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Unique structural and mechanistic properties of mycobacterial F-ATP synthases:

Implications for drug design

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Abstract

The causative agent of Tuberculosis (TB) *Mycobacterium tuberculosis* (*Mtb*) encounters unfavourable environmental conditions in the lungs, including nutrient limitation, low oxygen tensions and/or low/high pH values. These harsh conditions in the host triggers *Mtb* to enter a dormant state in which the pathogen does not replicate and uses host-derived fatty acids instead of carbohydrates as an energy source. Independent to the energy source, the bacterium's energy currency ATP is generated by oxidative phosphorylation, in which the F_1F_0 -ATP synthase uses the proton motive force generated by the electron transport chain. This catalyst is essential in *Mtb* and inhibition by the diarylquinoline class of drugs like Bedaquiline, TBAJ-587, TBAJ-876 or squaramides demonstrated that this engine is an attractive target in TB drug discovery. A special feature of the mycobacterial F-ATP synthase is its inability to establish a significant proton gradient during ATP hydrolysis, and its latent ATPase activity, to prevent energy waste and to control the membrane potential. Recently, unique epitopes of mycobacterial F_1F_0 -ATP synthase subunits absent in their prokaryotic or mitochondrial counterparts have been identified to contribute to the regulation of the low ATPase activity. Most recent structural insights into individual subunits, the F_1 domain or the entire mycobacterial enzyme added to the understanding of mechanisms, regulation and differences of the mycobacterial F_1F_0 -ATP synthase compared to other bacterial and eukaryotic engines. These novel insights provide the basis for the design of new compounds targeting this engine and even novel regimens for multidrug resistant TB.

1. Introduction

During infection, *Mtb* inhabits a wide range of intracellular and extracellular environments, which the pathogen has to address by redirecting its metabolic activity commensurate with either replicative growth or nonreplicative persistence (Rao et al., 2008). A fundamental feature in this adaptation is the ability of mycobacteria to respire, regenerate reducing equivalents, and to generate adenosine triphosphate (ATP) via oxidative phosphorylation by the enzyme ensemble of the electron transport chain (ETC). Mycobacteria harbor dehydrogenases to fuel the ETC, and two terminal respiratory oxidases an *aa3*-type cytochrome *c* oxidase (*cyt-bc1-aa3*) and a bacterial specific cytochrome *bd*-type menaquinol oxidase (*cyt-bd*). These are present for dioxygen reduction coupled to the generation of a proton motive force (PMF, the sum of proton-gradient and the membrane potential), which is finally used for the condensation of ADP + P_i to form ATP by the engine F₁F₀-ATP synthase (F-ATP synthase) (Cook et al., 2014). Despite being obligate aerobes, mycobacteria survive under low oxygen tension (hypoxia), by exiting the cell cycle and entering a dormant state. Hypoxic non-replicating *Mtb* displays a reduced pool of ATP, making them exquisitely sensitive to any further ATP depletion, and susceptible to drugs targeting the maintenance of ATP homeostasis (Rao et al., 2008). This observation suggests that drugs that inhibit oxidative phosphorylation could shorten the time of therapy for drug-resistant tuberculosis which is supported by the clinical use of Sirturo® (bedaquiline, BDQ), an anti-TB drug targeting the *MtF*-ATP synthase (Andries et al., 2005).

In contrast to other prokaryotes, the mycobacterial F-ATP synthase has been shown to be essential for growth and survival, which is important for the identification of novel antitubercular targets and agents (Sasseti et al., 2003). Another special feature of the mycobacterial F-ATP synthase is its inability to establish a significant proton gradient during ATP hydrolysis, and its

low or latent ATPase activity in the fast- or slow-growing form (Haagsma et al., 2010; Hotra et al., 2016). Recent genetic, biochemical and structural approaches identified that at least three subunits (α , γ and ϵ) contribute to the important enzymatic differences of mycobacterial F-ATP synthases in suppression of proton pumping and PMF formation (Ragunathan et al., 2017; Hotra et al., 2016; Joon et al., 2018), which is essential because dissipating the PMF is lethal to mycobacteria (Cook et al., 2014), as well as in ATP formation, employing them as potential drug targets.

