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Overexpression, purification, enzymatic and microscopic characterization of recombinant mycobacterial F-ATP synthase

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Abbreviations: BDQ, Bedaquiline; CTF, contrast transfer function; EGCG, Epigallocatechin gallate; MDR-TB, Multidrug-resistant tuberculosis; PMF, Proton motive force; SQA, Squaramides; TB, Tuberculosis; XDR-TB, Extensively drug-resistant tuberculosis

Abstract

The F-ATP synthase is an essential enzyme in mycobacteria, including the pathogenic *Mycobacterium tuberculosis*. Several new compounds in the TB-drug pipeline target the F-ATP synthase. In light of the importance and pharmacological attractiveness of this novel antibiotic target, tools have to be developed to generate a recombinant mycobacterial F₁F₀ ATP synthase to achieve atomic insight and mutants for mechanistic and regulatory understanding as well as structure-based drug design. Here, we report the first genetically engineered, purified and enzymatically active recombinant *M. smegmatis* F₁F₀ ATP synthase. The projected 2D- and 3D structures of the recombinant enzyme derived from negatively stained electron micrographs are presented. Furthermore, the first 2D projections from cryo-electron images are revealed, paving the way for an atomic resolution structure determination.

1. Introduction

In October 2019, a \$14 Bi Global Fund to fight Tuberculosis (TB), Malaria and Aids was initiated [1] and the WHO reported 1.4 million deaths in the 2018 due to TB, reflecting the need to tackle these important disease [2]. The current treatment of TB mainly utilizes a combination of antibiotics that requires long-term therapy [3]. An additional problem to treat TB lies in the emergence of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) [4,5].

A fundamental feature for the survival of *Mycobacterium tuberculosis* (*Mtb*), is the ability to generate ATP by the F-ATP synthase via oxidative phosphorylation [6]. Bedaquiline (BDQ, Sirturo[®]), which inhibits specifically the mycobacterial F-ATP synthase, brought the enzyme into the focus as an attractive target for TB drug development [7]. A new generation of 3,5-dialkoxypyridine analogues of BDQ with improved potency and physico-chemical properties were developed thereafter [8,9] and characterized [10]. The discovery of squaramides (SQA) [11], compound 5228485 [12] or epigallocatechin gallate (EGCG) [13] as potent ATP synthesis inhibitors underline the relevance of the enzyme as a drug target.

The mycobacterial F-ATP synthase, composed of the subunit $\alpha_3:\beta_3:\gamma:\delta:\epsilon:a:b:b':c_9$, differs from its host homologue [14], the human mitochondrial F-ATP synthase ($\alpha_3:\beta_3:\gamma:\text{OSCP}:\epsilon:a:b:d:c_8:F_6:f:g:e:\text{ATP6}:\text{ATP8}:\text{DAPIT}:6.8\text{PL}$) in subunit composition, stoichiometry and amino acid sequence of their respective homologue subunits [15]. This is reflected by the specific binding of the inhibitors BDQ to the mycobacterial subunits *c* and ϵ [16,17], SQA to the *a-c* interface [11] and EGCG to subunit ϵ [13]. The F-ATP synthase is essential in mycobacteria, which is different in other prokaryotes, and this is attributed to the extraordinarily high amount of ATP required to synthesize the mycobacterial cell envelope [6]. Essentiality of the enzyme is supported by the phenotypes of missense mutations in the mycobacterial ϵ subunit, revealing slower growth rates, an altered colony morphology and

hypersusceptibility to cell wall-acting antimicrobials [13]. Uniquely, the mycobacterial enzyme is unable to establish a significant proton gradient during ATP hydrolysis, and shows low or latent ATPase activity in fast- as well as slow-growing mycobacterial species [18,19]. Recent studies demonstrated that at least the subunits α , γ and ϵ contribute to the suppression of proton pumping and PMF formation [19-21], which is essential as dissipation the PMF is lethal to mycobacteria [6].

