Structure and function of *Mycobacterium*-specific components of F-ATP synthase subunits α and ϵ

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Running title: Insights into coupling of mycobacterial F-ATP synthase

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Abstract

The *Mycobacterium tuberculosis* (*Mtb*) F₁F₀-ATP synthase ($\alpha_3:\beta_3:\gamma:\delta:\epsilon:a:b:b':c_9$) is an essential enzyme that supplies energy for both the aerobic growing and the hypoxic dormant stage of the mycobacterial life cycle. Employing the heterologous F-ATP synthase model system $\alpha^{chi}_3:\beta_3:\gamma$ we showed previously, that transfer of the C-terminal domain (CTD) of *Mtb* subunit α (*Mt* $\alpha_{514-549}$) to a standard F-ATP synthase α subunit suppresses ATPase activity. Here we determined the 3D reconstruction from electron micrographs of the $\alpha^{chi}_3:\beta_3:\gamma$ complex reconstituted with the *Mtb* subunit ϵ (*Mt* ϵ), which has been shown to crosstalk with the CTD of *Mt* α . Together with the first solution shape of *Mtb* subunit α (*Mt* α), derived from solution X-ray scattering, the structural data visualize the extended C-terminal stretch of the mycobacterial subunit α . In addition, *Mt* ϵ mutants *Mt* ϵ R62L, *Mt* ϵ E87A, *Mt* ϵ_{6-121} , and *Mt* ϵ_{1-120} , reconstituted with $\alpha^{chi}_3:\beta_3:\gamma$ provided insight into their role in coupling and in trapping inhibiting MgADP. NMR solution studies of *Mt* ϵ E87A gave insights into how this residue contributes to stability and crosstalk between the N-terminal domain (NTD) and the CTD of *Mt* ϵ . Analyses of the N-terminal mutant *Mt* ϵ_{6-121} highlight the differences of the NTD of mycobacterial subunit ϵ to the well described *Geobacillus stearothermophilus* or *Escherichia coli* counterparts. These data are discussed in context of a crosstalk between the very N-terminal amino acids of *Mt* ϵ and the loop region of one *c* subunit of the *c*-ring turbine for coupling of proton-translocation and ATP synthesis activity.

1. Introduction

Tuberculosis disease (TB) is caused by species of the *Mycobacterium tuberculosis* complex, the most relevant being *M. tuberculosis*. Owing to their inherent metabolic flexibility, mycobacteria can exist in both extracellular and intracellular environments during an infection. The bacteria are also capable of surviving under low-oxygen conditions, albeit in a non-replicative state, despite being obligate aerobes (Dick et al., 1998; Berney and Cook, 2010). Depending on the available energy sources, mycobacteria can modify their metabolism to regulate the consumption of substrates necessary for respiration in order to ensure their survival (Berney and Cook, 2010). Such metabolic plasticity is paramount for bacterial survival under adverse environments as it enables a reasonable level of adenosine triphosphate (ATP) synthesis to maintain viability (Rao et al., 2008).

The biological energy currency ATP in mycobacteria is mainly synthesized by the bipartite enzyme F_1F_0 ATP synthase (Fig. 1A). This enzyme is composed of a membrane-bound F_0 section (subunits $a:b:b':c_9$), and a water-soluble F_1 portion (subunits $\alpha_3:\beta_3:\gamma:\delta:\epsilon$), oriented towards the cytoplasm. The catalytic $\alpha_3:\beta_3$ -hexamer is connected via a central (subunits γ and ϵ) and a peripheral stalk (subunits b, b' and δ) that converts rotational energy from the nine subunit c (Preiss et al., 2015), known as the c -ring, of F_0 to the nucleotide-binding sites within the $\alpha_3\beta_3$ -headpiece of F_1 (Lu et al., 2014); it has been well-established that movement of protons across the F_0 region generates a proton-motive force that fuels the rotation of the c -ring, which interacts with both the N-terminal domain of subunit ϵ and the globular domain of subunit γ to transfer rotational energy to the catalytic α - and β -subunits for ATP synthesis (Capaldi et al. 1996; Noji et al., 1997; Diez et al., 2004; Nakanishi-Matsui et al., 2016). A special feature of the mycobacterial

F-ATP synthases is its inability to establish a significant H⁺-gradient during ATP hydrolysis, and its low or latent ATPase activity (Haagsma et al., 2010; Hotra et al., 2016).

Recent studies showed that the unique 36 amino acid C-terminal extension of the catalytic *Mta* (*Mta*₅₁₄₋₅₄₉) is involved in the suppression of ATP hydrolysis and H⁺-pumping. Deletion of *Mta*₅₁₄₋₅₄₉ increased ATP hydrolysis and H⁺-pumping activity of the mycobacterial enzyme. When *Mta*₅₁₄₋₅₄₉ was genetically inserted into the *Geobacillus stearothermophilus* (formerly *Bacillus* PS3) $\alpha_3\beta_3\gamma$ complex (*Gsa* $\alpha_3\beta_3\gamma$) the ATPase rate of this chimeric protein, called $\alpha^{chi}_3\beta_3\gamma$, was reduced by an identical rate (Ragunathan et al., 2017). Single-molecule rotation experiments of $\alpha^{chi}_3\beta_3\gamma$ indicated that the introduction of this *Mycobacterium*-specific domain decreased the angular velocity of the power-stroke after ATP binding, proposed to be due to an interaction between *Mta*₅₁₄₋₅₄₉ of subunit α^{chi} and subunit γ (Ragunathan et al., 2017).

The mycobacterial ϵ subunit, composed of an N-terminal β -barrel domain (NTD, residues 1-87) and a C-terminal domain (CTD; residues 90-121) connected by the linker residues 88-89, also harbours a unique feature in the form of a shorter CTD when compared to subunit ϵ from other bacteria, such as *G. stearothermophilus* and *Escherichia coli* or the human counterpart subunit δ (Fig. 1B). As shown by the solution NMR structure of the *Mtb* subunit ϵ (*Mt* ϵ , 121 amino acids (Joon et al., 2018)), the two α -helices of the CTD of *Mt* ϵ are significantly shorter compared to other pro- or eukaryotic ϵ subunits (Uhlén et al., 1997; Wilkens and Capaldi, 1998; Yagi et al., 2007; Ferguson et al., 2016). Based on NMR relaxation measurements a novel mechanistic model of coupling *c*-ring- and ϵ rotation was proposed to occur via the N-terminal domain of *Mt* ϵ , the linker residues S88-E89, as well as the *Mt* ϵ CTD residues I90, D91, A112, A116, V117, and R113 to the catalytic $\alpha_3\beta_3$ -headpiece (Joon et al., 2018). In addition, studies of an *Mt* ϵ D121C mutant revealed that this very C-terminal residue can be cross linked with the C-

terminal DELSEED-region of subunit α , reflecting that the $Mt\epsilon$ CTD can undergo a swap from a compact- to an extended conformation (Joon et al., 2018).

To shed more light into the structural and mechanistic functions of the unique mycobacterial subunits α and ϵ , $Mt\epsilon$ was included in the chimeric $\alpha^{chi}_3:\beta_3:\gamma$ complex to form $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon$ and its structure was determined by electron microscopy. In parallel, the low-resolution solution shape of *Mtb* subunit α ($Mt\alpha$) was determined using small angle X-ray scattering (SAXS). Mutant-, ATPase activity-, and NMR studies provide insight into critical amino acids involved in the coupling network of $Mt\epsilon$. Deleting the N-terminal amino acids 1MAELN5 of $Mt\epsilon$ significantly affected protein stability and complex formation with $\alpha^{chi}_3:\beta_3:\gamma$. The structural and enzymatic data are discussed in light of the regulatory role of subunit ϵ .

2. Material and Methods

2.1 Biochemicals

Pfu DNA polymerase was purchased from Thermo Scientific (Massachusetts, USA) and Ni^{2+} -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, production and purification of *Mta* as well as *Mt\epsilon* and its mutant forms

The DNA coding for *M. tuberculosis* subunit α ($Mt\alpha$) was amplified using the primers 5'-CATCATGCCATGGCTGAGTTGACAATCCCC-3' and 5'-

CATGGATCCTTATTTCTTCTTCTTCGGCGC-3' as forward and reverse, respectively, which include the *Nco*I and *Bam*HI restriction sites (underlined). The PCR product was further subcloned into pET-9d(+) (Grüber et al., 2002) and the plasmid DNA was then introduced into *Escherichia coli* DH5 α cells for plasmid amplification. Plasmids encoding the gene for *Mt α* were transformed into *E. coli* OverExpress C41(DE3) cells (Lucigene) (Miroux and Walker, 1996) for protein production.

Subunit ϵ of the *M. tuberculosis* F-ATP synthase consists of 121 residues. Recently, the sequence of the *atpC* gene of *M. tuberculosis* was amplified and inserted into the pET-9d(+) vector (Biuković et al., 2013; Grüber et al., 2002). This plasmid was used as a template for generation of the mutant forms using the site-directed mutagenesis approach.

The single amino acid substitution mutant *Mt ϵ R62L* was done by amplification of the entire plasmid using the following forward primers GACCTGCTCATCGCGGTTCGACGGCG and the reverse GCGATGAGCAGGTCCTTTTCTCCCTCCCGC, while residue E87 was substituted with alanine using the forward primer AGTTCGCGTCGGAGATCGACGAGGCCG and the reverse TCCGACGCGAACTCGGCAGATTCGGC to generate the mutant *Mt ϵ E87A*.

