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The stimulating role of subunit F in ATPase activity inside the $A_1$-complex of the $Methanosarcina mazei$ Gö1 $A_1A_0$ ATP synthase

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Abstract

$A_1A_0$ ATP synthases couple ion-transport of the $A_0$ sector and ATP synthesis/hydrolysis of the $A_3B_3$-headpiece via their stalk subunits D and F. Here, we produced and purified stable $A_3B_3$D- and $A_3B_3$DF-complexes of the *Methanosarcina mazei* Gö1 A-ATP synthase as confirmed by electron microscopy. Enzymatic studies with these complexes showed that the *M. mazei* Gö1 A-ATP synthase subunit F is an ATPase activating subunit. The maximum ATP hydrolysis rates ($V_{max}$) of $A_3B_3$D and $A_3B_3$DF were determined by substrate-dependent ATP-hydrolysis experiments resulting in a $V_{max}$ of 7.9 s$^{-1}$ and 30.4 s$^{-1}$, respectively, while the $K_M$ is the same for both. Deletions of the N- or C-terminus of subunit F abolished the effect of ATP hydrolysis activation. We generated subunit F mutant proteins with single amino acid substitutions and demonstrated that the subunit F residues S84 and R88 are important in stimulating ATP hydrolysis. Hybrid formation of the $A_3B_3$D-complex with subunit F of the related eukaryotic V-ATPase of *Saccharomyces cerevisiae* or subunit ε of the F-ATP synthase from *Mycobacterium tuberculosis* showed that subunit F of the archaea and eukaryotic enzymes are important in ATP hydrolysis.
1. Introduction

Unlike energy conservation mechanisms in eukaryotes and bacteria, the metabolism in archaea, like methanogens, is coupled to the generation of an H\(^+\)- and/or Na\(^+\)-gradient across the membrane, and both ion gradients drive the synthesis of ATP \([1]\). The A\(_1\)A\(_O\) ATP synthase, catalyzing the synthesis of ATP, is composed of nine subunits in a proposed stoichiometry of A\(_3\)B\(_3\):C:D:E\(_2\):F:G\(_2\):a:c\(_x\) (Fig. 1). Whereas the related F\(_1\)F\(_O\) ATP synthases in prokaryotes and eukaryotes catalyze ATP synthesis at the expense of an electrochemical ion gradient, the evolutionary related eukaryotic V\(_1\)V\(_O\) ATPases function as ATP-driven ion pumps, unable to synthesize ATP under physiological conditions \([2-5]\). Although the cellular function of archaeal ATP synthases is to synthesize ATP by ion gradient-driven phosphorylation, they also work as ATP-driven ion pumps to generate an ion gradient under fermentative conditions \([1,2]\).

Like bacterial F-ATP synthases (α\(_3\)β\(_3\):γ:δ:ε:a:b\(_2\):c\(_{9-15}\)) and eukaryotic V-ATPase (A\(_3\)B\(_3\):C:D:E\(_3\):F:G\(_3\):H:a:c\(_X\):c\(_Y\):c\(_Z\):d:e), the A-ATP synthases possess a water-soluble (A\(_1\)) sector, containing the catalytic sites, and an integral membrane (A\(_O\)) domain, involved in ion translocation \([2-5]\) (Fig.1). The catalytic A\(_3\)B\(_3\)-center of the A\(_1\)-sector is connected to the A\(_O\)-part by two peripheral stalks, composed of subunits E and G, and a central stalk which consists of the subunits C, D and F \([6,7]\). Coupling of ATP synthesis/hydrolysis in the α\(_3\)β\(_3\)- or A\(_3\)B\(_3\)-headpieces with ion-transport in the F\(_O\)- (F-ATP synthases) or V\(_O\)/A\(_O\) parts of V-ATPases and A-ATP synthases, respectively, occurs via the central stalk subunits γ and ε (F-ATP synthases) or the related subunits C, D and F (V-ATPases, A-ATP synthases) \([2-5]\).

The 101 amino acids subunit F of the *Methanosarcina mazei* Gö1 A-ATP synthase (MmF) exhibits a distinct two-domain structure in solution, with the N-terminal globular region having 78 residues and the residues 79-101 forming the flexible and positively charged C-terminal part (\([8,9]\); Fig. 1). The flexible, C-terminal tail enables this subunit to
undergo up and down movements relative to the nucleotide-binding subunit B [8-11]. In the entire A-ATP synthase subunit F can be cross-linked in a nucleotide-dependent way to subunits B through their C-terminal domains [8,12].

In the A-ATP synthase subunit F is in close contact to the central stalk subunit D ([2,9]; Fig. 1A). The four-stranded β-sheet in the N-terminal part forms a hydrophobic surface that mediates the interaction of both subunits ([9]; Fig. 1B). The positively and negatively charged surface at the bottom of the N-terminal domain of subunit F is oriented toward the central stalk subunit C - a subunit absent in F-ATP synthases - and thereby toward the membrane side [2,9]. As described recently, subunits D, C and F form a cross-linked product inside the A1 ATPase [8,12], giving the central stalk a significant length of 84 Å in solution [13].

Besides the structural and biochemical characteristics, detailed information about the mechanistic function of subunit F of methanogenic A-ATP synthases is lacking. Here, we show for the first time that subunit F activates ATP hydrolysis in the A3B3-headpiece. Single mutation and truncated forms of subunit F reveal that the N- and C-termini of subunit F couple the ATPase activating effect.

