Mg-ADP inhibition in the $A_{(P235A, S238C)}B_3DF$ complex, a trait not observed in the MmA_3B_3DF complex (Figure 3.24A and B). Mg-ADP inhibition is a common phenomenon observed in F-ATP synthases and A-ATP synthases during ATP hydrolysis, when inorganic phosphate is released before MgADP. Therefore, MgADP gets trapped in the catalytic site and the enzyme goes in an MgADP inhibited state [4, 166-170]. The MgADP inhibited state was observed in MmA_3B_3DF , where ATPase activity decreases with time progressively and reaches saturation after a period of ~ 3 min, although the availability of substrate is not limited. This inhibited state has not been observed in case of $A_{(P235A, S238C)}B_3DF$, indicating that the P-loop mutation has an effect on relieving the inhibition. Further kinetic studies with $A_{(P235A, S238C)}B_3DF$ revealed a high K_m (0.95 ± 0.07 mM) compared to MmA_3B_3DF (0.15 ± 0.04 mM) and the V_{max} for $A_{(P235A, S238C)}B_3DF$ was $32.9 \pm 0.8 \text{ s}^{-1}$, which is similar to the one of MmA_3B_3DF ($30.4 \pm 0.9 \text{ s}^{-1}$). The decrease in binding affinity for MgATP is similar in case of $A_{(P235A, S238C)}B_3D$ and $A_{(P235A, S238C)}B_3DF$, whereas the V_{max} in $A_{(P235A, S238C)}B_3D$ increased compared to MmA_3B_3D . The V_{max} value of $A_{(P235A, S238C)}B_3DF$ was comparable to MmA_3B_3DF (Figure 3.24C) (Table 3.1). The decrease in binding affinity could be the result of alterations in the binding pocket of $A_{(P235A, S238C)}B_3DF$, influencing ATP binding, without further change in catalysis and release of the product.

Table 3.1: Summary of kinetics measurement for MmA_3B_3D , MmA_3B_3DF and mutants.

Proteins	K_m (mM)	V_{max} (s^{-1})
MmA_3B_3D	0.19 ± 0.04	7.9 ± 0.3
MmA_3B_3DF	0.15 ± 0.04	30.4 ± 0.9
$A_{(P235A, S238C)}B_3D$	1.07 ± 0.08	22.8 ± 0.5
$A_{(P235A, S238C)}B_3DF$	0.95 ± 0.07	32.9 ± 0.8

Figure 3.24: Enzyme kinetics of MmA_3B_3DF and $A_{(P235A, S238C)}_3B_3DF$. (A) ATP hydrolysis of MmA_3B_3DF and $A_{(P235A, S238C)}_3B_3DF$ at 2 mM of ATP at 37 °C. Decrease in NADH absorption at 340 nm is plotted against progressing time in seconds. Black line indicates MmA_3B_3DF and the red line represents $A_{(P235A, S238C)}_3B_3DF$. (B) Comparison of ATPase activity of MmA_3B_3DF and $A_{(P235A, S238C)}_3B_3DF$. $A_{(P235A, S238C)}_3B_3DF$ shows an ATPase activity of 3.9 $\mu\text{mol}/\text{min}/\text{mg}$ protein, which is similar to the ATPase activity of MmA_3B_3DF (4.2 $\mu\text{mol}/\text{min}/\text{mg}$ protein). (C) Michaelis-Menten plot of MmA_3B_3DF (○) with $A_{(P235A, S238C)}_3B_3DF$ (□) at varying concentrations of MgATP (0.1 mM to 6 mM) performed at 37 °C. The calculated K_m and V_{max} of $A_{(P235A, S238C)}_3B_3DF$ was 0.95 ± 0.07 mM and 32.9 s^{-1} , respectively. In comparison, the K_m and V_{max} of MmA_3B_3DF was determined to be 0.15 ± 0.04 mM and $30.4 \pm 0.9 \text{ s}^{-1}$, respectively.

In another set of experiments, the $A_{(P235A, S238C)}_3B_3DF$ complex was pre-incubated with different concentrations of MgADP before the ATPase activity experiment and compared with the similarly treated MmA_3B_3DF . The aim was to understand the process of relieving MgADP inhibited state in $A_{(P235A, S238C)}_3B_3DF$. As shown in figure 3.25, the activity of $A_{(P235A, S238C)}_3B_3DF$ was not inhibited by 10 μM of MgADP, whereas MmA_3B_3DF showed

a significant decrease in hydrolytic activity when pre-incubated with 10 μ M MgADP (Figure 3.25).

Figure 3.25: ATP hydrolysis of MmA_3B_3DF and $A_{(P235A, S238C)}_3B_3DF$. MmA_3B_3DF (A) and $A_{(P235A, S238C)}_3B_3DF$ (B) at 2 mM of ATP. The protein complexes were pre-incubated with different concentrations of MgADP before the measurements. Activities of MmA_3B_3DF (C) and $A_{(P235A, S238C)}_3B_3DF$ (D) complexes when pre incubated with different concentration of MgADP prior hydrolysis measurements.

3.2.4 Role of subunit F in relieving MgADP inhibition

As shown above $A_{(P235A, S238C)}_3B_3DF$ does not show a MgADP inhibition state, whereas $A_{(P235A, S238C)}_3B_3D$ becomes inhibited with progressive time (Figure 3.24A). In addition, a role of subunit F in stimulating ATPase activity has been established (section 3.1). In order to understand the role of subunit F in relieving MgADP inhibition, the $A_{(P235A, S238C)}_3B_3D$ complex was reconstituted with MmF and ATP hydrolysis of reconstituted $A_{(P235A, S238C)}_3B_3DF$ was measured.

The results shown in figure 3.26 indicate that MgADP inhibition, which was seen for $A_{(P235A, S238C)}_3B_3D$ (Figure 3.23A) has been relieved after the addition of MmF . Also, the activity of $A_{(P235A, S238C)}_3B_3D$ was increased from 2.4 μ mol/min/mg protein to 3.3 μ mol/min/mg protein in reconstituted $A_{(P235A, S238C)}_3B_3DF$. The data confirms again that

subunit F is the stimulator of ATPase activity and also signifies the role of subunit F in releasing the enzyme $A_{(P235A, S238C)}B_3D$ from the MgADP inhibited state.

Figure 3.26: Role of subunit F in relieving MgADP inhibition. (A) ATP hydrolysis of MmA_3B_3D , $A_{(P235A, S238C)}B_3D$, $MmA_3B_3D + MmF$ and $A_{(P235A, S238C)}B_3D + MmF$ at 2 mM of MgATP. (B) ATP hydrolysis activity shown as bar diagram for MmA_3B_3D , $A_{(P235A, S238C)}B_3D$, $MmA_3B_3D + MmF$ and $A_{(P235A, S238C)}B_3D + MmF$.

Our study demonstrated that both, P-loop mutations (P235A and S238C) and presence of subunit F are essential for relieving MgADP inhibited state in case of the A-ATP synthase from *M. mazei* Gö1. The relieving of MgADP inhibition by mimicking the P-loop of eukaryotic V-ATPases supports the fact that V-ATPases are dedicated ATP-driven proton pumps and they are regulated by reversible disassembly of the V_O - from V_1 -domain. This reversible disassembly protects the cytosolic pool of ATP in case of glucose deprived condition of the cell, which in F- and A-ATP synthase is done by ADP inhibition [129, 171-173]. Since the MgADP inhibition is observed in the rotor enzymes functioning in both direction of ATP synthesis and-hydrolysis, by mimicking the P-loop of V-ATPases in A-ATP synthase, we could relieve the inhibition, hence making the A-ATP synthase a more efficient hydrolytic enzyme like eukaryotic V-ATPases.

3.2.5 Cloning, expression and purification of P-loop double mutant ($PhA_{P235A, S238C}$) of subunit A

To study the role of these mutations in the single catalytic subunit A, the P-loop mutations (P235A and S238C) were incorporated in the subunit A of *P. horikoshii* OT3 A-ATP synthase. The subunit A of *P. horikoshii* OT3 A-ATP synthase was chosen because of its known structural information and its 100 % identity with the P-loop of subunit A of *M.*

mazei Gö1 A-ATP synthase. The mutant containing point mutations P235A and S238C is referred as “*PhA*_{P235A, S238C}” and used for further functional and structural studies.

The P-loop mutant (*PhA*_{P235A, S238C}) of subunit A from the A-ATP synthase of *Pyrococcus horikoshii* OT3 was generated and successfully cloned in pET-22b(+) expression vector as described in section 2.2.15. The two DNA fragments were amplified using PCR from the template containing subunit A (WT) gene in pET-22b (+). These two fragments were fused by a subsequent overlap extension PCR which incorporated the desired mutations. The fused DNA fragment was digested with *NdeI* and *SalI* restriction enzymes and ligated into *NdeI/SalI*-digested pET-22b(+) vector (Novagen).

Figure 3.27: PCR amplification of subunit A mutant *PhA*_{P235A, S238C}. (A) PCR I products; Marker DNA (1kb, Fementas) (lane 3); *PhA*_{P235A, S238C} fragment 1 and fragment 2 (lane 1 and 2). (B) PCR II product; Marker DNA (1 kb, Fermentas) (lane 1); *PhA*_{P235A, S238C} (lane 2).

The *PhA*_{P235A, S238C} protein was purified successfully by following the earlier protocol (Maegawa *et al.*, 2004 and Kumar *et al.*, 2010) (Section 2.2.16). The purification includes loading of the protein onto a Resource Q column (GE Healthcare) equilibrated with buffer (50 mM Tris-HCl, pH 8.5, 1 mM DTT), followed by the applying the peak fractions onto a c 26/60 Superdex200 (GE Healthcare) column equilibrated with buffer (50 mM Tris-HCl, pH 8.5, 1 mM DTT, 50 mM NaCl).

In case of the anion exchanger, bound protein eluted at 150 mM NaCl, using a linear NaCl gradient (0.05 - 1.0 M) (Figure 3.28A). The pooled fractions were applied on the gel filtration column (26/60 Superdex200) and the subunit A mutant eluted at 15 ml (Figure 3.28B). The protein was concentrated using 50 kDa concentrators (Amicon). Buffer exchange was performed during the process of protein concentration. The pH of the buffer was gradually reduced to 7.8 and finally to 7.5, to obtain the conditions for crystallization (50 mM Tris-HCl, pH 7.5) [118]. The purity of the protein was ascertained by SDS-PAGE (Figure 3.28B).

