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Review

ATP synthase in mycobacteria: Special features and implications for a function as drug target☆

Ping Lu, Holger Lill, Dirk Bald *

Department of Molecular Cell Biology, AIMMS, Faculty of Earth- and Life Sciences, VU University Amsterdam, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands

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ABSTRACT

ATP synthase is a ubiquitous enzyme that is largely conserved across the kingdoms of life. This conservation is in accordance with its central role in chemiosmotic energy conversion, a pathway utilized by far by most living cells. On the other hand, in particular pathogenic bacteria whilst employing ATP synthase have to deal with energetically unfavorable conditions such as low oxygen tensions in the human host, e.g. *Mycobacterium tuberculosis* can survive in human macrophages for an extended time. It is well conceivable that such ATP synthases may carry idiosyncratic features that contribute to efficient ATP production. In this review genetic and biochemical data on mycobacterial ATP synthase are discussed in terms of rotary catalysis, stator composition, and regulation of activity. ATP synthase in mycobacteria is of particular interest as this enzyme has been validated as a target for promising new antibacterial drugs. A deeper understanding of the working of mycobacterial ATP synthase and its atypical features can provide insight in adaptations of bacterial energy metabolism. Moreover, pinpointing and understanding critical differences as compared with human ATP synthase may provide input for the design and development of selective ATP synthase inhibitors as antibacterials. This article is part of a Special Issue entitled: 18th European Bioenergetic Conference.

1. Introduction

ATP synthase is a ubiquitous key enzyme in energy metabolism of virtually all living cells. The enzyme utilizes the energy stored in a trans-membrane electrochemical potential difference of a coupling ion for production of ATP. Bacterial ATP synthase is composed of a membrane-embedded F_o sector with subunit composition $a_1b_2c_{10-15}$ and a hydrophilic F_1 part, consisting of subunits $\alpha_3\beta_3\gamma\delta\varepsilon$ (Fig. 1A). In mitochondrial ATP synthase several more peripheral subunits are found in addition to the eight core subunits also present in bacteria (Fig. 1B) (for review see [1–3].

Passage of ions, in most cases protons, through F_o triggers rotation of the oligomeric subunit c ring, which is coupled to rotation of subunit γ and subunit ε. Rotation of subunit γ within the $\alpha_3\beta_3$ hexamer of F_1 in turn drives synthesis of ATP [4–8]. While the basic structure of ATP synthase and its mechanism of rotary catalysis apparently are conserved across the three kingdoms of life, ATP synthases from certain organisms show specialized features that may represent adaptations to specific environments encountered. As an example, ATP synthases from photosynthetic organisms carry an inserted sequence of some 35 amino acids in subunit γ, which allows for redox regulation, adjusting the enzyme's activity to the available light intensity [9–11]. ATP synthase from thermoalkaliphilic bacteria displays conserved basic residues in subunit a, facilitating capture and flow of protons under alkaline conditions [12].

ATP synthesis in pathogenic bacteria may face exceptional challenges, as a variety of pathogens need to deal with "low energy" conditions, such as low oxygen tensions and/or nutrient limitation, e.g. in remote parts of the human lungs or inside phagosomes of human macrophages. In particular *Mycobacterium tuberculosis* and related strains of the *Mycobacterium* genus, such as *M. leprae* and *M. ulcerans*, can reside in the human host for many years [13,14]. These mycobacterial strains can further shift-down their metabolism entering a so-called dormant state. In this dormant state bacterial replication virtually ceases, cell wall thickness is increased, protein and nucleic acid syntheses are significantly down-regulated, and lipid metabolism appears to be the primary energy source [15,16]. Typically, dormant mycobacteria display only very low susceptibility towards currently used antibacterials [17–20]. Specialized niches in which mycobacteria reside may require adaptations in the energy metabolism in order to provide sufficient amounts of ATP and/or to generate a proton motive force (PMF) [19,21]. As such, ATP synthase in these bacteria may carry special features facilitating survival under conditions encountered in the human host.

ATP synthase is reported to be essential in *M. tuberculosis* for optimal growth [22] and in the non-pathogenic model strain *Mycobacterium smegmatis* [23]. Likely, the essentiality stems from a need for ATP synthase for production of ATP and/or for maintenance of a minimal level of respiratory electron flow [19,24]. For *M. tuberculosis*, a PMF of −110 mV has been reported, with the membrane potential and the

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* Corresponding author. Tel.: +31 205986991; fax: +31 205987136.
E-mail address: d.bald@vu.nl (D. Bald).

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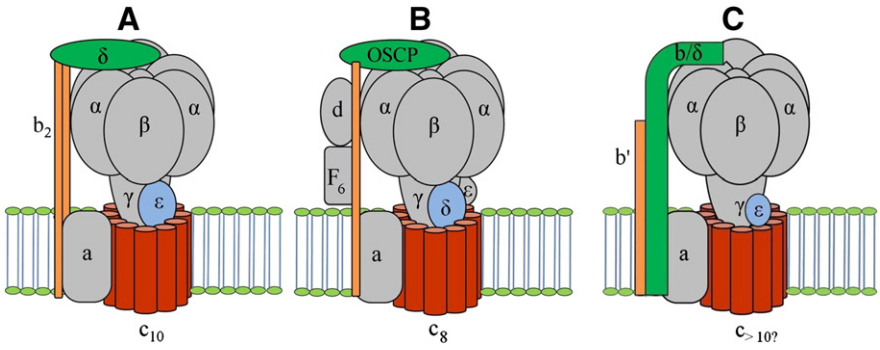

Fig. 1. Subunit composition of ATP synthase. (A) "Standard" composition of bacterial ATP synthase, as found in *E. coli*. (B) ATP synthase from human mitochondria, several small subunits such as e, f, g, and 6 L are not shown (C) ATP synthase in mycobacteria. In *M. tuberculosis*, ATP synthase comprises a fusion of subunits b and subunit δ and a second, shorter, b′-type subunit in the stator stalk. A C-terminal helix in subunit ε is lacking. The oligomeric state of the c-ring is unknown, it likely consists of >10 monomers.

ΔpH contributing roughly equally [18]. Typically, in other bacteria a PMF of ~− 200 mV is found. The low PMF in *M. tuberculosis* likely represents an adaptation to low nutrient and/or electron acceptor availability. ATP synthase is essential not only in replicating mycobacteria, but also in the dormant state [18,25].

ATP synthase in *M. tuberculosis* has recently been validated as a highly promising target of antibacterials. Its activity is efficiently blocked by the diarylquinoline class of drugs [26–28]. A deeper understanding of ATP synthase function in mycobacteria may thus not only shed light on molecular adaptations allowing for survival under conditions of low energy supply, but also allow for utilizing differences in mycobacterial ATP synthase as compared with the human homologue for the design and development of new drug candidates.