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 the A₂B₃D- and A₂B₃DF-complex

The A-ATP synthase genes *ahaA*, *ahaB*, and *ahaD*, encoding the subunits A, B and D of *M. mazei* Gö1, were cloned into the pGEM-4Z vector [14] with *XbaI* and *KpnI* restriction sites at the 5'- and 3'-end, respectively. The clone was kindly provided by Prof. V. Müller (Johann-Wolfgang Goethe University, Frankfurt, Germany).

For the cloning of A₂B₃DF, the fragment containing genes *ahaA*, *ahaB*, *ahaF*, and *ahaD* were synthesized and provided by DNA 2.0 in the vector pJ367 (in-house cloning vector). The fragment was further subcloned into pGEM-4Z [14].

All constructs contained a His₈-tag at the N-terminus of subunit A and the respective proteins were produced in *E. coli* DK8 cells. Protein complex production and Ni-NTA chromatography for A₂B₃D was performed as described previously [15]. In the purification protocol presented the A₂B₃D containing fractions, which eluted from the Ni-NTA affinity column after addition of 100 mM to 400 mM imidazole, were pooled and diluted with 50 mM Tris/HCl, pH 7.5 to a final salt concentration of 50 mM NaCl, and applied to an anion-exchange chromatography column (Resource™Q, GE-Healthcare). After washing the column with increasing salt concentrations the protein was finally eluted at 200 mM NaCl. Protein containing fractions were pooled, concentrated (spin concentrator, molecular cut-off: 100 kDa, Millipore) and applied to a size exclusion column (Superdex 200 HR 10/30, GE-Healthcare). The protein complex was eluted with buffer A (50 mM Tris/HCl, pH 7.5, 200 mM NaCl, 0.8 mM DTT). The purity of the samples was checked with SDS-PAGE analysis [16].

Cells containing recombinant A₂B₃DF were lysed on ice by sonication for 3 x 1 min in buffer B (50 mM Tris/HCl, pH 7.5, 200 mM NaCl, 1 mM PMSF and 2 mM PefablocSC (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride) (BIOMOL)). Cell debris was separated by centrifugation at 10,000 x g for 35 min. The supernatant was then filtered (0.45 µm;
Millipore), and proteins were allowed to bind to a Ni\(^{2+}\)-NTA matrix. Bound proteins were eluted with an imidazole gradient (0 - 600 mM) in buffer B. Fractions containing A\(_3\)B\(_3\)DF were identified by SDS-PAGE [16], pooled together and diluted with 50 mM Tris/HCl (pH 7.5) and 0.8 mM DTT to reduce the salt concentration to 50 mM NaCl and applied onto an anion exchange column (Resource Q, 6 ml, GE-Healthcare). The A\(_3\)B\(_3\)DF-complexes eluted with a NaCl concentration of 200 mM. These fractions were concentrated and applied onto a Superdex 200 HR 10/30 column (GE-Healthcare). The pure protein was eluted with buffer A.

2.3 Cloning and purification of subunit F and its mutant forms

To obtain *M. mazei* Gö1 subunit F mutant F(R88L), the forward 5'-GCTCATGCCATGGAGTTAGCAGTGATCGGAAAGACGAATTC-3' and the reverse primer

5'-GCCCGAGCTCTTTACTTCCACAGATCAACACCTACCGCTTTGTTTTTATTTTTTCAA GTAAACTCGTGGAGCC-3' were used for the amplification of the subunit F insert in pET9d1 [17] as a template. The subunit F mutant K92L, F(K92L), was cloned using the forward primer 5'-GCTCATGCCATGGAGTTAGCAGTGATCGGAAAGACGAATTC-3' and the reverse primer

5'-GCCCGAGCTCTTTACTTCCACAGATCAACACCTACCGCTTTGGAAGTATTTTTTCTC TTAAACTCGTGGAGCC-3'. The subunit F mutant S84A, F(S84A) was generated by overlap extension PCR using the forward primer 5'-GCTCATGCCATGGAGTTAGCAGTGATCGGAAAGACGAATTC-3' and the reverse primer 5'-GCCGAGCTCTTTACTTCCACAGATCAACACCTACCGCTTTGGAAGTATTTTTTCTC TTAAACTCGTGGAGCC-3', two internal primers 5'-AGCGGATCAGGCGCGACGAGTTTAAGA-3' (forward primer) and 5'-TCTTAAACTCGTGCACGCCCTGATCCGCT-3' (reverse primer) were used for the amplification of the gene.
C-terminal truncated subunits F₁₋₈₇, F₁₋₉₁ and F₁₋₉₅ were amplified with the forward primer 5'-GCTCATGCCATGGAGTTAGCAGTGATCGGAA-3' and reverse primers 5'-TTACGAGCTCTTTATAAAACTCGTTGAGCCTGATCCGCT-3', 5'-CTACGAGCTCTTTATATTTTTCTCTTAACCTCGTTGAGGCTGATCCGC-3' and 5'-ACAGGAGCTCTTTATACCGCTTTATTTTTCTCTTAACCTCG-3', respectively.