Figure 3.28: Purification of subunit A mutant *PhA*_{P235A, S238C}. (A) Purification of subunit A mutant *PhA*_{P235A, S238C} by a linear gradient of NaCl (0.05 - 1.0 M) on an anion exchange column Resource Q (6 ml). (B) The fractions containing *PhA*_{P235A, S238C} were applied onto a 26/60 Superdex200 column (GE Healthcare) equilibrated with buffer (50 mM Tris-HCl, pH 8.5, 1 mM DTT, 50 mM NaCl). The protein eluted at 15 ml. Inset shows a protein ladder, lane 1 and lane 2 represents 1 μ l of purified protein (12 mg/ml) on an SDS-PAGE gel.

3.2.6 Nucleotide binding studies on subunit A-WT (*PhA*-WT) and its mutant *PhA*_{P235A, S238C} using fluorescence correlation spectroscopy

Fluorescence correlation spectroscopy (FCS) is a tool to study protein-ligand interactions in solution at extremely low protein concentrations. Minute spontaneous fluctuations occur during the protein-ligand interactions and are reflected by fluorescence emission of the molecules, which are then recorded and studied by FCS, making FCS a highly sensitive technique. To determine the nucleotide binding constants of the *PhA*-WT and its mutant *PhA*_{P235A, S238C}, FCS experiments were performed using the fluorescent AMP-PNP and ADP derivatives (Mg-AMP-PNP ATTO-647N and Mg-ADP ATTO-647N) (Figure 3.29). The nucleotides bound to the dye (Mg-AMP-PNP ATTO-647N and Mg-ADP ATTO-647N) have low diffusion time t_D , which increases, if these fluorescent labelled nucleotides binds to the protein. This was seen in the presence of *PhA*-WT and its mutant *PhA*_{P235A, S238C}. In general, when fluorescent labelled nucleotides bind to the protein, the mass of the diffusing particle increases, resulting in an increased diffusion time of heavier particles. When Mg-AMP-PNP ATTO-647N was bound to either *PhA*-WT or the mutant protein *PhA*_{P235A, S238C}, a binding constant of 4.4 μ M and 13.1 μ M was calculated, respectively (Figure 3.29A-B).

Figure 3.29: Concentration-dependent binding of subunit *PhA*-WT and its mutant *PhA*_{P235A, S238C} with the nucleotides. (A) & (B) Comparison of the binding of *PhA*-WT and its mutant *PhA*_{P235A, S238C} with Mg-AMPPNP-ATTO-647N respectively. The bound protein fraction was plotted against the concentration of protein in μM . (C) & (D) Comparison of the binding of *PhA*-WT and its mutant *PhA*_{P235A, S238C} with Mg-ADP-ATTO-647N respectively. The percentage of bound nucleotides was analyzed using a triplet model (ConfoCor3, Zeiss). Best fits yielding the binding constants are represented as fitted lines.

The measurements with Mg-ADP-ATTO-647N showed a very high binding affinity for both *PhA*-WT and its mutant *PhA*_{P235A, S238C}, reflected by the obtained binding constants of $0.08 \mu\text{M}$ and $0.18 \mu\text{M}$ for *PhA*-WT and its mutant *PhA*_{P235A, S238C}, respectively (Figure 3.29C-D). The results here indicate that both, *PhA*-WT and *PhA*_{P235A, S238C} have high affinity for MgADP. In case of MgAMPPNP, the binding affinity of *PhA*_{P235A, S238C} decreased 3-fold, indicating the importance of P235A and S238C in the P-loop sequence GPFGSGKTV. These results are in line with the K_m values obtained from $A_{(P235A, S238C)3}B_3D$ and $A_{(P235A, S238C)3}B_3DF$ in sections 3.2.2 and 3.2.3.

3.2.7 Crystallization of apo- *PhA*_{P235A, S238C} and co-crystallization with Mg-AMP, MgADP, Mg-ATP and Mg-AMPPNP

To further study the possible conformational change in the P-loop caused by the double mutation (P235A and S238C), reflected in the binding of nucleotides in single subunit A (*PhA*_{P235A, S238C}) and in *A*_{(P235A, S238C)3B3D} and *A*_{(P235A, S238C)3B3DF}, a structure based approach was used. Therefore, the purified *PhA*_{P235A, S238C} was setup for crystallization following the conditions published in Kumar *et al.* (2010). The crystals for apo-*PhA*_{P235A, S238C} were obtained by the hanging-drop vapor diffusion method at 18 °C from a solution containing 50% (v/v) 2-Methyl-1,3Propanediol (MPD) and 0.1 M sodium acetate, pH 4.5 with an overlay of paraffin and silicon oil (1: 1) (Figure 3.30A). This protocol of crystallization resulted in the generation of large crystals of apo-*PhA*_{P235A, S238C} and the MgAMP bound form of *PhA*_{P235A, S238C}, with the final concentration of MgAMPPNP in the drop as 2 mM (Figure 3.30B).

For co-crystallization with nucleotides, *PhA*_{P235A, S238C} was divided into batches of 4 mg/ml each and incubated separately with 2 mM MgAMP, MgADP, MgATP and – MgAMPPNP, respectively, at 4 °C for 30 minutes on a sample rotator. This mixture was concentrated to 10 - 12 mg/ml and the protein-ligand complexes were then set up for crystallization in the same optimized conditions in hanging drop plates and incubated at 18 °C. In contrast to the empty form and the MgAMP bound form, the crystallization setup for the nucleotides bound form (MgADP, MgATP and MgAMPPNP) did not result in bigger crystals, instead crystalline precipitate was observed after 3 days of the setup. Several attempts were made for co-crystallization by varying protein (5-20 mg/ml) and the precipitant concentration (MPD- 10-60 %), but all of them resulted in crystalline precipitates.

Figure 3.30: Crystals of apo-*PhA*_{P235A, S238C} and co-crystallized with Mg-AMP. (A) Large crystal of empty form of *PhA*_{P235A, S238C}. (B) Co-crystal of *PhA*_{P235A, S238C} with MgAMP.

3.2.8 Data collection and analysis for apo- *PhA*_{P235A, S238C} and its MgAMP bound form

Apo-*PhA*_{P235A, S238C} and its MgAMP bound crystals were obtained in the condition as mentioned in section 3.2.7. The crystals were then picked from the crystallization liquid and frozen in liquid nitrogen at 100 K. Single wavelength datasets were collected for *PhA*_{P235A, S238C} and MgAMP bound form at 140 K on beamline 13B1 at the National Synchrotron Radiation Research Center (NSRRC, Hsinchu, Taiwan). Using the ADSC Quantum 315 CCD detector, 120° of data were collected with 0.5° oscillation (20 s per 0.5° frame). The crystals of *PhA*_{P235A, S238C} nucleotide free empty form diffracted to a resolution of 2.35 Å, while the AMP-bound form diffracted to a maximum resolution of 3 Å (Figure 3.31) (Table 3.1). Analysis of data including indexing, integration and scaling were performed using HKL2000 and the data collection statistics are shown in table 3.1

Figure 3.31: Diffraction pattern of apo-form and nucleotide bound form *PhA*_{P235A, S238C}. (A) X-ray diffraction pattern of *PhA*_{P235A, S238C} nucleotide free crystal at a resolution of 2.35 Å and (B) AMP-PNP-bound crystals at a resolution of 3 Å.

The *PhA*_{P235A, S238C} empty form was crystallised in a tetragonal system with the space group $P4_32_12$ and unit cell parameters $a = 127.46$, $b = 127.46$ and $c = 105.34$ Å. The crystals diffracted upto 2.35 Å resolution. The solvent content for the crystal was 62%, assuming one molecule in the asymmetric unit (Matthews, 1968). Molecular replacement method was used to obtain initial phases against the crystal structure of previously solved subunit A mutant from *P. horikoshii* OT3 carrying a single substitution of P235A [121]. The translational function (TFZ) and fast rotational function (RFZ) were high and indicated a good solution by program PHASER.

Table 3.2: Summary of the crystallographic data collection for *PhA*_{P235A, S238C}.

Data collection statistics	<i>PhA</i> _{P235A, S238C}
Wavelength	1.00
Space group	P4 ₃ 2 ₁ 2
Unit cell parameter (Å)	
<i>a</i>	127.46
<i>b</i>	127.46
<i>c</i>	105.34
Resolution range (Å)	30-2.35
Solvent content (%)	62
Total number of reflections	346 584
Unique Reflections	36 816
I/σ	24.07 (2.83)
Completeness (%)	99.7 (100)
R merge (%)	7 (61.9)
Redundancy	9.4 (9.7)
CC1/2	91.1
CC*	97.6

The preliminary structure was refined by 30 cycles of rigid body refinement using the program Refmac, obtaining an overall R-factor of 24 % and free R-factor of 27 %. The 2F_o-F_c maps of *PhA*_{P235A, S238C} show electron density for the amino acids in P-loop (Figure 3.32). Iterative cycles of model building and refinement were performed in a combined manner using the programs COOT [145] and REFMAC [142] of the CCP4 suite [148].

3.2.9 Crystal structure of empty form of *PhA*_{P235A, S238C} at 2.35 Å resolution

The crystal structure was solved at 2.35 Å and is similar to the wild type subunit A structure from *P. horikoshii* in terms of overall secondary structures. The structure can be divided into three domains: An N-terminal domain comprised of loops and β-sheet (1-199 amino acids), a nucleotide-binding α-β domain (200-437) and a C-terminal α-helical domain (438-588). The N-terminal domain is flexible with a high B-factor (above 100). Most of the residues (1-51) could not be assigned because of a lack of clear electron density in that region. Residues 122- 210 are part of the Non-Homologous Region (NHR) which is comprised of 90 amino acids. The NHR region is conserved in the nucleotide binding subunit

A of A-ATP synthases and V-ATPases, but not present in the homologous catalytic subunit β of F-ATP synthases. The nucleotide-binding α - β domain has a glycine rich phosphate binding loop (P-loop) known as a Walker A motif ($_{234}\text{GPFGSGKT}_{241}$), which is conserved throughout A-ATP synthases and binds to the phosphates of nucleotides (ATP and ADP). The detailed statistics of structure refinement is provided in table 3.2. A total of 165 water molecules, two acetate, a 2-methyl-2, 4 pentanediol (MPD), and a Tris molecule could be assigned in the structure.

Table 3.3: Refinement statistics of *PhA*_{P235A, S238C}.