2. Regulation of mycobacterial ATP synthase

Mycobacterial ATP synthase (Fig. 1C) is similar to other, homologous, bacterial ATP synthases regarding the subunit composition and the ability to reversibly separate into F_1 and F_o parts [29–32]. In the catalytic core ($\alpha_3\beta_3$) the sequences of subunits α and β, which house the nucleotide binding sites, are largely conserved in mycobacteria as compared with the homologue in *E. coli* and human mitochondria, respectively. Subunit α from *M. tuberculosis* displays 52% and 55% sequence identity compared with the homologue in *E. coli* and human mitochondria, respectively, the corresponding values for subunit β are 59% and 61%. Functional sequence motifs typically found in F_1-ATPases are conserved in mycobacteria, such as the P-loop for nucleotide binding (βG171–T178 in *M. tuberculosis*), the acidic residue acting as nucleophile for the ATP hydrolysis reaction (βE183) [5], the DELSEED sequence (βD403–D409) for torque transmission [33], and the arginine finger involved in stabilization of the ATP hydrolysis transition state (αR376) [5,34,35] (for sequence alignments of subunits α and β please see Supplementary Figs. 1 and 2). The main differences likely are not found in the central catalytic mechanism of ATP synthesis or hydrolysis, but are associated with the regulation of ATP synthase activity.

Synthesis of ATP is in principle reversible; under conditions of low PMF many ATP synthases can catalyze hydrolysis of ATP, yielding ADP and inorganic phosphate, thereby pumping protons across the membrane. Some bacteria can use this reversed reaction to maintain the PMF under energetically unfavorable conditions, whereas in certain other bacteria this inversion of function is blocked [36]. In ATP synthase from chloroplasts and mitochondria, elaborate regulatory mechanisms suppress hydrolysis of ATP [3,10]. In mycobacteria pronounced suppression of the ATP hydrolysis function has been reported for *M. phlei* [29], for *M. smegmatis* [37] and for *M. bovis* BCG [37]. The latent ATP hydrolysis activity by inverted membrane vesicles of *M. smegmatis* and *M. bovis* BCG could be activated, in particular by transient membrane energization [37]. *M. bovis* BCG and *M. smegmatis* are non-pathogenic mycobacterial strains widely used as model systems for *M. tuberculosis*. Suppression of ATP hydrolysis activity appears to be a general feature in mycobacteria, found both in strains residing in mammalian hosts, such as *M. tuberculosis and M. bovis*, as well as in saprophytic strains, such as *M. smegmatis*. For *M. tuberculosis* and other pathogenic mycobacterial strains blocking of ATP hydrolysis is of particular interest as it may represent an adaptation to its "lifestyle" in and outside human phagosomes, where ATP, once produced, must not be wasted.

2.1. Regulation by Mg-ADP

Suppression of ATP hydrolysis activity can be due to multiple factors, such as inhibitory binding of Mg-ADP, inhibition by subunit ε, or by binding of additional effectors, such as the inhibitory protein subunit ζ. Mg-ADP inhibition can occur during hydrolysis of ATP, when the produced ADP does not dissociate from the enzyme, but becomes entrapped in a catalytic nucleotide binding site [38–42]. Mg-ADP inhibition is found in ATP synthases from a broad range of organisms and can be relieved by proton motive force [43–45]. The PMF may relieve Mg-ADP inhibition by moving subunit γ away from its position arrested by Mg-ADP [46–48]. Mg-ADP inhibition can also be relieved by binding of ATP to the non-catalytic nucleotide binding sites, which are located mainly in the α-subunits [5,49–51]. As such, Mg-ADP inhibition is an important regulatory feature of the ATP hydrolysis reaction, in particular under circumstances of low ATP concentrations.

The observed strong suppression of ATP hydrolysis in mycobacteria may thus be an intrinsic feature of the catalytic core ($\alpha_3\beta_3\gamma$), caused by pronounced Mg-ADP inhibition. Residues pinpointed as important for ATP binding to the non-catalytic sites in other species, such as αK175, αT176, αD261 and αD262 in *Bacillus* PS3 [49–52] are conserved between *E. coli*, *Bacillus PS3* and mycobacteria, (αK178, αT179, αD272, αD273 in *M. tuberculosis*), indicating no specialized adaptations in mycobacteria in this regard. Moreover, βT165, the C-terminal residue of the P-loop in the catalytic site, for which a role in release of inhibitory ADP has been reported [53], is conserved (βT178 in *M. tuberculosis*). Two tyrosine and one arginine residue in subunit α, which are thought to form a link between catalytic and non-catalytic sites in *Bacillus* PS3 [54], are conserved in mycobacteria (αY241, αY303 and αR307 in *M. tuberculosis*).

Previously, mutation studies in *Schizosaccharomyces pombe* suggested a role of αQ173 and βQ170 in facilitating nucleotide binding to the non-catalytic sites [55]. αQ173 and the adjacent αR172 are also thought to contact residues in neighboring β subunits (βT354 and βD352 respectively), thereby stabilizing the α/β interface and contributing to crosstalk between catalytic and non-catalytic nucleotide binding sites [55]. In mycobacteria, αQ173 is replaced by a lysine (αK175 in *M. tuberculosis*), potentially enhancing formation of a salt bridge to a bound nucleotide, whereas βD352 is replaced by an alanine (βA361 in *M. tuberculosis*), possibly preventing formation of a salt bridge with

the arginine (αR174 in *M. tuberculosis*). The potential implications of these polymorphisms for crosstalk between nucleotide binding sites and concomitantly on control of Mg-ADP inhibition in mycobacteria need to be investigated. In this regard, functional experiments on over-expressed and purified $\alpha_3\beta_3\gamma$ from mycobacterial species in order to reveal the extent of Mg-ADP inhibition in the catalytic core are lacking.

As compared with the homologous *E. coli* and *Bacillus* PS3 sequence, mycobacterial subunit α carries an insertion of eight residues, βN192-K199. Moreover, the C-terminus displays an extension of 35 amino acid residues, αT514-K548 in *M. tuberculosis*. The potential influence of these extensions, if any, on function, regulation or stabilization of mycobacterial ATP synthase is not known.

2.2. Regulation by subunit ε

The inability of mycobacterial ATP synthase to hydrolyze ATP may alternatively be due to inhibition by subunit ε, known as a key regulator of the ATP synthase complex [56,57]. This subunit consists of an N-terminal β-barrel domain and a C-terminal helical domain [58]. The two carboxy terminal α helices can either fold into a compact form, referred to as hairpin state [58–60], or can adopt an extended conformation [61,62] (Fig. 2A). The reversible transition between these two conformations constitutes a key element for regulation of ATP hydrolysis activity. Subunit ε in the extended conformation suppresses ATP hydrolysis (but not ATP synthesis) activity, whereas the hairpin conformation is not inhibitory [63–74]. In the extended state the C-terminal helices, which carry a strongly positive charge mainly due to the presence of arginine residues [75], can interact with the negatively charged DELSEED sequence in subunit β, acting as a brake arresting rotary catalysis [76]. The crystal structure of *E. coli* F_1-ATPase with subunit ε in the extended state revealed the interaction of the C-terminal helices with subunits α, β and γ [62,77]. The helix at the C-terminal tip (residues ε115–133 in *E. coli*) deeply inserts into the central cavity of the $\alpha_3\beta_3$ hexamer whereas the second helix binds to the DELSEED sequence. As such, the helical domain interacts with the catalytic sites in subunits β and can execute its intrinsic inhibitory effect on ATP hydrolysis activity [62].

