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**Crystallographic and enzymatic insights into the mechanisms of Mg-ADP inhibition in
the A₁ complex of the A₁A₀ ATP synthase**

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Abstract

F-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. Knowledge about the existence and regulation of the related archaeal-type A_1A_0 ATP synthases ($A_3B_3CDE_2FG_2ac$) is limited. We demonstrate MgADP inhibition of the enzymatically active A_3B_3D - and A_3B_3DF complexes of *Methanosarcina mazei* Gö1 A-ATP synthase and reveal the importance of the amino acids P235 and S238 inside the P-loop (GPFGSGKTV) of the catalytic A subunit. Substituting these two residues by the respective P-loop residues alanine and cysteine (GAFGCGKTV) of the related eukaryotic V-ATPase increases significantly the ATPase activity of the enzyme variant and abolishes MgADP inhibition. The atomic structure of the P235A, S238C double mutant of subunit A of the *Pyrococcus horikoshii* OT3 A-ATP synthase provides details of how these critical residues affect nucleotide-binding and ATP hydrolysis in this molecular engine. The qualitative data are confirmed by quantitative results derived from fluorescence correlation spectroscopy experiments.

1. Introduction

F₁F₀ ATP synthases (F₁ with a subunit composition and stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$; and F₀: *ab₂c₉₋₁₅*) in prokaryotes and eukaryotes, A₁A₀ ATP synthase (A₁: A₃B₃CDE₂FG₂; and A₀: *ac*) in archaea couple the energy provided by the generation of an H⁺- and/or Na⁺- potential across the membrane by the electron transport chain with the condensation of ADP and Pi to ATP (Stewart et al., 2014; Grüber et al., 2014). Whereas these two enzyme complexes synthesize ATP, the evolutionarily related V₁V₀ ATPases (V₁: A₃B₃CDE₃FG₃H; and V₀: *ac_{xc}'_{yc}''_{zde}*) function as ATP-driven ion pumps, and are unable to synthesize ATP under physiological conditions (Huss et al., 2011; Marshansky et al., 2014).

As the names are indicating, all three enzymes are composed of an A₀/F₀/V₀ sector, which is membrane-embedded, and fulfills ion-translocation. The A₁/F₁/V₁ sector contains three catalytic interfaces of subunits A-B (A₁/V₁) and α - β (F₁) inside the A₃B₃- (A₁/V₁) or $\alpha_3\beta_3$ hexamers in which ATP synthesis or –hydrolysis occurs (Stewart et al., 2014). The central and rotating stalk subunits γ and ϵ (F-ATP synthases) or the related subunits C, D and F (V-ATPases, A-ATP synthases) couple ATP synthesis/hydrolysis in the $\alpha_3\beta_3$ - or A₃B₃- headpieces with ion-transport in the F₀- (F-ATP synthases) or V₀/A₀ parts of V-ATPases and A-ATP synthases, respectively (Stewart et al., 2014; Grüber et al., 2014; Huss et al., 2011; Marshansky et al., 2014). As shown for the A₃B₃DF-complex of the *Thermus thermophilus* (Imamura et al., 2004) and *Enterococcus hirae* (Minagawa et al., 2013) and *Methanosarcina mazei* Gö1 A-ATP synthase (Sielaff et al., 2016) the DF-ensemble rotates in 120° degree steps at saturating MgATP concentrations. Besides rotation of the central stalk unit, coupling of ion-translocation and ATP synthesis (or *vice versa* in case of ATP hydrolysis) inside the *M. mazei* Gö1 A-ATP synthase involves the communication of the 101 amino acid residue subunit F, which is an ATPase activating subunit (Sielaff et al., 2016). Its C-terminal residues interact with the C-terminus of the catalytic subunit A-B pairs (Sielaff et al., 2016 ;

Raghunathan et al., 2010) and was discussed to affect the transition state and with this, cleavage of ATP to ADP and Pi, and/or the release of the products (Raghunathan et al., 2010).

The residues present at the interface of subunits A and B are involved in ATP synthesis and -hydrolysis, with subunit A contributing the major part (Kumar et al., 2010a; Schäfer et al., 2006; Kumar et al., 2008; Kumar et al., 2009; Manimekalai et al., 2011; Kumar et al., 2010b). Homologous to the phosphate binding loop (P-loop) of catalytic β -subunit (GGAGVGKT) of F-ATP synthases and subunit A (GAFGCGKT) of V-ATPases, subunit A of A-ATP synthase also possesses a glycine rich P-loop with the sequence GPFGSGKT (Kumar et al., 2010a). As shown for the *Pyrococcus horikoshii* OT3 subunit A, the P-loop has an arched form, which is unique to the catalytic subunit A of A-ATP synthases (Kumar et al., 2010a). Crystallographic studies of substrate-bound forms of subunit A revealed the important role of the amino acids G234, F236 and S238 in maintaining the arched P-loop, the formation of bifurcated hydrogen bonding of S238 with the γ -phosphate as well as interaction of S238 with the α and β -phosphate of the nucleotide via a water molecule. Along with S238, the unique P-loop residue P235 also interacts with γ -phosphate of the nucleotide via a water molecule, indicating the importance of both residues in catalysis (Kumar et al., 2010a).