R factor (%)	23
R free (%)	27
Ramachandran statistics	
Residues in most favored (%) region	95.06
Residues in additionally allowed (%) region	4.94
Residues in disallowed (%) region	0.0
R.M.S. Deviations	
Bond length (Å)	0.006
Bond angle (°)	1.07
Mean atomic B values	73
Number of water molecule	165

The electron density map of the final structure has visible density for the P-loop region and major residues except for the residues 1-50, 345-354 and 394-408. This loss of density would have been because of high flexibility in those regions and yet resulted in the disordered state. The density for extreme N-terminal region was missing in all the subunit A wild type and mutant structures published previously [55, 121]. The difference map ($2F_o - F_c$) shows clear electron density in the P-loop region (Figure 3.32B). Superimposition of the *PhA*_{P235A, S238C} structure on subunit A-WT shows that the arched P-loop of subunit A-WT has flattened in the mutant *PhA*_{P235A, S238C}, which was seen earlier in the P235A mutant indicating the importance of proline residue in maintaining the unique arched conformation (Figure 3.33) [121].

Figure 3.32: The crystal structure of *PhA*_{P235A, S238C}. (A) Structure solved at 2.35 Å with the water molecules represented by red spheres, the MPD molecule is shown in red stick. (B) The $2F_O - F_C$ map of the P-loop of the *PhA*_{P235A, S238C}. The mutated amino acids (P235A and S238C) are marked with an asterisk. The $2F_O - F_C$ density is contoured at 1 σ .

The AMPPNP-bound structure and the ADP-bound structure of subunit A-WT were also superimposed with the *PhA*_{P235A, S238C} structure and it revealed that the γ -phosphate of AMPPNP has steric clashes with the flattened P-loop of the *PhA*_{P235A, S238C}, which was not the case in the AMPPNP-bound ADP-bound structures of subunit A-WT (Figure 3.33). This correlates with the FCS data (section 3.2.6) which showed that the mutation (P235A and S238C) decreases the binding affinity of Mg-AMPPNP by 3 folds but did not alter the binding of Mg-ADP. Also this is in accordance with the kinetic data obtained from the *A*_{(P235A, S238C)3B3D} and the *A*_{(P235A, S238C)3B3DF} complexes, where the K_m values were 5 times higher than the *MmA*_{3B3D} and *MmA*_{3B3DF} complexes indicating that the affinity for ATP has decreased due to the P-loop mutations (P235A, S238C) in the complexes.

Figure 3.33: Superimposition of nucleotide bound *PhA*-WT with *PhA*_{P235A} S_{238C}. (A) *PhA*-WT structure (Orange) bound to AMPPNP is superimposed with the apo- *PhA*_{P235A} S_{238C} structure (Cyan). (B) The superimposed P-loop of subunit A-WT bound AMPPNP with *PhA*_{P235A}, S_{238C} is zoomed and the bound AMPPNP is shown as sticks. (C) Superposition of subunit *PhA*-WT structure (Orange) bound to ADP with the empty *PhA*_{P235A},S_{238C} structure (Cyan). (D) The superimposed P-loop of subunit A-WT bound AMPPNP with *PhA*_{P235A},S_{238C} is zoomed into and the bound ADP is shown in sticks.

Conformational changes in the P-loop residues have been observed in the *PhA*_{P235A, S238C} structure where A235 and C238 moved inward when superimposed on the subunit A-WT bound to AMPPNP as shown in figure 3.34. Other amino acids like F236, G237, G239, K240, T241, V242 and T243 also showed a shift when superposed on subunit *PhA*-WT bound to AMPPNP. The importance of P235 and S238 residues in maintaining the arched conformation of the P-loop and the possibilities of alteration in the nucleotide binding in *PhA*_{P235A, S238C} has been discussed in section 4.4.

Figure 3.34: Superimposition of the P-loops of *PhA*-WT (Orange) bound to AMPPNP and *PhA*_{P235A, S238C} (Cyan), shown in stick conformation. The AMPPNP is shown in grey spheres.

3.3 Crystallization of A₃B₃D of the A-ATP synthase from *M. mazei* Gö1

3.3.1 Crystallization of *Mm*A₃B₃D

X-ray crystallography is the oldest method for structural determination of very large macromolecules at atomic resolution. However, it is difficult to crystallize these large assemblies; the reason is because they exhibit a high degree of structural and conformational heterogeneity. In spite of the challenges, it's worthwhile studying such complexes, since they provide an incredible amount of information. The structures of individual subunits of the A-ATP synthase have been studied well (Figure 1.7) but to understand the interface of these subunits inside the enzyme it is essential to study the entire enzyme complex. Keeping that in mind, crystallization setups for the *Mm*A₃B₃D complex were made in hanging drops using 5.3 mg/ml protein. Initial crystals were observed in wizard 1 #1 (20% (v/v) polyethylene glycol 8000 (PEG 8000) and 0.1 M CHES (pH 9.5)), wizard 1 #29 (10 % (v/v) PEG 8000, 0.2 M NaCl and 0.1 M CHES (pH 9.5)) and wizard 2 # 39 (20 % (v/v) PEG 8000, 0.2 M NaCl and 0.1 M CAPS (pH 10.5)) screens from Emerald Biosciences.

These crystals were small in size and appeared in a day in the conditions containing 20 % (v/v) PEG 8000 (Figure 3.35A-B). The quick appearance of crystals was speculated to be because of the higher concentration of PEG (precipitant). With condition 29 from wizard screen 1, crystals appeared after 14 days and they were comparatively bigger in size (Figure 3.35 C). All these conditions have a common precipitant and similar alkaline pH, which provides a hint that this protein may like to crystallize at alkaline pH.

Figure 3.35: Initial *Mm*A₃B₃D crystals. Crystallization buffer conditions used are (A) Wizard 1 #1 (20% (v/v) PEG 8000 and 0.1 M CHES (pH 9.5)). (B) Wizard 2 # 39 (20 % (v/v) PEG 8000, 0.2 M NaCl and 0.1 M CAPS (pH 10.5)). (C) Wizard 1 #29 (10 % (v/v) PEG 8000, 0.2 M NaCl and 0.1 M CHES (pH 9.5)), these crystals appeared after 14 days. All crystals were grown at 18 °C.

Single crystals were directly picked from the screening plate (10 % (v/v) PEG 8000, 0.2 M NaCl and 0.1 M CHES (pH 9.5)) and tested for diffraction in the in-house machine. Single crystals did not show any diffraction but multiple crystals showed two spots on the diffraction screen, which was speculated to be a size dependent behaviour.

A lot of nucleation was observed which could be responsible for the small size of crystals. Further optimizations were performed with variation in the PEG-% and salt concentration (NaCl). Different percentages of PEG 8000 (8-15%) were added keeping the rest of the parameters unchanged (Figure 3.36 A-C). Also the NaCl concentration was varied from 100 mM to 300 mM (3.37 A-B).

Figure 3.36: Crystals with different percentages of PEG 8000. (A) Crystallization drop in the condition of 8% PEG 8000, 0.2 M NaCl and 0.1 M CHES (pH 9.5). (B) Crystallization drop in the condition of 10% PEG 8000, 0.2 M NaCl and 0.1 M CHES (pH 9.5). (C) Crystallization drop in the condition of 15% PEG 8000, 0.2 M NaCl and 0.1 M CHES (pH 9.5).

Figure 3.37: Crystals with different concentrations of NaCl. (A) Crystallization drop in the condition of 10 % PEG 8000, 0.1 M NaCl and 0.1 M CHES (pH 9.5). (B) Crystallization drop in the condition of 10% PEG 8000, 0.3 M NaCl and 0.1 M CHES (pH 9.5).

Crystal drops were set up at different temperatures (6 °C, 12 °C and 25 °C), but no further improvement was observed in the size and quality of crystals. Macroseeding was performed simultaneously to improve the quality and size of crystals. In macroseeding experiments, one small crystal was transferred to the equilibrated crystallization drop (equilibrated for 1 h, 2 h and overnight), which has been setup at low protein concentration (0.5 mg/ml and 1 mg/ml) at 18 °C. But there was no growth of the transferred crystals. Further slowing down of the nucleation by overlaying the reservoir solution with paraffin and

combination of paraffin and silicon oil resulted in bigger phase separated drops but they did not attain the proper shape (Figure 3.38).

Figure 3.38: Crystal pictures of MmA_3B_3D after overlaying the reservoir solution with paraffin and silicon. (A) Formation of MmA_3B_3D crystals in the condition of 10% PEG 8000, 0.2 M NaCl and 0.1 M CHES (pH 9.5) (condition without overlay of paraffin and silicon oil). (B) Formation of MmA_3B_3D crystals in the condition of 10% PEG 8000, 0.2 M NaCl and 0.1 M CHES (pH 9.5) with paraffin and silicon (4:1). Concentration of protein was 6.9 mg/ml in both (A) and (B). (C) Formation of MmA_3B_3D crystals in the condition of 10% PEG 8000, 0.2 M NaCl and 0.1 M CHES (pH 9.5) (condition without overlay of paraffin and silicon oil). (D) Formation of MmA_3B_3D crystals in the condition of 10% PEG 8000, 0.2 M NaCl and 0.1 M CHES (pH 9.5) with paraffin and silicon (4:1). Concentration of protein was 9.5 mg/ml in both (C) and (D).

Setting up crystallization drops by sitting drop method, as shown in figure 3.39A (where vapour diffusion is indirect and it slows the process of crystallization) resulted in visually good crystals (Figure 3.39 B). In addition, higher concentrations of protein were used in sitting drops, which has improved the size of the crystals (Figure 3.39 B).

Figure 3.39: Overview of sitting drop method and crystals produced by sitting drop method. (A) A diagram showing the setup for sitting drop crystallization, where the protein drop is not in direct contact with the reservoir solution. (B) Crystal picture of *MmA₃B₃D* with higher concentration of protein used (9.5 mg/ml) in sitting drop setup, condition of 10% PEG 8000, 0.2 M NaCl and 0.1 M CHES (pH 9.5).

Additive screening resulted in a condition (0.3 M Glycyl-glycyl-glycine), where bigger crystals were observed, diffracting with few spots in the in-house machine (Figure 3.40).

Figure 3.40: Crystals and Diffraction pattern after the addition of 0.3 M Glycyl-glycyl-glycine. (A) Picture of crystallization drop with appearance of bigger crystals after the addition of 0.3 M Glycyl-glycyl-glycine in the existing condition of 10% PEG 8000, 0.2 M NaCl and 0.1 M CHES (pH 9.5) (B) Diffraction pattern of the *MmA₃B₃D* crystals with the addition of 0.3 M Glycyl-glycyl-glycine (In-house machine), showed resolution up to 28 Å.

