Structure of mycobacterial ATP synthase with the TB drug bedaquiline

Hui Guo^{1,2,*}, Gautier M. Courbon^{1,2,*}, Stephanie A. Bueler¹, Juntao Mai³, Jun Liu³, and John L. Rubinstein^{1,2,3,4}

¹ Molecular Medicine Program, The Hospital for Sick Children, Toronto, Ontario, Canada

² Department of Medical Biophysics, The University of Toronto, Ontario, Canada

³ Department of Molecular Genetics, The University of Toronto, Ontario, Canada

⁴ Department of Biochemistry, The University of Toronto, Ontario, Canada

* These authors contributed equally to this work

Correspondence: john.rubinstein@utoronto.ca

ORCID: HG (0000-0001-7007-2876), GMC (0000-0001-6628-9087), JM (0000-0002-8754-3627), JL (0000-0002-0522-6733), JLR (0000-0003-0566-2209).

Summary

Tuberculosis (TB), the leading cause of death by infectious disease worldwide, is increasingly resistant to first line antibiotics. Developed from a screen against *Mycobacterium smegmatis*, bedaquiline can sterilize even latent *M. tuberculosis* infections that may otherwise persist for decades and has become a cornerstone of treatment for multidrug resistant and extensively-drug resistant TB. Bedaquiline targets mycobacterial ATP synthase, an essential enzyme in the obligate aerobic *Mycobacterium* genus. However, how the drug binds the intact enzyme is unknown. We determined the structure of *M. smegmatis* ATP synthase with and without bedaquiline. The drug-free structure reveals hook-like extensions from the enzyme's α subunits that inhibit ATP hydrolysis in low-energy conditions, such as during latent infections. Bedaquiline binding induces global conformational changes in ATP synthase, creating tight binding pockets at the interface of subunits a and c. These binding sites explain the drug's structure-activity relationship and its potency as an antibiotic for TB.

Key words: Bedaquiline, Sirturo, TMC207, T207910, ATP synthase, mycobacteria, tuberculosis, cryoEM, structure

Introduction

Mycobacterium tuberculosis and closely related species cause the disease tuberculosis (TB), which resulted in 1.5 million deaths in 2018 alone (World Health Organization, 2019a). The same year, 0.5 million TB cases showed resistance to first line antibiotics, indicating a rise in drug resistance (World Health Organization, 2019a). The diarylquinoline drug bedaquiline (BDQ, also known as R207910, TMC207, and Sirturo) kills the mycobacteria that cause TB and other mycobacterial pathogens (Andries et al., 2005) at nanomolar concentrations. BDQ was approved by the United States Food and Drug Administration in 2012 as part of combination therapy in adults with pulmonary multi-drug resistant TB making it the first new TB drug in more than 40 years (Wellington and Hung, 2018). The World Health Organization subsequently added BDQ to its Model List of Essential Medicines (World Health Organization, 2019b). As of 2019, all seven ongoing phase III clinical trials of combination therapies for drug resistant TB include BDQ as a core component (World Health Organization, 2019a), with one trial reporting favorable outcomes in 90% of patients with extensively-drug resistant TB or complicated forms of multi-drug resistant TB (Conradie et al., 2020). It has been estimated that 80% of people with drug resistant TB would benefit from BDQ (Médecins Sans Frontières, 2019).

M. tuberculosis can survive low-energy conditions, allowing infections to remain dormant for extended periods and decreasing susceptibility to many antibiotics (Lu et al., 2014). BDQ can sterilize even these latent infections (Koul et al., 2008) by targeting the mycobacterial adenosine triphosphate (ATP) synthase (Andries et al., 2005), which is an essential enzyme in both *M. tuberculosis* and *M. smegmatis* (Andries et al., 2005; McNeil et al., 2020). However, affinity measurements between the drug and the isolated c subunit of ATP synthase, which was identified as part of its binding site, showed only micromolar affinity (Haagsma et al., 2011; Koul et al., 2007) suggesting that binding of the drug to the intact enzyme involves additional subunits. ATP synthases use energy stored in a transmembrane proton motive force established during respiration to synthesize ATP from adenosine diphosphate (ADP) and inorganic phosphate. In the absence of a proton motive force during low-energy conditions, the ATP synthase must be prevented from running in reverse and hydrolyzing the cell's supply of ATP. How this inhibition of ATP hydrolysis occurs in mycobacteria is unclear. In order to understand the structural basis by which ATP hydrolysis by ATP synthase is inhibited in mycobacteria, and

how BDQ targets mycobacterial ATP synthase, we isolated the *M. smegmatis* ATP synthase and used electron cryomicroscopy (cryoEM) to determine its structure with and without BDQ bound.