The N-terminal truncated subunit F₅₋₁₀₁ was amplified using the forward primer 5'-GAGTCCATGGTGATCGGAAAGAGCGAATTC-3' and the reverse primer 5'-GCCAGAGCTCTTTACTTCCACAGATCAACACCTACC-3'. The mutants and the truncated F subunits were cloned into the pET-9d1 vector [17] via their NcoI and SacI restriction sites and transformed into E. coli DH5α cells for plasmid amplification and purification. Verified plasmids were transformed into E. coli BL21 (DE3) cells (Stratagene) for protein production.

Cells, containing the plasmid for the respective subunit F mutant, were grown in 30 μg/ml kanamycin-containing LB-medium at 37 °C. Protein production was induced by adding 1 mM of isopropyl-β-D-thiogalactopyranoside at an OD₆₀₀ of 0.6. Cells were harvested 4 h after induction, frozen by liquid nitrogen and stored at -80 °C until further use.

Subunit F and its mutants were purified using Ni-NTA chromatography as described recently [9]. Afterwards, fractions containing protein were pooled, concentrated (spin concentrator, molecular cut-off: 3 kDa, Millipore) and applied to a size exclusion (Superdex 200 HR 10/30, GE Healthcare) column. The protein was eluted with buffer C containing 50 mM Tris/HCl, pH 8.5, and 100 mM NaCl.

2.4 Electron microscopy and 2D image analysis of M. mazei Gö1 A₃B₃D and A₃B₃DF

A₃B₃D and A₃B₃DF protein complexes were diluted to a final concentration of 36 μg/ml in buffer A. 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 A₃B₃D and A₃B₃DF subcomplexes, respectively. A total of 3619 particles for A₃B₃D and 1360 particles for 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 [18].

2.5 Reconstitution of M. mazei Gö1 sub-complex A₃B₃D with subunit F

For reconstitution of M. mazei Gö1 A₃B₃D with subunit F freshly purified A₃B₃D was incubated with a 3-fold molar excess of freshly purified wild type (WT) or mutant subunit F at 4 °C for 4 h in 50 mM Tris/HCl, pH 7.5, 100 mM NaCl and 0.4 mM DTT, followed by size exclusion chromatography (Superdex 200 HR 10/30, GE-Healthcare) and subsequent ATP hydrolysis activity measurements.

2.6 ATP hydrolysis assay

A continuous ATP hydrolysis assay was applied to measure the specific ATP hydrolysis activity of all 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. The change in absorbance was measured for 250 s in 2 s intervals at 37 °C after adding 50 µg 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.
2.7 Densitometric analysis of the wild type and reconstituted A₃B₃DF-complexes

Reconstitution of *M. mazei* Gö1 A₃B₃D with wild-type F, F(R88L), F₁₈₇ and F₅₋₁₀₁, respectively, was performed as mentioned in the section 2.5. After elution from the size exclusion column (Superdex 200 HR 10/30, GE-Healthcare) the reconstituted complexes were applied on a 17% SDS-gel and 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 [19]. The intensity of subunit F is given relative to subunit D, which in case of the wild-type A₃B₃DF complex is 1:03.

3. Results

3.1 Isolation and EM-analysis of the *M. mazei* Gö1 A₃B₃D- and A₃B₃DF-complexes

The modification and new purification protocol of the *M. mazei* Gö1 A₃B₃D- and A₃B₃DF-complexes resulted in highly pure complexes as shown by SDS-PAGE analysis (Fig. 2A-D). Electron microscopy was performed to visualize the proper complex formation of both proteins. A typical electron microscopy raw image of the negatively stained *M. mazei* Gö1 A₃B₃D- or A₃B₃DF-complex yielded homogenous particles with almost no contamination by smaller particles (Fig. 2E). 3619 particles of A₃B₃D were selected and a 2D-self-organizing map was calculated (Fig. 3A). The 2D-classes are mainly composed of the top and the bottom views, revealing the hexagonal pattern of the alternating nucleotide-binding subunits A and B (Fig. 3A-B). The projection of class 11 (Fig. 3A) reveals a bottom view of A₃B₃D, where apart from the six masses of the nucleotide-binding subunits, a seventh mass is visible in the middle of the hexagonal pattern, which is mainly formed by the central stalk subunit D. The highlighted projection in Figure 3B represents a top view of A₃B₃D with the alternating bigger masses of subunit A, composed of the additional 90 amino acid (Non-
homologous region) of the N-terminus of subunit A [20,21]. The top view reveals a clear ‘crown’ like arrangement of subunits A and B.

Similarly, for *M. mazei* Gö1 A3B3D 1360 particles were selected, processed and 26 2D-classes were generated (Fig. 3C). These classes show a similar hexagonal pattern for the subunits A and B with a seventh massed made-up of the stalk subunits D and F. The data presented confirm that stable, highly pure and enzymatically active A3B3D- and A3B3DF-complexes were produced.