Most recent developments in producing a stable recombinant *M. smegmatis* $\alpha_3\beta_3\gamma\epsilon$ complex [22] as well as an active endogenous *M. smegmatis* F_1F_O ATP synthase (*MsF₁F_O* ATP synthase) [23] provided a moderate resolution crystal structure (4 Å) and a low resolution 3D structure from negatively stained images, respectively, with insights into the subunit ensemble and arrangement of this engine. Unraveling the structural details of the entire mycobacterial complex is not only required for identifying the mycobacterial specific structural features of the enzyme, but also to exploit these for the development of new antibacterial agents as well as for medicinal chemistry efforts. Here, we engineered a new construct for the expression, production and purification of an active *MsF₁F_O* ATP synthase. Negative-stained and cryo-electron micrographs reveal the molecular engine with its membrane-embedded F_O domain, the central- and peripheral stalks, and the $\alpha_3\beta_3$ -headpiece, in which ATP synthesis and –hydrolysis occur.

2. Materials and methods

2.1 Construction of vector encoding the *atp* operon of *M. smegmatis*

The *atp* operon encoding the genes of the *MsF₁F₀* ATP synthase (*atpBEFJAGDC*) was amplified from the genomic DNA of *M. smegmatis* (strain mc² 155; GenBank: NC_008596) using the forward primer 5'-TTA AGA AGG AGA TAT ACC ATG GTG CTG GCC GCT GAA GAG GG-3' and the reverse primer 5'-AGC TCG AAT TCG GAT CCC TAG TCG ATC TGG CCG AGG G-3', with the incorporation of overhang sequences that overlap with the vector (underlined). The linearized pYUB1049 vector [24] was prepared by amplifying the vector using the forward primer 5'- GGA TCC GAA TTC GAG CTC CGT C -3' and the reverse primer 5'- CCA TGG TAT ATC TCC TTC TTA AAG TTA AAC AA -3'. Both DNAs were assembled using NEBuilder HiFi DNA Assembly Master Mix (NEB) and transformed into *Escherichia coli* TOP10 cells. The vectors were extracted from the cells and the DNA assembly was confirmed by DNA sequencing (BioBasic). To ease the purification of this complex using affinity chromatography, the vector with the confirmed sequences was used as the template, and the DNA sequences encoding a His₆-tag were introduced to the 5'-end of *atpD* (subunit β) by site-directed mutagenesis using the primers 5'- AGC AGT ATG ATG GTG ATG GTG ATG CAT TCT CTC TTC GCT TCC TGA AAG GGG C -3' and 5'- AGA ATG CAT CAC CAT CAC CAT CAT ACT GCT ACT GCA GAA AAG ACC GCG -3'. The template DNA was then removed from the amplified DNA product by DpnI (NEB) treatment, and the treated DNA sample was transformed into *E. coli* TOP10 cells. The presence of His₆-tag DNA sequences was screened by DNA sequencing, and final full DNA sequencing was performed to confirm the presence of the full *atp* operon.

2.2 Purification of *MsF₁F₀* ATP synthase

The vector encoding the *atp* operon of *M. smegmatis*, was transformed into the *M. smegmatis* strain mc² 4517 [25] for protein production and purification. The cells were grown in an auto-induction medium (ZYP-5052;[26]) supplemented with 0.05% Tween-80 and 50 µg/ml hygromycin at 37 °C for 48 h. Finally, cells were harvested by centrifugation at 4,000 rpm for 20 min at 4 °C. The cells were resuspended in buffer A (50 mM Tris/HCl, pH 7.5, 10 mM MgCl₂, 10% glycerol, 1 mM pheynylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich) and 2 mM Pefabloc^{SC} (BIOMOL)), and disrupted on ice by sonication. The cell lysate was first cleared by centrifugation at 12,500 x g for 25 min, followed by ultracentrifugation at 180,000 x g for 45 min. The resulting pellet was re-suspended in buffer A in the presence of 0.5% DDM (n-Dodecyl-β-D-Maltoside; ThermoFisher Scientific), followed by ultracentrifugation at 180,000 x g for 45 min. The resulting supernatant was incubated with Ni-NTA Agarose (Qiagen) for 1 h at 4 °C. Then, the His₆-tagged enzyme was eluted with an imidazole gradient from 20 mM to 500 mM in buffer B (50 mM Tris/HCl, pH 7.5, 300 mM NaCl, 5 mM MgCl₂, 10% glycerol, 0.1% DDM). The fractions containing the protein were pooled and applied to a size-exclusion column (SuperdexTM 200 10/300 column, GE Healthcare) in buffer C (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 0.05% DDM). Fractions containing the recombinant enzyme were pooled and concentrated using an Amicon Ultra-4 Centrifugal Unit (100 kDa molecular mass cut off; Millipore). The purity of the *MsF₁F₀* ATP synthase was evaluated by polyacrylamide gel electrophoresis (SDS-PAGE; [27]), while the protein concentration was determined with UV absorption at 280 nm using a BioSpec-nano spectrophotometer (Shimadzu). The extinction coefficient and molecular weight of the protein were calculated using the online tool ProtParam [28].