The N-terminal truncated subunit *Mt ϵ ₆₋₁₂₁* was amplified using the forward primer GTTGAGATCGTCGCCGTCGACCGGAAC and the reverse GGCGACGATCTCAACGGGGTGATGGTGATG. The entire plasmid amplification was performed using *Phusion* DNA polymerase, resulting in the plasmid with the desired mutations in the respective gene as well as the sequence encoding an N-terminal His₆-tag. Subsequently, the plasmid DNA was introduced into *E. coli* DH5 α cells for plasmid amplification. Plasmids encoding the gene for *Mt ϵ R62L*, *Mt ϵ E87A* or *Mt ϵ ₆₋₁₂₁* were transformed into *E. coli* OverExpress C41(DE3) cells (Lucigene) (Miroux and Walker, 1996) for protein production.

For the cloning of the *Geobacillus stearothermophilus* subunit ϵ (*Gse*), the fragment containing the gene *atpC* from *G. stearothermophilus* with restriction sites *NcoI* and *SacI* was synthesized and provided by *DNA 2.0* in the vector pJ367 (in-house cloning vector). The fragment was further sub-cloned into pET-9d(+) (Grüber et al., 2002).

To produce the recombinant His-tagged *Mt ϵ* , *Mt ϵ R62L*, *Mt ϵ E87A*, *Mt ϵ ₆₋₁₂₁*, the recently generated mutant, *Mt ϵ ₁₋₁₂₀* (Joon et al., 2018), lacking the very terminal amino acid D121, or *Gse* liquid cultures were incubated in kanamycin-positive (30 mg/ml) LB medium at 37 °C with shaking of 180 rpm, until an optical density (OD₆₀₀) of 0.6 - 0.7 was achieved. Production of the recombinant proteins was obtained by adding isopropyl (thio)- β -D-galactopyranoside (IPTG) to a final concentration of 1 mM. Following overnight incubation at 15 °C, the cells were harvested at 8,500 x g for 13 min, 6 °C. Subsequently, they were lysed on ice by sonication for 3 x 1 min in buffer A (50 mM Tris/HCl, pH 7.5, 200 mM NaCl, 2 mM phenylmethane sulfonyl fluoride (PMSF) and 2 mM Pefabloc^{SC} (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride) (BIOMOL) and 10% glycerol. Precipitated material was separated by centrifugation at 10,000 x g for 35 min. The supernatant was filtered (0.45 μ m; Millipore) and the His-tagged protein was allowed to bind to the matrix for 1.5 h at 4 °C. The recombinant protein eluted with an imidazole-gradient (0 - 400 mM) in buffer A, whereby fractions of 75 – 125 mM imidazole were concentrated using Centricon YM-3 (3 kDa molecular mass cut-off) spin concentrators (Millipore), and subsequently applied on a gel filtration chromatography column (Superdex 75 HR 10/30 column (GE Healthcare)). The eluting buffer consisted of 50 mM Tris/HCl (pH 7.5), 200 mM NaCl and 10% glycerol.

In case of producing ¹⁵N-labelled *Mt ϵ E87A* for NMR spectroscopy, freshly transformed *E. coli* C41 cells were plated on LB-agar, from which a single colony was selected to prepare a 15

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3 ml LB seed culture. After overnight growth, the culture was centrifuged at 3,000 x g for 5 min at
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5 4 °C to pellet out the cells. The cells were washed and subsequently suspended in 1 litre M9-
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7 minimal media supplemented with MgSO₄, CaCl₂, thiamine, FeCl₃, trace elements and
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9 kanamycin.
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12 Isolation and purification was done according to the protocol described for the non-labelled
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14 subunit with the following modification: after Ni-NTA affinity step, fractions containing the
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16 protein were combined and diluted to contain 100 mM NaCl with buffer A and were applied on
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18 an ion-exchange column (Resource^{TM} Q column, 6 ml; GE Healthcare). The proteins were
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20 separated by a salt gradient using buffer A (50 mM Tris/HCl (pH 7.5), 10% glycerol) and -B (50
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22 mM Tris/HCl (pH 7.5), 1 M NaCl and 10% glycerol). The flow-through included the recombinant
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24 protein, whereby the contaminants bound to the column. *MtεE87A* was concentrated using
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26 Centricon YM-3 (3 kDa molecular mass cut-off) spin concentrators (Millipore) and subsequently
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28 applied on a gel filtration chromatography column (Superdex 75 HR 10/30 column (GE
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30 Healthcare)). The eluting buffer consisted of 50 mM Tris/HCl (pH 7.5), 200 mM NaCl and 10%
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32 glycerol.
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40 For purification of recombinant *Mtα*, the cells were lysed by sonication 3-times for 1 min in
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42 Buffer A (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM PMSF, 0.8 mM DTT and 1 mM
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44 Pefabloc^{SC} (BIOMOL)). Centrifugation at 10,000 x g for 35 min was employed to allow the cell
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46 debris to settle down. The filtered supernatant was incubated with Ni²⁺-NTA agarose beads for 1
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48 hour at 4 °C to bind the His₆-tagged proteins, which were later eluted with a linear imidazole
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50 gradient (0-400 mM) in Buffer A. The eluted fractions were applied on a 17% SDS-PAGE
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52 (Laemmli, 1970) to check for the presence of the desired protein. The appropriate fractions were
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54 pooled, diluted with Buffer B (50 mM Tris/HCl, pH 7.5 and 1 mM DTT) to reduce the salt
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concentration to 50 mM, and subsequently applied onto an anion-exchange column (Mono Q, 8 ml, GE-Healthcare). As a final step of purification, gel filtration chromatography using a Superdex 200 HR 10/30 column (GE Healthcare) was performed with buffer containing 50 mM Tris-HCl, pH 7.5 and 200 mM NaCl. The fractions containing recombinant $Mt\alpha$ were pooled and concentrated as required using Centricon YM-30 (30 kDa molecular mass cut off) spin concentrators (Millipore).

2.3 Reconstitution of the $\alpha^{chi}_3:\beta_3:\gamma$ complex with $Mt\epsilon$, its mutant forms or Gse

For reconstitution of the $\alpha^{chi}_3:\beta_3:\gamma$ complex with subunit $Mt\epsilon$, its mutant forms or Gse , $\alpha^{chi}_3:\beta_3:\gamma$ was purified as described recently (Ragunathan et al., 2017), and then incubated with a 3-fold molar excess of freshly purified $Mt\epsilon$, its mutant subunit or Gse at 4 °C for 1 h in 50 mM Tris/HCl, pH 7.5, 200 mM NaCl, followed by size exclusion chromatography (Superdex 300 HR 10/30, GE Healthcare). The effect of reconstituted subunit ϵ was analysed by ATP hydrolysis activity measurements as described recently (Ho et al., 2015). To verify the reconstitution efficiency, the ratio of subunit γ to subunit ϵ was estimated from the 12% SDS gels (Laemmli, 1970), processed in the ImageJ software (Schneider et al., 2012). In short, the band intensities were converted into the 2D peaks, whose integrals were calculated. The integrated area below the peak of subunit γ was normalized to 1 and compared to the integral value fetched for the peak corresponding to subunit ϵ . In the case of the reconstitution of $Mt\epsilon$ with $\alpha^{chi}_3:\beta_3:\gamma$, the ratio of $\gamma:Mt\epsilon$ was 1:0.5.

2.4 Electron microscopy, 2D and 3D reconstruction

The freshly reconstituted complex was diluted to a final concentration of 40 µg/ml in buffer containing 50 mM Tris/HCl, 200 mM NaCl, pH 7.5. 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 120 kV Tecnai spirit T12 transmission electron microscope (FEI) equipped with a 4 K CCD camera (FEI) at a calibrated magnification of 66,350 x under low-dose conditions. A total of 28,349 particles were selected. The selection criteria were clear visibility of single molecules and separation from neighbouring particles. The particle selection and processing was performed using EMAN2 (Tang et al. 2007). Subsequent 2D-, 3D classification and class refinement were performed in *RELION* v1.4 (Scheres et al., 2012). A reference density map filtered to 30 Å was generated using the structural coordinates of *Gs* $\alpha_3:\beta_3:\gamma$ (PDB code 4XD7; A:B:C:D:E:F:G; Shirakihara et al., 2015). Fitting and the visualizing of the experimentally acquired surface density map of the $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon$ complex were all achieved within UCSF Chimera.

2.5 NMR spectroscopy data acquisition and assignments

Sample for NMR experiment contained 0.25 mM protein in 20 mM Tris, pH 7.5, 200 mM NaCl, 10% glycerol and 10% D₂O. Conventional ¹⁵N-HSQC NMR data were collected using uniformly ¹⁵N-labeled samples on a Bruker Avance 700 MHz spectrometer equipped with a 5 mm cryoprobe at 293K. Assignments of the backbone of WT form of subunit *Mtε* were described earlier (Joon et al., 2018) and the acquired *Mtε* ¹⁵N-HSQC spectrum was used as a reference for the *Mtε*E87A peak assignment. Chemical shifts were monitored and the weighted chemical shift perturbations (CSPs) between *Mtε* and mutant *Mtε*E87A for backbone ¹⁵N and ¹HN were

calculated by the formula $\Delta\delta = [(\Delta N/5)^2 + (\Delta HN)^2]^{0.5}$. NMR data were processed with NMRPipe (Delaglio et al., 1995) and analyzed using SPARKY (Goddard and Kneller, 2007).