### 3.2 Kinetic properties of the *M. mazei* Gö1 A3B3D- and A3B3DF-complexes

Bulk substrate-dependent ATP hydrolysis experiments with varying concentrations of 0.025 - 4 mM ATP were performed to determine the ATP hydrolysis activity and consequently the maximum rate ($V_{\text{max}}$) and the Michaelis-constant ($K_M$) of the complexes (Fig. 4A-C). Prior to this, we confirmed that the specific activity is independent of the protein concentration in the assay (data not shown). In the following we used 50 µg of protein to measure the ATP hydrolytic activity in the enzymatic assay. At 2 mM Mg-ATP (ratio 1:1) the *M. mazei* Gö1 A3B3D- and A3B3DF-complexes revealed an ATPase activity of about 1.2 and 4.6 U/mg protein (Fig. 5C), respectively, indicating the stimulating effect of subunit F in the catalytic process of ATP hydrolysis in the enzyme. The calculated $V_{\text{max}}$ of *M. mazei* Gö1 A3B3D (1.3 U/mg protein or $7.9 \pm 0.3$ s$^{-1}$) is four-times slower than the $V_{\text{max}}$ of *M. mazei* Gö1 A3B3DF (4.8 U/mg protein or $30.4 \pm 0.9$ s$^{-1}$), while the $K_M$-values were the same for both (0.19 ± 0.04 mM and 0.15 ± 0.02 mM, respectively). We calculated the second order binding rate constant $V_{\text{max}}/K_m$ for both complexes and found that *M. mazei* Gö1 A3B3DF ($2.0 \cdot 10^5$ M$^{-1}$s$^{-1}$) is catalytically five-times more effective than A3B3D ($0.4 \cdot 10^5$ M$^{-1}$s$^{-1}$).
3.3 Importance of the N- and C-termini of subunit F in ATPase activity

The ATP synthase/ATPase family members, A- and F-ATP synthases and V-ATPase have structural similarities, composed of a catalytic portion (A/F/V₁) and a membrane-embedded portion (A₀/F₀/V₀) [2,3]. Presently, it is unclear how the individual subunits may independently affect the interplay in ion-pumping and/or energy conversion. As demonstrated above, subunit F of *M. mazei* Gö1 A₁A₀ ATP synthases stimulates ATP hydrolysis activity in A₃B₃DF by a factor of four. The C-terminus of subunit F is flexible, enabling it to switch between an elongated and retracted state [9-11]. Here, we have used the recombinant form of subunit F(WT) [8], the truncated forms F₁₋₈₇, F₁₋₉₁ and F₁₋₉₅, and the subunit F mutant proteins F(S₈₄A), F(R₈₈L), F(K₉₂L), F(W₁₀₀I) and F(W₁₀₀Y) (Fig. 5A), to investigate the role and the critical residues of the C-terminal region of subunit F in a reconstituted A₃B₃DF complex. Residues R₈₈ and K₉₂ belong to the sequence ⁸⁶SLREKIKQA⁹⁴, revealing a propensity to form a helical structure [9,11]. S₈₄ was found to interact with the phosphate moiety of ATP [11] and residue W₁₀₀ has been identified to interact with the adenine ring of ATP [11].

For reconstitution, *M. mazei* Gö1 A₃B₃D and recombinant subunits F were incubated for 4 hours, followed by size exclusion chromatography (Superdex 200 HR 10/30, GE-Healthcare) and subsequent ATP hydrolysis activity measurements. As shown by the respective gel of the eluted protein sample a reconstituted A₃B₃D+F(WT)-complex with the correct subunit stoichiometry was formed (Fig. 5B). The ability of A₃B₃D and F to form a stable complex is also reflected by fluorescence spectroscopy data (FCS), revealing that the binding of subunit F to A₃B₃D reaches its saturation point already at 100 nM A₃B₃D (Supplementary Figure 1). The estimated *Kd* from the fit with the Hill equation resulted in a value of about 2 nM (Supplementary Figure 1).

The truncated forms of subunit F, F₁₋₈₇, F₁₋₉₁ and F₁₋₉₅, and mutant proteins F(S₈₄A), F(R₈₈L), F(K₉₂L), F(W₁₀₀I) and F(W₁₀₀Y) were incubated with A₃B₃D following the
same protocol, respectively. ATPase activities of 3.4, 3.1 and 3.2 U/mg protein were observed for the reconstituted A₃B₃D+F(K92L), A₃B₃D+F(W100I) and A₃B₃D+F(W100Y) mutant forms (Fig. 5C), respectively, which are similar to the reconstituted wild-type A₃B₃D+F(WT)-complex (see above). In comparison, reconstitution of A₃B₃D with the C-terminal truncated F₁₋₈₇, F₁₋₉₁ and F₁₋₉₅ did not reveal an increase of ATPase activity (0.6, 1.0 and 1.6 U/mg protein, respectively). FCS experiments have been done, to prove whether the truncation of the C-terminus of recombinant F₁₋₈₇ may alter significantly the binding to A₃B₃D. Like for the A₃B₃D+F(WT)-complex, binding of F₁₋₈₇ to A₃B₃D reached its saturation point already at low A₃B₃D concentration (Supplementary Figure 1), and the estimated $K_d$-value was determined to be 32 nM. Therefore, the data above indicate the importance of the C-terminus of subunit F for ATP hydrolysis stimulation (Fig. 5C-D). This is confirmed by the 20% drop in hydrolytic activity of the A₃B₃D+F(R88L) mutant protein (2.6 U/mg protein; Fig. 5C). As shown in the densitometric comparison of the bands in the wild-type A₃B₃DF- and the reconstituted A₃B₃D+F(R88L)-complex, the subunit D:F ratio is correct for both complexes, indicating that the R88L mutation in subunit F did not alter the binding and the drop in activity is caused by the mutation. These data are of interest since F(R88) is part of a peptide sequence, which forms a zero-length cross-link with the nucleotide-binding subunit B in the related entire Methanococcus jannaschii A-ATP synthase in the presence of MgAMP-PNP or MgATP [8]. Substitution of serine to alanine in F(S84A) caused a slight increase in activity (3.6 U/mg protein) in the reconstituted A₃B₃D+F(S84A)-complex.