The characteristic residues P235 and S238 of A-ATP synthases are absent in the P-loop of V-ATPases (GAFGCGKT) and are substituted by an alanine and cysteine in V-ATPases, respectively (Kumar et al., 2010a). The role of the alanine and cysteine residues in ATP hydrolysis, and/or the release of the Pi and ADP are not known. However, knowledge about the product release mechanism is fundamental as described for F-ATP synthases in which P_i-release precedes the release of ADP from the catalytic site, resulting in a stochastic entrapment of ADP in the catalytic site and inhibiting the enzyme in a MgADP inhibited state (Yoshida et al., 1983). Together with the intrinsic subunit ϵ , ADP-inhibition prevents

bacterial F-ATP synthase from wasteful hydrolysis during the ATP limited state, which is obligatory to conserve the ATP pool (reviewed in Nakanishi-Matsui et al., 2016). Currently, MgADP inhibition is not observed in the dedicated eukaryotic V-ATPases and details about the mechanisms of MgADP inhibition of A-ATP synthases are still missing.

Here we show the effect of MgADP inhibition of the A₃B₃D- and A₃B₃DF complexes of the *M. mazei* Gö1 A-ATP synthase and demonstrate the importance of the P-loop residues P235 and S238 (GPFGSGKTV) as well as stalk subunit F in this mechanism. We demonstrate that substituting these two residues by the respective P-loop residues alanine and cysteine (GAFGCGKTV) of the eukaryotic V-ATPase increases significantly the ATPase activity of the enzyme variant and abolishes MgADP inhibition. The atomic structure of the P235A, S238C double mutant of subunit A provides insights for the effect seen in ATP hydrolysis and nucleotide-binding, with the latter being studied by fluorescence correlation spectroscopy experiments.

2. Material and Methods

2.1 Biochemicals

Pfu DNA polymerase was purchased from Thermo Scientific (Massachusetts, USA) and Ni-NTA chromatography resin was obtained from Qiagen (Hilden, Germany). Enzymatic digestion was performed using restriction enzymes from New England BioLabs. Chemicals from Bio-Rad (California, USA) were used for SDS-PAGE. All other chemicals of analytical grade were obtained from Biomol (Hamburg, Germany), Merck (Darmstadt, Germany), Sigma, or Serva (Heidelberg, Germany).

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

Full plasmid amplification method was used to incorporate the mutation (P235A, S238C) in subunit A of *MmABD* and *MmABFD* operon, which was already cloned in the pGEM-4Z plasmid (Singh et al., 2016). The *MmABD* and *MmABFD* genes in plasmid pGEM-4Z were used as the template for the mutagenic PCR. This plasmid was kindly provided by Prof. Volker Müller, Johann Wolfgang Goethe University, Frankfurt, Germany. The double mutants will be referred as $A_{(P235A,S238C)3}B_3D$ and $A_{(P235A,S238C)3}B_3DF$. The mutation in the P-loop was incorporated using forward primer 5'-CTGCTGCAATTCCAGGAGCTTTCGGGTGTGGCAAAACCGTTACTCAGCAGC- 3' and the reverse primer 5'-GCTGCTGAGTAACGGTTTTGCCACACCCGA AAGCTCCTGGAATTGCAGCAG- 3'.

The PCR products were purified followed by *DpnI* digestion and transformation in *E. coli* DH5 α cells. *E. coli* DK8 cells were used for production of the recombinant protein. Protein expression and purification was performed as mentioned in Singh et al., 2016 except for skipping the step of ion-exchange chromatography.

2.3 Cloning and purification of the P235A,S238C mutant of subunit A

To engineer the P235A, S238C mutant of subunit A the gene encoding subunit A of the A_1A_0 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 (+) (Kumar et al., 2010a) and is devoid of the intein region. The double mutations were incorporated by site directed mutagenesis using overlap extension method. In this case, two separate PCR reactions were setup, one with a flanking primer (forward primer *a*: 5'-TATACATATGGTGGCGAAGGGGAGGATAATTAGGGTTACTGGACC- 3') and an internal primer (internal reverse primer *b*:

5'-CACCGTCTTACCACAGCCGAAAGCCCCAGGAATTGC- 3') and the second PCR with the internal primer (internal forward primer *c*: 5'-GCAATTCCTGGGGCTTTTCGGCTG TGGTAAGACGGTG- 3') and flanking primer (reverse primer *d*: 5'-GCTTGTCGACTCACGCCCCATACTTCTTGAAGAGCTCC- 3').