Transition between the two conformations of subunit ε is controlled by the cellular energy status. ATP can trigger the transition into the hairpin state, thereby relieving inhibition of ATP hydrolysis [69,78]. Subunit ε can monitor cellular ATP levels in two ways, in addition to sensing the ATP occupancy of the nucleotide binding sites in subunits β subunit ε has the intriguing ability of directly binding ATP, although lacking typical ATP-binding domains and motifs [79–83].

The regulatory role of this subunit may vary considerably between different species, allowing for fine-tuning the activity of ATP synthase to the physiological needs of each individual species. Accordingly, the helical domain of subunit ε differs considerably between organisms [75]. Subunit ε in several anaerobic bacteria such as *Chlorobium* lack the C-terminal helical domain, which may facilitate inversion of ATP synthase function for maintenance of the PMF [75,84]. Mutagenesis experiments on subunit ε in *E. coli* did not reveal a distinct role for the C-terminal helical domain [85] and subunit ε in *Bacillus subtilis* was reported to relieve inhibition by Mg-ADP [86]. In mycobacteria the need to prevent excess ATP consumption under low oxygen tensions may be exceptionally strict, consequently subunit ε may play an exceptionally important role in order to adapt to the environment encountered in the human host. Genetic data indicated that while the N-terminal β-barrel region of subunit ε is largely conserved in

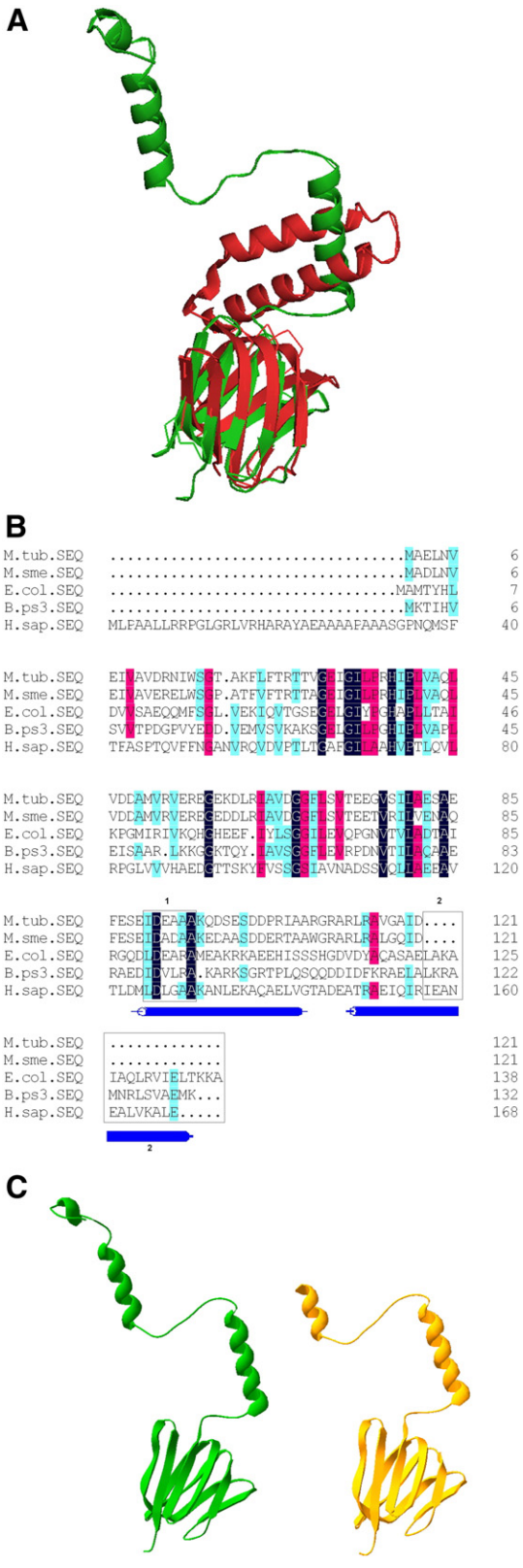

Fig. 2. The key regulator subunit ε in mycobacteria. (A) Structure of *E. coli* ATP synthase subunit ε in extended state (green) as compared with the hairpin state (red). The structures were obtained from the Protein Data Bank (PDB IDs 3oaa and 1aqt, respectively). (B) Sequence alignment of mycobacteria ATP synthase subunit ε with the homologue in *E. coli* and in human mitochondria (referred to as subunit δ in mitochondria). Dark blue indicates conserved across all shown species, red indicates 75% identity, light blue indicates 50% identity. M.tub: *M. tuberculosis*, M.sme: *M. smegmatis*, E.col: *Escherichia coli*, B. ps3: *Bacillus* PS3 and H.sap: *Homo sapiens*. Blue rods below the sequences indicate the C-terminal α-helices. Boxed region 1 highlights ATP-binding motif region, boxed region 2 indicates the lacking C-terminal α-helix in mycobacteria. (C) Homology model for the structure of *M. tuberculosis* ATP synthase subunit ε (orange) as compared with the structure of subunit ε from *E. coli* (green). The homology model was made using the Swiss-Model server [162] using the structure of *E. coli* subunit ε (PDB ID 3oaa [62]) as template.

mycobacteria as compared with the homologue in *E. coli* and in human mitochondria (Fig. 2B), interestingly mycobacteria lack the helix at the C-terminal tip (Fig. 2B,C). Recently, subunit ε from *M. tuberculosis* was over-expressed and functionally characterized. This subunit was capable of blocking ATP hydrolysis activity by the isolated catalytic $\alpha_3\beta_3\gamma$ core from *Bacillus* PS3, however, it was found unable of binding ATP [87].

In subunit ε from *Bacillus* PS3 binding of ATP is facilitated by the motif I(L)DXXRA (ε88–93), a highly atypical ATP binding motif (Fig. 2B). In addition, the side chains of three additional arginine residues (εR100, εR121, and εR125) coordinate the phosphate group of ATP, with εE83 involved in formation of the adenine-binding pocket [81]. In the homologous ATP binding motif in mycobacterial subunit ε the arginine is replaced by alanine (IDEAAA, ε90–95), which may impede efficient ATP binding. Moreover, mycobacterial subunit ε lacks the residues corresponding to εR121 and εR125 due to the lacking C-terminal helix and εR100 is not conserved in mycobacteria (Fig. 2B). Taken together, these differences may explain the lack of ATP binding by mycobacterial subunit ε.