Unraveling the structural details of the mycobacterial F-ATP synthase is not only required for identifying the mycobacterial specific structural features of the enzyme, but also to exploit these specific features for the development of new antibacterial agents against the enzyme. Previous attempts have been made to understand the structure of the isolated subunits ϵ and c (Preiss et al., 2015; Joon et al., 2018) (Fig. 1). Most recent developments in producing a stable and enzymatically active recombinant *M. smegmatis* $\alpha_3\beta_3\gamma\epsilon$ complex (referred to as *MsF*₁-ATPase throughout the text (Zhang et al., 2019)) as well as an active endogenous *M. smegmatis* F₁F₀ ATP synthase (*MsF*-ATP synthase) (Kamariah et al., 2019) provided structural insights into the subunit ensemble and arrangement of this engine (Fig. 1). Like other F-ATP synthases, the mycobacterial assembly consists of a water soluble F₁ part that synthesizes ATP and a membrane embedded F₀ part that allows proton translocation from the periplasmic to the cytosolic side. The F₁ part contains the nucleotide-binding subunits $\alpha_3\beta_3$, forming the catalytic and hexameric headpiece, which sits on top of the rotating central stalk subunits $\gamma\epsilon$ (Fig. 1). The F₀ complex (subunits $a:b:b':c_9$) includes a rotary c -ring of nine c subunits (Preiss et al., 2015), where the loop docks to the bottom of the N-terminal domain of subunit ϵ and the globular domain of γ (Hotra et al., 2016; Joon et al., 2018), which both rotate and enable the coupling to the F₁ portion to transfer torque, derived by H⁺-transport via subunits a and c , to the catalytic $\alpha_3\beta_3$ -headpiece. The peripheral stalk subunits b and

b' , whose N-termini are membrane-embedded, extend and become attached via subunit δ to the top of the F_1 headpiece (Kamariah et al., 2019). Together the peripheral- and central stalks establish two connections between the F_1 and F_0 domains.

The discovery of BDQ, which is active against *Mtb* and its multi drug-resistant (MDR) strains, validated *MtF*-ATP synthase for drug development for tuberculosis (Andries et al. 2005; Andries et al., 2014). Most recent medicinal chemistry campaigns resulted in a new generation of 3,5-dialkoxypyridine (DARQ) analogues of BDQ (Tong et al., 2017; Choi et al., 2017; Sutherland et al., 2018; Blaser et al., 2019; Sutherland et al., 2019). In addition, the discovery of squaramides (SQA; Tantry et al., 2019), the compounds 5228485 and 5220632 (Kumar et al., 2018) as potent ATP synthesis inhibitors underline the attractiveness of the mycobacterial F-ATP synthase as a drug target. Therefore, the implementation of specific drug-mediated inhibition of the mycobacterial enzyme could increase efficacy, possibly decrease treatment time of a rational drug combination, and kill MDR- and extensively drug-resistant (XDR) bacterial strains of *Mtb*. In this review, we focus on developments in the understanding of the structure, function and regulation of mycobacterial F-ATP synthases in the past few years, whose outcomes will provide a first step toward structure-based drug design for the development of novel *MtF*-ATP synthase inhibitors.

2. Mycobacterial subunit α reveals a C-terminal extension important in regulation

Nucleotide-binding and ATP synthesis occurs in the interface of a subunit α - β pair. The mycobacterial subunit α (549 amino acid) includes a unique C-terminal stretch of about 3.5 kDa (*Mt*_{514–549}; according to *Mtb* numbering), which is not found in other bacteria, chloroplast or mitochondrial sequences (Ragunathan et al., 2018). Secondary structural prediction revealed that this additional stretch is either entirely unstructured like in *M. smegmatis*, *M. phlei* and *M. ulcerans*,

while in *M. bovis* or *Mtb* (Fig. 2; Zhang et al., 2019) it is proposed to include an α -helix, as shown for the NMR structure of the α -helical residue 526–539 within the C-terminal peptide of *Mtb* (*Mt* $\alpha_{520-540}$) (Ragunathan et al., 2018). These studies open a new window for a specific structure-based design of the *Mt* $\alpha_{514-549}$ stretch, since its deletion decreases drastically ATP-synthesis activity in inside-out vesicles (IMVs) and increases ATP hydrolysis going along with ATP-driven H^+ -translocation (Ragunathan et al., 2018). The studies clearly demonstrated that the C-terminal stretch contributes in part to the latent ATP hydrolysis-, its linked ATP-driven proton-translocation activity and thereby in wasting ATP reserves to alter the PMF. The mechanistic role of *Mt* $\alpha_{514-549}$ was demonstrated by single-molecule rotation experiments, indicating that this C-terminal extension decreases the angular velocity of the power-stroke after ATP binding (Ragunathan et al., 2018). Small angle X-ray scattering (SAXS) data of the *Mtb* subunit α (*Mt* α) as well as recent 3D reconstructions derived from the *Ms*F-ATP synthase locate the C-terminal 3.5 kDa stretch at the bottom of mycobacterial α subunit (Bogdanović et al., 2018; Kamariah et al., 2019) (Fig. 2), providing short distances to the rotary stalk subunits γ - ϵ .