The amplified fragments were fused together via a second PCR using the primers *a* and *d*, incorporating *NdeI* and *SalI* restriction sites and were ligated in pET-22b(+) vector followed by transformation in *E. coli* DH5 α cells. The protein was produced in *E. coli* (BL21–CodonPlus (DE3)–RIL) cells and purified following the protocol of Kumar et al. (Kumar et al., 2010a).

2.4 ATP hydrolysis assay

A continuous ATP hydrolysis assay was applied to measure the specific ATP hydrolysis activity of all ATP synthase constructs (Ho et al., 2015). In this assay, ATP was constantly regenerated by an enzymatic reaction, while the consumption of NADH was measured spectroscopically at 340 nm. The change in absorbance was measured for 250 s in 2 s intervals at 37 °C after adding 50 μ g of protein complex to 1 ml reaction solution (25 mM 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.

2.5 Nucleotide-binding studies of the P235A, S238C mutant of subunit A

In order to determine the binding affinity of nucleotides with the P235A,S238C mutant of subunit A, fluorescence correlation spectroscopy (FCS) was performed according to Cs et al. (Ho et al., 2015) and Hunke et al., 2007 at 25 °C on a ConfoCor 3 (Zeiss, Jena, Germany) using the AMP-PNP- and ADP-analogues EDA-AMP-PNP-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 labelled 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 P235A, S238C mutant. The calculations of the bound fractions and the dissociation constants were done by the ConfoCor 3-software 4.2 and Origin 7.5.

2.6 Crystallization and structure determination of the P235A, S238C mutant

Diffraction crystals of the subunit A mutant P235A,S238C 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 of protein solution were 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.

Single-wavelength datasets for the subunit A mutant 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. All the diffraction data were indexed, integrated and scaled using the HKL2000 suite program. All the crystals belong to tetragonal space group $P4_32_12$ and have similar unit cell parameters as wild type subunit A. The Matthew's coefficient (Matthews et al., 2011) was determined, which revealed one molecule in the asymmetric unit.

The structure of subunit A mutant (P235A; PDB: 3M4Y; (Priya et al., 2011) from *P. horikoshii* OT3 (*Ph*) was used as model for structure determination by molecular replacement method using the programs PHASER (McCoy et al., 2007) and MOLREP (Vagin et al., 2010). 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 (Emsley et al., 2010) and REFMAC5 (Murshudov et al., 2011) of the CCP4 suite (Winn et al., 2011). The template of wild type (WT) subunit A used for cloning had a mutation at position 79 from glycine to arginine, which has been reported in earlier structures (Kumar et al., 2010a) 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 (Laskowski et al., 1993) and MOLPROBITY (Chen et al., 2010) and the figures were drawn using the program PyMOL (DeLano, 2003). Structural comparison analysis was carried out using the program SUPERPOSE (Krissinel and Henrick; 2004) as included in the CCP4 suite. The

atomic coordinates and structure factors have been deposited in the Protein Data Bank (PDB ID: 5X09).

3. Results

3.1 Effect of MgADP inhibition in the *M. mazei* Gö1 A₃B₃D- complex

Substrate-dependent ATP hydrolysis experiments of the A₃B₃D- and A₃B₃DF complexes of the *M. mazei* Gö1 A-ATP synthase with varying concentrations of 0.025 - 6 mM ATP were performed to determine the ATP hydrolysis activity and consequently the maximum rate (V_{max}) and the Michaelis-constant (K_M) of the complexes (Fig. 1). The ATP hydrolysis profile of *MmA*₃B₃D was lower and rapidly decayed (after 30 sec.; Fig. 1A) compared to the *MmA*₃B₃DF complex which was active for longer duration (Fig. 1D). The ATPase activity for *MmA*₃B₃D and *MmA*₃B₃DF was determined to be 1.0 μ mol/min/mg protein and 4.2 μ mol/min/mg protein, confirming the recently described stimulating effect of subunit F in ATP hydrolysis (Singh et al., 2016). The respective K_m -values of *MmA*₃B₃D and *MmA*₃B₃DF were similar with 0.19 ± 0.04 mM and 0.15 ± 0.04 mM (Fig. 1C and 1F). We recently proposed that higher V_{max} of the subunit F bound form of A₃B₃D may indicate that subunit F affects the transition state and with this, cleavage of ATP to ADP and Pi, and/or the release of the products Pi and ADP (Singh et al., 2016). In order to prove whether the activity increase and longer duration of *MmA*₃B₃DF maybe caused at least in part by subunit F driven product release of ADP, the complex was pre-incubated with varying MgADP concentrations after the last gel-filtration step, followed by ATP hydrolysis measurements. *MmA*₃B₃DF pre-incubated with 10 μ M of MgADP, a concentration above the K_d (1.5 μ M) of the catalytic A subunit (Kumar et al., 2010a), showed a significant decrease in hydrolytic activity (2.0 μ mol/min/mg protein) when compared to the non-treated complex (4.2 μ mol/min/mg protein), whose activity became even abolished in the presence of 100 μ M of MgADP (Fig.