Further optimization led to the inclusion of an additional step of gel filtration chromatography (Superdex 200) subsequent to ion exchange chromatography. This resulted in crystals with better diffraction pattern (Figure 3.41). Crystal drops of different volume (0.5 μ l to 6 μ l) were setup and 4.8 μ l drop (2 μ l protein + 2 μ l precipitant and 0.8 μ l of 0.3 M Glycyl-glycyl-glycine) showed bigger crystals, which were analyzed at the National synchrotron radiation research center (NSRRC Taiwan), and diffracted up to 10 Å (Figure 3.42).

Figure 3.41: Diffraction pattern of MmA_3B_3D crystals after the introduction of Gel filtration chromatography (A) Gel-filtration profile subsequent to the Ion-exchange chromatography. The protein after ion-exchange was applied onto a superdex 200 column using buffer (50 mM Tris/HCl (pH 7.5), 200 mM NaCl, 0.8 mM DTT) at a flow rate of 0.5 ml/min. (B) On top is the picture of crystallization drop with appearance of bigger crystals with the introduction of gel-filtration step in the purification protocol. Crystallization condition used was 0.3 M Glycyl-glycyl-glycine, 10% PEG 8000, 0.2 M NaCl and 0.1 M CHES (pH 9.5). Diffraction pattern of the MmA_3B_3D crystal showing spots upto 17 Å (In-house machine) is shown in the lower part of the figure.

Figure 3.42: Crystal picture and the diffraction pattern upto 10 Å (A) Picture of an MmA_3B_3D crystal from the 4.8 μ l crystallization drop grown in condition of 0.3 M Glycyl-glycyl-glycine, 10% PEG 8000, 0.2 M NaCl and 0.1 M CHES (pH 9.5) (B) Diffraction pattern of the MmA_3B_3D crystal showing spots upto 10 Å (National synchrotron radiation research center, Taiwan).

The data was processed using the CCP4 suite, and resulted in a cubic space group with the unit cell parameters being: $a=b=c=322$ Å, and a Matthew's coefficient [159] resulted in one molecule per asymmetric unit. Further optimization did not result in high resolution datasets and molecular replacement was not possible at such a low resolution. To improve the

resolution, co-crystallization of MmA_3B_3D with nucleotides was performed as described in section 3.3.2.

3.3.2 Crystallization of MmA_3B_3D in the presence of MgADP, MgATP and MgAMP-PNP

Keeping crystallization conditions identical with the one used for the crystallization of MmA_3B_3D (0.3 M Glycyl-glycyl-glycine, 10% PEG 8000, 0.2 M NaCl and 0.1 M CHES (pH 9.5)), attempts were made to crystallize MmA_3B_3D with 2 mM of MgADP, MgATP and MgAMP-PNP. Crystals appeared in the presence of MgADP and MgAMP-PNP, but only a single crystal was obtained in the presence of MgATP in one drop and was not reproducible (Figure 3.43A, B and C). The co-crystals with MgATP and MgAMP-PNP did not improve further diffraction and AMP-PNP co-crystals diffracted upto 14 Å, when analysed at the in-house machine (Figure 3.43D).

Figure 3.43: Crystals of MmA_3B_3D co-crystallized with 2 mM MgADP, 2 mM MgAMP-PNP and 2 mM MgATP (A) Picture of a crystal drop, where complex MmA_3B_3D was co-crystallized with 2 mM MgADP. (B) Crystal picture of complex MmA_3B_3D , which was co-crystallized with 2 mM MgATP. (C) Picture of a crystal drop, where MmA_3B_3D was co-crystallized with 2 mM MgAMP-PNP. Crystallization conditions were identical, comprising 0.3 M Glycyl-glycyl-glycine, 10% PEG 8000, 0.2 M NaCl and 0.1 M CHES (pH 9.5). (D) Diffraction pattern of the MmA_3B_3D with AMP-PNP crystal showing spots upto 14 Å (In-house machine).

3.4 Rotational studies on complex A_3B_3DF of the A-ATP synthase from *M. mazei* Gö1

3.4.1 Rotation of central stalk subunits D and F in the A_3B_3DF complex of the A-ATP synthase from *M. mazei* Gö1

As described in section 2.2.25, the *M. mazei* Gö1 A_3B_3DF mutant (MmA_3B_3DF -M1) was generated to study the rotation of the central stalk, composed of subunits D and F. The MmA_3B_3DF -M1 mutant included the amino acid substitutions C36V, C72V, C181A and C380V in subunit A as well as the substitutions C67S and A71C in subunit B and D, respectively. MmA_3B_3DF -M1 was expressed and purified according to the protocol described in section 3.1.2 and its purity was analysed by SDS-PAGE (see Figure 3.44 A). The mutant revealed an ATPase activity of 2.1 $\mu\text{mol}/\text{min}/\text{mg}$ protein, which is 55 % lower than the ATPase activity of MmA_3B_3DF -WT (4.6 $\mu\text{mol}/\text{min}/\text{mg}$ protein), indicating the importance of cysteine residues in the catalytic A subunit for hydrolysis. The significance of cysteine residues inside subunit A was also shown in previous studies, in which cysteine substitutions to alanine in the related V-ATPases of *S. cerevisiae* affected ATP hydrolytic activity [174].

Figure 3.44: Purification and ATPase activity of rotation mutants. (A) The SDS-PAGE gel confirms the purity of MmA_3B_3DF -M1 and MmA_3B_3DF -M2 with respect to MmA_3B_3DF . (B) NADH absorbance curves showing the ATPase activity of MmA_3B_3DF -M1, MmA_3B_3DF -M2 and MmA_3B_3DF complexes. (C) Comparison of ATPase activity of MmA_3B_3DF , MmA_3B_3DF -M1 and MmA_3B_3DF -M2 as bar diagram.

With the enzymatic insight and the structural information that cysteines in subunit A and B are not accessible for the 300 nm streptavidin coated beads doped with quantum-dots, a second *MmA₃B₃DF* mutant, called *MmA₃B₃DF-M2*, was generated. The His₈-tagged *MmA₃B₃DF-M2* complex included only the single substitution A71C in subunit D and revealed an ATPase activity of 4.5 $\mu\text{mol}/\text{min}/\text{mg}$ protein, which is close to the one of *MmA₃B₃DF-WT* (Figure 3.44 C). This mutant was used for single molecule rotation experiment and will be referred to as the *MmA₃B₃DF*-complex in the following chapters. Residue A71C of subunit D was biotinylated, in order to bind a streptavidin coated bead ($\varnothing=300$ nm) to the protein. The bead was further doped with biotinylated quantum dots to visualize its possible movement in a TIRF microscope. The beads were bound to the protein and fluctuations were observed in some cases, but no rotating molecules (Figure 3.45). The fluctuations could be a result of the large size of the streptavidin beads ($\varnothing=300$ nm) which might be hampering the rotation or by unstable binding of streptavidin beads to the protein, since *MmA₃B₃DF-M2* had only one cysteine in subunit D for binding the streptavidin bead. Therefore, a second cysteine at position 131 (E131C) was engineered and the mutant was called *MmA₃B₃DF-M3* (see section 2.2.27).

Figure 3.45: Pictorial representation of the rotation assay of *MmA₃B₃DF*. (A) The setup of rotation assay performed with streptavidin beads coated with quantum dots. The *MmA₃B₃DF* molecule was bound to the coverslip with the His₈-tags and the biotin labelled cysteine (C71) is bound to the streptavidin beads. (B) The image was recorded with a TIRF microscope and shows the fluorescent beads representing the single protein molecules. (right inset) A zoom-in of the fluorescent bead.

Although the rationale for introducing a second cysteine in subunit D was to enhance the binding of streptavidin beads, it did not result in the visualization of any rotating molecule. Even when smaller streptavidin beads ($\varnothing=110$ nm) were attached to the biotinylated *MmA₃B₃DF-M3*, only fluctuating molecules could be observed.

Since the rotation cannot be investigated with this assay, as the movement of subunit D may be damped by the viscous drag of the large bead, we further moved towards another technique of studying the rotation of molecules. Here, the biotinylated C71 of subunit D of

MmA₃B₃DF-M2 was docked with a neutravidin-coated gold nanorod (40 x 76 nm), and the change in intensity of scattered polarized light was measured and analysed (Figure 3.46 A) [3]. Nanorods were illuminated by white light passing through a darkfield condenser. Polarized red light scattered from a nanorod attached to single *MmA₃B₃DF* passed through a polarizing filter prior to detection by an avalanche photo diode (APD). Under these conditions, rotation of the nanorod was observed as sinusoidal changes in light intensity. This rotation assay was performed and analyzed by Dr. Hendrik Sielaff of our laboratory and the studies were done in the laboratory of our collaborator Prof. Wayne Frasch, Arizona State University, USA.

Figure 3.46: Experimental setup and rotation analysis of *MmA₃B₃DF*. (A) Adapted schematic model of the microscope setup with a protein-gold nanorod complex [3]. The mutant protein *MmA₃B₃DF*-M2 (subunits A, B, D, and F in orange, green, yellow, and purple, respectively) is attached via its His₈-tags in subunit A to a cover slide, while a neutravidin coated nanorod is attached to the opposing biotinylated cysteine (red) in subunit D. Gold-nanorods were illuminated by a dark-field condenser. Red scattered polarized light was recorded by an APD after passing through a polarizer and a band pass filter to block shorter wavelengths of light. (B) Angular velocity of *MmA₃B₃DF* (blue) at 1 mM ATP was plotted against the rotational position from catalytic dwell [3].

The generation of *MmA₃B₃DF*-M2, which will be referred as *MmA₃B₃DF* led to the single molecule measurements of rotating *MmA₃B₃DF*-gold nanorod complexes. The rotation of *MmA₃B₃DF* is described in figure 3.46B by plotting the angular velocity of power strokes for 120° degrees. The data shown in figure 3.46B were retrieved from 25 *MmA₃B₃DF* molecules with an average of 2319 power strokes. As shown in the figure, the angular velocity accelerated to ~300°ms⁻¹ at 7° and then decelerated to a steady-state rate of ~200°ms⁻¹ between 25° and 60°, followed by a second acceleration at 88° reaching upto 500°ms⁻¹. At an angular position of ~90°, there was a steep acceleration to a maximum of 1200°ms⁻¹ which decelerated to 210°ms⁻¹ at 94°. The angular velocity accelerated again to a maximum of

$245^{\circ}\text{ms}^{-1}$ at 100° , before the power stroke decelerated as it approached the next catalytic dwell at 120° [3].