Results

Overall structure of mycobacterial ATP synthase includes an unusual peripheral stalk

Similar to other bacterial ATP synthases (Guo et al., 2019; Sobti et al., 2020), the mycobacterial ATP synthase consists of a soluble cytosolic F_1 region and a membrane-embedded F_0 region (Lu et al., 2014). To isolate endogenous ATP synthase from *M. smegmatis*, we introduced a sequence into the chromosomal DNA encoding 3×FLAG affinity tags at the C termini of the β subunits in the F_1 region. The resulting strain was cultured, its membranes harvested, and the ATP synthase purified by affinity chromatography. CryoEM of the preparation (Fig. S1 and S2) yielded three three-dimensional (3D) maps, corresponding to the main rotational states of the enzyme (Guo et al., 2019; Zhao et al., 2015), at nominal resolutions of 3.4 to 3.7 Å. Focused refinement of the F_0 region (Fig. S1 and S2) produced a map at 3.5 Å resolution (Fig. 1A, *left*). Together, these maps allowed construction of an atomic model for almost all of the complex (Fig. 1A, *right*, Fig. S3, Tables S1-S5).

The *M. smegmatis* F_0 region contains nine copies of subunit c (Fig. 1A and B, *grey*), similar to the isolated c ring from *M. phlei* (Preiss et al., 2015). The proton conducting subunit a (Fig. 1A and B, *green*) has the same fold found in the mammalian (Zhou et al., 2015), yeast (Guo et al., 2017), algal (Klusck et al., 2017), and chloroplast (Hahn et al., 2018) ATP synthases and aligns well with the *Bacillus* PS3 and *E. coli* subunits (Fig. S4) (Guo et al., 2019; Sobti et al., 2020). During ATP synthesis, the transmembrane proton motive force drives protons through the F_0 region, inducing rotation of the $\gamma\epsilon c_9$ rotor subcomplex (Junge et al., 1997; Vik and Antonio, 1994). This rotation of γ within the catalytic $\alpha_3\beta_3$ subcomplex of the F_1 region leads to synthesis of ATP from ADP and inorganic phosphate. The $\alpha_3\beta_3$ subcomplex is prevented from rotating in sympathy with the rotor by a peripheral stalk, which consists of two copies of subunit b and subunit δ in most bacteria (Guo et al., 2019; Sobti et al., 2016) but is formed from a b- δ fusion protein and subunit b' in mycobacteria (Lu et al., 2014). The structure shows that the transmembrane α helices of subunit b' and subunit b- δ sandwich the N-terminal α helix of subunit a, replacing the two identical b subunits found in the canonical bacterial ATP synthase

(Fig. 1B) (Sobti et al., 2016). Subunit b' is packed tightly against the second and third transmembrane α helices of subunit a while subunit b- δ forms fewer contacts. These interactions explain the observation that subunit b- δ is responsible for mediating attachment with the F₁ region while subunit b' is more important for binding the F_O region (Gajadeera and Weber, 2013). The δ region of the b- δ fusion subunit differs dramatically from the canonical bacterial ATP synthase δ subunit (Fig. S5) (Guo et al., 2019; Sobti et al., 2016).

Hook-like structures from the α subunits prevent ATP hydrolysis

The F₁ regions of ATP synthases have three catalytic nucleotide-binding sites, primarily within the β subunits, and three non-catalytic sites, primarily within the α subunits (Fig. 1C and D) (Abrahams et al., 1994). These catalytic sites are known as β_{TP} (indicating 'ATP-bound'), β_{DP} (indicating 'ADP-bound'), and β_E (indicating 'Empty'), based on their expected nucleotide content. Because ATP synthases can function as ATPases that deplete cellular energy reservoirs, these enzymes require a mechanism to inhibit ATP hydrolysis in the absence of a transmembrane proton motive force. Inhibition of ATP hydrolysis is thought to be particularly important in pathogenic mycobacteria due to low-energy challenges faced within hosts (Lu et al., 2014). In other bacteria (Cingolani and Duncan, 2011; Guo et al., 2019; Shirakihara et al., 2015), subunit ϵ blocks ATP hydrolysis by adopting an inhibitory 'up' conformation where its C-terminal α helix inserts into a catalytic α/β interface. However, as seen previously (Zhang et al., 2019), the purified mycobacterial ATP synthase has only negligible ATP hydrolysis activity (Fig. 2A, *left*) even though subunit ϵ adopts a non-inhibitory 'down' conformation (Fig. 1A, *right, red oval*). The structure of the *M. smegmatis* F₁ region purified in the presence of ADP showed MgADP in both the β_{TP} and β_{DP} sites and a weak density, consistent with phosphate or sulfate, in the β_E site (Zhang et al., 2019). This unusual nucleotide occupancy was suggested as an explanation for inhibition of ATP hydrolysis activity (Zhang et al., 2019). For the present study no exogenous nucleotide was added. In this structure (Fig. 1C and D), only the β_{TP} site has density for ADP, while the β_E site is empty and density consistent with phosphate or sulfate is found in the β_{DP} site, indicating that a different explanation for inhibition of hydrolysis must exist.