2A). These data demonstrate that MgADP has an inhibitory effect to the ATPase activity of the complex and the higher ATPase of *MmA*₃B₃DF pre-incubated with 10 μ M of MgADP (2.0 μ mol/min/mg protein) compared to *MmA*₃B₃D in the absence of MgADP (1.0 μ mol/min/mg protein) confirms that subunit F abolishes at least in part the inhibitory effect of the nucleotide.

3.2 Effect of the critical P-loop residues in ATPase activity and nucleotide-binding

Because of the importance of P-loop residue P235 and S238 in maintaining the arched P-loop, the transition-state formation and nucleotide-binding (Kumar et al., 2010a; Manimekalai et al., 2011), we addressed the questions, whether these residues may contribute in the MgADP inhibition described above and whether substituting these conserved A-ATP synthase residues by the respective conserved P-loop residues alanine and cysteine of eukaryotic V-ATPases may at the same time provide information about the related V-ATPase, which is primarily an ATPase. Therefore, the mutant complexes A_(P235A, S238C)₃B₃D and A_(P235A, S238C)₃B₃DF of the *M. mazei* Gö1 A-ATP synthase were generated and purified to high quality using affinity- and size exclusion chromatography (Fig. 3A-B).

Substitution of the P-loop residues P235A and S238C in the A_(P235A, S238C)₃B₃D complex resulted in an increase in ATP hydrolysis (2.4 μ mol/min/mg protein) when compared to the wild type (wt) *MmA*₃B₃D complex (1.0 μ mol/min/mg protein), indicating that the double mutation in the P-loop plays an important role in ATPase activity (Fig. 1A-B). Further kinetic studies with A_(P235A, S238C)₃B₃D revealed that the V_{max} for A_(P235A, S238C)₃B₃D is 3.7 μ mol/min/mg protein or $22.8 \pm 0.5 \text{ s}^{-1}$, which is three times higher than the one of *MmA*₃B₃D (1.3 μ mol/min/mg protein or $7.9 \pm 0.3 \text{ s}^{-1}$) (Fig. 1C). Interestingly, the K_m of A_(P235A, S238C)₃B₃D ($1.07 \pm 0.08 \text{ mM}$) was much higher than the K_m of *MmA*₃B₃D ($0.19 \pm 0.04 \text{ mM}$),

reflecting that the binding affinity for MgATP has been decreased by the incorporation of the P-loop mutations (Table 1).

In comparison, the $A_{(P235A, S238C)3}B_3DF$ mutant complex showed an ATPase activity of 3.9 $\mu\text{mol}/\text{min}/\text{mg}$ protein (Fig. 1D-E), similar to the of MmA_3B_3DF complex (4.2 $\mu\text{mol}/\text{min}/\text{mg}$ protein). A K_m -value of 0.95 ± 0.07 mM was calculated for the mutant $A_{(P235A, S238C)3}B_3DF$, which is six times higher compared to wt MmA_3B_3DF (0.15 ± 0.04 mM) (Fig. 1F). The respective V_{max} -value was similar for $A_{(P235A, S238C)3}B_3DF$ (32.9 ± 0.8 s⁻¹) and MmA_3B_3DF (30.4 ± 0.9 s⁻¹). It is interesting to note, that although the MgATP-affinity decreases in both mutants ($A_{(P235A, S238C)3}B_3D$ and $A_{(P235A, S238C)3}B_3DF$) compared to the WT complexes, the $A_{(P235A, S238C)3}B_3DF$ mutant has a higher V_{max} compared to the $A_{(P235A, S238C)3}B_3D$ one. (Fig. 1C and 1F) (Table 1).

The ATP hydrolysis profile in figure 1D demonstrates that the $A_{(P235A, S238C)3}B_3DF$ complex was active for longer duration than the one of MmA_3B_3DF . In order to confirm whether MgADP-binding may in part cause this difference, $A_{(P235A, S238C)3}B_3DF$ and MmA_3B_3DF were pre-incubated with varying MgADP concentrations. As shown in figure 2B ATPase activity of $A_{(P235A, S238C)3}B_3DF$ was not inhibited by 10 μM of MgADP (3.6 $\mu\text{mol}/\text{min}/\text{mg}$ protein compared to 3.9 $\mu\text{mol}/\text{min}/\text{mg}$ protein without preincubation with MgADP as observed for the MmA_3B_3DF -complex above (Fig. 2A). A significant decrease in ATPase activity of this mutant enzyme was only revealed at MgADP concentration above 100 μM . These data confirm that inhibitory MgADP becomes released in the $A_{(P235A, S238C)3}B_3DF$ (Fig. 2D).