3.4.2 Effect of nucleotides on *MmA*₃B₃DF rotation

To understand the effect of ATP concentration on the rotation of *MmA*₃B₃DF, limiting concentration (0.1 mM) of ATP was used. It was established previously that at 0.1 mM of ATP the rate of hydrolysis of *MmA*₃B₃DF is limited [2]. Even though the concentration of ATP was limiting, the ATP binding dwell was not observed for *MmA*₃B₃DF at 40° after the catalytic dwell, as shown in figure 3.47. It is known that the ATP binding dwell decreases the angular velocity at the rotational position, which was not seen in the first phase (0° - 75°) of the angular velocity plot of *MmA*₃B₃DF at 0.1 mM of ATP compared to the plot at 1 mM ATP (Figure 3.47). The comparison of the data of *MmA*₃B₃DF at 1 mM and 0.1 mM ATP indicates that the ATP-binding dwells do not interrupt the power strokes of the *MmA*₃B₃DF-complex and ATP binding dwells in this complex must occur at the same rotary position as the catalytic dwells.

Figure 3.47: The effects of ATP concentration on angular velocity of *MmA*₃B₃DF complex. The effects of ATP concentrations that limit the rate of ATPase activity on the average angular velocities of the ATPase-dependent power strokes from the *MmA*₃B₃DF complex. (A) Average angular velocity as a function of rotational position during the power stroke of the *MmA*₃B₃DF complex at 1 mM ATP (blue) versus 0.1 mM ATP (red) (B) Dwell abundance as a function of rotational position during the power stroke of the *MmA*₃B₃DF complex at 1 mM MgATP (blue) versus 0.1 mM MgATP (red).

The effect of ADP was also studied on the *MmA*₃B₃DF complex, since it has been reported earlier that ADP induces dwells in *Eca*₃B₃ γ between 20° to 60° of the power strokes [163]. The occurrence of dwells results in decreasing the angular velocity. The ADP binding

dwells between 20° to 60° were not observed for the MmA_3B_3DF complex, indicating that ADP concentrations upto 250 μM does not affect the angular velocity of the MmA_3B_3DF complex (Figure 3.48).

Figure 3.48: The effect of ADP concentration on the angular velocity of the MmA_3B_3DF complex. (A) Average angular velocity of the power stroke as a function of rotational position of the MmA_3B_3DF complex at 1 mM MgATP in the presence of 5 μM (blue), 50 μM (red), and 250 μM (orange) MgADP. (B) Dwell abundance as a function of rotational position during the power stroke of the MmA_3B_3DF complex at 1 mM MgATP in the presence of 5 μM (blue), 50 μM (red), and 250 μM (orange) MgADP.

4. Discussion

4.1. Structural and functional characterization of *MmA₃B₃D* and *MmA₃B₃DF*

The newly developed three step purification protocol (section 3.1.1) for *MmA₃B₃D* and *MmA₃B₃DF* resulted in homogenous protein, which was further used for structural and functional studies. The 2D reconstruction of *MmA₃B₃D* and *MmA₃B₃DF* showed a pseudo-hexagonal arrangement of alternating nucleotide binding subunits A and B, which is similar to the F-ATP synthases and V-ATPases [41, 84, 175-180]. The top view in the 2D classes reveal a crown like arrangement of the alternating subunits A and B representing a bigger and a smaller mass, respectively (Figure 4.1). The bigger size of subunit A is a trait only seen in A-ATP synthases and V-ATPases, because of the presence of a 90 amino acid insertion in the N-terminus of subunit A, known as the non-homologous region (NHR). The subunit β of F-ATP synthases, though homologous to subunit A, lacks the NHR region [181, 182]. The bottom view of the obtained 2D classes shows a seventh globular mass proposed to be subunit D (Figure 4.1). After confirming the assembly of the *MmA₃B₃D* and *MmA₃B₃DF* complex with electron microscopy, the functionality of these enzymes were detected by NADH-coupled ATPase assay.

Figure 4.1: Surface view of *MmA₃B₃D* complex and comparison with the assembled model. The top and bottom view of *MmA₃B₃D* are compared with the assembled model generated by combining subunit A (orange; PDB 3I72) of *P. horikoshii* OT3, subunit B (green; PDB 2C61) of *M. mazei* Gö1 and subunit D (grey; PDB 3A5C) of *T. thermophilus* [2].

The ATPase activities for *MmA₃B₃D* and *MmA₃B₃DF* were calculated to be 1.2 and 4.6 $\mu\text{mol}/\text{min}/\text{mg}$ protein, respectively. The higher activity of *MmA₃B₃DF* was hinting towards the designation of subunit F as an ATPase stimulating subunit in A-ATP synthases, the role of subunit F in generating torque was also mentioned for prokaryotic V-ATPases [183]. The 4 times slower V_{max} of *MmA₃B₃D* compared to *MmA₃B₃DF* and similar Michaelis constants (K_m) for both the complexes (*MmA₃B₃D* and *MmA₃B₃DF*) indicated that the presence of subunit F does not affect the binding of the substrate (MgATP), but might affect the step of cleavage of ATP to ADP and Pi, and/or the release of the products.

4.2. The C-terminus of subunit F is involved in ATPase stimulation

The proposed ATPase stimulator subunit F (*MmF*) from the A-ATP synthase of *M. mazei* Gö1 exhibits a well-ordered N-terminal and a highly flexible C-terminal domain [57]. It is proposed in section 3.1 that the full length subunit F increases ATPase activity in *MmA₃B₃D*F and was confirmed with the reconstitution experiments. Whereas, the C-terminal truncated forms *MmF*₁₋₈₇, *MmF*₁₋₉₁, and *MmF*₁₋₉₅ reconstituted with *MmA₃B₃D* did not enhance ATPase activity, indicating that the C-terminus of subunit F plays an important role in stimulation of ATPase activity. The low ATPase activities of the reconstituted *MmA₃B₃D* + *MmF*₁₋₈₇ and *MmA₃B₃D* + *MmF*₁₋₉₁, suggests the importance of the α -helical structure formed by the peptide ₈₈REKIKQAV₉₅ in ATP hydrolysis. The other C-terminal truncated form (*MmF*₁₋₉₅) also did not increase the ATPase activity of *MmA₃B₃D*, signifying the presence of important residues in peptide ₉₆GVDLWK₁₀₁. Earlier studies from our laboratory revealed that the residue W100 of *MmF* is spatially close to W430 of subunit B, allowing a π - π interactions of the two indole-rings [57]. Furthermore, the interactions between the side chains of *MmF* (V95) and *MmB* (K399), *MmF* (K101) and *MmB* (Q440), and between the backbone and side chains of *MmF* (K101) and *MmB*(G442) were revealed by molecular dynamics studies from our laboratory [126]. It has been proposed by cross linking experiments that subunit F could only interact with subunit B in the extended state and the interaction gets demolished in the presence of MgADP [134].

The structural similarity between the subunit F of *M. mazei* Gö1 and subunit F of the related V-ATPase from yeast (*ScF*) could be reflected from the successful reconstitution of *MmA₃B₃D* + *ScF* complex [71, 155]. The ATP hydrolysis experiments with the reconstituted *MmA₃B₃D* + *ScF* complex demonstrated that subunit F of the eukaryotic V-ATPase also activates ATP hydrolysis of the related archaeal A₁ complex, but to a lower degree than *MmF*. In addition, the experiment with *ScF*₁₋₉₄ reveals that the C-terminus of *ScF* is required to form an assembly with *MmA₃B₃D*. The formation of *MmA₃B₃D* + *MmF*₁₋₈₇, *MmA₃B₃D* + *MmF*₁₋₉₁ and *MmA₃B₃D* + *F*₁₋₉₅ indicate that the structure of the N-terminal domain is not altered due to truncations and the loss of stimulation is a result of structural changes in *MmF*₁₋₈₇, *MmF*₁₋₉₁, and *MmF*₁₋₉₅. The FCS experiments presented and the densitometric analysis showed that the full length subunit F (*MmF*) and C-terminal truncated subunit F (*MmF*₁₋₈₇) have similar binding affinity towards *MmA₃B₃D*. The reason could be the involvement of hydrophobic amino acids of the N-terminus of subunit F for binding with subunit D (Figure 4.2). These hydrophobic residues are unaltered in the C-terminal truncated forms (*MmF*₁₋₈₇, *MmF*₁₋₉₁, and *MmF*₁₋₉₅). The homologous coupling subunit ϵ from F-ATP synthases of *Mycobacterium*

tuberculosis did not form a complex with the *MmA*₃B₃D, supporting the fact that A-ATP synthase subunits are specific and could only assemble and function within their respective A-ATP synthases and V-ATPases.

Figure 4.2: The charge distribution on the surface of subunit F (PDB: 2OV6) and subunit D (PDB: 3AON) of A-ATP synthases. Red and blue areas are negatively and positively charged areas, respectively. The arrow in subunit F indicates the very positively charged C-terminus. The hydrophobic area can be seen in grey colour. The dashed circle on the N-terminal domain of subunit F and D represents the hydrophobic amino acids involved in the binding of both subunits [2].

Apart from the C-terminal truncations, the N-terminal truncated F₅₋₁₀₁ also revealed the importance of the peptide ₁MELA₄ in ATPase activity. When the structure of the subunit DF-heterodimer of the related *Enterococcus hirae* (*Eh*) ATPase was superimposed on the structure of subunit F, it was observed that the residues ₁MELA₄ and the peptide ₄₆GILVMHND₅₃ of *MmF* are in close proximity to the peptide ₈₈VSIS₉₁ of the β -hairpin region of the subunit D (Figure 4.3). The high degree of conformational mobility of ₁MELA₄ and the peptide ₄₆GILVMHND₅₃ might affect the movement of the β -hairpin in subunit D by interacting with peptide ₈₈VSIS₉₁, leading to conformational changes on the N- and C-terminal helices of subunit D, which is speculated to affect the catalytic interfaces of subunit A and B.