2.3 ATP hydrolysis assay

A continuous ATP hydrolysis assay [18] was used to measure ATP hydrolytic activity of the complex. The effect of trypsin on the ATP hydrolysis of the enzyme was studied following the protocol according to [22]. The complex was incubated with porcine trypsin protease (1 mg/ml; PierceTM) at a molar ratio of 50:1 (w/w) for 10 min at 37 °C. The digestion was terminated with 2-fold molar excess of trypsin inhibitor from soybean (Merck). All data were collected in triplicate.

2.4 Negative-staining EM

MsF-ATP synthase was diluted in buffer C to 20 µg/ml. 4 µl of the sample was applied to a glow-discharged carbon-coated copper grid. The grid was stained with 2% (w/v) uranyl acetate for 1 min and blotted, then air-dried before imaging. Micrographs were collected on a FEI Tecnai T12 transmission electron microscope equipped with a 4 K CCD camera, and operated at 120 kV with magnification of 43,000 x under low-dose condition.

All the image processing steps were performed using the image processing framework, SCIPION v2.0 [29]. The contrast transfer function (CTF) estimation was first performed on the micrograph using CTFFIND4 [30]. Particles were manually picked from raw micrographs using RELION v3.0 [31], and used for 2D classification and 3D reconstruction using Xmipp3 with the algorithm CL2D (Clustering in 2D) [32] and RELION v3.0, respectively. The visualization and superimposition of models were done using UCSF Chimera v1.13.1.

2.5 Cryo-electron microscopy

The *MsF*-ATP synthase was concentrated to 1 mg/ml. 4 µl of the sample were applied to a glow-discharged Quantifoil® 300 mesh R2/1 copper grid coated with 2 nm carbon. The grids were blotted for 2 s at 100% humidity, 4 °C and plunge-frozen using FEI Vitrobot. The

prepared grids were screened on a FEI Talos Arctica TEM equipped with a Falcon3EC detector, and operated at 200 kV with magnification of 78,000 x. Each movie was collected in 40 frames with dose per frame of $1.05 \text{ e}^- \text{ \AA}^2$.

All the data processing steps were performed using RELION v3.0 [31]. The raw movies were first corrected for beam-inducing motion and dose weighted using RELION's own implementation of MOTIONCOR2 [33]. The CTF estimation was performed on the corrected and dose weighted micrographs using CTFFIND4 [30]. The particles were manually picked from 80 micrographs and used for 2D classification. The particles from good 2D classes were selected to build a 3D initial model, which was used as the template in auto-picking mode. Then the particles were extracted for 2D classification.