3.3 The concerted action of P-loop residues and subunit F in relieving MgADP inhibition

In order to understand the role of subunit F in relieving MgADP inhibition in more depths, MmA_3B_3D and its mutant $A_{(P235A, S238C)3}B_3D$ was reconstituted with MmF according

to our recent protocol (Singh et al., 2016), respectively. As revealed in figure 4A the ATP hydrolysis profile of reconstituted MmA_3B_3DF and $A_{(P235A, S238C)}B_3DF$ was comparable to the one of the recombinant MmA_3B_3DF (Fig. 2C) and $A_{(P235A, S238C)}B_3DF$ complex (Fig. 2D), respectively, indicating that the reconstitution was successful and that subunit F increases ATPase activity (3.3 $\mu\text{mol}/\text{min}/\text{mg}$ protein). Importantly, although the reconstituted MmA_3B_3DF complex showed an increase in ATPase activity (3.1 $\mu\text{mol}/\text{min}/\text{mg}$ protein) its profile is comparable to the recombinant MmA_3B_3DF (Fig. 2C) but different to the one of $A_{(P235A, S238C)}B_3DF$. These data demonstrate the concerted effect of the P-loop residue substitutions in subunit A and coupling subunit F regarding MgADP release and ATPase hydrolysis increase. As confirmed also in this experiment, the MmA_3B_3D profile (1.0 $\mu\text{mol}/\text{min}/\text{mg}$ protein) reflects a MgADP inhibited enzyme, which became stimulated in the $A_{(P235A, S238C)}B_3D$ mutant (2.4 $\mu\text{mol}/\text{min}/\text{mg}$ protein).

3.4 Crystal structure of subunit A mutant $A_{P235A, S238C}$

The crystallographic structure of the *Pyrococcus horikoshii* OT3 A-ATP synthase subunit A as well as its P-loop mutants have contributed to the understanding of the evolutionary differences of this P-loop in A- and F-ATP synthase (Kumar et al., 2010a). To extend this knowledge by looking into possible structural differences of the P-loop of catalytic subunit A of A-ATP synthases and their related V-ATPases, and to shed light into the catalytic phenomena we observed in the $A_{(P235A, S238C)}B_3D$ and $A_{(P235A, S238C)}B_3DF$ above, we used the *P. horikoshii* subunit A (*PhA*) as a model system and generated the $PhA_{P235A, S238C}$ mutant. Crystals of the recombinant protein were grown and the crystallographic structure of the $PhA_{P235A, S238C}$ mutant was solved at 2.35 Å (Fig. 5A). Like for the WT subunit A structure (Kumar et al., 2010a), the $PhA_{P235A, S238C}$ mutant 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). Residues 122- 210 are part of the Non-Homologous Region (NHR) which is comprised of 90 amino acids (Fig. 5A). The detailed statistics of structure refinement are provided in Table 2. A total of 165 water molecules, two acetate, a 2-methyl-2, 4-pentanediol (MPD), and one Tris molecule could be assigned in the structure (Fig. 5A). 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. The difference map ($2F_o - F_c$) shows clear electron density in the P-loop region (Fig. 5B).

Superimposition of the *PhA*_{P235A, S238C} structure on WT subunit A shows that the arched P-loop of subunit A has flattened in the mutant *PhA*_{P235A, S238C}, which has been seen earlier in the subunit A mutant *PhA*_{P235A} (Priya et al., 2011), indicating the importance of the proline residue in maintaining the unique arched conformation (Fig. 5C). A comparison of the mutant *PhA*_{P235A, S238C} structure with the one of the AMPPNP-bound- and the ADP-bound form of WT subunit A revealed that the γ -phosphate of the non-hydrolysable AMPPNP has slight steric clashes with the flattened P-loop of the *PhA*_{P235A, S238C} (Fig. 5D-F). In addition, the superimposition highlights also the inward movements of substituted amino acids A235 and C238 in the *PhA*_{P235A, S238C} structure when compared with the WT subunit A bound to AMPPNP (Fig. 5D and 5F). 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 (Fig. 5G). The conformational changes in the P-loop of *PhA*_{P235A, S238C} signifies the importance of P235 and S238 in maintaining the arched conformation of the P-loop and phosphate-binding.