Recent NMR-experiments of M. mazei Gö1 subunit F showed that the N-terminal residues have a degree of conformational mobility [9], and have been proposed to be in vicinity with subunit D, leading to further alterations of the nucleotide-binding sites A and B via the N- and C-terminal helices of subunit D. Therefore, a recombinant F₅₋₁₀₁ has been
generated, which missed the first four amino acids (Fig. 5A), and enabled reconstitution of an A$_3$B$_3$D+F$_{5,101}$-complex in the right stoichiometry (Fig. 6C). When compared to the WT-complex, the hydrolytic activity of the A$_3$B$_3$D+F$_{5,101}$-complex was reduced to 1.5 U/mg protein (Fig. 5C), showing the importance of the four N-terminal residues for proper ATP-hydrolysis activity.

3.4 Effect of the related coupling subunit of V-ATPase and F-ATP synthase

Subunit F of the eukaryotic V-ATPase shares a sequence (<23%) and structural similarity to subunit F of A-ATP synthases [22]. Here, we took advantage of the recombinant subunit F of Saccharomyces cerevisiae V-ATPase (ScF) [22] to generate an A$_3$B$_3$DF-ScF-hybrid complex and to investigate the effect of eukaryotic ScF in ATPase activity. As shown in Figure 7A an A$_3$B$_3$DF-ScF(WT)-hybrid complex in correct stoichiometry was generated, and an ATP hydrolytic activity of 1.6 U/mg protein was measured (Fig. 7D). The successful hybrid formation demonstrates nicely the likeness of the archaea and eukaryotic proteins and that both subunits cause an increase in ATPase activity of the A-ATP synthase. In analogy to the C-terminal truncated F$_{1,87}$ the homologue C-terminal truncated ScF$_{1,94}$, whose crystallographic structure has been determined recently [22,23], was incubated with A$_3$B$_3$D. As shown by the elution profile and SDS-PAGE (Fig. 7B), no A$_3$B$_3$DF-ScF$_{1,94}$-hybrid complex could be assembled, which was also reflected by the ATPase activity of 1.2 U/mg protein, which is similar to the one of A$_3$B$_3$D (Fig. 7D). The data indicates that in contrast to the F$_{1,87}$ truncation the deletion of the C-terminus of the V-ATPase subunit F prevents formation of an A$_3$B$_3$DF-ScF$_{1,94}$-hybrid complex.

To obtain more details about the specificity of ATPase activation and assembly formation of A$_3$B$_3$DF we studied the possible reconstitution of the related coupling subunit ε from F-ATP synthases, in particular ε of Mycobacterium tuberculosis (Mtc), described to play an
essential role in ATP hydrolysis and -synthesis of the MtF-ATP synthase [24,25]. Following
the same protocol as for the complexes described above, even with a 5:1 molar ratio of
Mtε:A3B3D, the elution profile, SDS-PAGE and ATPase measurement revealed that Mtε and
A3B3D did not form a complex (Fig. 7C). In line with this we found the hydrolytic activity of
the peak fraction obtained from the size exclusion chromatography to be the same as for
A3B3D alone (1.1 U/mg protein; Fig. 7D).

4. Discussion

4.1 The elements of subunit F that are responsible for ATPase activity stimulation

Subunit F (101 residues) in solution exhibits a well ordered N-terminal domain (78
residues) and a flexible C-terminal part [9]. The data presented here demonstrate that the
entire subunit F increases ATPase activity in the A3B3DF-complex of the M. mazei Gö1 A-
ATP synthase. Comparison of the hydrolytic activity of the reconstituted A3B3D with subunit
F or its C-terminal truncated forms F1-87, F1-91 and F1-95 revealed that the C-terminal domain is
mainly responsible for the increase in hydrolytic activity of the enzyme (Fig. 5C).
In particular the deletion of the C-terminal peptide 96GVDLWK101 caused a decrease of
50% in hydrolytic activity. It was shown earlier by intrinsic tryptophan fluorescence
spectroscopy that residue W100 of subunit F is spatially close to residue W430 of subunit B
[9], which allows π-π interactions of the two indole-rings. In addition, molecular dynamics
studies revealed intermolecular interactions between the side chains of F(V95) and B(K399),
F(K101) and B(Q440), and between the backbone and side chains of F(K101) and B(G442)
[10]. However, these interactions occur only when subunit F is in its extended form, and not
in the presence of MgADP or MgADP+Pi, due to a relative movement of subunit F [8].
Furthermore, the deletion mutants F1-87 and F1-91 demonstrate that the secondary structure of
the α-helical peptide 88REKIKQAV95 is essential for proper ATPase activity. Superposition
of *M. mazei* Gö1 subunit F of the related V-ATPase from yeast (ScF) as well as the C-terminal truncated subunit F, ScF₁₋₉₄, revealed that the structure of the N-terminal domain is not altered due to truncations [22,23], reflecting that the loss of stimulation is not caused by structural changes in F₁₋₈₇, F₁₋₉₁ and F₁₋₉₅.