3. Implications of an inhibited state of mycobacterial subunit β

Although each α - β heterodimer comprises a catalytic site, the majority of residues that contribute to nucleotide binding reside on the β subunit. Each β subunit has a catalytic- and a ‘lever’ domain, including the C-terminal called DELSEED-region. During ATP synthesis and hydrolysis, the central stalk of an F-ATP synthase rotates clockwise and counterclockwise, respectively, relative to the surrounding $\alpha_3\beta_3$ -ring (Noji et al., 1997; Diez et al., 2004; Spetzler et al., 2010). Each of the three catalytic sites in the $\alpha_3\beta_3$ -ring works in succession such that at saturating ATP concentrations, the drive shaft is driven in 120° power strokes that are separated by catalytic dwells

when ATP is hydrolyzed. In most available crystal structures, one catalytic site lacks bound nucleotide (β_E), while ATP (β_T) and ADP (β_D) are bound to the other sites. The most prominent conformational change between the empty and nucleotide-occupied sites is that the lever domain is open in the former and closed in the latter when nucleotide is bound. At concentrations that limit the rate of ATP hydrolysis, each 120° power stroke of prokaryotic F_1 complexes is interrupted ~80° prior to the catalytic dwell by an ATP-binding dwell that has a duration proportional to the time required for ATP to bind to the empty catalytic site (Yasuda, et al., 2001; Hirona-Hara, et al., 2001; Sielaff et al., 2008; Martin et al., 2014; Sielaff et al., 2016).

Although at moderate resolution, the β_D - and β_T subunits in the *MsF₁*-ATPase crystal structure appear to be occupied by an ADP and a magnesium ion (Mg^{2+}), while β_E was interpreted to have the usually empty β_E subunit site occupied by ADP and phosphate but without Mg^{2+} , even though the crystallization medium contained excess Mg^{2+} (Zhang et al., 2019). Such ADP plus P_i occupation of the *M. smegmatis* β_E (*Ms* β_E) is similar to the one of the *Caldalkalibacillus thermarum* enzyme (Ferguson et al., 2016), representing also a poorly hydrolysing F_1 -ATPase (Ferguson et al., 2016), and has been discussed to represent an inhibited state (Ferguson et al., 2016; Zhang et al., 2019; Duncan, 2019). The difference in occupation of the *M. smegmatis* β_D - (*Ms* β_D) and β_T (*Ms* β_T) subunits to *Ms* β_E are also reflected by the movements of the phosphate-binding loop (P-loop) in the catalytic center as well as the C-terminal lever domain of *Ms* β_E relative to *Ms* β_D - and *Ms* β_T (Fig. 3A).

Interestingly, when the three *Ms* β subunits are superimposed on the β_E - (*Gs* β_E), β_D - (*Gs* β_D) and β_T (*Gs* β_T) subunits of the ATPase active enzyme from *Geobacillus stearothermophilus* (formerly *Bacillus* PS3; Guo et al., 2019), no structural difference can be observed between *Gs* β_T and *Ms* β_T , while the structure of *Gs* β_D and *Ms* β_D as well as *Gs* β_E and *Ms* β_E show movements in the P-loop-

and lever domain, respectively (Fig. 3B). Such differences may reflect conformational alterations of an inhibitory state within the *M. smegmatis*, potentially caused by cross talks with the rotary central stalk subunits γ and ϵ being in vicinity to the lever region (see chapters below).

4. Mycobacterial subunit γ represents a specific TB drug target

The 310-amino acid mycobacterial subunit γ consists of the N- and C-terminal α -helices, forming an antiparallel coiled coil domain that protrudes into the catalytic $\alpha_3\beta_3$. The N-terminal amino acids 24-34 of the *M. smegmatis* γ subunit interact with the C-terminal domains of the α - and β -subunits, and act like a rigid body (Zhang et al., 2019). Like in the crystal structure of the poorly hydrolysing F_1 -ATPase of *C. thermarum* (Ferguson et al., 2016), this rigid-body region of subunit γ rotates only 10.5° in *M. smegmatis*. In comparison, the rotation angles of this region in ATPase-active F_1 -ATPases like the *Escherichia coli* enzyme are significantly larger (50° ; Cingolani and Duncan, 2011). It was speculated that the lower rotation angle of the rigid-body region of the *C. thermarum* (Ferguson et al., 2016) and *M. smegmatis* (Zhang et al., 2019) may reflect a common mechanistic feature in latent F_1 -ATPases (Zhang et al., 2019).