3.5 Nucleotide binding studies on subunit *PhA* and its mutant *PhA*_{P235A, S238C}

In order to quantify the structural changes of the P-loop mutant *PhA*_{P235A, S238C} the nucleotide binding constants of WT *PhA* and *PhA*_{P235A, S238C} were determined by FCS

experiments using the fluorescent AMP-PNP and ADP derivatives (Mg-AMP-PNP-ATTO-647N and Mg-ADP-ATTO-647N) (Fig. 6A-B). The characteristic diffusion times τ_D of Mg-AMP-PNP-ATTO-647N and MgADP-ATTO-647N, determined from a single component fit of the resulting autocorrelation function, were about 70 μ s. After addition of proteins τ_D increased due to the binding of fluorescently labelled nucleotides to the proteins. The characteristic diffusion times of Mg-AMP-PNP-ATTO-647N and MgADP-ATTO-647N bound to (i) *PhA* were $310 \pm 24 \mu$ s and $323 \pm 11 \mu$ s, and (ii) *PhA*_{P235A,S238C} were $294 \pm 23 \mu$ s and $318 \pm 22 \mu$ s, respectively. From the respective autocorrelation curves (*insets* in Fig. 6) the fractions of bound nucleotides were derived, plotted against the rising protein concentrations, and fitted with the Hill equation to determine the dissociation constants (K_D). The K_D -values of *PhA* and *PhA*_{P235A,S238C} for the binding of Mg-AMP-PNP-ATTO-647N were $4.4 \pm 0.33 \mu$ M and $13.1 \pm 0.85 \mu$ M, respectively. The respective K_D -values for Mg-ADP-ATTO-647N were $0.08 \pm 0.006 \mu$ M and $0.18 \pm 0.01 \mu$ M. The data indicate that both proteins have a preference for the ADP- over the ATP analogue and that mutating the critical P-loop residues P235A and S238C reduces significantly binding of ATP, which is in line with the observed increase of the K_m values for *A*_{(P235A,S238C)3}*B*₃*D* and the *A*_{(P235A,S238C)3}*B*₃*DF* mutants described in sections 3.1-3.2 above, and the structural alteration determined in the *PhA*_{P235A,S238C} structure (Fig. 5D- and 5F).

4. Discussion

4.1. The decreased ATP binding affinity and role of subunit *F* in relieving 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)3}*B*₃*D* indicates that step 3 (release of product (ADP)) might be faster in

case of $A_{(P235A, S238C)3}B_3D$ compared to WT MmA_3B_3D , leading to the increase of ATPase activity. The phenomenon of MgADP-inhibition is well-studied in the related F-ATP synthases (Weber et al., 2000; Creczynski-Pasa et al., 1994), and 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 *Geobacillus 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 (Feniouk et al., 2007). This inhibited state was shown to be relieved by applying external force by magnetic tweezers which pushed the F_1 in 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 (Watanabe et al., 2010).

Furthermore, rotation of γ is affected due to the interaction of the rotary subunit ϵ with the so-called C-terminal DELSEED-region of subunit β , which stabilizes subunit γ in an angular position corresponding to the inhibited state (Nakanishi-Matsui et al., 2016; Cingolani et al., 2011; Shirakihara et al., 2015; Sobti et al., 2016). The inhibitory subunit ϵ has subunit F as a counterpart in A-ATP synthases, which in contrast to subunit ϵ is involved in stimulating the ATPase activity (Singh et al., 2016). 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 the catalytic A_3B_3 -headpiece. This is seen in the experiments in which MgADP-inhibition of $A_{(P235A, S238C)3}B_3D$ was observed after the reconstitution of MmF (Fig. 4B). The other set of experiments where MgADP was incubated with MmA_3B_3DF and $A_{(P235A, S238C)3}B_3DF$ prior to the ATPase assay showed that MmA_3B_3DF was inhibited even with lower concentrations of MgADP compared to $A_{(P235A, S238C)3}B_3DF$ (Fig. 4). These studies indicate the importance of the P-loop mutations (P235A and S238C) and subunit F in relieving MgADP-inhibition.

4.2 Nucleotide-binding and structural alterations in the P-loop of subunit A

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 analogue of ATP) compared to WT subunit A (PhA -wt). This result is in line with the K_m -values of MgATP in the $A_{(P235A, S238C)3}B_3D$ and $A_{(P235A, S238C)3}B_3DF$ complexes. The binding affinity for MgADP is similar in WT PhA and $PhA_{P235A, S238C}$. The crystal structure of $PhA_{P235A, S238C}$ revealed a flattened conformation of the P-loop as seen earlier in the P235A mutant (Ragunathan et al., 2011), indicating the importance of proline in maintaining the arched conformation unique to P-loop of A-ATP synthases.

This flattened P-loop has slight steric clashes with the γ -phosphate of AMPPNP, suggesting that the decrease in binding affinities of ATP or AMPPNP to $A_{(P235A, S238C)3}B_3D$, $A_{(P235A, S238C)3}B_3DF$ and $PhA_{P235A, S238C}$ is a result of structural alterations (Fig. 7A-B). 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 7A. The γ -phosphate interaction is disrupted in $PhA_{P235A, S238C}$, and polar interactions are observed between the γ -phosphate and the main chain of residues C238 and G234. The loss of P235 and S238 in mutant $PhA_{P235A, S238C}$ could be a reason for the reduced binding affinities of AMPPNP and ATP in $PhA_{P235A, S238C}$ and the $A_{(P235A, S238C)3}B_3D$, $A_{(P235A, S238C)3}B_3DF$ complexes, respectively.