Figure 4.3: The solution structure of subunit F (PDB: 2OV6; *magenta*) is superimposed on the crystal structure of the subunit DF-heterodimer of the related *E. hirae* ATP synthase (PDB: 3AON; *yellow*). The peptide $_{88}\text{VSIS}_{91}$ (*red*) in the β -hairpin region of the *EhD*, proposed to be involved in coupling, is in close proximity to the very N-terminal residues $_{1}\text{MEL}_3$ (*blue*) and the peptide $_{46}\text{GILVMHND}_{53}$ (*green*) of subunit F [2].

4.3. The interaction of C-terminus of subunit F with subunit B

The regulatory subunit B of A-ATP synthases and V-ATPases has a unique phosphate binding loop (P-loop) with a conserved peptide sequence (SASGLPHN) [61]. It binds MgATP with a two-fold higher affinity compared to MgADP [113], whereas the homologous α subunit of F-ATP synthases has similar binding affinities for MgATP and MgADP [184]. Subunit B (Earlier referred as non-catalytic subunit) does influence the ATPase activity as seen in the related V-ATPase from *S. cerevisiae*, where the mutation of H156 in the P-loop ($_{175}\text{SASGLPHN}_{182}$) resulted in 25% loss of hydrolytic activity and proton translocation [125]. Previous studies on subunit B of *M. mazei* Gö1 ATP synthase, revealed that ATP binds to the final binding pocket of subunit B by passing through two intermediate states (site 1 and site 2) [124, 126, 127]. It was also confirmed by molecular dynamics simulations that the interaction of the C-termini of subunit F and B, leads to the migration of the nucleotide from site 1 via site 2 to its final binding site 3 (Figure 1.11) [127].

The formation of a cross-link between the C-terminus of subunit F and subunit B in the presence of MgATP or MgAMP-PNP has already been established in A-ATP synthases [133, 134]. The zero-length cross-link involves the C-terminal peptide of subunit F and C-terminus region of subunit B. The nucleotide-dependent movement of C-terminal of subunit F is also observed in the smFRET experiments as mentioned in section 3.1.11, confirming the flexibility of the C-terminus of subunit F. The C-terminal part of subunit B, which is involved in cross linking, is similar to the DELSEED-region of the nucleotide-binding subunits α and

β of F-ATP synthases [185]. The involvement of R88 of *MmF* in intermolecular interaction with the C-terminal residues of subunit B (E391 and E402) justifies the 20% drop in hydrolytic activity of the reconstituted *MmA*₃*B*₃*D*+ *MmF* (R88L) complex.

The binding of nucleotides occurs at the interface of subunits A and B, indicating the importance of subunit B in nucleotide binding. Amino acid R349 of subunit B of *M. mazei* Gö1 ATP synthase is homologous to R373 of subunit α of the mitochondrial bovine F-ATP synthase, known to stabilize the negative charge of the terminal phosphate during the penta-coordinated transition state [84]. The other important residue of subunit B is L347, which is in vicinity of the P-loop residue K240 of the catalytic subunit A. The importance of R349 in subunit B has also been confirmed by the crystallographic structure of the *A*₃*B*₃*DF*-complex of the *E. hirae* V-ATPase. Previous studies revealed that the protein–protein interactions between the *DF*-ensemble and the C-terminal domains of subunits A and B lead to a closer movement of the homologue R350 residue of subunit B towards the γ -phosphate, bringing the AMP-PNP γ -phosphate closer to the conserved E261, which is an important residue in ATP hydrolysis [178].

The similar K_m values of the *MmA*₃*B*₃*D* and *MmA*₃*B*₃*DF* and a 4-times higher V_{max} of *MmA*₃*B*₃*DF* described in section 3.1.5, indicate that the interaction of subunit F with subunit B might affect the transition state stabilized by the critical residue R349 of *MmB* and with this, cleavage of ATP to ADP and P_i , and/or the release of the products. It has been studied earlier in F-ATP synthases that the release of P_i from the active site generates a torque which drives the 40° rotational sub-step of the central stalk subunits $\gamma\epsilon$ [186]. Similarly, it has been proposed that the release of P_i from the *A*₃*B*₃-headpiece of *MmA*₃*B*₃*DF* might cause structural changes that can lead to the rotation of the *DF*-ensemble and result in higher ATPase activity of *MmA*₃*B*₃*DF* [2].

4.4. The importance of the P-loop in V-ATPases

V-ATPases are dedicated ion pumps in eukaryotic cells and an efficient ATP-driven proton-pump to maintain pH-homeostasis in lysosomes, Golgi-derived vesicles, endosomes, synaptic vesicles, etc. [14-16]. This class of ATPases is regulated by a variety of mechanisms [1], including the mechanism of reversible disassembly of the *V*₁ and *V*_O domains [187]. ATPase activity in the homologous F- and A-ATP synthases is regulated by MgADP. The mutations in the P-loop of the catalytic A subunit of the *M. mazei* Gö1 A-ATP synthase to mimic the P-loop of V-ATPase, resulted in increased ATPase activity of the mutant complex *A*_(P235A, S238C)*B*₃*D* and relieving of MgADP inhibition.

The catalytic process of ATPase activity can be divided in at least three steps: 1) binding of Mg: ATP, 2) hydrolysis of ATP to ADP and Pi, and 3) release of ADP, Pi and the bivalent ion from the catalytic site. The decreased binding affinity reflected by the increased K_m in case of $A_{(P235A, S238C)}B_3D$ indicates that step 3 [release of product (ADP)] might be faster in case of $A_{(P235A, S238C)}B_3D$ compared to wild type MmA_3B_3D , leading to the increase of ATPase activity. The relieving of MgADP inhibition in $A_{(P235A, S238C)}B_3DF$ could be a combined effect of decreased binding affinity of ATP and the presence of subunit F, which plays a role in torque generation [188].

MgADP-inhibition is a well-studied phenomenon in F-ATP synthases and as discussed earlier, it occurs when the P_i -release precedes the release of ADP from the catalytic site, resulting in a stochastic entrapment of ADP in the catalytic site. It is known from the *G. stearothermophilus* (formerly *Bacillus* PS3) F-ATP synthase that the rotation of the central stalk subunit γ can shift the enzyme from an ADP-inhibited state to the active form [189]. This inhibited state was shown to be relieved by applying external force by magnetic tweezers which pushed the F_1 in the forward direction, thus decreasing the affinity of ADP in the catalytic site and activating the enzyme. The experiment indicated the importance of rotation angle and showed the tight coupling between the rotation and catalysis.

In F-ATP synthases, the rotation of γ , which is needed for relieving ADP-inhibition, is hindered due to subunit ϵ by interacting with the DELSEED-region of subunit β and stabilizing subunit γ in an angular position corresponding to the inhibited state [190]. The inhibitory subunit ϵ has subunit F as a counterpart in A-ATP synthase, which is involved in stimulating the ATPase activity, contradictory to subunit ϵ [2]. This subunit F might play an important role in providing the force for the rotation and relieving ADP-inhibited enzyme by interacting with the C-terminus of subunit B. This is seen in the experiments in which MgADP-inhibition of $A_{(P235A, S238C)}B_3D$ was observed with the incubation of *MmF*. The other set of experiments where MgADP was incubated with *MmA*₃*B*₃DF and $A_{(P235A, S238C)}B_3DF$ prior to ATPase assay showed that *MmA*₃*B*₃DF was inhibited even with lower concentration of MgADP compared to $A_{(P235A, S238C)}B_3DF$. These studies concluded the importance of the P-loop mutations (P235A and S238C) and subunit F in relieving MgADP-inhibition and the model is as proposed in figure 4.4.

The P-loop substitutions (P235A and S238C) in the single catalytic subunit A (*PhA*_{P235A, S238C}) of the *P. horikoshii* A-ATP synthase showed also a decreased binding affinity of Mg-AMPPNP (non-hydrolyzable homologue of ATP) compared to wild type subunit A (*PhA*-WT). This result is in line with the K_m -values of MgATP in the $A_{(P235A, S238C)}B_3D$

Figure 4.4: The model proposing the sequence of MgADP inhibition in A-ATP synthases similar to F-ATP synthase [4]. Once the ATP is hydrolysed into ADP and Pi and if the Pi release precedes ADP release, ADP gets entrapped and catalytic sites become inactive. The rotation of central stalk subunits DF can relieve the inhibition as seen in case of $A_{(P235A, S238C)}B_3DF$. This is similar to the rotation of γ subunit in F-ATP synthases. The subunit ϵ in F-ATP synthases stabilizes the inhibited state, here the homologous subunit F might provide the torque for rotation of subunit D and relieves the inhibition. The P-loop mutations (P235A, S238C) in cooperation with subunit F is important for relieving the MgADP inhibition.

and $A_{(P235A, S238C)}B_3DF$ complexes. The binding affinity for MgADP is similar in *PhA*-WT and *PhA*_{P235A, S238C}. The crystal structure of *PhA*_{P235A, S238C} revealed a flattened conformation of P-loop as seen earlier in P235A mutant, indicating the importance of proline in maintaining the arched conformation unique to P-loop of A-ATP synthases [121].

This flattened P-loop has steric clashes with the γ -phosphate of AMPPNP, suggesting that the decrease in binding affinities of ATP or AMPPNP to $A_{(P235A, S238C)}B_3D$, $A_{(P235A, S238C)}B_3DF$ and *PhA*_{P235A, S238C} is a result of steric clash. Also the side chain of S238 and main chain of P235 are involved in hydrogen bonding with the γ -phosphate of AMPPNP as shown in figure 4.5A [55, 121]. This interaction of γ -phosphate is disrupted in *PhA*_{P235A, S238C}, and polar interaction is observed between γ -phosphate and main chain of residues C238 and G234. The loss of P235 and S238 in mutant *PhA*_{P235A, S238C} could be a reason for decrease in binding affinities of AMPPNP and ATP in *PhA*_{P235A, S238C} and $A_{(P235A, S238C)}B_3D$, $A_{(P235A, S238C)}B_3DF$ complexes respectively.

Figure 4.5: AMPPNP bound *PhA*-WT and *PhA*_{P235A, S238C}. (A) The P-loop of subunit A (orange) from *P. horikoshii* A-ATP synthase bound to AMPPNP (stick) (PDB: 3I4L). The γ -phosphate of AMPPNP is in hydrogen bonding directly with S238 and with P235 via water molecule (grey, sphere). (B) The P-loop of subunit A mutant (*PhA*_{P235A, S238C}) superposed on wild type subunit A and modelled to show the binding of AMPPNP molecule. The γ -phosphate of AMPPNP is interacting with the main chain of C238 and G234. The flattened P-loop of *PhA*_{P235A, S238C} shows steric clashes with the AMPPNP molecule.