2.6 Small angle X-ray scattering (SAXS) studies

SAXS-data were measured with the BRUKER NANOSTAR SAXS instrument equipped with a Metal-Jet X-ray source (Excillum, Germany) and VÅNTEC 2000-detector by the method described in details elsewhere (Dip et al., 2014; Kamariah, et al., 2015). Briefly, SAXS data of *Mta* (0.7, 1.0 and 1.2 mg/ml) were measured at 15 °C in buffer containing 50 mM Tris-HCl (pH 7.5), 200 mM NaCl and 1 mM DTT for 30 min (6 frames of 5 min each) in a vacuum tight quartz capillary. The scattering of the buffer was subtracted from the scattering of the sample. All the data processing steps were performed using the program package PRIMUS (Konarev et al., 2003). The experimental data obtained for different concentrations were analyzed for aggregation and folding state using Guinier (Guinier and Fournet, 1955) and Kartky plots (Glatter and Kratky, 1982), respectively. The forward scattering $I(0)$ and the radius of gyration R_g were evaluated using the Guinier approximation (Guinier and Fournet, 1955). These parameters were also computed from the entire scattering patterns using the indirect transform package GNOM (Svergun, 1992), which also provided the distance distribution function $p(r)$, which gives the maximal particle diameter, D_{max} . Low-resolution models of *Mta* were built using GASBOR (Svergun et al., 2001). *Ab initio* solution shapes were obtained by superposition of ten independent model reconstructions with the program package SUBCOMP (Kozin and Svergun, 2001) and building an averaged model from the most probable one using DAMAVER (Volkov and Svergun, 2003). Comparison of the experimental scattering curves with the theoretical

scattering curve calculated from high resolution structures were performed with CRY SOL (Svergun *et al.*, 1995).

3. Results

3.1 Reconstitution and EM-analysis of the $\alpha^{chi}_3\beta_3\gamma:Mt\epsilon$ complex

Reconstitution of $\alpha^{chi}_3\beta_3\gamma$ and $Mt\epsilon$ in a molar ratio of 1:3 followed by a size exclusion chromatography step resulted in a highly pure and stoichiometric $\alpha^{chi}_3\beta_3\gamma:Mt\epsilon$ complex as shown by SDS-PAGE analysis and densitometric analysis of the protein bands with a correct intensity ratio of 1:0.5 for subunit γ to ϵ (Fig. 2A). Electron microscopy was performed to visualize formation of the correct complex. A typical electron microscopy raw image of the negatively stained $\alpha^{chi}_3\beta_3\gamma:Mt\epsilon$ complex yielded homogenous particles with almost no contamination by smaller particles (Fig. 2B). 28,349 particles of $\alpha^{chi}_3\beta_3\gamma:Mt\epsilon$ were selected and a 2D-self-organizing map was calculated (Fig. 2C). The 2D-classes are composed of top-, bottom- and side views, revealing the hexagonal pattern of the alternating nucleotide-binding subunits α^{chi} and β (Fig. 2C-D) as well as the central stalk domain (Fig. 2C).

In order to determine the 3D reconstruction of the $\alpha^{chi}_3\beta_3\gamma:Mt\epsilon$ complex, a reference map using the atomic coordinates of the subunits α , β and γ of the *G. stearothermophilus* $\alpha_3\beta_3\gamma:\epsilon$ complex (PDB code 4XD7; Shirakihara *et al.*, 2015) was filtered to 30 Å. The final reconstruction shows a resolution of 18 Å with the crown, nucleotide-binding- and C-terminal domains of the $\alpha_3\beta_3$ -headpiece, and the central stalk, which accommodates the central stalk subunits γ and $Mt\epsilon$ (Fig. 2D-E). Superimposition of the *G. stearothermophilus* $\alpha_3\beta_3\gamma:\epsilon$ complex (PDB code 4XD7; Shirakihara *et al.*, 2015) highlights the lacking space at the bottom of α^{chi} inside the 3D reconstruction of the $\alpha^{chi}_3\beta_3\gamma:Mt\epsilon$ complex (Fig. 2D; 2nd row #1-2), indicating the

increase of the overall length of the chimeric α subunit. The lacking space was filled, when the recently determined NMR structure of the mycobacterial α -helical extension $Mt\alpha_{521-540}$ (Ragunathan et al., 2017) was fitted within this bottom segment of α^{chi} (Fig. 2D; 1st row #3). A cross-section at the distinct C-terminal region of α^{chi} reflects nicely that the additional mass is visible due to the presence of the additional mycobacterial C-terminal stretch (Fig. 2D; 2nd row #3). The data presented confirm that a stable, highly homogenous and well assembled $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon$ complex was produced.

3.2 Solution structure of *Mtα* studied by SAXS

To confirm that the mycobacterial subunit α is longer compared to its counterparts from other bacteria, and that this is due to its unique C-terminal stretch, subunit α of the *Mtb* F-ATP synthase, *Mtα*, was generated and purified (Fig. 3A). The freshly eluted protein was concentrated to 0.7, 1.0 and 1.2 mg/ml and used in SAXS experiments to determine its low-resolution solution structure. The final composite scattering patterns recorded at 0.7, 1.0 and 1.2 mg/ml are shown in figure 3B. The Guinier plots at low angles appeared linear and confirmed good data quality with no indication of protein aggregation (inset in Fig. 3B). The normalized Kratky plots (Fig. 3C) of *Mtα* along with the one of *Gsa* (Ragunathan et al., 2017) and lysozyme revealed the typical bell-shaped profile of the compact lysozyme, which is shifted away in case of the *Gsa* profile, reflecting its extended form in solution. In comparison, the profile of *Mtα* shifted even more outwards, suggesting that *Mtα* might be more extended in solution than the related *Gsa*. The radius of gyration (R_g) of *Mtα* determined from the Guinier approximation was $31.25 \pm 1.4 \text{ Å}$, which is larger than the one of *Gsa* (28.1 ± 0.3 ; Ragunathan et al., 2017) (Table 1). The maximum particle dimension (D_{max}) of *Mtα* and *Gsa*, computed from the distribution functions,

$p(r)$ (Fig. 3D), were 105 ± 10 Å and 90.0 ± 10 (Table 1), respectively. These data underline that *Mtα* is an elongated molecule in solution and longer compared to *Gsα*. The gross shapes of the proteins were *ab initio* reconstructed and had a good fit to the experimental data in the entire scattering range discrepancies of $\chi^2 = 0.66$. Ten independent reconstructions produced similar envelopes with a normalised spatial discrepancy (NSD) of 1.16 ± 0.04 . The average structure is shown in figures 3E. The theoretical scattering curve for the *Gsα* structure (PDB code 1SKY) was computed and compared with the experimental scattering curves of *Gsα* and *Mtα* using CRY SOL with resulting discrepancies of $\chi^2 = 1.44$ and $\chi^2 = 1.65$, respectively (Fig. 3B). Superimposition of the *Mtα* shape with the crystal structure of *Gsα* revealed an extra domain of 15×29 Å, indicating the additional 36 residues of *Mtα*. Furthermore, the comparison demonstrated that the C-terminal extension *Mtα*₅₁₄₋₅₄₉ is located below the very C-terminus of *Gsα*, in which the NMR-derived α-helix *Mtα*₅₂₁₋₅₄₀ (Ragunathan et al., 2017) can be well accommodated (Fig. 3E).

3.3 Kinetic properties of the $\alpha^{chi}_3:\beta_3:\gamma$ - and $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon$ -complexes

$\alpha^{chi}_3:\beta_3:\gamma$ and $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon$ revealed an ATPase activity of 4.7 ± 0.01 μmol/min/mg protein and 8.4 ± 0.04 μmol/min/mg protein (Fig. 4A), respectively, indicating a stimulating effect of subunit *Mtε* in the catalytic process of ATP hydrolysis at the physiological nucleotide concentration of 2 mM MgATP. The comparison of ATP hydrolysis activities of both complexes at 200 μM, 60 μM, 20 μM, and 6 μM MgATP demonstrated that the stimulating effect of *Mtε* on ATPase activity reduces in an ATP concentration dependent manner (Fig. 4B).

In a control experiment, subunit ε of the *G. stearothermophilus* F-ATP synthase was generated, purified (Fig. 4C) and reconstituted with *G. stearothermophilus* $\alpha_3:\beta_3:\gamma$ (*Gsα*₃:β₃:γ)

according to the same reconstitution protocol as $\alpha^{chi}_3\beta_3\gamma:Mt\epsilon$ (Fig. 4C). The complex, which revealed the right subunit stoichiometry of $Gs\alpha_3\beta_3\gamma:\epsilon$, showed an ATPase activity of 12.7 ± 0.03 $\mu\text{mol}/\text{min}/\text{mg}$ protein, which is 2.3 times higher compared to the recently described activity of $Gs\alpha_3\beta_3\gamma$ (Ragunathan et al., 2017). In addition, the $Gs\alpha_3\beta_3\gamma:\epsilon$ showed in the presence of 1 mM and 0.5 mM MgATP an ATPase activity of 9.5 ± 0.03 $\mu\text{mol}/\text{min}/\text{mg}$ protein and 6.9 ± 0.04 $\mu\text{mol}/\text{min}/\text{mg}$ protein, respectively, indicating an ATP concentration dependence of ATPase activity similar to $\alpha^{chi}_3\beta_3\gamma:Mt\epsilon$ (Fig. 4D). The data demonstrate that under physiological ATP concentrations and correct subunit stoichiometry, subunit ϵ is a prerequisite for maximum ATPase activity of the $Gs\alpha_3\beta_3\gamma:\epsilon$ -subcomplex.