3. Results and discussion

3.1 Purification of recombinant *MsF₁F₀* ATP synthase

Previously, the endogenous *MsF₁F₀* ATP synthase could be purified and allowed negative-stained EM to be performed [22]. The drawback of this purification was the low yield of the complex and a 54 kDa contamination of the mycobacterial glutamine synthetase. Therefore, with the aims of improving the yield and purity of the enzyme, as well as for performing future mutational studies, recombinant *MsF₁F₀* ATP synthase was constructed and purified. Since the production of soluble mycobacterial proteins in *E. coli* cells was shown to be challenging [34,35], the *M. smegmatis* strain mc² 4517 [25], which utilizes T7 expression system, was selected to produce this complex at good yield. The pYUB1049 shuttle plasmid [24] was chosen to generate the vector that carries the *M. smegmatis* *atp* operon. The plasmid presents a T7 promoter-based vector, which can be induced in an auto-induction medium or by Isopropyl β -d-1-thiogalactopyranoside, and contains *oriE* for replication in *E. coli* and *oriM* for replication in mycobacteria (Fig. 1A). With these features, the vector that carries the desired genes can be prepared in *E. coli*, then the selected vector can be transferred into *M. smegmatis* strain mc² 4517 for high-level protein production. To prepare the construct, the linearized vector (without the N-terminal His-tag, trypsin cleavage site and *cmaA2* gene) and amplified *M. smegmatis* *atp* operon were assembled using a seamless assembly method. The His₆-tag was then introduced to the N-terminus of subunit β (*atpD*) of the successful cloned vector (Fig. 1A). The final vector (pYUB1049-*MsF₁F₀* ATP synthase) (Fig. 1A) was then transformed into *M. smegmatis* strain mc² 4517 for protein production.

The recombinant enzyme was purified using a two-step purification method. Ni-NTA affinity chromatography was employed as the first purification step, and the fractions containing the desired protein were identified through SDS-PAGE, combined and applied

into a gel filtration column. One fraction that corresponded to the highest peak (*grey shaded area* in Fig. 1B) was kept and used only for EM grid preparation, while the top 40% of the peak fractions (*light grey shaded area* in Fig. 1B) were pooled and used for enzymatic assays. The purity of the purified sample was assessed through SDS-PAGE (Fig. 1C). The identity of the subunit b - δ , γ , b' and ϵ was established using MALDI mass spectrometry (Supplementary Tables S1A-D). These studies revealed that the 48 kDa, 34 kDa, 18 kDa, and 13 kDa band corresponds to the peripheral stalk subunits b - δ , b' and the rotary subunits γ and ϵ .

The low ATP hydrolytic activity of membrane- [17,18] and detergent solubilized mycobacterial F-ATP synthase [22] and recombinant F_1 -ATPase [22] were previously described. Even though this protein complex has very low ATP hydrolytic activity, this activity can be stimulated by trypsinolysis as shown for the recombinant F_1 -ATPase [22] and the native MsF_1F_O ATP synthase [23]. Therefore, ATPase activity measurements of the MsF_1F_O ATP synthase were performed after trypsinolysis. As shown in figure 2, recombinant MsF_1F_O ATP synthase revealed an ATP hydrolytic activity of $0.041 \pm 0.001 \mu\text{mol min}^{-1} (\text{mg of protein})^{-1}$ when assayed with 25 μg of the enzyme, which is comparable to the activities determined using inverted membrane vesicles of *M. smegmatis* ($0.0375 \pm 0.0013 \mu\text{mol min}^{-1} (\text{mg of total protein})^{-1}$) [21] and recombinant F_1 -ATPase of *M. smegmatis* ($0.07 \mu\text{mol min}^{-1} (\text{mg of protein})^{-1}$) [22]. After trypsin treatment, the ATP hydrolytic activity of the MsF_1F_O ATP synthase increased 18-fold to $0.76 \pm 0.03 \mu\text{mol min}^{-1} (\text{mg of protein})^{-1}$, which is similar to the reported activity of trypsin-digested native *M. smegmatis* F-ATP synthase ($0.8 \pm 0.1 \mu\text{mol min}^{-1} (\text{mg of total protein})^{-1}$) [23]. The assay and trypsinolysis were repeated with 50 μg of recombinant enzyme and similar results were obtained (Fig. 2B). The results show that the recombinant MsF_1F_O ATP synthase is enzymatically active.

3.2 EM data analysis of negatively stained *MsF₁F_O* ATP synthase

After protein purification, the purified *MsF₁F_O* ATP synthase was applied to the EM grid and stained. The raw micrographs showed that the protein complexes were dispersed on the EM grid, and that the four domains *F_O*, the central- and peripheral stalks (*yellow arrow*) and the *F₁*-headpiece were visible (Fig. 3A), indicating that the whole *MsF₁F_O* ATP synthase was intact. That allowed the micrographs to be used for particle picking. After CTF estimation, 77 out of 89 micrographs were selected for particle picking. 5,500 particles were picked for the 2D classification. To remove the false-positive picked particles or artifacts due to staining, two rounds of 2D classifications were performed. The remaining 3,800 particles were sorted into 10 classes and shown in figure 3B. The densities of the *F₁* and *F_O* parts of the *MsF₁F_O* ATP synthase can be clearly visualized from all 2D averaged classes, as well as the central stalk. The tip part can be clearly seen on top of the *F₁*-headpiece from the 2D averaged classes, which is formed by the δ region of the fused subunits *b*- δ of the peripheral stalk.