As ATP binding facilitates the transition of subunit ε from the extended inhibitory conformation towards the folded hairpin state, the observed lack of ATP binding may indicate strong, continuous inhibition of ATP hydrolysis by subunit ε in mycobacteria. In line with this finding, lack of ATP-dependency of inhibition has previously been reported for subunit ε from spinach chloroplast and from the cyanobacterium *Thermosynechococcus elongates* [56,88], species, which like mycobacteria may need to efficiently prevent wasteful ATP consumption. However, it cannot be excluded that the lack of the C-terminal helix allows for only minor inhibition of ATP hydrolysis in mycobacterial ATP synthase and that the observed suppression of ATP hydrolysis is caused by other features, e.g. in the catalytic core. Additional effectors, as exemplified by subunit ζ in *P. denitrificans* and other α-proteobacteria [89], may also contribute to activity modulation of mycobacterial ATP synthase.

Mycobacterial subunit ε may constitute an interesting tool for investigation of the working of bacterial ATP synthase regulation, in particular the respective contributions of each of the two C-terminal helices as well as the function of individual amino acid residues in the helical domain. Furthermore, in case subunit ε is responsible for the observed suppression of ATP hydrolysis by mycobacterial ATP synthase, activation of the "latent" ATP hydrolysis functionality could constitute an attractive approach for development of new antibacterial entities. This subunit may serve as an interesting epitope for small-molecule compounds to bind and to stimulate ATP hydrolysis. Fragment-based approaches for drug discovery [90] may provide small-molecules mimicking the lacking C-terminal tip of subunit ε, potentially relieving its inhibitory effect. Activators specific for mycobacterial ATP synthase may have the ability to deplete bacterial ATP pools and may represent powerful drugs, in particular on bacteria in metabolic states with low cellular energy reserves.

3. Coupling proton flow with ATP synthesis

M. tuberculosis can survive with a low PMF of −110 mV [18], suggesting that ATP synthase needs to be adapted for efficient energy usage and is able of using this low proton motive force for synthesis of ATP. Activity of ATP synthase significantly depends on the PMF, with considerable variation between different organisms [91]. As hypothesized previously, ATP synthase of *M. tuberculosis* may turn out to be active at lower PMF as compared to most bacteria or mitochondria [19]. This ability apparently is closely linked to the proton translocating subunit c. Subunit c is an integral membrane protein, which consists of two transmembrane helices. This subunit assembles as an oligomeric ring, with the N-terminal helix (helix 1) located towards the center of the ring and the C-terminal helix (helix 2) facing the outside. Protons from the periplasmatic side of the membrane can bind the carboxylate side chain of a highly conserved acidic residue located in the membrane-spanning region of helix 2 (cD61 in *E. coli*, cE61 in *M. tuberculosis*) [92,93]. This protonation allows rotational movement of the c-ring within the hydrophobic core region of the membrane, after which the proton is released into the cytoplasm [94,95]. The number of subunit c monomers in the c-ring apparently is fixed in a given species, but this stoichiometry varies considerably between different species as detailed below.

Whereas 10 monomers have been found in c-rings from *E. coli* and *Bacillus* PS3 [96,97] in the alkaliphilic *Bacillus* sp. strain TA2.A1, which has to cope with a low PMF under alkaline conditions, the subunit c complex is composed of 13 monomers [98,99]. A larger number of monomers per subunit c oligomer may increase the H^+/ATP ratio and thus facilitate proton flow and synthesis of ATP under low PMF conditions [98]. No structural data are available for the c-ring from human mitochondria, however, in ATP synthase from bovine heart mitochondria, eight monomers were found in the c-ring [100]; conceivably, the human homologue may show the same stoichiometry.

For mycobacteria the number of monomers in the c-ring is unknown. However, as mycobacterial ATP synthase has to cope with a low PMF in order to synthesize ATP, mycobacteria may employ a strategy similar to alkaliphilic bacteria. A large c-ring (number likely >10) would allocate more steps of subunit c to the synthesis of one ATP, supporting production of ATP at low PMF. A comparison of c-rings from several cyanobacterial species revealed that subtle sequence differences apparently determine the oligomeric state of the c-ring [101]. In *Ilyobacter tartaricus*, directed mutagenesis of a glycine-repeat motif in helix 1 of subunit c, cG25–G33, allowed for engineering of c-rings with defined stoichiometry (12 instead of 11 monomers) [102]. The mutants with increased numbers of monomers in the c-ring indeed displayed higher activity at lower PMF, as compared with the wild type [102]. In mycobacterial subunit c the region containing the glycine-repeat (cG23–G29 in *M. tuberculosis*) is conserved, however, in the flanking regions a higher content of glycine and alanine residues (e.g. cG19, cG20, cA31) is found as compared with the *E. coli* homologue (Fig. 3A). Helix 1 is thought to be involved in establishing helix/helix contacts in the center of the c-ring [102,103]; the large number of small residues in mycobacterial subunit c may thus facilitate a dense packing of the monomers in the ring. If the c-ring in *M. tuberculosis* indeed is comprised of >10 monomers then a significantly different topology of this key subunit in proton translocation can be expected as compared with the human homologue (Fig. 3B).

The c-ring is the target of several small molecule inhibitors, such as dicylcohexyl carbodiimide, oligomycin and venturicidin [104–107]. Dicylcohexyl carbodiimide directly binds to the conserved acidic residue located in the central part of helix 2 of the c-ring. For venturicidin no structural data are available, mutations influencing venturicidin sensitivity have been mapped to the membrane-spanning part of helix 1 in subunit c (G24 and A27 in *S. cerevisiae*) [108]. Recently, the structure of the c-ring from yeast mitochondria complexed with oligomycin has been determined [107]. Olimomycin makes contact with the carboxyl side chain of the essential acidic residue in helix 2 (Glu59 in *S. cerevisiae*), with the bulk of the inhibitor bound via hydrophobic interactions to the membrane-spanning regions of subunit c. As the oligomycin binding site overlaps with residues involved in resistance to venturicidin and ossamycin, a common drug-binding niche in the central part of subunit c was proposed [107]. Whereas DCCD is a non-selective inhibitor that blocks ATP synthase function in many species, oligomycin displays increased affinity for eukaryotic ATP synthases as compared with bacterial ones [107].