The Michaelis-Menten constant (K_m) and the maximum rate (V_{max}) of $\alpha^{chi}_3\beta_3\gamma$ - and $\alpha^{chi}_3\beta_3\gamma:Mt\epsilon$ were determined from the substrate-dependent ATP-hydrolysis experiments described above (Fig. 4B). The calculated V_{max} values of $\alpha^{chi}_3\beta_3\gamma$ and $\alpha^{chi}_3\beta_3\gamma:Mt\epsilon$ were 4.97 ± 0.12 s^{-1} and 8.63 ± 0.25 s^{-1} , respectively, while the K_m values were similar with 0.107 ± 0.01 mM and 0.164 ± 0.02 mM, respectively (Fig. 4E), reflecting that the presence of subunit $Mt\epsilon$ in the $\alpha^{chi}_3\beta_3\gamma$ complex does not significantly alter the substrate binding affinity (K_m). However, the maximum rate is 1.8-times lower in the complex lacking subunit $Mt\epsilon$, indicating its' role in another step of the ATP hydrolysis reaction, possibly relieving of MgADP inhibition, cleavage of the ATP to ADP and P_i and/or release of the products.

In order to test whether MgADP-binding may in part cause this difference, $\alpha^{chi}_3\beta_3\gamma$ - and $\alpha^{chi}_3\beta_3\gamma:Mt\epsilon$ were pre-incubated with varying MgADP concentrations. As shown in figure 5A and -C the presence of 10 μM and 100 μM of MgADP reduced the ATPase activity of $\alpha^{chi}_3\beta_3\gamma$ (4.7 ± 0.01 $\mu\text{mol}/\text{min}/\text{mg}$ protein) slightly to 4.3 ± 0.05 $\mu\text{mol}/\text{min}/\text{mg}$ protein and 4.0 ± 0.02 $\mu\text{mol}/\text{min}/\text{mg}$ protein, respectively. In comparison, a significant decrease in ATPase activity was

observed in the $-\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon$ complex (8.0 ± 0.02 $\mu\text{mol}/\text{min}/\text{mg}$ protein) after preincubation of 10 μM MgADP (Fig. 5B-C), causing a reduction of 21% in ATPase activity (6.3 ± 0.02 $\mu\text{mol}/\text{min}/\text{mg}$ protein), which was comparable to the value determined in the presence of 100 μM of MgADP (6.1 ± 0.01 $\mu\text{mol}/\text{min}/\text{mg}$ protein). These data confirm that inhibitory MgADP becomes in part trapped by the reconstituted $Mt\epsilon$ within the $-\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon$ complex.

3.4 Effect of amino acid 121 of $Mt\epsilon$ in ATPase inhibition

Recently, we demonstrated in mycobacterial inverted-membrane vesicles (IMVs) that deletion of the last amino acid D121 of subunit ϵ resulted in a 13% increase in ATP hydrolysis activity (Joon et al., 2018). Here we purified (Fig. 6A) and reconstituted recombinant mutant $Mt\epsilon_{1-120}$ lacking the last amino acid with $-\alpha^{chi}_3:\beta_3:\gamma$ (Fig. 6B). The densitometric analysis (Fig. 6B) of the SDS gel for the reconstituted $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon_{1-120}$ complex revealed a ratio of 1:0.5 for subunits $\gamma:\epsilon$, indicating that the D121 deletion mutant in subunit ϵ did not alter binding. As demonstrated in Figures 6C-D, the $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon_{1-120}$ complex revealed an ATP hydrolysis activity of 9.5 ± 0.04 $\mu\text{mol}/\text{min}/\text{mg}$ protein, resulting in a similar 13% increase in ATP hydrolysis activity as determined for the D121 deletion mutant of subunit ϵ of mycobacterial IMVs (Joon et al., 2018), which confirms that the increase in activity is caused by the mutation. These data also underline, that $\alpha^{chi}_3:\beta_3:\gamma$ plus $Mt\epsilon$ is a good model to study structural and mechanistic differences of the specific mycobacterial domains and subunits. To address, whether D121 may contribute in the tight binding of the inhibitory MgADP of $-\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon$ and therefore reduction in ATP hydrolysis described above, $-\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon_{1-120}$ was pre-incubated with 10 μM of MgADP. The ATPase activity data in Figures 6E-F show that the ATP hydrolysis value of the Mg-ADP pre-incubated $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon_{1-120}$ complex dropped by about 10% (8.7 ± 0.01 $\mu\text{mol}/\text{min}/\text{mg}$ protein) in

the direct comparison to the one without pre-incubated nucleotide ($9.7 \pm 0.03 \mu\text{mol}/\text{min}/\text{mg}$ protein). When compared with the MgADP inhibiting effect of about 21% of the $-\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon$ complex shown in Figures 5B-C, the results indicate that residue D121 of *Mt* ϵ is in part involved in trapping the inhibitory MgADP.

3.5 Critical residues of *Mt* ϵ in structure and catalysis

The recent *Mt* ϵ solution structure revealed interactions between the N-terminal residues A10-W16 with the epitopes L61-A64 and A81-I90, respectively (Fig. 7A). To shed more light into these interacting residues, E87 was replaced by an alanine (*Mt* ϵ E87A). The purified *Mt* ϵ E87A mutant was purified (Fig. 6A) and reconstituted to an $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon$ E87A complex (Fig. 7B). As revealed in figures 6C-D, ATP hydrolysis of the $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon$ E87A complex ($6.6 \pm 0.02 \mu\text{mol}/\text{min}/\text{mg}$ protein) dropped by 21.5% compared to $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon$ ($8.4 \pm 0.04 \mu\text{mol}/\text{min}/\text{mg}$ protein). In order to understand the reduction in ATPase activity from a structural perspective, ^1H - ^{15}N HSQC spectra of the recombinant mutant *Mt* ϵ E87A were collected in NMR experiments and compared with the spectrum of recombinant *Mt* ϵ (Fig. 7C). The key residues undergoing significant chemical shift perturbation included W16, V51, V53, I63, E85 of the NTD, the linker residue S88 as well as the C-terminal residues R113, R115, A116, G118, I120 (Fig. 7D). These residues were mapped onto the NMR solution structure of *Mt* ϵ (Fig. 7E) and belong to the amino acid epitope providing the interaction of the NTD and CTD (Joon et al., 2018).

To investigate a possible polar effect of R62 of *Mt* ϵ , (which is in close proximity to the NTD residue D60 and the CTD residue A116 (Fig. 7A)), this amino acid was replaced by a leucine. The generated mutant protein *Mt* ϵ R62L was purified (Fig. 6A) and reconstituted to an $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon$ R62L complex in the correct stoichiometry (Fig. 7F). In comparison to the

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3 $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon$ complex (8.4 ± 0.04 $\mu\text{mol}/\text{min}/\text{mg}$ protein), the R to L substitution within the
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6 $Mt\epsilon R62L$ mutant did not alter the ATPase activity of $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon R62L$ complex (8.7 ± 0.02
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8 $\mu\text{mol}/\text{min}/\text{mg}$ protein) (Fig. 6C-D).
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10 The sequence alignment in figure 1B shows that the N-terminus of mycobacterial subunit ϵ
11 has no similarity with ϵ subunits of other prokaryotic species or the counterpart of the human δ
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13 subunit. According to the structural model in figure 8A, the N-terminal residues 1MAELN5 of
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15 subunit. According to the structural model in figure 8A, the N-terminal residues 1MAELN5 of
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17 $Mt\epsilon$ are in neighbourhood to the loop amino acids Q46, E48, and A49 of one c subunit of the
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19 rotary c -ring. In order to test, whether the very N-terminus is important in structure, assembly
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21 and/or enzymatic traits, a mutant with a deletion of the first five amino acids of $Mt\epsilon$ ($Mt\epsilon_{6-121}$)
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23 was engineered. Such deletion prevents disruption within the following $\beta 1$ -sheet of the $Mt\epsilon$
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25 structure. The size exclusion chromatogram in figure 8B represents with peaks I and -II at 9.5 ml
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27 and 11.5 ml higher oligomers of recombinant $Mt\epsilon_{6-121}$ as demonstrated in the SDS-PAGE,
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29 whereby the peak at 13.8 ml (peak III), representing a monomeric $Mt\epsilon$, is not defined. The
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31 respective sample of a fraction at 13.8 ml includes a lower amount of monomeric $Mt\epsilon_{6-121}$. These
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33 data demonstrate that deletion of the N-terminal 1MAELN5 peptide led to a shift of monomeric
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35 to an oligomeric form. Monomeric $Mt\epsilon_{6-121}$ eluting at 13.8 ml was used for the formation of an
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37 $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon_{6-121}$ complex. The SDS gel and its respective densitogram reflect that a sub-
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39 stoichiometric $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon_{6-121}$ complex ($\gamma:Mt\epsilon_{6-121}$ ratio of 1:0.14) was generated (Fig. 8C) with
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41 an ATPase activity of 5.7 ± 0.01 $\mu\text{mol}/\text{min}/\text{mg}$ protein (Fig. 8D-E). Although a sub-
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43 stoichiometric complex was formed, these observations underline that binding of $Mt\epsilon_{6-121}$
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45 increases ATP hydrolysis (5.7 ± 0.01 $\mu\text{mol}/\text{min}/\text{mg}$ protein) when compared to $\alpha^{chi}_3:\beta_3:\gamma$ ($4.7 \pm$
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47 0.01 $\mu\text{mol}/\text{min}/\text{mg}$ protein; Fig. 8E), and that the first five residues of $Mt\epsilon$ are essential for
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49 structural traits and mono-dispersity of the protein to form a proper ϵ -bound $\alpha^{chi}_3:\beta_3:\gamma$ complex.
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4. Discussion