Next, a 3D reconstruction of *MsF₁F_O* ATP synthase was generated from the negative stained EMs. First, the initial 3D model was built using all the picked particles. To ensure all projections belonged to a single structure, the initial 3D model was refined with the particles used in last 2D classification, and a negative-stained 3D model (Fig. 3C) with a final resolution of 30 Å (Supplementary Fig. S1) was generated. Overall, the dimensions of the 3D model are approximately 250 x 124 Å (Fig. 3C). The structural model of the mycobacterial *F₁F_O* ATP synthase was computed previously using computational methods [23]. This model superimposed well with the 3D EM model. The volume of the tip part (*white surface* in Fig. 3C) can be accommodated well by the δ -region of subunit *b*- δ .

3.3 Cryo-EM studies of recombinant *MsF₁F₀* ATP synthase

To achieve a good quality grid preparation condition for cryo-EM, different conditions were tested, and the best condition for this complex is as described in Materials and Methods. 611 movies were collected from the selected grid. After beam-inducing motion correction and CTF estimation, the micrographs with unclear or missing thin rings were discarded, and 564 micrographs were selected for further data analysis. To prepare the template for auto-picking step, the particles from 80 micrographs were manually picked and classified into 2D. Then an initial 3D model was built from good 2D classes, which was used as the template to auto-pick particles from all micrographs, resulting in the extraction of 137,932 particles. After elimination of junk particles from five rounds of 2D classification, 29,117 particles were selected for the final 2D classification (Fig. 4). The 2D averaged classes displayed different orientations of the particles that adsorbed to the grid surface, indicating no preferential orientation. The densities of the central stalk, F_1 and F_0 domains of *MsF₁F₀* ATP synthase can be clearly seen from the 2D averaged classes. Importantly, the peripheral stalk (*yellow arrow*, Fig. 4), which is formed by subunit b' and b - δ , became visualized in the 2D averaged classes c1, c3, and c4 (Fig. 4), with the δ region (*magenta arrow*, Fig. 4) attached to the top of the F_1 -headpiece.

Taken together, the data presented, provide a platform for a high-resolution structure determination of this essential complex using cryo-EM in the future in the absence and presence of compounds as well as for structure-activity relationship studies of existing mycobacterial F-ATP synthase inhibitors [7-12] and those being in the development. Furthermore, the genetic engineered construct containing the entire *MsF₁F₀* ATP synthase genes and the developed protocol of the first highly pure recombinant mycobacterial F_1F_0 ATP synthase opens the door for the generation and characterization of mutants to design in future, and to unravel the diversities in regulation of this essential energy converter.