Instead, the mycobacterial ATP synthase appears to have a unique mechanism for inhibiting ATP hydrolysis (Video 1). Mycobacterial α subunits possess ~35 residue C-terminal extensions not found in other ATP synthases (Zhang et al., 2019). These extensions were previously proposed to form α helices involved in inhibition (Ragunathan et al., 2017), but this model was questioned because the extensions were not observed in a crystal structure of the F_1 region and were predicted to be disordered (Zhang et al., 2019). However, in the present study a single α extension is resolved in the map for each rotational state, forming intimate contacts with the γ subunit (Fig. 2B, *red*). The remaining two α extensions are not resolved, suggesting that they are disordered when not bound to subunit γ . An evolutionarily conserved region of the α extension (Zhang et al., 2019) forms a β strand that creates a parallel β sheet with a β strand from subunit γ and possesses multiple charged residues that appear to stabilize this α/γ interaction (Fig. 2C). Consequently, the C terminus of the α extension is trapped under the β strand from subunit γ , forming the hook of a ratchet that only allows unidirectional rotation of subunit γ in the ATP synthesis direction and preventing ATP hydrolysis.

To test this model of inhibition, an *M. smegmatis* strain was constructed with a 3 $\times$ FLAG tag inserted after residue Ser518 of subunit α (Fig. 2B, *red arrow*). This construct truncates the α subunits, removing the C-terminal extensions. The resulting complex had strong ATP hydrolysis activity (Fig. 2A, *middle*), which could be inhibited by ~60% upon addition of bedaquiline (BDQ; also known as R207910, TMC207, and Sirturo) (Fig. 2A, *right*). Rotational state 2 also revealed an alternative conformation with the α extension from a different α subunit bent in the opposite direction to attach to the central rotor (Fig. 2D, *centre*, Fig. S6). The existence of this conformation suggests the sequence of events that occurs as the α extension binds subunit γ to block ATP hydrolysis or is released from subunit γ during ATP synthesis (Fig. 2D, Video 1). While binding of an α extension prevents rotation in the ATP hydrolysis direction (Fig. 2D, *left*), rotation in the ATP synthesis direction causes the α extension to fold back on itself, as seen in the alternative conformation of rotational State 2 (Fig. 2D, *middle*). Further rotation in the ATP synthesis direction pulls the C terminus of the α extension out from under subunit γ , breaking the interaction between the two proteins (Fig. 2D, *right*).

Bedaquiline binds three distinct sites in ATP synthase

In vitro selection and sequencing of three resistance mutants established that the mechanism of action of BDQ (Fig. 3A) involves the mycobacterial ATP synthase c subunits (Andries et al., 2005; Koul et al., 2007). However, only micromolar affinity was measured between BDQ and recombinant subunit c (Haagsma et al., 2011; Koul et al., 2007), which typically assembles into detergent-resistant rings upon expression (Preiss et al., 2015). This micromolar binding affinity is inconsistent with the drug's nanomolar inhibition of mycobacterial ATP synthesis (Koul et al., 2007; Preiss et al., 2015) and nanomolar inhibition of *M. tuberculosis* growth (Andries et al., 2005). Consequently, it has been suggested that subunit a in the F_O region also contributes to BDQ binding in the intact complex *in vivo* (Haagsma et al., 2011). To determine how BDQ interacts with intact mycobacterial ATP synthase, the enzyme was incubated with 200 μM drug and again subjected to cryoEM structure determination. This BDQ concentration corresponds to a ~40-fold molar excess over the enzyme but only a ~4-fold molar excess relative to the c subunits in the complex. Image analysis produced maps of the three rotational states at nominal resolutions of 3.2 to 3.4 Å with focused refinement of the F_O region providing a map at 3.4 Å resolution (Fig. S1 and S2). The maps revealed seven BDQ molecules, one interacting with each c subunit in the ring except for the two c subunits occluded by subunit a (Fig. 3B, Video 2). No density was found to support the proposed binding of BDQ to subunit ε (Kundu et al., 2016). Five of the binding sites, which we refer to as 'c-only sites', correspond to sites described previously from a structure of isolated *M. phlei* c ring crystallized in the presence of millimolar BDQ (Preiss et al., 2015) (Fig. 3B-C, *yellow*). Two previously unobserved sites also involve interactions with subunit a. Considering the direction of rotation of the c ring during ATP synthesis (Fig. 3B, *purple arrow*), one of these sites, which we name the 'leading site' (Fig. 3B-C, *pink*) involves a c subunit that has just interacted with subunit a and picked up a proton from the periplasmic half channel. The other site, which we designate the 'lagging site' (Fig. 3B-C, *blue*) is poised to interact with subunit a to deposit a proton into the cytoplasmic half channel.