The remaining residues 33-326 of the mycobacterial γ subunit reside outside of the $\alpha_3\beta_3$, forming the globular foot domain and composed of three helices, five strands forming a Rossmann fold, and the connecting loops (Fig. 4; Zhang et al., 2019). Although the five strands and the connecting loops were not resolved in the 4 Å resolution structure, they have been assigned based on the crystallographic structures of other species (Zhang et al., 2019; Yang & Zhang., 2015) (Fig. 4). The foot domain and the N-terminal domain of subunit ϵ provide the structural and mechanistic link with the *c*-ring (Capaldi et al., 1996). Rotation of the *c*-ring drives proton-transfer between the F_0 -subunits *a* and *c* and rotation of the central stalk subunits $\gamma\epsilon$. This torque energy causes

sequential conformational changes in the catalytic α - β interfaces via the N- and C-terminal α -helices of γ , resulting in ATP synthesis.

A mycobacterial specific peptide (γ 166-179 *M. smegmatis* numbering) within the foot domain has been shown to be in part responsible for latent ATPase activity and the lack of ATP hydrolysis-driven H^+ -pumping (Priya et al., 2013, Hotra et al., 2016). Deletion of this γ 166-179-loop, which is shown as a red loop in Figure 4, led to ATP-driven H^+ -pumping (about 61%) in IMVs (Hotra et al., 2016). It has been proposed that this unique polar loop (Priya et al., 2013) of the mycobacterial subunit γ interacts with the *c*-ring in a manner that affects rotation, inhibits proton-pumping and thereby regulating the PMF, which enables the pathogenic bacterium to survive (Priya et al., 2013, Hotra et al., 2016). Most importantly, ATP synthesis of such $\Delta\gamma$ 166-179-loop mutant is drastically diminished (Hotra et al., 2016). As recently being confirmed by the F-ATP synthases from *Acetobacterium woodii*- (Bogdanović et al., 2019) and chloroplast (*S. oleracea*; (Hahn et al., 2018) (Fig. 4), such species-specific extra loop elements in the central rotating γ subunit are essential for the regulation of ion-conduction, ATP hydrolysis and -synthesis. These examples demonstrate the variety of regulatory elements for niche adaptation, whose insights open an avenue for novel and specific drug targets. As the mycobacterial γ -loop is unique, absent in the human homologue and other prokaryotes, as well as important for the regulation of ATP synthesis, -hydrolysis and proton pumping, it fulfils fundamental criteria to investigate this loop as a potential TB-drug target.

5. Variations of mycobacterial subunit ϵ , its interaction with the TB-drug BDQ and its traits for a novel drug target

Previous trypsin proteolysis of IMVs from *M. smegmatis*, proposed to cleave subunit ϵ (Haagsma et al., 2010), as well as of recombinant *MsF*₁-ATPase, demonstrating rapid cleavage of ϵ (Zhang et al., 2019), stimulated ATP hydrolysis. The importance of the mycobacterial subunit ϵ

(121 residues) and in particular its C-terminus in regulating ATP hydrolysis became defined by a deletion mutant of the last and conserved residue D121 (mutant ϵ_{1-120}) which exuded increased ATP hydrolysis activity in IMVs (Joon et al., 2018). It is noteworthy that the deletion of D121 inhibited ATP synthesis, thus reflecting its importance in coupling and regulation (Joon et al., 2018). Substitution of D121 to a cysteine and reconstitution of this mycobacterial ϵ mutant with the mechanistically well understood hybrid $\alpha^{\text{chi}}_3\beta_3\gamma$ -complex (Ragunathan et al., 2018) in which the subunit α of *G. stearothermophilus* is extended by the mycobacterial subunit α , showed that the C-terminus of mycobacterial ϵ can move up to its counterpart of the nucleotide-binding subunit α (Fig. 5A), a prerequisite for communication of the rotary subunit ϵ with the catalytic $\alpha_3\beta_3$ headpiece (Joon et al., 2018).