The diarylquinolines represent a class of ATP synthase inhibitors that selectively inhibit ATP synthase in mycobacteria by binding to subunit c [27,28,109]. Diarylquinolines are potent antibacterials that effectively kill *M. tuberculosis* [26]. The lead compound of this drug class, bedaquiline, has recently received approval from the US Food & Drug Administration [110,111]. Residues in mycobacterial ATP synthase important for sensitivity to bedaquiline are located in the membrane spanning part of subunit c, both in helix 1 (cD28) as well as in helix 2 (cL59,

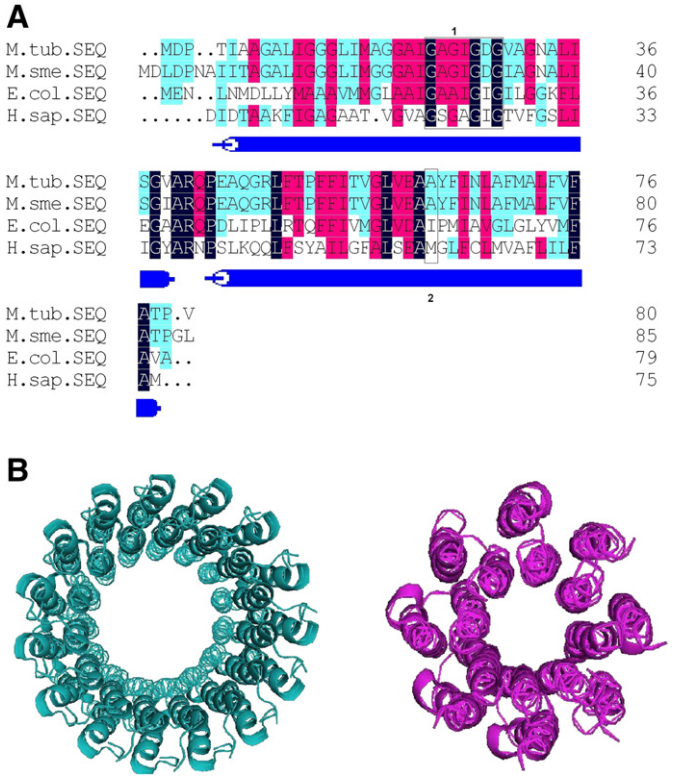

Fig. 3. The proton translocating subunit c in mycobacteria. (A) Sequence alignment of ATP synthase subunit c in mycobacteria compared with the homologue from *E. coli* and from human mitochondria. Dark blue indicates conserved across all shown species, red indicates 75% identity, light blue indicates 50% identity. M.tub: *M. tuberculosis*, M.sme: *M. smegmatis*, E.col: *Escherichia coli* and H.sap: *Homo sapiens*. Blue rods below the sequences indicate the two membrane-spanning α-helices. Box region 1 highlights the highly conserved glycine-repeat region in mycobacteria. Box region 2 shows cA63 in mycobacteria, a residue modulating sensitivity for diarylquinolines (B) The structure (top view) of the c-ring from *Arthrospira platensis* (15 monomers, cyan) and the c-ring from bovine heart mitochondria (8 monomers, magenta, 100% sequence identity with the human homologue). The structures of c-rings from *Arthrospira platensis* [163] and from bovine heart mitochondria [100] were obtained from the Protein Data Bank (PDB IDs 2wie and 2xnd, respectively).

cE61, cA63, cI66) [26,112–114]. Biochemical assays and binding studies suggest a defined binding site in subunit c for bedaquiline [28]. Modeling studies predict a binding niche for bedaquiline at the interface between subunit a and subunit c, close to the conserved acidic residue in helix 2 [115,116]. Alternatively, based on binding studies involving mycobacterial subunit ε, a binding site for diarylquinolines at the interface between subunit c and subunit ε was proposed [87].

The molecular basis for the selectivity observed for bedaquiline [26,109] still needs to be elucidated. Amino acid polymorphisms in the membrane-spanning part of subunit c may act as key determinants for size and shape of a putative drug-binding pocket. Replacement of a small residue in mycobacterial ATP synthase by a bulkier one in less susceptible ATP synthases such as the *E. coli* or human mitochondrial homologue, e.g. substitution of cA63 by an isoleucine in *E. coli* or by a methionine in human mitochondria (Fig. 3A), may impose steric hindrance on drug binding [109,115]. Bedaquiline likely binds to a niche overlapping with the oligomycin-binding site [107]. In both cases the hydrophilic parts of the inhibitors may interact with the essential acidic residue in helix 2, providing specificity, whereas the hydrophobic bulk makes extensive van-der-Waals contacts and/or π–π interactions with the adjacent unipolar residues, enhancing the free binding energy.

As mentioned above, spontaneous mutations in mycobacterial ATP synthase can decrease sensitivity for diarylquinolines [26,114]. These mutants retain their fitness under optimal growth conditions [27], however, they have not been systematically characterized for fitness under conditions mimicking the environment in the human host. As such, it is unknown if ATP synthase in the mutated mycobacterial strains remains fully functional under conditions of physiological stress. The polymorphisms may at least in part represent adaptations needed for optimal (long-term) survival chances for *M. tuberculosis* in the human host.

Polymorphisms in subunit c may not only directly contribute to a drug-binding site, but may also affect the general structure of the c-ring, thereby indirectly influencing the drug-binding site. This has been proposed for resistance mutations against oligomycin in helix 1 of subunit c, which are located at the inner surface of the c-ring, whereas the inhibitor binds to the outer surface [107]. Similarly, mutations of cD28, also located in helix 1, may influence the general structure of the binding pocket for diarylquinolines. As polymorphisms/point mutations can even change the oligomeric state of the c-ring [102] they may in this way contribute to shaping the surface curvature of the c-ring and concomitantly modulate potential binding sites for small molecule inhibitors (Fig. 3B). As an example, cD28 in *M. tuberculosis* is located in the glycine-rich stretch thought to be involved in helix/helix contacts (Fig. 3A), mutations at this position may influence the density of helix packing or even the oligomeric state of the c-ring.

Next to the protein components of the proton-translocating machinery in ATP synthase in addition lipids may play an important role for ATP synthase function and potentially also for drug binding. Lipids bound to distinct sites in oligomeric membrane proteins can contribute to protein stability and regulation. As an example, cardiolipin has been shown to closely interact with respiratory complexes in mitochondria [117] and this lipid activates purified ATP synthase from yeast [118]. The cytoplasmatic membrane in mycobacteria displays an unusual lipid composition with a large percentage of cardiolipin and less phosphatidyl glycerol, as compared with other bacteria [119]. The importance of lipids, in particular of cardiolipin, in mycobacterial energy metabolism needs to be investigated.

The c-ring is closely connected to subunit γ, which connects the F_o rotary motor to the nucleotide binding sites in the $\alpha_3\beta_3$ catalytic unit. Subunit γ consists of an extended coiled-coil made up by the N-terminal and C-terminal helical regions, which penetrates into the cavity of the $\alpha_3\beta_3$ hexamer [5], and a globular mixed helical/sheet domain, which is located at the bottom, close to the c-ring (Fig. 4A) [62,120]. The coiled-coil region displays a higher degree of sequence identity/similarity among across species than the mixed globular domain. This likely reflects evolutionary constraints in closely fitting the coiled-coil region into the $\alpha_3\beta_3$ cavity, whereas the bottom part of subunit γ may need to be adapted to the size and oligomeric state of the c-ring in each particular organism. Subunit γ from *M. tuberculosis* shows 37% and 25% similarity compared with its homologue *E. coli* and in human mitochondria, respectively (Fig. 4B) [121]. In line with findings on other species, the highest degree of sequence similarity is found in the N-terminal and C-terminal coiled-coil region (Fig. 4B).