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Reference

- [1] A. Gulland, World leaders heed Macron's call and pledge \$14bn to fight killer diseases, The Telegraph, The Telegraph, London, United Kingdom, 2019.
- [2] WHO, Global tuberculosis report 2019, World Health Organization, Geneva, 2019.
- [3] Z.S. Bhat, M.A. Rather, M. Maqbool, *et al.*, Drug targets exploited in *Mycobacterium tuberculosis*: Pitfalls and promises on the horizon, Biomed. Pharmacother. 103 (2018) 1733-1747. <https://doi.org/10.1016/j.biopha.2018.04.176>.
- [4] N.R. Gandhi, P. Nunn, K. Dheda, *et al.*, Multidrug-resistant and extensively drug-resistant tuberculosis: a threat to global control of tuberculosis, Lancet. 375 (2010) 1830-1843. [https://doi.org/10.1016/S0140-6736\(10\)60410-2](https://doi.org/10.1016/S0140-6736(10)60410-2).
- [5] WHO, Multidrug and extensively drug-resistant TB (M/XDR-TB): 2010 global report on surveillance and response, World Health Organization, Geneva, 2010.
- [6] G.M. Cook, C. Greening, K. Hards, *et al.*, Chapter One - Energetics of Pathogenic Bacteria and Opportunities for Drug Development, in: R.K. Poole (Ed.) Adv. Microb. Physiol., Academic Press 2014, pp. 1-62.
- [7] K. Andries, P. Verhasselt, J. Guillemont, *et al.*, A Diarylquinoline Drug Active on the ATP Synthase of *Mycobacterium tuberculosis*, Science 307 (2005) 223. <https://doi.org/10.1126/science.1106753>.
- [8] A. Blaser, H.S. Sutherland, A.S.T. Tong, *et al.*, Structure-activity relationships for unit C pyridyl analogues of the tuberculosis drug bedaquiline, Bioorg. Med. Chem. 27 (2019) 1283-1291. <https://doi.org/10.1016/j.bmc.2019.02.025>.
- [9] H.S. Sutherland, A.S.T. Tong, P.J. Choi, *et al.*, Structure-activity relationships for analogs of the tuberculosis drug bedaquiline with the naphthalene unit replaced by bicyclic heterocycles, Bioorg. Med. Chem. 26 (2018) 1797-1809. <https://doi.org/10.1016/j.bmc.2018.02.026>.

- [10] J.P. Sarathy, P. Ragunathan, J. Shin, *et al.*, TBAJ-876 retains Bedaquiline's activity against subunits c and ϵ of *Mycobacterium tuberculosis* F-ATP synthase, *Antimicrob. Agents Chemother.* 63 (2019) e01191-01119. <https://doi.org/10.1128/aac.01191-19>.
- [11] S.J. Tantry, S.D. Markad, V. Shinde, *et al.*, Discovery of Imidazo[1,2-a]pyridine Ethers and Squaramides as Selective and Potent Inhibitors of Mycobacterial Adenosine Triphosphate (ATP) Synthesis, *J. Med. Chem.* 60 (2017) 1379-1399. <https://doi.org/10.1021/acs.jmedchem.6b01358>.
- [12] S. Kumar, R. Mehra, S. Sharma, *et al.*, Screening of antitubercular compound library identifies novel ATP synthase inhibitors of *Mycobacterium tuberculosis*, *Tuberculosis* 108 (2018) 56-63. <https://doi.org/10.1016/j.tube.2017.10.008>.
- [13] W.-G. Saw, M.-L. Wu, P. Ragunathan, *et al.*, Disrupting coupling within mycobacterial F-ATP synthase subunit ϵ causes dysregulated energy production and cell wall biosynthesis, *Sci. Rep.* (2019) in press. <https://doi.org/10.1038/s41598-019-53107-3>.
- [14] D. Bald, C. Villellas, P. Lu, *et al.*, Targeting Energy Metabolism in *Mycobacterium tuberculosis*, a New Paradigm in Antimycobacterial Drug Discovery, *mBio* 8 (2017) e00272-00217. <https://doi.org/10.1128/mBio.00272-17>.
- [15] J. He, H.C. Ford, J. Carroll, *et al.*, Assembly of the membrane domain of ATP synthase in human mitochondria, *Proc. Natl. Acad. Sci.* 115 (2018) 2988. <https://doi.org/10.1073/pnas.1722086115>.
- [16] G. Biuković, S. Basak, M.S.S. Manimekalai, *et al.*, Variations of subunit ϵ of the *Mycobacterium tuberculosis* F₁F₀ ATP synthase and a novel model for mechanism of action of the tuberculosis drug TMC207, *Antimicrob. Agents Chemother.* 57 (2013) 168-176. <https://doi.org/10.1128/aac.01039-12>.