The mechanistic features enabling such movements are displayed by the solution NMR structure of the *Mtb* subunit ϵ (*Mt* ϵ ; Joon et al., 2018) (Fig. 5B), whose C-terminus is in a down-position as determined in the *MsF*₁-ATPase (Zhang et al., 2019). The subunit is divided into an N-terminal β -barrel domain (NTD), connected via the linker residues S88-E89 to the C-terminal domain (CTD). The latter consists of a helix-loop-helix with helix 1 and -2 being shorter compared to their bacterial and human mitochondrial counterparts (Fig. 5A-B). The C-terminal amino acids are oriented to the NTD, forming a domain-domain interface between the NTD and CTD. The *Mt* ϵ is characterized by a number of hydrophobic interactions and hydrogen bonds between the N-terminal residues A10-W16 with the epitopes L61-A64 and A81-I90 (Fig. 5C). Such arrangement allows an ideal molecular communication between the NTD and CTD of *Mt* ϵ . It was proposed that rotation of the *c*-ring derived by ion transport affects at least in part the interaction between those residues of the *c*-ring loop and the bottom of the *Mt* ϵ NTD (Joon et al., 2018). Like in a spring, the interaction of the epitopes formed by residues A10-W16 and L61-A64 would translate the coupling

events between the *c*-ring and the amino acids at the bottom of the *Mtε*NTD, which then become coupled to the epitope of amino acids A81-I90. Here, residue F86 of the *Mtε*NTD and E87-I90 of the *Mtε*CTD form a hinge region, which would allow the up- and down movements of the CTD (Fig. 5C). Such a spring-like mechanism would compensate for the non-binding of ATP to *Mtε* (Biuković et al., 2013), which in bacteria like *G. stearothermophilus* seems to trigger up- and down movements of the CTD (Yagi et al., 2007). Furthermore, the importance of the A10-W16 epitope has been also confirmed by the increase in ATPase hydrolysis of IMVs, including the F-ATP synthase mutant ϵ W16A and in particular the inhibition of ATP synthesis by 83% (Kundu et al., 2016; Joon et al., 2018), reflecting that W16 and the hydrophobic patch of *Mtε* play a pivotal role in coupling the events of F_0 rotation with ATP synthesis via the ϵ NTD to the ϵ CTD.

The *Mtε* became of pharmacological interest after *in vitro* (Biuković et al., 2013) and *in vivo* (Kundu et al., 2016) studies which identified this subunit as a second BDQ-binding site, besides the rotary *c* subunit ring (Koul et al., 2007; Preiss et al., 2015). This resembles the phenomenon of the F-ATP synthase inhibitor N,N'-Dicyclohexylcarbodiimide (DCCD), which binds to the catalytic β subunits of F_1 (Yoshida et al., 1981) and the *c* subunit of F_0 (Fillingame, 1975). The multitude of aromatic, hydrophobic and hydrogen bonding interactions of BDQ with *Mtε* are mainly within the A10-W16-, L61-A64-, and the A81-I90 epitopes. As discussed above, these three epitopes were shown to have inter-domain interactions and enable the transfer of conformational alterations between the three epitopes, thereby regulating the up- and down movements of the CTD. Recent discoveries of DARQ analogs of BDQ including the molecules TBAJ-876 and the three analogues 5366, 5316 and 5307 (Tong et al., 2017; Choi et al., 2017; Sutherland et al., 2018; Blaser et al., 2019; Sutherland et al., 2019) revealed improved potency in ATP synthesis and -hydrolysis of fast- and slow growing mycobacteria (Sarathy et al., 2019).

Interestingly, like BDQ, these potent TB-inhibitors show the same dual-subunit mechanism by binding to mycobacterial subunit *c* and ϵ (Sarathy et al., 2019). In the future, the novel insights into the structure, regulation, spring mechanism, BDQ and 3,5-dialkoxypyridine analogues described above will not only pave the way for the development of new classes of BDQ analogs but also for new vaccines targeting the dynamic coupling by *Mt* ϵ as indicated by recent structure-based approaches (Shahbaaz et al., 2019).