4.1 Implications of the unique C-terminal extension of mycobacterial subunit α

Recent studies showed that deletion of the unique C-terminal stretch of the mycobacterial α subunit ($\alpha_{514-549}$) increased ATP hydrolysis and H^+ -pumping activity of the mycobacterial F-ATP synthase within IMVs (Ragunathan et al., 2017). Consistently, fusion of $Mt\alpha_{514-549}$ to the $Gs\alpha$ subunit and insertion of the chimeric α^{chi} protein into $Gs\beta_3\gamma$ to generate the chimeric $\alpha^{chi}_3:\beta_3:\gamma$ complex reduced ATP hydrolysis activity. This reduction in enzymatic activity appeared to be due to a reduction of the angular velocity of the rotating $\alpha^{chi}_3:\beta_3:\gamma$ complex after binding of ATP (Ragunathan et al., 2017). The presented 3D reconstruction $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon$ as well as the solution low resolution $Mt\alpha$ structure visualize that this C-terminal extension is allocated at the bottom of mycobacterial subunit α , resulting in a significantly longer subunit ($105 \pm 10 \text{ \AA}$), when compared to the related $Gs\alpha$ ($90 \pm 10 \text{ \AA}$) (Fig. 3D-E). In addition, due to the $Mt\alpha_{514-549}$ extension in α^{chi} the alternating arrangement of the two nucleotide-binding subunits α^{chi} and the shorter nucleotide-binding subunit β are clearly demonstrated in the hexameric ensemble of the $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon$ 3D reconstruction (Fig. 2D). The recently determined solution NMR structure of the mycobacterial C-terminal helix $\alpha_{521-540}$ fits nicely into the extension volume observed both in the 3D reconstruction of $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon$ (Fig. 2D) as well as inside the solution shape of $Mt\alpha$ (Fig. 3E). This arrangement brings this $\alpha_{521-540}$ helix in a close proximity to the short γ -helix including γ C109 (Fig. 2D), explaining the recently described crosslink between residue α C525 and γ C109 (Ragunathan et al., 2017). As proposed previously, at a 60° -degree rotation of the central $\gamma\epsilon$ -stalk, the α -helical residues $_{90}AYNSNVLRLVYQT_{102}$ of subunit γ come into the vicinity of subunit α residues $_{396}AQFGSDLK_{404}$ (Ragunathan et al., 2017). In addition, at this rotary angle the polar residues Q104, R106 and C109 of subunit γ are in proximity to the extended C-terminal α

residues D522, E523 and V525 within the $\alpha^{chi}_3:\beta_3:\gamma$ complex. Such additional interactions due to the extended α subunit have been proposed to influence the rate of catalysis observed in the chimeric $\alpha^{chi}_3:\beta_3:\gamma$ complex (Ragunathan et al., 2017).

4.2 MgATP-dependent specific ATPase activity of the $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon$ complex

While the mycobacterial subunit α shows an elongated C-terminus, mycobacterial subunit ϵ reveals a gap of about 16 amino acids, resulting in two α -helices, which are significantly shorter compared to subunit ϵ of other F-ATP synthases (Biuković et al., 2013; Joon et al., 2018). The 3D reconstruction of $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon$ demonstrates that the recently determined NMR structure of $Mt\epsilon$ (Joon et al., 2018) can be well positioned inside the complex (Fig. 2B), and that a complex with the right stoichiometry (Fig. 2A) and enzymatic activity (Fig. 4A-B) was generated, enabling to extend the former successful chimeric $\alpha^{chi}_3:\beta_3:\gamma$ (Ragunathan et al., 2017) by $Mt\epsilon$, and to deepen the understanding of the unique coupling mycobacterial ϵ subunit. The significant MgATP-dependent reduction in specific ATPase activity of $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon$ (Fig. 4B) is in line with the inhibitory effect of $G\epsilon$ reported for the *G. stearotheophilus* $\alpha_3:\beta_3:\gamma:\epsilon$ complex at lower ATP concentrations, whereby at physiologically relevant concentrations of >1 mM MgATP, this complex is believed to exist in an active form (Kato et al., 1997). Rotation experiments of $\alpha_3:\beta_3:\gamma:\epsilon$ have shown that MgATP concentrations above 2 mM do not affect rotation of the complex, whereby at 200 μ M of MgATP, subunit ϵ prolongs the pause of rotation caused by MgADP inhibition (Tsumuraya et al., 2009). The correlation of MgATP concentration and ATPase inhibition of $\alpha_3:\beta_3:\gamma:\epsilon$ has in part been attributed to MgATP-binding to $G\epsilon$ and switching from a compact to an extended form (Kato et al., 1997). $Mt\epsilon$ differs from $G\epsilon$ in its inability to bind ATP (Biuković et al., 2013). Therefore, the mechanism(s) behind the observed

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3 inhibition in specific ATPase activity of $\alpha^{chi}_3\beta_3\gamma:Mt\epsilon$ (Fig. 4B) may partially be caused by the
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5 extra mycobacterial C-terminus $\alpha_{521-540}^{chi}$ of α^{chi} and/or $Mt\epsilon$, which makes it an additional
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7 interesting aspect to probe into (see below).
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10 11 12 13 *4.3 Critical amino acids of mycobacterial subunit ϵ for enzymatic coupling* 14

15 The $Mt\epsilon$ structure has been described to include a number of hydrophobic interactions and
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17 hydrogen bonds between the N-terminal residues A10-W16 with the epitopes L61-A64 and A81-
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19 I90, respectively (Fig. 7A), enabling communication between the NTD and CTD of $Mt\epsilon$ (Joon et
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21 al., 2018). A mechanistic model was proposed in which the loop residues R45, Q46 and E48 of
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23 one c -subunit interact with the amino acids F24 and K21 of the $Mt\epsilon$ NTD, respectively, and that
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25 rotation of the c -ring derived by ion transport would affect the interaction between those residues
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27 of the c -ring loop and the residues at the bottom of $Mt\epsilon$ NTD (Joon et al., 2018). Like in a spring,
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29 the interaction of the epitopes formed by residues V51-V53, L61-A64, I8-A10, and D12-N14,
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31 would propagate the coupling events between the c -ring and the amino acids at the bottom of the
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33 $Mt\epsilon$ NTD, which then become coupled to the epitope of amino acids A81-I90 that is closer to the
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35 $Mt\epsilon$ CTD (Fig. 1A and 7A). In this model, residues F86-I90 form a hinge region, allowing up- and
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37 down movements of the CTD (Fig. 1A; Joon et al., 2018). Here, the generated mutant $Mt\epsilon$ E87A,
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39 reconstituted to an $\alpha^{chi}_3\beta_3\gamma:Mt\epsilon$ E87A complex caused a 21.5% reduction of ATP hydrolysis
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41 activity, reflecting the importance of this residue for enzyme activity. The identified residues of
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43 the $Mt\epsilon$ E87A undergoing significant chemical shift perturbation (V51, V53, I63, E85 of the
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45 NTD, the linker residue S88, and R113, R115, A116, G118, I120 of the CTD (Fig. 7D)), were
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47 similar to the once belonging to the amino acid epitope providing the communication of the NTD
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49 and CTD (Joon et al., 2018), underlining the importance of E87 in NTD and CTD coupling.
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3 In comparison, when mutant *Mtε*R62L was reconstituted to an $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon R62L$ complex
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5 (Fig. 7F) no effect in ATP hydrolysis activity was observed (Fig. 6C-D), demonstrating that
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7 eliminating the charge and keeping the length of the side chain by the R to L substitution, does
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9 not alter the coupling efficiency within subunit ϵ . At the same time this result underlines the
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11 importance of amino acid E87, whose E to A substitution reduced the enzyme activity of
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13 $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon E87A$ complex (see above).
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17 As demonstrated recently, deletion of the last C-terminal residue (D121) of mycobacterial
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19 subunit ϵ was found to increase the rate of ATP cleavage and to decrease ATP synthesis of IMVs
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21 (Joon et al., 2018). In addition, substitution of D121 to a cysteine, and reconstitution of this
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23 *Mtε*D121C mutant within an engineered $(\alpha^{chi}\text{-S400C})_3:\beta_3:\gamma$ resulted in a crosslink product formed
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25 by residues C121 of *Mtε*D121C and amino acid C400 of the DELSEED-region of the nucleotide-
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27 binding subunit $\alpha^{chi}\text{-S400C}$ (Joon et al., 2018). These data (i) confirmed that the CTD of *Mtε* is
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29 able to extend, (ii) that the very C-terminus of mycobacterial subunit ϵ comes in proximity of the
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31 DELSEED-region of the nucleotide-binding subunit α , and (iii) that an extended CTD of *Mtε*
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33 with a proposed total length of 42 Å would be too short to reach into the upper region of the
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35 catalytic $\alpha_3:\beta_3$ -headpiece like the CTD of *E. coli* and *G. stearothermophilus* ϵ , whose extended
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37 CTD has a total length of about 67 Å (Joon et al., 2018). The $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon_{1-120}$ complex showed
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39 a similar increase in ATP hydrolysis activity as the D121 deletion mutant of subunit ϵ
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41 mycobacterial IMVs (Fig. 6C-D; Joon et al., 2018), confirming that this residue is important in
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43 the activity regulation of the catalytic headpiece. Moreover, the new data presented (Fig. 6E-F)
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45 also add to the picture that residue D121 of mycobacterial subunit ϵ is in part being involved in
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47 trapping the inhibitory MgADP.
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4.4 Importance of the N-terminus for *Mtε* stability