The important role of S238 in facilitating the MgADP inhibition can be concluded from the fact that the related bacterial ATPase from *E. hirae* is not regulated by MgADP as observed recently (Suzuki et al., 2016). This is of interest, since the *E. hirae* enzyme has an alanine residue in the position of the archaeal S238. Furthermore, when the P-loop serine residue of the bacterial ATPase from *T. thermophilus* was substituted to an alanine, the

mutant enzyme showed a decrease in binding affinity of MgADP and a drop of its V_{max} , indicating that one of the step(s) during ATP hydrolysis was affected (Uner et al., 2012).

To understand the importance of the serine residue in more depth, the crystal structure of *PhA*_{P235A,S238C} was superimposed on the sulfate (phosphate analog) bound subunit A structure (PDB: 3I72) (Kumar et al., 2010a) (Fig. 7C-D). As demonstrated, residue S238 of subunit A is in hydrogen bonding with the sulfate via a water molecule (Fig. 7C). In comparison, in the *PhA*_{P235A,S238C} structure, the sulfate is predicted to be directly bound to A235 by polar interactions (Fig. 7D). Therefore, the binding affinity of phosphate might have increased in the *PhA*_{P235A,S238C} mutant as well as in the *A*_{(P235A,S238C)3}*B*₃*D* and *A*_{(P235A,S238C)3}*B*₃*DF* complexes, leading to the delayed release of P_i from the active site of *A*_{(P235A,S238C)3}*B*₃*D* and *A*_{(P235A,S238C)3}*B*₃*DF* complexes, allowing ADP to release before P_i and avoiding the stochastically entrapment of ADP in the active site. In line with this model is the recently determined vanadate (V_i)-bound structure of subunit A of *P. horikoshii* ATP synthase (Manimekalai et al., 2011), demonstrating that V_i had a preferred binding over ADP, and indicating that during ATP synthesis binding of ADP precedes P_i binding. This order is proposed to be reversed during ATP hydrolysis.

5. Conclusion

The data presented demonstrate, that both, P-loop mutations (P235A and S238C) and the presence of subunit F are essential for relieving a 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 at least one of the facts that V-ATPases are dedicated ATP-driven proton pumps and they are regulated by reversible disassembly of the V_1 - from the V_O domain. Since MgADP-inhibition is observed in the rotor enzymes functioning in both directions of ATP synthesis and -hydrolysis, by mimicking the P-loop of V-ATPases in

A-ATP synthase, we could relieve ATP-inhibition, hence making the A-ATP synthase a more efficient hydrolytic enzyme like eukaryotic V-ATPases.

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Figure Legends

Fig. 1. Enzyme kinetics of MmA_3B_3D , MmA_3B_3D and its mutants $A_{(P235A,S238C)}B_3D$ and $A_{(P235A,S238C)}B_3DF$. (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 $\mu\text{mol}/\text{min}/\text{mg}$ protein which is much higher than the ATPase activity of MmA_3B_3D (1.0 $\mu\text{mol}/\text{min}/\text{mg}$ 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. (D) ATP hydrolysis of MmA_3B_3DF and $A_{(P235A,S238C)}B_3DF$ 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 $A_{(P235A,S238C)}B_3DF$. (E) Comparison of ATPase activity of MmA_3B_3DF and $A_{(P235A,S238C)}B_3DF$. $A_{(P235A,S238C)}B_3DF$ 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). (F) Michaelis-Menten plot of MmA_3B_3DF ($\circ$) with $A_{(P235A,S238C)}B_3DF$ ($\square$) at varying concentrations of MgATP (0.1 mM to 6 mM) performed at 37 °C. The calculated K_m and V_{max} of $A_{(P235A,S238C)}B_3DF$ 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.

Fig. 2. Effect of MgADP on the ATP hydrolysis of MmA_3B_3DF (A) and $A_{(P235A,S238C)}B_3DF$ (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 $A_{(P235A,S238C)}B_3DF$ (D)

complexes when pre-incubated with different concentrations of MgADP prior ATP hydrolysis measurements.

Fig. 3. *Purification of the mutants $A_{(P235A,S238C)}B_3D$ and $A_{(P235A,S238C)}B_3DF$ complexes.* (A) Elution profile of complex $A_{(P235A,S238C)}B_3D$ 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 $A_{(P235A,S238C)}B_3DF$ complex. The elution profile was similar to the one of $A_{(P235A,S238C)}B_3D$. The purity of the mutant was analysed on a 17% SDS-gel (shown in *inset*).