6. Proton-transfer via the α -*c* domain and mechanism of action of BDQ within subunit *c*

The two entirely membrane-embedded mycobacterial F_0 subunits *a* and *c* build the core for proton translocation from the periplasmic- to the cytoplasmic side. So far only the *M. phlei c* (*Mpc*) subunit structure has been determined at atomic resolution (Preiss et al. 2015; PDB ID: 4v1f). Nine *c* subunits form the *c*-ring (Fig. 1), leading to a proton-to-ATP ratio of 3.0, whereby each *Mpc* N-terminus forms an α -helix at the inner ring and the C-terminal α -helix forms the outer ring side. Both helices are connected by a 14 amino acids loop. The mycobacterial *c* subunits are characterized by a significant content of aromatic amino acids (Preiss et al., 2015), providing hydrophobic interactions with aromatic molecules like BDQ. Two adjacent subunits of the *c*-ring are involved in extensive hydrophobic and hydrophilic interactions with BDQ during drug binding (Fig. 1). The residues G62, E65, A66, A67, Y68, F69 and L72 from one subunit and L63, A66, A67 and I70 from another subunit are involved in these interactions. Residue E65, whose counterpart in *Mtb* is the E61 residue, is involved in hydrogen bond interactions with the dimethylamino moiety and hydroxyl group of BDQ. Binding of the drug presumably results in stalling of *c*-ring rotation (Preiss et al., 2015).

Based on the atomic models of subunit *a* from other species (Hahn et al., 2018; Guo et al., 2019), one can propose, that proton conduction between subunits *a* and *c* begins with entrance of protons through a hydrophilic pore behind tilted α -helices of subunit *a* at the periplasmic side, which are adjacent to the *c*-ring. They next bind and protonate E65 of a *Mpc*, whereby the glutamate neutralizes its negative charge. Rotation of the *c*-ring, mainly driven by the electric field generated by membrane potential (Miller et al., 2013), allows the protonated E65 to reach the cytoplasmic half channel. It then releases its proton to a nearby glutamate or aspartate residue.

Because of the importance of the *a-c* interface in proton conduction, a high-resolution structure of the mycobacterial *a-c* heterodimer is a prerequisite for the next generation of drug targets. The recent discovery of squaramides (SQA) as inhibitors of mycobacterial ATP synthesis and the medicinal chemistry exploration underline this important *a-c* interface (Tantry et al., 2017). So far docking- and mutational studies propose the binding site interaction of the most active SQAs at the *a-c* interface of the *MtF*-ATP synthase model (Subramanyam et al., 2017). An atomic structure of the mycobacterial *a-c* heterodimer would contribute to an improved medical chemistry approach and structure-activity relationship studies (SAR) might result in a next generation of SQAs with attractive pharmacological properties.

7. The evolutionary variation within the elastic mycobacterial peripheral stalk subunits

The flexibility within the mycobacterial peripheral stalk subunits *b*, *b'* and δ is proposed to smoothen transmission of power between the rotary *c*-ring and the F_1 domain with significant conformational variability of the C-terminal part of subunit *b*, which is in proximity to subunit δ (Kamariah et al., 2019). The gene encoding the *b*- δ subunits of mycobacterial F-ATP synthase is

unique, in that the putative *b* subunit (*atpF*) and δ subunit (*atpH*) genes were fused to form a single gene (*atpH*) (Gajadeera and Weber, 2013; Kamariah et al., 2019). In addition, the *atpF* gene of mycobacteria encodes the information for subunit *b'* (18 kDa), which is a shorter *b*-type subunit, without the C-terminus (Fig. 1). Interestingly, *Mycobacteria* species possess a more elongated δ subunit, including a canonical δ subunit consisting of 179 amino acids, which is similar in length to other prokaryotic δ subunits, and an N-terminal 111 amino acid extension, resulting in a 272 amino acids protein. Due to the fusion between the extended δ and the *b* subunits, the 446 residues (according to *M. tuberculosis*) fusion protein has evolved during evolution (Gajadeera and Weber, 2013; Kamariah et al., 2019). A recent *ab-initio* structural model of the extended mycobacterial δ subunit revealed a similar bundle of six short α -helices ($\alpha 7$ - $\alpha 12$), followed by the four-stranded mixed β -sheet ($\beta 1$ - $\beta 4$) and two α helices ($\alpha 13$ - $\alpha 14$) at the C-terminal domain (Fig. 6) (Kamariah et al., 2019) as described for the *E. coli*- (Sobti et al., 2016) and chloroplast subunit δ (Hahn et al., 2018). Importantly, the unique N-terminal 111-residues extension consists of six α -helices ($\alpha 1$ - $\alpha 6$) (Kamariah et al., 2019).