Systematic studies on the *E. coli* ϵ subunit showed that deletion of the first six amino acids does not alter the ATPase activity of *E. coli* membranes (Jounouchi et al., 1992). The sequence alignment in figure 1B shows that the N-terminus of mycobacterial subunit ϵ has no similarity with the related *Ecε*, *Gsε* or the counterpart of the human δ subunit. Deletion of the N-terminal peptide 1MAELN5 of *Mtε* resulted in the destabilization of the protein, which manifests as oligomerization (Fig. 8B), and affects the reconstitution of an $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon_{6-121}$ complex (substoichiometric of $\gamma:Mt\epsilon_{6-121}$ of 1:0.14; Fig. 8C). As pointed out above and by the structural model in figure 8A, peptide 1MAELN5 of *Mtε* is in neighbourhood of the loop amino acids Q46, E48, and A49 of one *c* subunit of the turbine *c*₉-ring. Based on our recent hypothesis, which described that one *c* subunit loop (residues R45, Q46 and E48) comes in close contact with the amino acids F24 and K21 of the *Mtε*NTD, respectively (Joon et al., 2018), we extend this model by proposing, that in addition to these interactions, part of the N-terminal peptide 1MAELN5 of *Mtε* interacts with the loop residues Q46, E48, and A49 of a second *c* subunit and that rotation of the *c*-ring derived by ion transport would affect, at least in part, the interaction between those residues of the two *c*-ring loops and the residues at the bottom of the *Mtε*NTD (Fig. 8A). These interactions would translate the coupling events between the *c*-ring and the amino acids at the bottom of the *Mtε*NTD, which than become coupled to the epitope of amino acids at the CTD of *Mtε* including D121, and finally via the C-termini of the $\alpha_3:\beta_3$ -headpiece to the catalytic side. In particular, the *c*-loop interactions with 1MAELN5 may via the β 1-sheet of *Mtε*, alter the interaction of amino acids 10AVDRN14 of *Mtε* and residues 40KSFV43 of subunit γ (Fig. 8A). Interestingly, direct measurements of backbone dynamics including R1 and R2 and two-dimensional ^1H - ^{15}N heteronuclear NOE experiments of *Mtε* demonstrated that the residues A10,

D12, and N14 have a high degree of conformational mobility (Joon et al., 2018). Furthermore, these residues are close to the ϵ -binding site of the TB drug Bedaquiline (Kundu et al., 2016; Joon et al., 2018), reflecting the importance of this *Mt* ϵ epitope.

In line with the proposed mechanism above are the structural and mechanistic data of subunit D and F of the A-type ATP synthases from *Methanosarcina mazei* Gö1 (Singh et al., 2016) and *Enterococcus hirae* (Suzuki et al., 2016) which are homologue subunits of subunit γ and ϵ of F-ATP synthases, respectively. Here the N-terminal residues of subunit F come in neighbourhood to the β -hairpin region of the rotating 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 of the A-ATP synthase (Suzuki et al., 2016). Deletion of four N-terminal residues of *M. mazei* Gö1 subunit F reduced ATPase activity of the *M. mazei* Gö1 A_3B_3DF -complex by about 77% (Singh et al., 2016).

5. Conclusion

The data presented visualize for the first time the $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon$ complex and the solution shape of the *Mtb* subunit α with its unique C-terminal stretch. Within this $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon$ complex the C-terminal D121 of *Mt* ϵ is involved in trapping the inhibiting MgADP. Amino acid E87 of *Mt* ϵ , which is close to the loop residues S88 and E89, was shown to be important for enzyme activity, and supports our recent proposal, that this residue belongs to a network of amino acids inside *Mt* ϵ which allows a spring-like mechanism inside the *Mt* ϵ NTD to couple *c*-ring rotation with up-side down movements of the *Mt* ϵ CTD, enabling ATP synthesis in the catalytic subunits. The presented data of the *Mt* ϵ_{6-121} have provided valuable information pertaining the importance of the N-terminal residues in structure and mechanism and highlighted the difference of the N-

terminal amino acid composition of mycobacterial ϵ subunits when compared to other bacterial species, which makes it an additional interesting epitope to probe as an attractive drug target for TB.

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Figure Legends

Fig. 1. (A) The structural model of the mycobacterial F-ATP synthase was generated based on the cryo-EM model of *E. coli* F-ATP synthase (PDB ID: 5T4O; Sobti et al., 2016). Subunits α (*green*) and β (*orange*) form the static hexameric catalytic part. The solution shape of α^{chi} is shown as *green sphere* (Ragunathan et al., 2017). For clarity one α - and β -subunit are removed. Subunit γ (*yellow*) has a unique γ -loop (Hotra et al., 2016) in case of the mycobacterial one which is highlighted in *red*. The compact *Mt ϵ* (PDB ID: 5YIO; Joon et al., 2018) is shown in *magenta*. The modelled extended ϵ subunit (*red*) of *M. tuberculosis* reaches in to the catalytic subunit. The *c*-ring loop residues of *M. phlei* (*wheat*; PDB ID: 4V1G; Preiss et al., 2015), proposed to interact with the rotating ϵ and γ stalks subunits are indicated. The subunits *a*, *b*, *b'* and δ are shown in the *dark blue*, *light blue* and *purple*, respectively. The dimer of subunit *b* was constructed into the EM map based on the NMR structure of the transmembrane domain *b*₁₋₃₄ (PDB ID: 1B9U; Dmitiriev et al., 1999), NMR structure of *b*₃₀₋₈₂ (PDB ID: 2KHK; Priya et al., 2009) and the crystal structure of *b*₆₂₋₁₂₂ (PDB ID: 1L2P; Del Rizzo et al., 2002) with additional *de novo* modeling for the remaining residues. Based on operon studies it was proposed that one of the mycobacterial *b*-subunits is fused to subunit δ , which is represented as transparent cylinder. (*inset; right*) The network of interactions, that is proposed to be involved in the coupling events between the *Mt ϵ* NTD and the *M. phlei* *c*-ring residues, and the up-down movement of *Mt ϵ* CTD. The *blue* arrow represents the proposed spring action of the *Mt ϵ* NTD. For clarity the γ -subunit is not shown. (B) Sequence alignment of F-ATP synthase subunit ϵ s from various *Mycobacterium* species with the ones of *E. coli*, *G. stearotheophilus* and the human related subunit δ .

Fig. 2. (A) Isolation of the reconstituted $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon$ complex using size-exclusion chromatography (*left*). The collected fractions at around 12 ml are shown in grey and labelled as *I*. The purity and subunit composition are shown by a 12% SDS gel (*right*). The intensity ratio 1:0.5 of subunit γ to $Mt\epsilon$ is derived from the peak integration in the densitogram (*right*), indicating complete ϵ binding. (B) The electron-micrograph of negatively-stained $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon$ represents well dispersed and individual particles (*white box*) used for further analysis. A scale bar is shown in the lower right corner. (C) Upper panel shows 50 classes separated from two rounds of particle elimination and classification. Most populated classes showing the prominent features of the complex are squared in *red* (1st row). Lower panel shows the enhanced view of the most populated classes 1-3. Panel one represents a class of side-views of $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon$ with a defined central stalk, which is highlighted by an arrow. The second panel shows the bottom view (membrane-view), and the third class reveals the top view with the crown-like domain formed by the N-termini of the six nucleotide-binding subunits $\alpha^{chi}_3:\beta_3$. (D) Surface representation of the 3D reconstruction of $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon$ refined to about 18 Å using *RELION 1.3* (Tang et al. 2007) with side views (Panel 1-2, top row), as well as top- (Panel 4, top row) and bottom view (Panel 5, top row). Panel 3 (top row) shows that the $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon$ complex model fitted into the surface density map obtained from the negative stained EM of the reconstituted $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon$ complex. Colour code: α -forest green, extended C-terminal NMR solution structure of $Mta_{521-540}$ light green; β -orange, γ -yellow, and extended $Mt\epsilon$ -red. In the bottom row, panels 1, 2, 4 and 5 display the cartoon representations of *G. stearothermophilus* $\alpha_3:\beta_3:\gamma:\epsilon$ (PDB code 4XD7; Shirakihara et al., 2015), including the $Gs\epsilon$ subunit in blue. The first panel shows a side view of the complex revealing the unoccupied densities of the subunit α that correspond to the extra C-terminal peptide of α^{chi} ; The second panel shows the model of the extended state of subunit ϵ , subunit β is

hidden for viewing convenience, and the arrow indicates unoccupied densities at the bottom of subunit α ; The third panel represents the cross section taken at the bottom of the $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon$ complex model in panel 3 (row 1, *yellow line*) displaying the extra C-terminal helices $Mt\alpha_{521-540}$ of α^{chi} nested in the density map in an alternating fashion. All the models and surface density maps were visualized using *UCSF CHIMERA*. (E) Fourier shell correlation graph of the negatively-stained reconstituted $\alpha^{chi}_3:\beta_3:\gamma:Mt\epsilon$ complex. Density map resolution calculated for the FSC value of approx. 0.143 (0.146). Global resolution based on FSC is $1/0.55 = 18 \text{ \AA}$.