γM23 (*E. coli* numbering) is located at the interface with subunit β [5]. Its substitution by lysine in *E. coli* and *Rhodobacter capsulatus* strongly impeded catalysis [48,122]. It has been suggested that the mutation arrests subunit γ due to electrostatic interaction with the DELSEED sequence in subunit β and that the PMF can activate ATP synthase from this arrested state [48]. In mycobacteria γM23 is replaced by a glutamine (γQ24 in *M. tuberculosis*) (Fig. 4B), which may potentially allow for formation of hydrogen bonds with the DELSEED sequence. If so, such an interaction may contribute to suppression of ATP hydrolysis activity in the absence of a PMF and may form an important element for regulation of ATP synthesis in mycobacteria.

Subunit γ from mycobacteria displays a unique insertion of 13 amino acid residues, γG165–D178 in *M. tuberculosis*, which is not present in known prokaryotic or eukaryotic homologues (Fig. 4A,B). NMR spectroscopy of a synthesized peptide corresponding to γG165–D178 indicated that this insert forms a loop of polar residues. Modeling of the peptide into the high-resolution structure of F_1 from *E. coli*

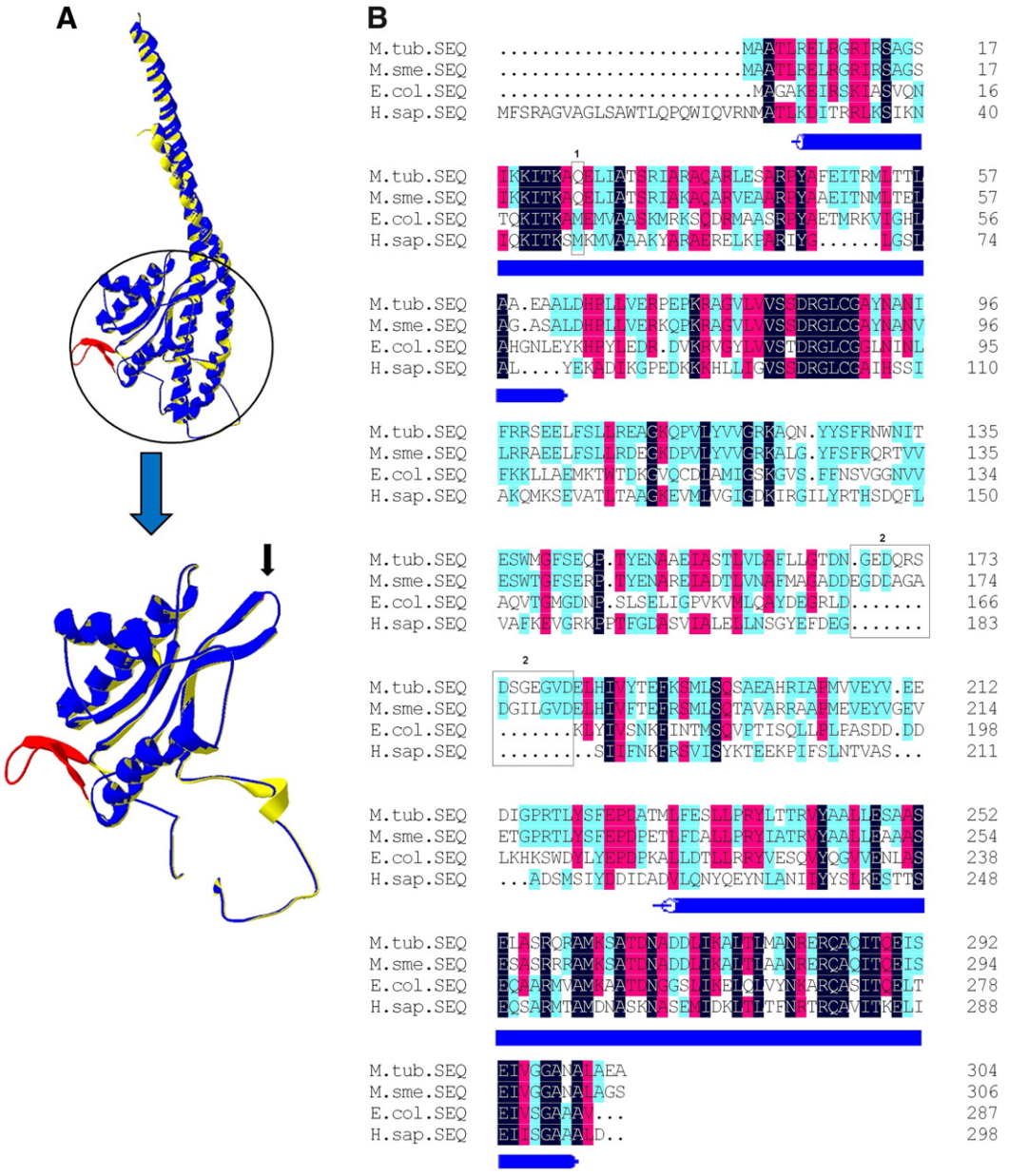

Fig. 4. The rotating subunit γ in mycobacteria. (A) Homology model for the structure of *M. tuberculosis* ATP synthase subunit γ (yellow) superimposed on the structure from the *E. coli* homologue (blue). Lower part: magnification of the mixed helical/sheet domain with the unique insertion of 13 amino acid residues in *M. tuberculosis* (red loop). The black arrow indicates the location of the regulatory insert found in photosynthetic ATP synthases. The homology model was made using the Swiss-Model server [162]. The structure of *E. coli* subunit γ [62] was obtained from the Protein Data Bank (PDB ID 3oaa). (B) Sequence alignment of mycobacteria ATP synthase subunit γ with the homologue in *E. coli* and in human mitochondria. Dark blue indicates conserved across all shown species, red indicates 75% identity, light blue indicates 50% identity. M.tub: *M. tuberculosis*, M.sme: *M. smegmatis*, E.col: *Escherichia coli* and H.sap: *Homo sapiens*. Blue rods below the sequences indicate the coiled-coil α-helices. Boxed region 1 shows the residue γ24 (*M. tuberculosis* numbering) thought to be involved in contact with subunit β. Boxed region 2 highlights the unique insertion found in mycobacteria subunit γ.

indicated a location of this loop in proximity of the cytoplasmic parts of subunits c [121]. Due to the presence of largely polar and highly acidic residues, γG165–D178 might interact with residues cR41, cQ42, cE44 and Q46 in the cytoplasmic loop between helices 1 and 2 of subunit c. For this loop of subunit c a function in gating of proton flow has been proposed [123]. cQ46 (replaced by an unpolar isoleucine in *E. coli*) has been proposed to interact with γE169 and γD170, enhancing the interaction between γ165–178 and the c-ring [121]. Alternatively it can be speculated that an additional connection between subunit γ and the c-ring, as potentially provided by the mycobacterial insert γG165–D178, may modulate the elastic properties of the γ/c rotating unit and as such improve the efficiency of the mycobacterial enzyme. Subunit γ has previously been pinpointed as a central elastic element in ATP synthase, coupling the non-integer ratio of proton transfer across subunit c with synthesis or hydrolysis of ATP in the catalytic sites [124,125]. If γG165–D178 plays a specific role in activity, stability or assembly of mycobacteria, it may prove a promising target for small-molecule compounds to bind and to regulate the enzyme's activity.