Like in other prokaryotic F-ATP synthases (Sobti et al., 2016; Guo and Rubinstein, 2019; Colina-Tenorio et al., 2018; Hahn et al, 2016), the peripheral stalk of the mycobacterial F-ATP synthase is attached to the top of the F_1 region by subunit δ (OSCP in mitochondria; Hahn et al, 2016; Srivastava et al. 2018; Colina-Tenorio et al., 2018) so that together, the central and peripheral stalks establish two connections between the F_1 and F_0 regions of the complex (Kamariah et al., 2019). In the *M. smegmatis* enzyme, the helices $\alpha 1$ and $\alpha 3$ of the 111-residues extension are proposed to reside in proximity to the interface of the N-termini of one α - β pair ($\alpha 1\beta 1$) (Kamariah et al., 2019). In addition, helix $\alpha 9$ and $\alpha 11$ are in the neighborhood to the N-termini of subunit β ($\beta 3$) and α ($\alpha 3$), respectively, while the α -helix 14 is in vicinity to subunit α

(α 1). In comparison, the canonical subunit δ within the chloroplast F-ATP synthase (Hahn et al., 2018) or its mitochondrial equivalent OSCP of *Saccharomyces cerevisiae* (Srivastava et al., 2018) interact only with the α subunits via helices corresponding to the mycobacterial helix bundle (α 7- α 12; Hahn et al., 2018).

Substantial flexibility in the peripheral stalk was observed between rotatory states as displayed in bacterial-, chloroplast- and mitochondrial F-ATP synthases, which confirms that the flexibility of the peripheral stalk is important for the smooth transmission of power between the F_1 and F_0 domains, which often have mismatched symmetries (Guo et al., 2019; Sobti et al., 2019; Hahn et al., 2018; Murphy et al., 2019). Interestingly, the recent mitochondrial F-ATP synthase structure of the green algae *Polytomella* revealed that the flexible coupling between the F_1 and F_0 motor is primarily provided by the hinge region of the δ subunit homologue OSCP that sits on top of the F_1 head by binding to the N-termini of subunit α (Murphy et al., 2019). The pivot point of this movement is the single peptide chain connecting the two OSCP domains, while the peripheral stalk remains mainly unchanged during rotation (Murphy et al., 2019). In comparison, the structurally simpler bacterial peripheral stalk subunit *b* flexes substantially, suggesting the different mechanism for stator flexes between eukaryotes and prokaryotes (Sobti et al., 2019). The mechanistic importance of the peripheral stalk and in particular the subunit δ homologue, OSCP in mitochondria is revealed to be a target of the insecticide dichlorodiphenyltrichloroethane (DDT) (Younis et al., 2011), the compound Bz-423, which induces apoptosis (Stelzer *et al.*, 2010), as well as the estrogen 17 β -estradiol (Zheng *et al.*, 1999). In this context, the mycobacterial δ subunit with its unique N-terminal extension and arrangement within the F-ATP synthase, promises to be an interesting drug target. Furthermore, in contrast to other F-ATP synthases the mycobacterial engine represents a class of F-ATP synthases in which no symmetry mismatch is required, since

the 120° steps in the F₁ motor and the 40° steps of the *c*₉-ring results in an average 3-step rotation, in line with three protons being required for the formation of one ATP.

Taken together, the aspects above do not only highlight that more structural and functional studies will be needed to understand the mechanism(s) of the mycobacterial peripheral stalk but also the possibility that this fused *b*- δ peripheral stator flexibility might be different to its counterpart in other F-ATP synthases. Finally, the mycobacterial δ subunit with its unique N-terminal extension and arrangement within the F-ATP synthase promises to be an interesting drug target.