The role of S238 in facilitating the MgADP inhibition can be concluded from the fact that ATPase from *E. hirae* does not get inhibited by MgADP and it has an alanine substituting the serine in the P-loop. Also, ATPase from *T. thermophilus* did not show MgADP inhibition when serine from its P-loop was substituted to alanine. This mutant showed a decrease in binding affinity of ADP and lowering of V_{max} , certainly affecting one of the step(s) of ATP hydrolysis [191]. The crystal structure of *PhA*_{P235A, S238C} was superposed on the sulfate (phosphate analog) bound subunit A structure (PDB: 3I72), and binding of sulfate with *PhA*_{P235A, S238C} was predicted. The residue S238 of subunit A (WT) revealed to be in hydrogen bonding with the sulfate via a water molecule (Figure 4.6A). Whereas, in the *PhA*_{P235A, S238C} the sulfate is predicted to be directly bound to the P235 by polar interactions (Figure 4.6B), therefore the binding affinity of phosphate might have increased in *PhA*_{P235A, S238C} and in *A*_(P235A, S238C)*B*₃D and *A*_(P235A, S238C)*B*₃DF complexes. This could have led to the delayed release of P_i from the active site of *A*_(P235A, S238C)*B*₃D and *A*_(P235A, S238C)*B*₃DF complexes, allowing ADP to release before P_i and avoiding the stochastically entrapment of ADP in the active site. It has also been supported by the vanadate (V_i)-bound structure of subunit A of *P. horikoshii* ATP synthase, where V_i had preferred binding over ADP, indicating that during ATP synthesis binding of ADP precedes P_i binding. This order is proposed to be reversed during ATP hydrolysis [119].

Figure 4.6: Sulphate bound *PhA*-WT and *PhA*^{P235A, S238C}. (A) The P-loop of subunit A (magenta) from *P. horikoshii* A-ATP synthase bound to sulfate (stick representation) (PDB: 3I72). The sulfate molecule interacts with S238 via a water molecule (grey, sphere). (B) The P-loop of subunit A mutant (*PhA*^{P235A, S238C}) superposed on wild type subunit A and modelled to show the binding of sulfate molecule. The sulfate here interacts with the A235 directly by polar interaction.

4.5. Purification and crystallization of the 370 kDa A₃B₃D-complex of A₁-ATP synthase from *M. mazei* Gö1

The structures of single subunits of an enzyme complex are informative, but the information generated from the structure of the entire protein complex could provide much more insight into the mechanism of the enzyme. The task of purifying and crystallising a huge protein complex is challenging but this endeavour could potentially unravel a complete picture about the functioning of the protein. The complex A₃B₃D of A-ATP synthase of *M. mazei* Gö1 (*MmA*₃B₃D) is the critical assembly required for the rotation of central stalk subunit D and for multi-state catalysis. The purification of *MmA*₃B₃D was challenging given that molecular mass of approximately 370 kDa.

After screening a lot of crystallization conditions, microcrystals appeared in three conditions, which had a common precipitant (PEG 8000). The condition with the lower percentage of precipitant resulted in comparatively bigger sized crystals. With the lower percentage of precipitant, the protein would have faced a delay to attain the super-saturation state and hence resulted in appearance of a few crystal nuclei's, which then grew bigger with time. Since the crystals did not diffract, further optimization steps to increase the size included the addition of glycerol in the crystallization condition. Glycerol increases the solubility of the protein and decreases the nucleation by delaying the time to reach super-saturation [192]. The addition of glycerol had no major effect on the size and diffraction of the crystals. The overlay of paraffin and silicon oil resulted in a bigger phase separated state but the protein

stayed in phase separated state and no proper shape was attained. The overlay of oil acts as a barrier for vapour diffusion between the reservoir and the drop and slows down the rate of vapour diffusion.

Finally, the sitting drop method resulted in slow growth of crystals and permitted to use high concentration of protein. Since the vapour diffusion is slow in case of sitting drops, bigger crystals were obtained. Further optimization using additive screening provided bigger crystals and drastically improved the diffraction with the cross-linking agent 'Glycyl-Glycyl-glycine'. The role of linkers (containing amino- and carboxy- groups) in stabilising the protein conformation and reducing the dynamic character is well known. They can also form intramolecular hydrogen bonding networks using surface polar groups [193, 194]. Certainly, the linker (glycyl-glycyl-glycine) improved crystal packing and resulted in better diffraction.

Along with the optimization of crystallization setup, an additional step of gel filtration was included in the purification protocol after ion-exchange chromatography, which resulted in crystals, which diffracted upto 10 Å at NSRRC, Taiwan. The additional step of purification may have enhanced the monodispersity of the protein, which is an important factor to be considered during crystallisation. To further improve the monodispersity, only 30% of the peak of the eluted protein was used for crystallization. The idea for using minimum fractions of the apical peak for crystallisation was stemmed from earlier work on subunit F of *S. cerevisiae* (SeMetF₁₋₉₄I69M) from the laboratory, where remarkable improvement in crystal growth was observed with minimizing the apical peak fractions [155]. Minimizing the apical fractions of the protein did not result in any further improvement in diffraction of *MmA₃B₃D*.

Binding of nucleotide does help in improving the diffraction as seen in case of *A₃B₃* and *A₃B₃DF* crystals of homologous V-ATPase from *E. hirae* [178], therefore the attempts were made to crystallize *MmA₃B₃D* in the presence of MgAMPPNP, MgADP and MgATP. Though crystals were obtained, no further improvement in the diffraction was observed. The crystals of nucleotide-free *MmA₃B₃D* were indexed at 10 Å and the solvent content of 64.82% indicated one molecule per asymmetric unit. Molecular replacement was not successful at such low resolution (10 Å). The three step purification developed for crystallization of *MmA₃B₃D* was used for other biochemical studies as mentioned in section 3.1. The steps involved in the optimization of crystallization of *MmA₃B₃D* are summarized as a flow chart in figure 4.7.

Figure 4.7: Flow chart of the steps involved in the crystallization of complex A_3B_3D of *M. mazei* Gö1. Important steps, which drastically changed the shape, size and diffraction of the crystals are marked with the numbers (1-4).

4.6. Rotation of A_3B_3DF of *M. mazei* Gö1 A-ATP synthase

Rotation of subunit D and F within MmA_3B_3DF complex using the gold nanorod probe as a probe (see chapter 3.4) is demonstrated for the first time, enabling the determination of the power strokes angular velocity of the MmA_3B_3DF complex. The power strokes of MmA_3B_3DF occurred at 120° steps at saturating MgATP concentrations. The mechanism for rotation of MmA_3B_3DF included the closure of the catalytic site-lever domain against the axle (subunits D and F) as a cam shaft, induced by the binding of MgATP [3]. Here, the angular velocity at a given rotational position is determined by the mechanical efficiency of the force of the lever acting as a cam shaft against the axle and the rotational drag imposed on the axle as it rotates inside the stator subunits (subunits A and B). The angular velocity profile of MmA_3B_3DF was comparable to the thermophilic motor $\alpha_3\beta_3\gamma$ from *G. stearothermophilus* and *E. coli* $\alpha_3\beta_3\gamma$ -complex of F-ATP synthases [3].

The comparison of angular velocity profiles of MmA_3B_3DF , $Ec\alpha_3\beta_3\gamma$ and $Gs\alpha_3\beta_3\gamma$ at saturating concentration of MgATP is shown in figure 4.8 and described most recently [3]. It has been observed that the average angular velocity powered by 1 mM MgATP is 28% higher during the first 70° in case of EcF_1 when compared with that of the $Gs\alpha_3\beta_3\gamma$ -complex of the F-ATP synthase and the MmA_3B_3DF -complex of the A-ATP synthase.

Figure 4.8: Angular velocity comparison of *Eca*₃β₃γ, *Gsa*₃β₃γ and *MmA*₃β₃DF (A) Angular velocities of *Eca*₃β₃γ (black), *Gsa*₃β₃γ (orange) and *MmA*₃β₃DF (blue) were plotted against the rotational position from catalytic dwell [3]. (B) The effects of ATP concentrations that limit the rate of ATPase activity on the average angular velocities of the ATPase-dependent power strokes from the *MmA*₃β₃DF and *Eca*₃β₃γ. Probability of a dwell occurring as a function of rotational position during the power stroke of *EcF*₁ at 1 mM ATP (black) versus 0.3 mM ATP (red) and (C) *MmA*₃β₃DF complex at 1 mM ATP (blue) versus 0.1 mM ATP (red). This figure has been adapted from Sielaff *et al.*, [3].

This suggests that the transfer of energy due to substrate binding in *EcF*₁ into angular velocity is more efficient than in *Gsa*₃β₃γ or in *MmA*₃β₃DF. The comparison also indicates that the angular velocity profiles of these rotary motors are similar, and exhibit a series of accelerations and decelerations that occur at nearly the same rotary positions [3]. It has been established from earlier studies with *EcF*₁ that the binding affinity of MgATP on the empty catalytic site influences the angular velocity significantly [163] and this has been observed in case of the *MmA*₃β₃DF-complex also, which shows similar angular velocities during the final 50° of the power strokes when compared with the α₃β₃γ-complex of *GsF*₁, and that of *EcF*₁ (Figure 4.8A) [3].

To study the ATPase-dependent rotation of *MmA*₃β₃DF, limiting MgATP concentrations were used and dwells were not observed at 0.1 M MgATP (Figure 4.8B). Whereas, in case of *EcF*₁ at limiting ATP concentrations, ATP-binding dwells were observed after the γ subunit had rotated ~40° from the catalytic dwell (Figure 4.8B) [3]. The non-occurrence of additional ATP-binding dwell in the *MmA*₃β₃DF complex at limiting ATP concentrations is in line with the rotation data of other bacterial V-ATPases of *T. thermophilus* and *E. hirae*, suggesting that in bacterial V-ATPase A₃β₃DF-complexes, ATP-binding occurs at the same rotary positions as the catalytic dwell [195, 196] The rotational studies with *MmA*₃β₃DF suggests A-ATP synthases are more related to V-ATPases than to F-ATP synthases.