Fig. 4. *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 $A_{(P235A,S238C)}B_3D + MmF$.

Fig. 5. *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 . (C) Superimposition of nucleotide bound WT PhA with $PhA_{P235A,S238C}$. The WT PhA structure (*orange*) bound to AMPPNP is superimposed with the nucleotide-free $PhA_{P235A,S238C}$ structure (*cyan*). (D) The superimposed P-loop of WT subunit A-bound AMPPNP with $PhA_{P235A,S238C}$ is zoomed and the bound AMPPNP is shown as sticks. (E) Superposition of the WT PhA structure (*orange*) bound to ADP with the empty $PhA_{P235A,S238C}$ structure (*cyan*). (F) The superimposed P-loop of WT subunit A-bound AMPPNP with $PhA_{P235A,S238C}$ is zoomed and the bound ADP is

shown in sticks. (G) Superimposition of the P-loops of WT *PhA* (*orange*) bound to AMPPNP and *PhA*_{P235A,S238C} (*cyan*), shown in stick conformation. The nucleotide AMPPNP is shown in *grey* spheres.

Fig. 6. *Binding properties of PhA and PhA_{P235A,S238C} to fluorescently labeled nucleotides.* (A) Results of FCS experiments, showing the binding of AMP-PNP-ATTO 647N to *PhA* and *PhA*_{P235A, S238C}. The upper left and lower right insets show the normalized autocorrelation curves of AMP-PNP-ATTO 647N bound *PhA* and *PhA*_{P235A,S238C}, respectively. The curve in the centre displayed complex formation between AMP-PNP-ATTO 647N bound *PhA* (*red*) and *PhA*_{P235A,S238C} (*blue*). The bound protein fraction was plotted against the concentration of protein in μM . The percentage of complex formation for each concentration was calculated using a two-component fitting model and the binding constant, K_D , was derived by fitting the data with the Hill equation. (B) Results of FCS experiments, showing the binding of ADP-ATTO 647N to *PhA* and *PhA*_{P235A,S238C}. The upper left and lower right insets show the normalized autocorrelation curves of ADP-ATTO 647N bound *PhA* and *PhA*_{P235A,S238C}, respectively. The curve in the centre displayed complex formation between ADP-ATTO 647N bound *PhA* (*red*) and *PhA*_{P235A,S238C} (*blue*).

Fig. 7. *Structural model of AMPPNP- and sulphate-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 wt 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. (C) The P-loop of subunit A (*magenta*) from *P. horikoshii* A-ATP synthase bound to sulfate (stick representation) (PDB: 3I72) (Kumar et al., 2010a). The sulfate molecule interacts with S238 via a water molecule (*grey*, sphere). (D) The P-loop of subunit A mutant (*PhA_{P235A,S238C}*) superposed on subunit A and modelled to show the binding of sulfate molecule. The sulfate here interacts with the A235 directly by polar interaction.

Table 1: ATPase kinetic values of MmA_3B_3D , MmA_3B_3DF and their mutant forms

Protein	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
$MmA_{(P235A, S238C)}_3B_3D$	0.15 ± 0.04	22.8 ± 0.5
$MmA_{(P235A, S238C)}_3B_3DF$	0.95 ± 0.07	32.9 ± 0.8

Table 2: Statistics of the *PhA*_{P235A,S238C} structure determination

Wavelength	1.00
Space group	P4 ₃ 2 ₁ 2
Unit cell parameter (Å)	$a = b = 127.46$, $c = 105.34$ $\alpha = \beta = \gamma = 90$ (°)
Resolution range (Å)	30-2.35
Solvent content (%)	62
Total number of reflections	346 584
Number of unique reflections	36 816
I/ σ	24.07 (2.83)
Completeness (%)	99.7 (100)
R-merge (%)	7 (61.9)
Redundancy	9.4 (9.7)

The values in the parentheses is for the last resolution shell (2.43 - 2.35Å)

Refinement Statistics:

R-factor (%)	23
R-free (%)	27
Number of amino acid residues	513
Ramachandran statistics	
Most favored (%)	95.06
Additionally allowed (%)	4.94
Generously (%)	0
Disallowed (%)	0
R.M.S. Deviations	
Bond lengths (Å)	0.006
Bond angles (°)	1.07
Mean atomic B-values	
Overall	73
Wilson B-Factor	61.3
Clash score	5.09
Molprobit score	1.71

Figure 1A-C

Figures 1D-F

Figures 2A-D

Figures 3A-B

(A)

(B)

Figures 4A-B

Figures 5A-B

(C)

(D)

(E)

(F)

Figures 5C-F

(G)

Figure 5G

(A)

(B)

Figures 6A-B

Figures 7A-D