γG165–D178 is located about 30 residues from the insert in photosynthetic organisms that allows for adjusting ATP synthase activity in response to environmental light intensity [126,127]. This regulatory insert (γ194–230 in spinach chloroplast) enables redox regulation of ATP synthase in plant chloroplasts [126], whereas in cyanobacteria it modulates inhibition by subunit ε, but does not facilitate redox-regulation [88]. Although both γG165–D178 and the insert in photosynthetic organisms are part of the mixed domain of subunit γ, sequence/structure data suggest they are located at opposite ends of one β-strand (Fig. 4A). These locations may reflect the likely respective roles of interaction with

F_o (γG165–D178 in mycobacteria) [121] and with F_1 (photosynthetic insert). The regulatory insert from spinach chloroplast ATP remains functional when introduced into ATP synthases from other species [128,129]. It would be interesting to test whether the mycobacterial insert can be transferred across species as well and to evaluate the impact of the insertion on enzyme stability and functionality.

Subunit γ occupies a central position in ATP synthase. This pivotal function may predetermine subunit γ, next to subunit ε, for special features assisting in optimizing efficiency during rotary catalysis.

4. The unusual stator stalk in mycobacteria

The non-rotating parts of F_1 ($\alpha_3\beta_3$) and F_o (subunit a) need to be well connected in order to prevent unproductive rotation of the whole ATP synthase complex during catalysis. This task is carried out by the stator stalk, an extended, largely hydrophilic structure at the periphery of the ATP synthase complex. In most bacteria the stator stalk comprises subunit δ, which is located on top of the $\alpha_3\beta_3$ hexamer, and a dimeric subunit b, which connects to δ and then reaches all the way down into the cytoplasmic membrane (Fig. 1A). In the mitochondrial enzyme, subunit δ is replaced by the homologous oligomycin-sensitivity-conferring protein (OSCP). In addition, only a single subunit b is found, accompanied by two smaller subunits called subunit d and F6 (Fig. 1B) (for review see [130–132]. In cyanobacteria, other photosynthetic bacteria and chloroplasts, as well as in the hyperthermophilic bacterium *Aquifex aeolicus* two different, but homologous subunits (called subunit b and subunit b′ in cyanobacteria and subunits I and II in chloroplasts) substitute for the subunit b homodimer [133–135].

Interestingly, in mycobacteria one of the subunits b is genetically fused with subunit δ. The two fusion partners are connected by a linker region of about 110 residues (Figs. 1C, 5A,B) [136,137]. The second mycobacterial subunit b lacks the C-terminal part typically found in other species (Fig. 5B), resembling the b′-type subunits in photosynthetic bacteria. In this paper we refer to these two mycobacterial ATP synthase subunits as subunit b/δ and as subunit b′. In subunit b/δ the C-terminus of the "subunit b part" is fused to the N-terminus of the "subunit δ part" (Fig. 5A,B). However, in *E. coli*, the N-terminus of subunit δ is thought to be located close to the top of the $\alpha_3\beta_3$ hexamer, whereas the C-terminus of subunit δ has been assigned as contact area with subunit b [138]. Moreover, in ATP synthase from bovine heart, the N-terminus of OSCP