Fig. 3. (A) SDS gel shows purified recombinant $Mt\alpha$ (lane 2) with molecular marker proteins (kDa) in the first lane. (B) Small angle X-ray scattering pattern of $Mt\alpha$ measured for the concentrations 0.7 mg/ml (*blue*), 1.0 mg/ml (*red*) and 1.2 mg/ml (*green*). Fitting of the theoretical scattering curve (—) computed by CRY SOL (Svergun *et al.*, 1995) with the experimental scattering pattern ($\circ$) resulted in χ^2 -values of 1.65. The curves are displayed in logarithmic units for clarity. (*inset*) Guinier plots show linearity indicating no aggregation. (C) Normalized Kratky plot of Gsa (*yellow*), $Mt\alpha$ (*green*) and lysozyme as control (*grey*), indicating the folded nature of the protein. (D) Pair-distance distribution function $P(r)$ of Gsa (*yellow*) and $Mt\alpha$ (*green*). (E) The average solution shape of $Mt\alpha$, was fitted with the crystal structure of Gsa (*dark green*; PDB code 1SKY), represented as cartoon (*left*). The empty space at the bottom of this $Mt\alpha$ shape (*left*) provides additional volume for the extra mycobacterial extension of $Mt\alpha_{514-549}$, and enables to fit the extra C-terminal helix structure $Mt\alpha_{521-540}$, derived by solution NMR spectroscopy (Ragunathan *et al*, 2017; *center and right*).

Fig. 4. (A) Continuous ATPase activity of $\alpha^{chi}_3\beta_3\gamma$ and $\alpha^{chi}_3\beta_3\gamma:Mt\epsilon$ plotted against the time. The *orange* and *turquoise* line represent the activity profile of $\alpha^{chi}_3\beta_3\gamma$ and $\alpha^{chi}_3\beta_3\gamma:Mt\epsilon$, respectively. (B) Bar diagram representing the specific ATPase activities of $\alpha^{chi}_3\beta_3\gamma$ and $\alpha^{chi}_3\beta_3\gamma:Mt\epsilon$ in the presence of varying substrate (MgATP) concentrations ranging from 2 mM down to 6 μ M. Values are mean $\pm$ SD based on more than ten determinations. (C) 17% SDS gel showing the purity and homogeneity of the purified $Gs\alpha_3\beta_3\gamma$ complex (*lane 1*) and subunit $Gs\epsilon$ (*lane 3*) as well as the reconstituted $Gs\alpha_3\beta_3\gamma:\epsilon$ complex (*lane 2*) in the correct $\gamma:\epsilon$ stoichiometry of 1:1. As described recently, $Gs\alpha$ and $Gs\beta$ run as a single band on a 17% SDS gel (Chaban et al., 2004). *Lane LM* shows molecular marker proteins (kDa). (D) Bar diagrams displaying the specific ATP hydrolytic activity of the $Gs\alpha_3\beta_3\gamma:\epsilon$ complex in the presence of varying MgATP concentrations ranging from 2 mM down to 0.5 mM. Values are mean $\pm$ SD based on six determinations. (E) Kinetic profiles of $\alpha^{chi}_3\beta_3\gamma$ and $\alpha^{chi}_3\beta_3\gamma:Mt\epsilon$ calculated based on substrate-dependent ATP-hydrolysis experiments using the Michaelis-Menten equation. Maximum velocity values, V_{max} , were determined to be $4.97 \pm 0.12 \text{ s}^{-1}$ and $8.63 \pm 0.25 \text{ s}^{-1}$, respectively, while the K_m values were similar with $0.107 \pm 0.009 \text{ mM}$ and $0.164 \pm 0.015 \text{ mM}$, respectively.

Fig. 5. Effect of MgADP on the inhibition of ATPase activity. Profiles of the ATPase activity of $\alpha^{chi}_3\beta_3\gamma$ (A) and $\alpha^{chi}_3\beta_3\gamma:Mt\epsilon$ (B) measured in the presence of 2 mM MgATP and varying MgADP concentrations; Complexes incubated without MgADP (*black*), 10 μ M MgADP (*red*) and 100 μ M MgADP (*blue*). (C) Bar diagrams showing the specific ATPase activity of $\alpha^{chi}_3\beta_3\gamma$ and $\alpha^{chi}_3\beta_3\gamma:Mt\epsilon$ in presence of 2 mM MgATP and varying MgADP concentrations. All values displayed are a mean value of three measurements.

Fig. 6. Reconstitution of $\alpha^{chi}_3\beta_3\gamma$ with various subunit *Mtε* mutants. (A) Purity of the subunit *Mtε* (WT and mutants) analysed on a 17% SDS-gel. Lanes *LM*, protein marker (kDa); lanes 1–5 show the recombinant proteins *Mtε*, *Mtε*₁₋₁₂₀, *Mtε*R62L, *Mtε*E87A, *Mtε*₆₋₁₂₁, respectively. Lane 5 shows that even in an SDS PAGE mutant *Mtε*₆₋₁₂₁ occurs still as an oligomer (*O*) in addition to a monomer (*M*). (B) Densitogram revealing the ratio of subunits $\gamma:Mtε_{1-120}$ of 1:0.5 within the reconstituted $\alpha^{chi}_3\beta_3\gamma:Mtε_{1-120}$ complex. ATPase activity profiles (C) and bar diagrams (D) revealing the activities of the complex $\alpha^{chi}_3\beta_3\gamma$ and reconstituted complexes $\alpha^{chi}_3\beta_3\gamma:Mtε$, (WT and mutants), *Mtε*, *Mtε*₁₋₁₂₀, *Mtε*R62L, *Mtε*E87A, respectively, in the presence of 2 mM ATP. Influence of MgADP inhibition on the ATPase activity of the reconstituted complex $\alpha^{chi}_3\beta_3\gamma:Mtε_{1-120}$ measured in the presence of 2 mM MgATP. Values are mean $\pm$ SD based on three determinations. Profiles (E) show the ATPase activity of the $\alpha^{chi}_3\beta_3\gamma:Mtε_{1-120}$ without MgADP (*black*) and pre-incubated with 10 μ M MgADP (*red*), as reflected in the bar diagrams (F). Values are mean $\pm$ SD based on three determinations

Fig. 7. (A) Surface representation of *Mtε* showing the formation of a network of residues connecting NTD to CTD. The residues partaking in the network formation are shown as sticks and are represented in *green* and *magenta* colour for the NTD and CTD, respectively. Left panel shows the detailed view of the amino acid residues in the network connecting NTD to CTD, represented as sticks. Right panel shows an expanded view of the interface between CTD and NTD, and important residues are labeled and inter-proton distances less than 5 Å are marked as dashed lines. (B) Densitogram of band intensities of subunits γ (*left peak*) and *Mtε*E87A (*right peak*) in the $\alpha^{chi}_3\beta_3\gamma:MtεE87A$ complex derived from the 12% SDS-gel (*inset*). Integrals of the peaks were used to determine the ratio of $\gamma:MtεE87A$ as 1:0.5. (C) Effect of the E87A mutation

Mtε revealed by NMR. The overlaid ^{15}N -HSQC-spectra of *Mtε* (*blue*) and the mutant E87A (*red*). (D) Weighted CSPs for the ^{15}N and ^1H resonance of *Mtε* and mutant *Mtε*E87A. Average and average plus standard deviation values are indicated as dotted and solid lines, respectively. CSPs residues showing CSP above average plus standard deviation (solid line) value are labelled as one-letter code. The weighted CSPs between *Mtε* and mutant *Mtε*E87A for backbone ^{15}N and ^1HN were calculated by the formula $\Delta\delta = [(\Delta\text{N})^2 + (\Delta\text{HN}/5)^2]^{0.5}$. (E) Perturbed residues mapped on the surface of *Mtε*; the residues having CSP higher than 0.1 ppm are represented in *red*, while the residues with CSP values between 0.05 and 0.1 are represented in *blue*. (F) The plot represents band intensities of subunits γ (*left peak*) and *Mtε*R62L (*right peak*) in the $\alpha^{chi}_3:\beta_3:\gamma:\textit{Mtε}R62L complex derived from the 12% SDS-gel (*inset*). Ratio of the $\gamma:\textit{Mtε}R62L is calculated to be 1:0.5.$$