To further extend this understanding, the *Eca* $\alpha_3\beta_3\gamma$ and *MmA* $\alpha_3\beta_3\text{DF}$ complexes have also been analyzed to understand their mechanistic differences (Figure 4.9) [3]. During the catalytic dwell, in both the complexes ATP hydrolysis and phosphate release are noted to occur. However, in the case of *Eca* $\alpha_3\beta_3\gamma$, these steps occur at the β_{DP} and β_{E} catalytic sites, respectively, with the phosphate release initiating the power stroke. This power stroke is halted in two probable instances, one being limiting ATP concentration and the other being elevated ADP concentration (not shown). In the first case, the power stroke is halted by an ATP binding dwell, when the axle reaches a 40° rotation from the initial 0° catalytic dwell. The nucleotide is unable to bind at this ATP binding dwell. The second case, where the catalytic dwell is halted at 40° is when an ADP binding dwell occurs. This is witnessed with ADP binding to β_{E} , when ADP out competes ATP at elevated ADP concentrations. Also affected, is the angular velocity of the power stroke, given that the release of ADP from the β_{D} site is dampened [3]. However, when ATP displaces ADP, the power stroke is resumed for 80° at the β_{E} site and the previously designated β_{TP} now assumes the role of β_{DP} , the site at which subsequent hydrolysis occurs. As mentioned earlier in the *MmA* $\alpha_3\beta_3\text{DF}$ complex, hydrolysis and Pi release occur at the catalytic dwell at 0° , similar to the *Eca* $\alpha_3\beta_3\gamma$. Analysis of the current rotational data reveals that no differences arise with the ATP binding to A_{E} (equivalent of β_{E}), that occurs at the same catalytic dwell. This suggests that the ATP binding dwell for *MmA* $\alpha_3\beta_3\text{DF}$ complex overlaps with the catalytic dwell at 0° . To further decode the exact sequence of occurrence for all these events during the catalytic dwell more work needs to be performed [3].

Taking together, the data for hydrolysis and rotation of *MmA* $\alpha_3\beta_3\text{DF}$ demonstrate that the rotation of this enzyme is comparable to that of the homologous $\alpha_3\beta_3\text{DF}$ sub-complex of *E. hirae* and *T. thermophilus*, suggesting similar rotary mechanisms in these enzymes.

Figure 4.9: Schematic representation of the sequence of events occurring during ATP hydrolysis of *Eca3β3γ* and *MmA3B3DF*. (I) Represents the $\alpha_3\beta_3$ ring of the *Eca3β3γ*, with α shown in green, β shown in orange and γ (composed of the coiled coil domain and a foot region) in yellow. At the time of ATP hydrolysis at β_D (A) represents the Pi release occurring at β_E . (B) Initiation of the power stroke to reach a catalytic dwell at 40° rotation. (C) ATP binding at β_E can help the power-stroke to resume for 80° rotation. (D) Delayed ADP release from β_{DP} . (E) Previous β_{TP} is converted to β_{DP} and hydrolysis ensues at this site. (II) Depicts the *MmA3B3DF* complex with A shown in orange, B shown in green and D in yellow and F in magenta. ATP hydrolysis occurs at the A_{DP} and the transition states are depicted as follows. (A) Hydrolysis occurring at A_{DP} . (B) Pi release at A_E . (C) ATP binding at A_E . (D) Previous A_{TP} is converted to A_{DP} and hydrolysis ensues during catalytic dwell at this site [3].

5. Conclusions

Conclusions

Being closer to the origin of life and the ability to thrive in hostile environment make archaea an unusually attractive class of organisms. The A-ATP synthases present in the archaea are structurally different compared to the F-ATP synthases present in the bacteria, chloroplasts and mitochondria [1]. In the present study, with the development of a new purification protocol, highly purified A_3B_3D - and A_3B_3DF -complexes from the A-ATP synthase of *M. mazei* Gö1 (*Mm*) were produced. The 2D-classification and 3D-reconstructions of negatively stained EM-images enabled the assignment of the subunits in the MmA_3B_3D and MmA_3B_3DF [2]. Efforts were also made to crystallize MmA_3B_3D (370 kDa), resulting in 3D crystals diffracting to 10 Å.

The four times higher ATPase activity of MmA_3B_3DF compared to MmA_3B_3D revealed the role of subunit *MmF* as an ATPase stimulator. The similar binding affinities (K_m) of both, MmA_3B_3D and MmA_3B_3DF towards ATP, and the four times slower V_{max} of MmA_3B_3D compared to MmA_3B_3DF indicated, that the presence of *MmF* does not affect the binding of the substrate (MgATP), but might affect the step of cleavage of ATP to ADP and Pi, and/or the release of the products. The higher ATPase activity of the recombinant MmA_3B_3DF complex compared to MmA_3B_3D , demonstrates the role of subunit F (*MmF*) in ATPase stimulation. The C-terminal truncated forms F_{1-87} , F_{1-91} , and F_{1-95} reconstituted with MmA_3B_3D did not enhance ATPase activity, indicating the importance of the C-terminus of subunit F in ATPase stimulation. Further, mutational studies have been used to map the critical epitopes confirming the role of R88 of subunit F in ATPase stimulation. Apart from the C-terminal truncations, the N-terminal truncated F_{5-101} also revealed the importance of N-terminal peptide $_1MELA_4$ of subunit F in ATPase activity [2]. Although a complex of MmA_3B_3D and yeast V-ATPase subunit F (*ScF*) was successfully reconstituted, its slight enhancement of ATPase activity indicated that the coupling effect of *MmF* is specific inside the A-ATP synthases. This is supported by the inability of homologous coupling subunit ϵ from F-ATP synthases of *Mycobacterium tuberculosis* to form a complex with MmA_3B_3D [2].

In addition, the importance of conserved alanine and cysteine residues in the phosphate binding loop (P-loop) of catalytic subunit A of V-ATPases ($_{257}G\text{A}\text{F}\text{G}\text{C}\text{G}\text{KTV}_{265}$) was investigated by incorporating the alanine and cysteine in the P-loop of subunit A of MmA_3B_3D and MmA_3B_3DF of A-ATP synthases ($_{234}G\text{P}\text{F}\text{G}\text{S}\text{G}\text{KTV}_{242}$) and thereby replacing residues proline and serine, respectively. The mutants of MmA_3B_3D and MmA_3B_3DF were termed as $A_{(P235A,S238C)}B_3D$ and $A_{(P235A,S238C)}B_3DF$ respectively, based on the mutations. The enzyme kinetics with the mutants revealed a five times higher K_m and a three times higher V_{max} for

$A_{(P235A,S238C)3}B_3D$ (22.8 s^{-1}) compared to MmA_3B_3D . The higher K_m ($0.95 \pm 0.07\text{ mM}$) was also observed in $A_{(P235A,S238C)3}B_3DF$ when compared with MmA_3B_3D , indicating that the binding affinity for ATP is decreased after P235A and S238C mutations.

Another important result was the relieving of Mg-ADP inhibition in $A_{(P235A,S238C)3}B_3DF$, suggesting the role of both subunit F and P-loop mutations in dismissing the Mg-ADP inhibition. This was confirmed by the reconstituted $A_{(P235A,S238C)3}B_3DF$. Similar mutations were incorporated in the individual catalytic subunit A of *P. horikoshii* A-ATP synthases and FCS studies revealed lower binding affinity of the mutant ($PhA_{P235A,S238C}$) for AMP-PNP. These data are in line with the enzyme kinetics data, where decreased binding of ATP was observed for $A_{(P235A,S238C)3}B_3D$ and $A_{(P235A,S238C)3}B_3DF$. The crystal structure of $PhA_{P235A,S238C}$ solved at $2.35\text{ \AA}$ revealed a flat conformation of the P-loop which resulted in steric clashes of P-loop residues with the γ -phosphate of AMP-PNP, justifying the lower binding affinity of ATP in $A_{(P235A,S238C)3}B_3D$ and $A_{(P235A,S238C)3}B_3DF$.

With the goal of understanding the mechanism of the A_1 -complex of A-ATP synthases, a cysteine residue was incorporated at the tip of subunit D (C71) of MmA_3B_3DF and the angular velocities of the complex was determined by analyzing the rotation of the central stalk docked with neutravidin-coated gold nanorod. The angular velocity profiles of MmA_3B_3DF was comparable to eubacterial F-ATP synthases, exhibiting a series of accelerations and decelerations at nearly the same rotary positions. The effects of limiting ATP concentration was also studied on MmA_3B_3DF and no additional ATP-binding dwell was observed, indicating that ATP-binding in MmA_3B_3DF occurs at the same rotary positions as the catalytic dwell [3]. This was comparable to the ATPases of *E. hirae* and *T. thermophiles*, suggesting that in bacterial V-ATPase A_3B_3DF -complexes, ATP-binding and product release steps occur at the same rotary positions as the catalytic dwell [3, 197].

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Singh, D., Sielaff, H., Sundararaman, L., Bhushan, S. and Grüber, G. (2016). *The stimulating role of subunit F in ATPase activity inside the A₁-complex of the Methanosarcina mazei Gö1 A₁AO ATP synthase*. Biochimica et Biophysica Acta (BBA) – Bioenergetics. 1857(2): 177-187.

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Conference attendance

“18th European Bioenergetics Conference”, Lisbon, Portugal (9th – 14th July, 2014). Singh, D., Bhushan, S., Sielaff, H. and Grüber, G. *Subunit F of A-ATP synthases is an ATPase stimulating subunit* (Poster Presentation).

“7th Annual Retreat”, organized by Konstanz Research School Chemical Biology, Germany (30th Aug- 02nd Sep, 2015). - Oral presentation.

Speaker invitation, Posters and Abstracts

18th European Bioenergetics Conference (EBEC), Lisbon, Portugal (9th – 14th July, 2014). *Subunit F of A-ATP synthases is an ATPase stimulating subunit*. Biochim. Biophys. Acta 1837: e21

18th European Bioenergetics Conference (EBEC), Lisbon, Portugal (9th – 14th July, 2014). *Single-molecule approach to compare the rotation of F₁-ATPase with the archaeal A₁-ATPase*. Biochim. Biophys. Acta 1837: e21

7th Annual Retreat of the Konstanz Research School Chemical Biology, Germany (30th Aug- 02nd Sep, 2015). *The importance of subunit F of the M. mazei Gö1 A₁AO ATP synthase for its ATPase activity*. - Oral presentation.

Abstract submitted to BiOS 2017, California, United States of America (28th Jan-2nd Feb, 2017). *Monitoring conformational dynamics of the rotary subunit F in the A₁-complex of archaeal ATP synthase by single-molecule FRET*.

Awards

Nanyang Technological University Research Scholarship (2015-2016).

Ministry of Education Tier 2 Research scholarship (2012-2015).