The data presented demonstrate that subunit F of the eukaryotic V-ATPase also activates ATP hydrolysis of the related archaea A₁ complex, but to a lower degree than subunit F. The reconstitution experiment of A₁B₃D with ScF₁₋₉₄ underlines the need of the C-terminus of the archaeal subunit F for proper assembly and enzyme activity. The specificity of the A-ATP synthase subunit F for function and complex formation is supported by the fact that it was not possible to form a complex between A₁B₃D and Mte, a subunit involved in coupling and rotation of bacterial F-ATP synthases [26-30].

When the solution structure of subunit F is superimposed on the crystal structure of the subunit DF-heterodimer of the related *Enterococcus hirae* (Eh) ATPase, the peptide^{88}VSIS^{91} in the β-hairpin region of the subunit EhD, proposed to be involved in coupling [31], is in close proximity to the very N-terminal residues^{1}MEL^{3} and the peptide^{46}GILVMHND^{53} of subunit F (Fig. 8A). Recent direct measurements of backbone dynamics including $R_1$ and $R_2$ and two dimensional $^1$H-$^{15}$N heteronuclear NOE experiments of subunit F showed that the subunit F peptides^{1}MEL^{3} and^{46}GILVMHND^{53} have a degree of conformational mobility [9]. Such mobility and close proximity indicate that the residues^{1}MEL^{3} and/or^{46}GILVMHND^{53} of subunit F may affect the movement of the β-hairpin in subunit D, leading to further alteration in the N- and C-terminal helices of subunit D, which are close to the catalytic interfaces of the subunits A and B in the A₁B₃DF-complex. This is in line with the present data, showing the importance of the peptide^{1}MELA^{4} for proper hydrolytic activity of an A₁B₃DF-complex, as its deletion does not increase ATP hydrolysis in a fully reconstituted A₁B₃D+F₅₋₁₀₁-complex (Fig. 5C).
4.2 Communication of the C-termini of subunits B and F

Subunit B, which has also been described as a regulatory subunit, binds MgATP with a two-fold higher affinity compared to MgADP [32]. These binding affinities of MgATP and MgADP are similar to those observed for the related α subunit in F-ATP synthases [33]. It has been suggested that the positive charge of the P-loop residue H156 of subunit B, the counterpart of the conserved lysine residues in the P-loop of F-ATP synthases, might cause this effect [32], which is supported by mutagenesis of a histidine residue at a corresponding position of the related V-ATPase from *Saccharomyces cerevisiae* (175SASGLPHN182), leading to about 25% loss of hydrolytic activity and proton translocation [34]. Crystallography, fluorescence spectroscopy and molecular dynamics simulations [10,11,35] have shown that binding of ATP to subunit B passes through two intermediate states (site 1 and site 2) to its final binding site 3 (Fig. 8B). Molecular dynamics simulations revealed that upon binding of the C-terminus of subunit F to the C-terminus of subunit B the nucleotide migrates from site 1 via site 2 to its final binding site 3. When the nucleotide occupies site 2, the complex assumes a transition state like character and the large stabilization of site 3 occupation, compared with site 1 occupation, appears to drive the reaction forward, i.e. the migration of ATP from site 2 to site 3 [11].

Inside the multi-subunit enzyme, the C-terminus of subunit F forms a cross-link with the nucleotide-binding subunit B in the presence of MgATP or MgAMP-PNP as shown in the A3B3DF-complex of the A1A0 ATP synthase from *M. mazei* Göl1 [12] and *M. jannaschii* [8], respectively. The zero-length cross-link is formed via the C-terminal peptide of subunit F and the C-terminal region of subunit B ([8], Fig. 8B), which is at a similar position to the so-called DELSEED-region of the nucleotide-binding subunits α and β of the F1F0 ATP synthases [26]. The 20% drop in hydrolytic activity of the A3B3D+F(R88L) mutant protein (Fig. 5C) indicates partial involvement of residue R88 in coupling and confirms that it is at
least one of the critical residues of the cross-link formation described above [8]. The data presented here support also recent MD simulations, revealing that intermolecular interactions arise between the side chains of F(R88) and B(E391) as well as B(E402) [10,11]. In comparison, mutating residue F(S84) increased the ATPase activity slightly by 10%. As shown in the structural model in Figure 8C, the side chain hydroxyl oxygen of F(S84) and the main chain oxygen of EhD(L146) interact with each other via a hydrogen bond. Altering S84 to an alanine might change the D-F interaction, leading to an increase in hydrolytic activity.