Fig. 8. (A) The network of interactions that is proposed to be involved in the coupling events between the *M. phlei* c-ring residues (*beige*) and the amino acids of the *Mtε*NTD as well as the crosstalk of *Mtε* residue A10 to N14 with amino acids K40 to V43 of subunit γ (indicated in *orange*). Subunit γ is shown in *yellow* and its mycobacterial specific loop (Hotra et al., 2016) is highlighted in *red*. (B) Purification of *Mtε*₆₋₁₂₁ via size exclusion chromatography. The fractions collected, marked in grey, at 9.5 ml, 11.5 ml and 13.8 ml were analyzed by a 17% SDS-gel (*inset*), where P1-P3 correspond to the same marks on the chromatogram. *M*, corresponds to monomeric form and *O* to oligomeric form. (C) The plot represents the densitometric analysis of $\alpha^{chi}_3:\beta_3:\gamma:\textit{Mtε}_{6-121}$. Similarly to the other analysed complexes, the integrals of the respective peaks of subunit γ and *Mtε*₆₋₁₂₁ show a ratio of 1:0.14, indicating incomplete complex formation. Taking into consideration that the stable, structured NTD is required for the association of subunit *Mtε* to the $\alpha^{chi}_3:\beta_3:\gamma$ complex, we propose that the deletion of the five N-terminal affects the binding to

the complex. ATPase activity profiles (*D*) and bar diagrams (*E*) represent the rate of ATP hydrolysis of the $\alpha^{chi}_3\beta_3\gamma$ - and $\alpha^{chi}_3\beta_3\gamma:Mt\epsilon_{6-121}$ complexes. Comparatively to the value obtained for $\alpha^{chi}_3\beta_3\gamma:Mt\epsilon$ (8.4 $\mu\text{g/ml/mg}$ of protein), the lower enzyme activity observed for $\alpha^{chi}_3\beta_3\gamma:Mt\epsilon_{6-121}$ corroborates the fact that a sub-stoichiometric complex was formed. Values are mean $\pm$ SD based on three determinations

Table 1: Data collection and scattering derived parameters for *Mta* compared with *Gsa*

Sample details	<i>Mta</i>	
Organism	<i>Mycobacterium tuberculosis</i> (<i>Mt</i>)	
GenBank	NC_000962	
Extinction coefficient (A_{280} , $M^{-1} \text{ cm}^{-1}$)	31,400	
MM from chemical composition (Da)	59,288	
Protein concentration (mg ml^{-1})	0.7 – 1.2	
Solvent	50 mM Tris/HCl, pH 7.5, 200 mM NaCl, 1 mM DTT	
Data collection parameters		
Instrument (source & detector)	Bruker NanoStar equipped with MetalJet eXcillum X-ray source and VÅNTEC-2000 detector	
Beam geometry	100 μm slit	
Wavelength (nm)	0.134	
Sample-to-detector distance (m)	0.67	
q range (nm^{-1})	0.16 – 4.0	
Normalization	To transmitted intensity by glassy carbon	
Monitoring for radiation damage	Data frame-by-frame comparison	
Exposure time (min)	30 (6 frames x 5 min)	
Sample configuration	Vacuum tight quartz capillary	
Sample temperature (K)	288.15	
Software employed		
Primary data reduction	BRUKER SAXS program suite	
Data processing	PRIMUSqt from ATSAS 2.8.2	
<i>Ab initio</i> analysis	GASBOR from ATSAS 2.8.2	
Validation and averaging	DAMAVAR from ATSAS 2.8.2	
Atomic structure modeling	CRY SOL from ATSAS 2.8.2	
3D graphics representations	PyMOL v1.5.0.4	
Structural parameters	<i>Mta</i>	<i>Gsa</i> *
I(0) (arbitrary unit) (from P(r))	47.7 ± 1.5	80.2 ± 0.7
R_g (Å) (from P(r))	32.6 ± 1.1	28.1 ± 0.3
I(0) (arbitrary unit) (from Guinier)	46.9 ± 1.4	80.3 ± 0.5
R_g (Å) (from Guinier)	31.3 ± 1.3	27.9 ± 0.3
D_{max} (Å)	105 ± 10	90.0 ± 10
Porod volume estimate (Å ³)	~95,845	~79,156
Dry volume calculated from sequence (Å ³)	71,736	66,050
Molecular mass determination		
Calculated monomeric <i>MM</i> (kDa) (from sequence)	~59	~55
Molecular mass <i>MM</i> (kDa) (from <i>Porod invariant</i>)	60 ± 6	49 ± 6
From SAXS MoW	59 ± 6	56 ± 6
From excluded volume	55 ± 6	51 ± 6
Shape model-fitting results		
GASBOR (default parameters, slow mode, 10 calculations)		
q range (Å ⁻¹)	0.02 – 0.2	0.022-0.2
Symmetry, anisotropy assumptions	<i>P1</i> , none	<i>P1</i> , none

NSD, No. of clusters	$1.16 \pm 0.04, 1$	$1.02 \pm 0.02, 1$
χ^2 range	$0.64 - 0.66$	$0.36 - 0.38$
Constant adjustment to intensities	0.41	0.33

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* Reported for data from Ragunathan et al. 2017

Figure 1A
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18	<i>H.sapiens</i>	I MLPAALLRRPGLGRLVRHARAYAEAAAAAPAAASGPNQMSFTEASPTQVFFNCANVRQVDVP ~~ELT~~ GAFGFLAAHVP ~~TLQV~~ ER - PGL ~~VV~~ 87
19	<i>E.coli</i>	I - - - - - MAMTYHLDV ~~VS~~ AEQQMF ~~SL~~ - LVEKIQVTGSE ~~GL~~ LG ~~LY~~ PGHAP ~~LT~~ AIK - PGM ~~I~~ I 53
20	<i>G.stearothermophilus</i>	I - - - - - MKT ~~I~~ H ~~VS~~ V ~~MT~~ P ~~D~~ GPVYED - DVEMVSVKAKS ~~DEL~~ GL ~~LP~~ GHIP ~~EV~~ AP ~~TE~~ - ISAA ~~H~~ - 51
21	<i>M.africanum</i>	I - - - - - MAELNVEIVAVDRNIWSG - TAK ~~FL~~ TRTTVGEIGILPRHIPLVAQEVDDAMV ~~EV~~ 53
22	<i>M.tuberculosis</i>	I - - - - - MAELNVEIVAVDRNIWSG - TAK ~~FL~~ TRTTVGEIGILPRHIPLVAQEVDDAMV ~~EV~~ 53
23	<i>M.bovis</i>	I - - - - - MAELNVEIVAVDRNIWSG - TAK ~~FL~~ TRTTVGEIGILPRHIPLVAQEVDDAMV ~~EV~~ 53
24	<i>M.alcerans</i>	I - - - - - MAELNVEIVAVDRKIWSG - AGT ~~FL~~ TRTTVGEIGILPNHIPLVAQEVDDAMV ~~EV~~ 53
25	<i>M.megmatitis</i>	I - - - - - MAELNVEIVAVIRELWSG - PAT ~~EV~~ TRTTAGEIGILPRHIPLVAQEVDDAMV ~~EV~~ 53
26	<i>H.sapiens</i>	88 HA ~~LD~~ GGTTSKYF ~~V~~ SS ~~GS~~ IA ~~V~~ NADSS ~~V~~ OLL ~~ALE~~ AVTLDML ~~DL~~ GA ~~AK~~ ANL ~~E~~ KAQAE ~~L~~ VGTADEAT ~~RAE~~ IQIRIEANI ~~AL~~ VKALE - - - - - 168
27	<i>E.coli</i>	54 VKQHGH ~~EEF~~ YLS ~~GH~~ ILEV - QP ~~GN~~ TV ~~L~~ ADTAIRGQDL ~~DE~~ ARAMEAKRKAE ~~EH~~ SSSHGDDVDYAQ ~~NS~~ AELAK ~~I~~ AQLRVIELTKKAM 139
28	<i>G.stearothermophilus</i>	52 LKKGG ~~KTQY~~ IAVSG ~~GLE~~ EN - RPD ~~NT~~ ILAQAA ~~IR~~ AEDIDVLRAKAR - KSGRTPLOSQQDDIDFK ~~AE~~ LALKRAMNRLSVAEMK - - - - 132
29	<i>M.africanum</i>	54 EREG ~~E~~ KDLRIAVDGG ~~F~~ LSV - TEEG ~~VS~~ ILAESAEFES ~~E~~ IDEAANKQDS ~~ES~~ DDPRIAA - - - - - RGHARL - - - RAVGAID - - - - - 121
30	<i>M.tuberculosis</i>	54 EREG ~~E~~ KDLRIAVDGG ~~F~~ LSV - TEEG ~~VS~~ ILAESAEFES ~~E~~ IDEAANKQDS ~~ES~~ DDPRIAA - - - - - RGHARL - - - RAVGAID - - - - - 121
31	<i>M.bovis</i>	54 EREG ~~E~~ KDLRIAVDGG ~~F~~ LSV - TEEG ~~VS~~ ILAESAEFES ~~E~~ IDEAANKQDS ~~ES~~ DDPRIAA - - - - - RGHARL - - - RAVGAID - - - - - 121
32	<i>M.alcerans</i>	54 ERDGD ~~K~~ DLRIAVDGG ~~F~~ MSV - TDAG ~~VI~~ ILAESAEFES ~~E~~ IDEAVARQDS ~~ES~~ DDPRIAA - - - - - RGHARL - - - RAVGAID - - - - - 121
33	<i>M.megmatitis</i>	54 EREG ~~E~~ DDLRIAVDGG ~~F~~ LSV - TEET ~~VR~~ ILEVNAQFES ~~E~~ IDEAANKEDAASDD ~~ER~~ TAA - - - - - WGRARL - - - RALGQID - - - - - 121

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