- [17] L. Preiss, J.D. Langer, Ö. Yildiz, *et al.*, Structure of the mycobacterial ATP synthase F_o rotor ring in complex with the anti-TB drug bedaquiline, *Sci Adv* 1 (2015) e1500106. <https://doi.org/10.1126/sciadv.1500106>.
- [18] A.C. Haagsma, R. Abdillahi-Ibrahim, M.J. Wagner, *et al.*, Selectivity of TMC207 towards Mycobacterial ATP Synthase Compared with That towards the Eukaryotic Homologue, *Antimicrob. Agents Chemother.* 53 (2009) 1290. <https://doi.org/10.1128/AAC.01393-08>.
- [19] A. Hotra, M. Suter, G. Biuković, *et al.*, Deletion of a unique loop in the mycobacterial F-ATP synthase γ subunit sheds light on its inhibitory role in ATP hydrolysis-driven H⁺ pumping, *FEBS J.* 283 (2016) 1947-1961. <https://doi.org/10.1111/febs.13715>.
- [20] S. Joon, P. Ragunathan, L. Sundararaman, *et al.*, The NMR solution structure of *Mycobacterium tuberculosis* F-ATP synthase subunit ϵ provides new insight into energy coupling inside the rotary engine, *FEBS J.* 285 (2018) 1111-1128. <https://doi.org/10.1111/febs.14392>.
- [21] P. Ragunathan, H. Sielaff, L. Sundararaman, *et al.*, The uniqueness of subunit α of mycobacterial F-ATP synthases: An evolutionary variant for niche adaptation, *J. Biol. Chem.* 292 (2017) 11262-11279. <https://doi.org/10.1074/jbc.M117.784959>.
- [22] A.T. Zhang, M.G. Montgomery, A.G.W. Leslie, *et al.*, The structure of the catalytic domain of the ATP synthase from *Mycobacterium smegmatis* is a target for developing antitubercular drugs, *Proc. Natl. Acad. Sci.* 116 (2019) 4206. <https://doi.org/10.1073/pnas.1817615116>.
- [23] N. Kamariah, R.G. Huber, W. Nartey, *et al.*, Structure and subunit arrangement of Mycobacterial F₁FO ATP synthase and novel features of the unique mycobacterial subunit δ , *J. Struct. Biol.* 207 (2019) 199-208. <https://doi.org/10.1016/j.jsb.2019.05.008>.

- [24] G. Bashiri, A.M. Rehan, D.R. Greenwood, *et al.*, Metabolic engineering of cofactor F420 production in *Mycobacterium smegmatis*, PLoS One 5 (2010) e15803-e15803. <https://doi.org/10.1371/journal.pone.0015803>.
- [25] F. Wang, P. Jain, G. Gulten, *et al.*, *Mycobacterium tuberculosis* Dihydrofolate Reductase Is Not a Target Relevant to the Antitubercular Activity of Isoniazid, Antimicrob. Agents Chemother. 54 (2010) 3776. <https://doi.org/10.1128/AAC.00453-10>.
- [26] F.W. Studier, Protein production by auto-induction in high-density shaking cultures, Protein Expression and Purification 41 (2005) 207-234. <https://doi.org/10.1016/j.pep.2005.01.016>.
- [27] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680-685. <https://doi.org/10.1038/227680a0>.
- [28] E. Gasteiger, C. Hoogland, A. Gattiker, *et al.*, Protein identification and analysis tools on the ExPASy server, in: J.M. Walker (Ed.) The Proteomics Protocols Handbook, Humana Press, Totowa, NJ, 2005, pp. 571-607.
- [29] J.M. de la Rosa-Trevín, A. Quintana, L. del Cano, *et al.*, Scipion: A software framework toward integration, reproducibility and validation in 3D electron microscopy, J. Struct. Biol. 195 (2016) 93-99. <https://doi.org/10.1016/j.jsb.2016.04.010>.
- [30] A. Rohou, N. Grigorieff, CTFFIND4: Fast and accurate defocus estimation from electron micrographs, J. Struct. Biol. 192 (2015) 216-221. <https://doi.org/10.1016/j.jsb.2015.08.008>.
- [31] J. Zivanov, T. Nakane, B.O. Forsberg, *et al.*, New tools for automated high-resolution cryo-EM structure determination in RELION-3, eLife 7 (2018) e42166. <https://doi.org/10.7554/eLife.42166>.