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Figure legends

Figure 1. Mesh representation of the entire *MsF₁F_O*-ATP synthase reconstructed using negative stain electron microscopy (Kamariah et al., 2019). The final EM map nicely aligns with the 4 Å structure of the *MsF₁*-ATPase (Zhang et al., 2019; PDB ID: 6FOC) and the *M. phlei* *c*₉-ring (beige; Preiss et al., 2015; PDB ID: 4v1f). The alternating subunits α (green) and β (orange) form the catalytic hexamer, which is linked by the rotary central stalk subunits γ (yellow) and ϵ (red). The remaining F_O volume becomes occupied by the *ab-initio* structures of mycobacterial subunits *a* (blue), the N-terminal and membrane-embedded parts of the mycobacterial subunits *b* (pink) and *b'* (in *turkish blue*). The C-terminal regions of *b'* and *b*, the latter being fused with the novel mycobacterial subunit δ (pink) form the peripheral stalk (Kamariah et al., 2019). Subunit δ occupies well the crown of the F₁ domain. The interaction profile of BDQ with the *Mtb* ϵ subunit, based on studies conducted by Joon et al. 2018, is shown in the top inset. The multitude of aromatic, hydrophobic and hydrogen bonding interactions stabilizing the binding of BDQ with the ϵ subunit are presented. Amino acid residues R13, N14 and I15 have hydrogen bond interactions with the quinoline moiety, dimethylamino group and hydroxyl group of BDQ respectively. Residue W16 has face-to-face stacked interaction with BDQ's naphthalene moiety and phenyl group. Residue F86 has an edge-to-face aromatic interaction with the phenyl group while residues A84 and S17 have hydrophobic interactions with the quinoline and naphthalene moieties of BDQ, respectively. The interaction profile of BDQ with the *M. phlei* *c*₉-ring, based on studies conducted by Preiss et al. 2015, is shown in the bottom inset. Two adjacent subunits of the *c*-ring are involved in extensive hydrophobic and hydrophilic interactions with BDQ during drug binding. The residues G62, E65, A66, A67, Y68, F69 and L72 from one subunit and L63, A66, A67 and I70 from another subunit are involved in these interactions. Residue E65, whose counterpart in *Mtb* is the E61 residue, is

involved in hydrogen bond interactions with the dimethylamino moiety and hydroxyl group of BDQ.

Figure 2. The average solution shape of *Mtα* (Bogdanović et al., 2018), which fits with the crystal structure of *Msα* (Zhang et al., 2019; PDB ID: 6FOC), provides space for the C-terminal extension *Mtα*₅₁₂₋₅₄₉ within *Mtα*. The extended helical structure of *Mtα*₅₂₁₋₅₄₀ was solved by solution NMR spectroscopy (Ragunathan et al., 2018).

Figure 3. (A) Comparison of β_E (green), β_D (cyan) and β_T (magenta) subunits of the *MsF*₁-ATPase crystal structure (Zhang et al., 2019). The *Msβ*_E subunit adopts an open conformation, while *Msβ*_D and *Msβ*_T reveal a closed one. A close view of the phosphate-binding loop (P-loop) highlights the difference in *Msβ*_D and *Msβ*_T to *Msβ*_E (green). (B) Superposition of the *Msβ*_D (deep teal) interface with the one of *G.s. stearothermophilus*, *Gsβ*_D (light blue; Guo et al., 2019), displaying the significant movements in the P-loop and C-terminal lever domain (arrows), respectively.

Figure 4. Comparison of a homology model of *Mtγ* (yellow) with the structures of *E. coli* (cyan; Cingolani & Duncan, 2011), chloroplast (magenta; Hahn et al., 2018) and a homology model of *A. woodii* (wheat; Bogdanović et al., 2019) highlights the unique loops in *Mtγ* (red), chloroplast (green) and *A. woodii* (blue). The mycobacterial subunit γ has an extra 14 amino acid stretch at a different position compared to the chloroplast- and *A. woodii* counterpart as indicated by the colored arrows.

Figure 5. (A) The cartoon representation of subunit $Mt\epsilon$ in the modeled extended conformation derived from the NMR solution structure spanning 42 Å. In comparison to the $Gs\epsilon$ (67 Å), the $Mt\epsilon$ is significantly shorter, and it cannot directly reach the nucleotide-binding site (P-loop, *green circles*) with its C-terminal helix. However, recent crosslinking data demonstrated that the D121C mutant $Mt\epsilon$ can reach the DELSEED region of the hybrid subunit α^{chi} of the engineered $\alpha^{\text{chi}}_3\beta_3\gamma$ -complex (Ragunathan et al., 2018), providing sufficient coupling. (B) Superposition of $Mt\epsilon$ (*red*), *E. coli* subunit ϵ (*pink*) and $Gs\epsilon$ (*blue*) underlining the short two C-terminal helices of $Mt\epsilon$. (C) Surface representation of $Mt\epsilon$ shows the formation of the hydrophobic cleft (*green*), which also belongs to the BDQ-binding site, that couples with residues in the L61-A64- and A81-I90 epitope.

Figure 6. The predicted structure of the mycobacterial peripheral stalk. The fused b- δ and the extended N-terminal domain (*magenta*) is unique for the mycobacterial peripheral stalk.

Figure 1

Figure 2

Figure 3A

Figure 3B

Figure 4

(A)

(B)

Figure 5A-B

(C)

Figure 5C

Figure 6