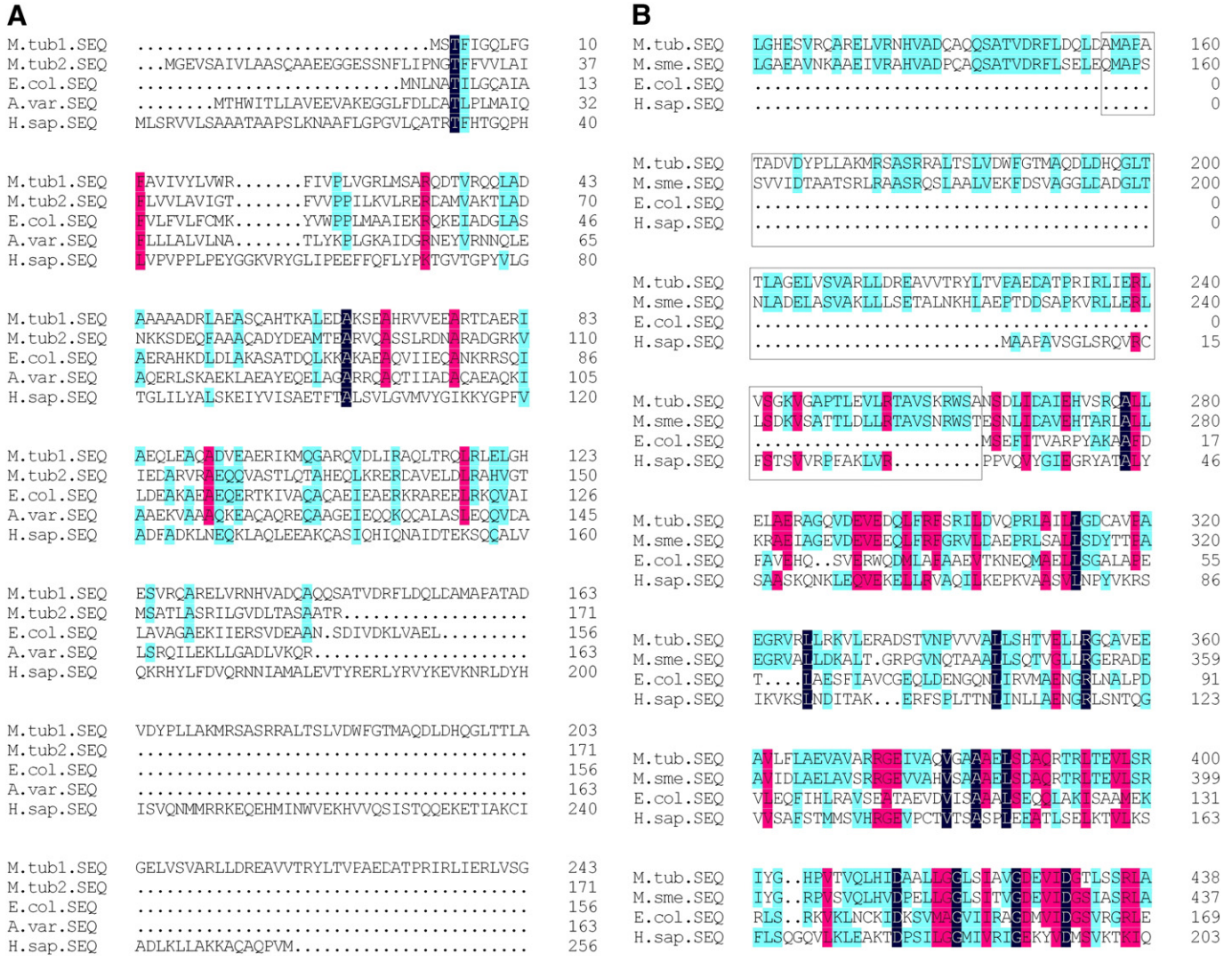

Fig. 5. The unusual stator stalk in mycobacteria. (A) Sequence alignment of mycobacterial ATP synthase subunits b/δ (N-terminal part)(M.tub1) and b′ (M.tub2) with the homologous subunits in *E. coli* (subunit b), *Anabaena variabilis* (subunit b′) and in human mitochondria (subunit b). Dark blue indicates conserved across all shown species, pink indicates 75% identity, light blue indicates 50% identity. M.tub: *M. tuberculosis*, M.sme: *M. smegmatis*, E.col: *Escherichia coli*, A.var: *Anabaena variabilis* and H.sap: *Homo sapiens*. (B) Sequence alignment of mycobacteria ATP synthase subunit b/δ (C-terminal part) with the homologue in *E. coli* (subunit δ) and in human mitochondria (OSCP). The boxed region indicates the unique 110-residue insertion in mycobacteria.

is not in close proximity to the C-terminus of subunit b [139]. Likely, the 110-residue linker region in the mycobacterial b/δ fusion allows for the connection of the two fusion partners [137].

An asymmetric stator stalk may have several potential advantages. For photosynthetic bacteria it has been suggested that subunit b′ may be an adaptation to optimize binding to F_1 or F_o. The regions of subunits b and b′ that are not involved in dimer formation may adopt different conformations and in this way improve interaction with the asymmetric subunit a and/or subunit δ [140]. In mycobacteria the b/δ fusion and the presence of subunit b′ may serve a similar purpose. This hypothesis has recently been supported by the generation of a chimeric *E. coli* ATP synthase carrying *E. coli* subunit b and subunit δ, which were connected by the b/δ linker region obtained from *Mycobacterium vanbaalenii* [137]. These experiments showed the presence of one b/δ fusion without the additional b- or b′-type subunit is not sufficient for proper functional holoenzyme assembly, extending previous work on the cyanobacterium *Synechocystis PCC6803*, where deletion of subunit b′ was lethal [141]. Deletion experiments using the same chimeric *E. coli* ATP synthase revealed that the b/δ fusion is responsible for binding to the $\alpha_3\beta_3$ hexamer, whereas the shorter b′-type subunit is required for connection with F_o [137]. The N-terminus of the b′-type subunit in mycobacteria shows an extension of ~25 residues as compared with the b/δ fusion (Fig. 5B), this extension may help in optimizing binding to the F_o part. Specialization between two subunits b, even in a homodimer, has been reported earlier based on different interaction with F_1 [142], an offset topology of the two subunit b copies [143,144], an asymmetric location relative to subunit a [145,146] and the necessity of the membrane spanning b′, but not its hydrophilic part, for ATP synthase assembly [141]. Taken together, these findings underscore the view of extensive specialization between two (different) b-type subunits in ATP synthase, a principle for which the mycobacterial enzyme may represent a prime example. Additionally, or alternatively, the b/δ fusion may increase the stiffness of the stator stalk, thereby improving power transmission within the ATP synthase complex. The presence of two different types of subunit b may also improve the mutual interaction between the two subunits, which form a closely intertwined coiled-coil [147,148].

The stator stalk fulfills a key role in this complex and dynamic enzyme, blocking its function most likely inactivates in ATP synthase. Efforts to find enzyme inhibitors usually are directed at blocking the active site or other dynamic parts of a target enzyme, not the rigid, static regions. However, considerable dynamics have been proposed for the stator stalk, to balance movements related to rotary catalysis [149]. It is unclear if (potential) dynamics of the stator stalk in the future can be exploited for inhibition by small molecules and if the subtle differences in stator stalk composition between prokaryotic and eukaryotic systems allow for selective inactivation of stator stalk function in bacteria. However, insight in stator stalk function in ATP synthase may in addition to the added value in basic knowledge, based on the homology observed between stator stalk subunits and components of bacterial secretions systems [136], also provide input for understanding of the complex and elaborate secretion systems in a variety of pathogenic bacteria.

5. Concluding remarks

Further investigations are required to gain insight into the working of the peculiar ATP synthase in mycobacteria. Over-production, purification and mutagenesis strategies need to be developed to characterize this protein in isolated form and to link unusual amino acid sequences to specialized function or adaptation.

The spectrum of antibacterials blocking energy metabolism, in particular in mycobacteria, is expected to expand in the near future, with small-molecules inhibitors targeting different components of the mycobacterial oxidative phosphorylation reported [19,20]. This includes inhibitors of the type-II NADH dehydrogenase [150,151], the cytochrome bc_1 complex [152,153], and compounds interfering with the PMF [154,155]. Moreover, energy metabolism has been validated as target of antibacterials in non-mycobacterial Gram-positive bacteria, such as *Staphylococcus aureus* and *Streptococcus pneumoniae*, with diarylquinoline-type inhibitors showing pronounced activity on *Staphylococcus* biofilms [156]. High-resolution structures of (parts of) mycobacterial ATP synthase with bound drugs are needed in order to gain insight into the mode of drug binding and to understand drug selectivity. New small-molecule entities acting on energy metabolism may not be restricted to enzyme inhibitors, but may also include activators, e.g. for depletion of cellular ATP pools by activation of latent ATP hydrolysis activity or by re-routing of electron flow to reactive oxygen species as reported for clofazimine [151].

ATP synthase and the related V-type ATPases are highly dynamic proteins whose rotary catalysis has been extensively characterized using a variety of single molecule techniques [6,8,157–160]. Single molecule analysis of inhibitors bound to ATP synthase may provide valuable insight into the modes of drug/target interaction, as recently demonstrated for inhibition of ATP synthase from *Bacillus* PS3 by dicylcohexylcarbodiimide [161].

Elucidating the working of ATP synthase from pathogenic bacteria can not only contribute to improved insight into the general working of this enzyme, but may also reveal molecular adaptations to the specific environments. A better understanding of these adaptations in pathogenic bacteria and of the differences as compared with the human homologue may provide input for a knowledge-based design of new inhibitors blocking ATP synthase or other components of bacterial energy metabolism.

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