In the five c-only sites, the dimethylamino group of BDQ penetrates into the ion-binding pocket of the subunit, interacting with the carboxyl group of the proton-carrying Glu65 residue, as described previously (Preiss et al., 2015) (Fig. 3Cii). This interaction is consistent with the finding that the tertiary amine of BDQ is necessary for the drug's antimycobacterial activity

(Lounis et al., 2010). Also seen previously (Preiss et al., 2015), Phe69 of subunit c moves from its orientation in the drug-free structure away from BDQ in the drug-bound structure (Fig. 3Cii, *red arrow*). This movement avoids a clash with BDQ's hydroxyl group and provides what has been called a 'hydrophobic platform' for the quinoline moiety (Preiss et al., 2015). The remainder of the c-only binding pocket is formed primarily from Ala66 and Tyr68 from the same c subunit as Glu65 as well as Leu63, Ala66, Ala67, Ile70 from the adjacent c subunit. BDQ's bromine group remains accessible on the surface of the complex, supporting the reliability of affinity measurements between BDQ and the c ring where the drug was tethered with a linker at position 6 of the quinoliny group (Fig. 3A, *green*) (Haagsma et al., 2011; Koul et al., 2007). An explanation for BDQ's activity at low concentration can be found in the binding sites that only exist in the intact ATP synthase. The lagging drug binding site is clockwise from subunit a when viewed from the F₁ region toward the F₀ region (Fig. 3B, *blue*). This binding pocket is formed from the c subunit residues described above, as well as Leu70, Pro172, Ile173, and Val176 from subunit a. In the lagging site, Phe69 of subunit c undergoes the same conformational change seen in the c-only sites. The additional interactions provided by subunit a in the lagging site produce a deeper binding pocket than the c-only pocket, which increases protein-drug Van der Waals contacts (Fig. 3Ci) but does not appear to induce additional side chain rearrangement in the protein. The leading site, which is likely the most important for BDQ's *in vivo* activity, is counter-clockwise from subunit a when the complex is viewed from F₁ toward F₀ (Fig. 3B, *pink*). In addition to the c subunit residues found in the c-only and lagging sites, the leading site binding pocket comprises residues Phe213, Pro214, Val217, Trp218, and Phe221 from subunit a. These residues create a particularly deep binding pocket that fits BDQ with extensive contacts (Fig. 3Ciii). The bromine group of BDQ interacts with Phe221 of subunit a in the leading site, with the side chain undergoing a conformational change to accommodate drug binding (Fig. 3Ciii, *red arrow*). This interaction explains structure-activity relationship studies of the diarylquinoline series that led to BDQ, where the bromine increased antimycobacterial activity (Guillemont et al., 2011). It is also consistent with the finding that halogens in ligands have a high propensity to interact with hydrophobic amino acids (Kortagere et al., 2008), with interactions between bromine and Phe residues common in drug binding (Sirimulla et al., 2013).

The leading and lagging binding sites explain bedaquiline's potency

Binding of BDQ to the leading and lagging sites induces a global conformational change in the enzyme, trapping the rotor in an intermediate position between the rotary states described above (Fig. 4A, Video 2). The direction of rotation in each state can be determined by inspecting the F_1 region, and amounts to a 28° rotation in the synthesis direction for State 1, 25° in the synthesis direction for State 2, and 8° in the hydrolysis direction for State 3 (Fig. 4B). This rotation is needed to produce both the leading and lagging binding sites. The extensive contact between the drug and protein suggests that the leading and lagging sites may have a higher affinity than the c-only sites. To test this hypothesis, which would explain the activity of BDQ at low concentration, we attempted to remove weakly-bound BDQ by washing the drug-bound complex in a desalting column with a drug-free buffer. CryoEM of the resulting preparation gave maps of the three rotational states at nominal resolutions of 3.3 to 3.5 Å resolution and the F_0 region at 3.7 Å resolution (Fig. S1 and S2). The conformations of the structures matched the drug-bound state rather than the drug-free state (Fig. S7). However, in the washed structure, density for BDQ in the c-only sites is extremely weak (Fig. 4C and D, *yellow*) indicating that most BDQ has been removed from these sites. Further, density for the Phe69 residues in the c-only sites more closely resembles the density in the drug-free state than the BDQ-saturated state (Fig. 4D, *red arrow*). In contrast, strong density for BDQ is found in the leading site (Fig. 4C and D, *pink*), with weaker density for BDQ in the lagging site (Fig. 4C and D, *blue*). Density for the Phe69 residues in the leading and lagging sites resembles the drug-saturated state. The clarity of Tyr68 and Phe64 in the maps support the ability to resolve side-chain detail in all three sites (Fig. 4D). Together, these observations suggest that the leading site binds BDQ the most tightly while the c-only sites bind it with lower affinity. The binding of BDQ in the leading and lagging sites, which are not present in the isolated c ring, explains the potent antimycobacterial activity of BDQ at low concentration.