The nucleotide-binding site as well as the catalytic side span the A-B subunit interface. As shown recently [21], the arginine residue 349 of the M. mazei Gö1 subunit B, which is homologous to R373 in subunit α of the mitochondrial bovine F-ATP synthase, is found to play an important role in catalysis by stabilizing the negative charge that develops on the terminal phosphate in a pentacoordinated transition state [32,36]. Furthermore, residue L347 of subunit B inside the catalytic interface is in vicinity of residue K240 of subunit A, both being important in the nucleotide binding process. These interactions of subunit B residues R349 and K347 reinstate the previous claim that mapped the peptide region G336 to R349 binding to 8-N3-3'-biotinyl-ATP [37]. The importance of R349 in subunit B has later been confirmed by the crystallographic structure of the A3B3DF-complex of the Enterococcus hirae V-ATPase, where the closer movement of the homologue R350 residue of subunit B to the γ-phosphate caused the rotation of the AMP-PNP γ-phosphate [38]. Subsequently, the γ-phosphate moved closer to the conserved E261, which is an important residue in ATP hydrolysis. Importantly, the movement of R350 is induced by extensive protein-protein interactions between the DF-ensemble and the C-terminal domains of subunits A and B, whose concerted actions inside the complex have been described above. In light of the importance of subunit B in regulation, ATP hydrolysis and in particular in the transition state, the similar $K_M$ values of the M. mazei Gö1 A3B3D and A3B3DF demonstrate that subunit F
may not affect the binding of ATP to the catalytic active nucleotide-binding site in subunit A. However, the four-times higher $V_{\text{max}}$ of the subunit F bound form of $A_3B_3D$ indicates that subunit F may affect the transition state stabilized by the critical R349 of the *M. mazei* Go1 subunit B and with this, cleavage of ATP to ADP and Pi, and/or the release of the products. In the related F-ATP synthases the cleavage of ATP in the nucleotide-binding site is independent of rotation, while the release of Pi from the active site is a major torque-generating step that drives the 40° rotational substep of the central stalk [39]. Thus, Pi release causes a structural change in the $\alpha_3\beta_3$-headpiece that is transferred to the central stalk subunits $\gamma\varepsilon$. In analogy, Pi release from the $A_3B_3$-headpiece would cause a structural change that causes the rotation of the DF-ensemble.

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5. References


M. Nakanishi-Matsui, M. Sekiya, S. Yano, M. Futai, Inhibition of $F_1$-ATPase Rotational Catalysis by the Carboxyl Terminal Domain of the $\varepsilon$ Subunit, J. Biol. Chem. 289 (2014) 30822-30831.


E. Vasilyeva, M. Forgac, 3'-O-(4-Benzoyl)benzoyladenosine-5'-triphosphate inhibits activity of the vacuolar (H$^+$)-ATPase from bovine brain clathrin-coated vesicles by modification of a rapidly exchangeable, noncatalytic nucleotide binding site on the B subunit, J. Biol. Chem. 271 (1996) 12775-12782.


Figure Legends

**Fig. 1.** Arrangement of the atomic structures of individual subunits in A-ATP synthases shown as Gaussian surfaces. (A) Subunits A (PDB ID 3I4L; orange) and B (PDB ID 2C61; dark green) alternate in the A$_3$B$_3$-hexamer. The NMR structure of subunit F ([9]; PDB ID 2OV6; magenta) and the crystal structures of subunits C (PDB ID 1R5Z; blue) and D (PDB ID 3AON; light yellow) form the central stalk. The structure of subunit a (yellow) was taken from PDB ID 3RRK and the c-ring (wheat) from PDB ID 2BL2. The *T. thermophilus* EG dimer (green) with straight peripheral stalk was taken from PDB ID 3K5B and the kinked second peripheral stalk (red) was modeled with the crystal structure of *P. horikoshii* OT3 subunit E (PDB ID 4DT0) and the NMR structure (PDB ID: 2KK7) along with the *T. thermophilus* G structure. The structurally unknown region of subunit a (345-668) is shown as yellow cylinder. On the right side, an enlarged picture of subunit F is shown. Important residues of the C-terminus of subunit F and the engineered mutations and truncations are labeled and shown in yellow. (B) The charge distribution on the surface of subunit F (PDB ID 2OV6) and subunit D ((PDB ID 3AON) of A-ATP synthases. Red and blue areas are negatively and positively charged areas, respectively. The arrow indicates the very positively charged C-terminus. The hydrophobic area can be seen in grey color. 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.

**Fig. 2.** Purification of A$_3$B$_3$D- and A$_3$B$_3$DF-complexes. Top: Anion-exchange chromatogram of A$_3$B$_3$D (A) and A$_3$B$_3$DF (B). The proteins eluted at 200 mM NaCl. Bottom: Size-exclusion chromatogram of A$_3$B$_3$D (C) and A$_3$B$_3$DF (D). The proteins eluted at about 12 ml. The insets show the respective coomassie stained SDS-gels after size exclusion chromatography. The shaded areas in each graph indicate the portion of the peak volume used for further
experiments. 

(E) Electron micrographs of negatively stained $A_3B_3D$ (left) and $A_3B_3DF$ (right) complexes at tilt angle of 0°.

**Fig. 3.** Visual representation of the $A_3B_3D$-complex. (A) 2D-class averages obtained from 3619 particles depicting various orientations of the $A_3B_3D$ complex. (I) and (II) represents the top and bottom view of the complex, respectively. (B) Comparison of the top and bottom view of $A_3B_3D$ with the assembled model generated by combining subunit A (orange; PDB 3I72) of *P. horikoshii* OT3, subunit B (green; PDB 2C61) of *M. mazei* Göl and subunit D (grey: PDB 3A5C) of *T. thermophilus*. (C) Class averages obtained from 1360 particles of $A_3B_3DF$, highlighted boxes 6 and 22 corresponds to I and III (bottom view), and box 11 represents II (top view), respectively.