- [32] C.O.S. Sorzano, J.R. Bilbao-Castro, Y. Shkolnisky, *et al.*, A clustering approach to multireference alignment of single-particle projections in electron microscopy, *J. Struct. Biol.* 171 (2010) 197-206. <https://doi.org/10.1016/j.jsb.2010.03.011>.
- [33] S.Q. Zheng, E. Palovcak, J.-P. Armache, *et al.*, MotionCor2: anisotropic correction of beam-induced motion for improved cryo-electron microscopy, *Nat. Methods* 14 (2017) 331. <https://doi.org/10.1038/nmeth.4193>.
- [34] Y. Xue, Y. Bai, X. Gao, *et al.*, Expression, purification and characterization of *Mycobacterium tuberculosis* RpfE protein, *J Biomed Res* 26 (2012) 17-23. [https://doi.org/10.1016/S1674-8301\(12\)60003-7](https://doi.org/10.1016/S1674-8301(12)60003-7).
- [35] G. Bashiri, C.J. Squire, E.N. Baker, *et al.*, Expression, purification and crystallization of native and selenomethionine labeled *Mycobacterium tuberculosis* FGD1 (Rv0407) using a *Mycobacterium smegmatis* expression system, *Protein Expression Purif.* 54 (2007) 38-44. <https://doi.org/10.1016/j.pep.2007.01.014>.

Figure legends

Figure 1. Construct design and protein purification of recombinant *MsF₁F_O* ATP synthase.

(A) Vector map of pYUB1049-*MsF₁F_O*. This vector is a Hygromycin-selectable, T7-promoter based vector and consists of *oriE* for replication in *E. coli* and *oriM* for replication in mycobacteria. The full *atp* operon containing *atpBEFJAGDC* was inserted into this vector. Upon induction, the *atp* operon will be expressed to produce subunits *a*, *c*, *b*, *b*- δ , α , γ , β and ϵ , according to the gene arrangement in the operon. A His₆-tag was introduced to the N-terminus of subunit β to use Ni-NTA affinity chromatography. (B) Size exclusion chromatogram of the *MsF₁F_O* ATP synthase. The fraction that corresponded to the highest peak (*grey shaded area*) was used in the EM study, while the top 40% of the peak fractions (*light grey shaded area*) were used for the enzymatic assay. (C) The SDS gel loaded with the sample after size exclusion chromatography. The subunits *b*- δ , γ , *b*' and ϵ were identified through MALDI-TOF/TOF.

Figure 2. ATP hydrolytic activity of recombinant *MsF₁F_O* ATP synthase.

(A) Continuous ATP hydrolytic activity of *MsF₁F_O* ATP synthase was measured at the protein amounts of 25 μ g (*red*) and 50 μ g (*blue*). Without trypsin (*solid line*), the *MsF₁F_O* ATP synthase showed latent ATP hydrolytic activity, and its activity could be stimulated by the presence of trypsin (*dashed line*). (B) Specific ATP hydrolytic activity of the enzyme. Values are means $\pm$ SD based on triplicate determinations.

Figure 3. Negative-stained EM of recombinant *MsF₁F_O* ATP synthase.

(A) EM raw micrograph showed the presence of particles that consist of two visible domains. One of these particles (*green box*) showed clearly the presence of peripheral stalk of F-ATP synthase, and this particle can overlap well with the structural model (*cartoon representation*) of *MsF₁F_O*

ATP synthase generated using computational method [23]. (B) 2D class averages resulting from the picked and screened particles. The δ portion (*magenta arrow*) of the fused subunit b - δ is attached to the top of the F_1 -headpiece. (C) Surface representation of the 3D reconstruction of the complex (*grey; left*) and the overlapped surface with structural model (*cartoon representation*) generated using computational method [23] (*center*) at the side view, and top view (*right*). Its overall dimensions are approximately 250 x 124 Å. The tip volume, which is occupied by the δ domain of subunit b - δ , is highlighted in *white* color.

Figure 4. Cryo-EM of recombinant MsF_1F_O ATP synthase. 2D class averages reveal the densities of the central stalk, the F_1 - and F_O domains, and the peripheral stalk (*yellow arrow*), which can be clearly seen from the 2D average classes c1, c3, and c4. The δ portion (*magenta arrow*) of subunit b - δ is attached to the top of F_1 .

Figures 1A-C

Figures 2A-B

(A)

(B)

Figures 3A-B

Figure 3C

Figure 4

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