**Fig. 4.** ATP hydrolysis of $A_3B_3D$ and $A_3B_3DF$ at varying ATP concentrations. Continuous ATPase activity of 50 µg $A_3B_3D$ (A) or $A_3B_3DF$ (B) measured at different concentrations of MgATP (0.05 to 4 mM). The decrease in NADH absorption at 340 nm is plotted against the progressing time in seconds. (C) Michaelis-Menten plot and kinetic parameters of $A_3B_3D$ (□) and $A_3B_3DF$ (○) at varying concentrations of MgATP (0.025 mM to 4 mM) performed at 37 ºC.

**Fig. 5.** Reconstitution of $A_3B_3D$ with various subunit F mutants. (A) Purity of the subunit F (WT and mutants) analyzed on an SDS-gel. *Lanes 1 and 12 reveal the protein marker (kDa); lanes 2-11* show the recombinant proteins F(R88A), F(K92L), F(W100I), F(W100Y), F(S84A), F(WT), F1-87, F1-91, F1-95, F5-101 respectively. (B) Elution profile (Superdex 200) of the reconstituted $A_3B_3D+F(WT)$-complex. The inset in the figure shows an SDS-gel of the pooled fractions of the reconstituted $A_3B_3D+F(WT)$-complex (shaded area in grey). Sizes of
the subunits are compared to a protein marker (kDa). (C) ATP hydrolysis activities of A3B3D with subunit F, its truncated forms or with single amino acid mutations. (D) After incubation of A3B3D with F1,91 or F1,95, the reconstituted sample was applied onto a Superdex 200 column. In both cases the shaded area (grey) of the major elution peak fractions was pooled and applied on an SDS-PAGE, which is shown in the inset. Lane 1, protein marker (kDa); lane 2, A3B3D+F1,91; lane 3 A3B3D+F1,95.

Fig. 6. Densitometric analysis of the A3B3DF-complex and the reconstituted A3B3D with mutant F forms. (A) The plot represents band intensities of subunits D (left peak) and F (right peak) in the A3B3DF(WT) complex derived from the 17% SDS-gel (inset). Integrals of the peaks were used to determine the ratio of subunit F to D. (B-D) The plots represent the densitometric analysis of the complexes A3B3D+F(R88L)-, A3B3D+F5,101- and A3B3D+F1,87, respectively. Like for the wild-type A3B3DF-complex, the integrals of the respective peaks of subunit D and F show a ratio of 1:0.3, indicating complete complex formation. The band intensities of reconstituted A3B3D+F1,87-complex revealed a ratio of the integral peaks of 1:0.1 for subunits D:F. Taking into consideration that the hydrophobic surface of the four-stranded β-sheet in the N-terminus of subunit F mediates the binding to the complex via the hydrophobic stretch of subunit D (Fig. 1B), we propose that the 1:0.1 ratio of D:F1,87 is caused by the lower overall mass of F1,87 and the loss of its highly rich positively charged C-terminal amino acids (see Fig. 1B), which have a higher affinity towards the negatively charged Coomassie Brilliant Blue G-250 dye molecule used for staining [40].

Fig. 7. Reconstitution of A3B3D with ScF(WT), ScF1,94 or Mtc. (A) After incubation of A3B3D with ScF(WT), the sample was applied onto a Superdex 200 column. The inset in the Figure shows an SDS-gel of the reconstituted complex A3B3D+ScF(WT), which corresponds to the
shaded area (grey) of the major elution peak, which was pooled and applied on the gel (right lane). (B) Final purification with Superdex 200 of the complex after reconstitution of A₃B₃D with ScF₁₋₉₄. The right lane of SDS-gel in the inset corresponds to the shaded area (grey) indicating the elution peak, which was pooled and applied on the gel. The data show that only A₃B₃D is present. The middle lane shows the purified recombinant ScF₁₋₉₄. (C) Final purification with Superdex 200 of the complex after reconstitution of A₃B₃D with Mτε. The gel shows that only A₃B₃D (middle lane) is present in the major peak (large grey area) and unbound Mτε (right lane) eluted as smaller peak (small grey area). The left lane in each gel shows the protein marker (kDa). (D) ATP hydrolysis activity of the hybrid complexes formed by A₃B₃D and ScF(WT), ScF₁₋₉₄ or Mτε.

**Fig. 8. Interaction of subunit F with its neighboring subunits B and D.** (A) When the solution structure of subunit F (PDB ID 2OV6; magenta) is superimposed on the crystal structure of the subunit DF-heterodimer of the related *E. hirae* ATP synthase (PDB ID 3AON; yellow) the peptide 88̶91 VSIS (red) in the β-hairpin region of the EhD, proposed to be involved in coupling [30], is in close proximity to the very N-terminal residues 1̶3 MEL (blue) and the peptide 46̶53 GILVMHND (green) of subunit F. (B) The C-terminus of subunit F forms a crosslink with the nucleotide-binding subunit B in the presence of MgATP or MgAMP-PNP [8]. (B) Binding of ATP to subunit B has been shown to go through two intermediate states (T1 and T2) to its final binding site (F). (C) A zoomed in view of EhD (PdB ID 3AON; light yellow) and F (PDB ID 2OV6; magents) in the A-ATP synthase, showing the interaction between S84 (red) of subunit F and L146 (red) of subunit D via the hydrogen bond (not shown) interaction of side chain hydroxyl oxygen of S84 and the main chain oxygen of L146.
Figure 1A-B
Figure 2A-E
Figure 3A-C
Figure 4A-C
Figure 5A-D
Figure 6A-D
Figure 7A-D
Figure 8A-C