Discussion

The newly-identified BDQ binding sites are formed by induced fit, with ATP synthase undergoing rotation of its c ring away from the position found in the resting drug-free structure. Rotation of the ring allows Glu65 of subunit c to interact with the drug's dimethylamino group while simultaneously creating close but distinct contacts between BDQ and subunit a in the leading and lagging sites. The sequences of mycobacterial ATP synthase subunits a and c differ

248 from the human ATP synthase in these binding pockets, explaining BDQ's specificity for
 249 mycobacteria (Haagsma et al., 2009; Preiss et al., 2015). Consequently, in addition to revealing
 250 the unique mechanism by which the ATP synthase prevents ATP hydrolysis in the low-energy
 251 conditions faced by mycobacterial pathogens, the structures provide information that can be used
 252 for further optimization of BDQ. They also show that as the c ring rotates it creates potential
 253 drug binding sites of varying size that bridge subunits c and a, which could be targeted by other
 254 compounds to inhibit ATP synthesis and kill mycobacteria.

Acknowledgements: We thank Voula Kanelis for suggestions on the manuscript. HG was supported by an Ontario Graduate Scholarship for International Students. JLR was supported by the Canada Research Chairs program. This work was supported by Canadian Institutes of Health Research grant PJT162186 (JLR) and PJT156261 (JL). CryoEM data was collected at the Toronto High-Resolution High-Throughput cryoEM facility, supported by the Canada Foundation for Innovation and Ontario Research Fund.

Author contributions: JLR conceived the project and supervised the research. JL advised on *M. smegmatis* cell culture and chromosomal tagging strategies. SAB prepared the a 3×FLAG ORBIT payload plasmid and an *M. smegmatis* strain used in the initial stages of the project with help from JM. GMC prepared the *M. smegmatis* strains used for structure determination and enzyme assays. GMC and HG purified the protein, HG performed the cryoEM and image analysis, and GMC and HG constructed the atomic models. JLR, HG, and GMC wrote the manuscript and prepared figures with input from the other authors.

Competing interests: The authors declare no competing interests

Data and materials availability: CryoEM maps are deposited in the Electron Microscopy Data Bank under accession numbers EMD-22311 to 22322. Atomic models are deposited in the Protein Data Bank under accession numbers 7JG5, 7JG6, 7JG7, 7JG8, 7JG9, 7JGA, 7JGB, 7JGC. Strains and plasmids are available from the corresponding author.

Methods

Plasmid and strains

M. smegmatis strains were generated by the ORBIT (oligonucleotide-mediated recombineering followed by Bxb integrase targeting) method (Murphy et al., 2018), which requires a plasmid with the Che9c phage RecT annealase and Bxb1 integrase, a payload plasmid with the desired insert, and an oligonucleotide that guides integration of the payload into the chromosomal DNA. The FLAG-His sequence in payload plasmid pKM491 (Addgene) was replaced with sequence for a 3×FLAG tag by Gibson Assembly (New England Biolabs) to create payload plasmid pSAB41. *M. smegmatis* strains were then constructed by transforming strain MC²155 bearing plasmid pKM444, which encodes the Che9c annealase and Bxb1 integrase, with plasmid pSAB41 and the targeting oligonucleotide. Strain GMC_MSM1 was generated with a 3×FLAG tag at the C terminus of the β subunits with oligonucleotide 5'-CGACGGCGACGATCTCGACGTTTCAGATCAGCCACACCACACCTTTCGGACGCTCAAGGACATGGCAATCAGGTTTGTACCGTACACCACTGAGACCGCGGTGGTTGACCA GACAAACCCAGCTTGGCGCCGAGGCTCTCGGCCTTCTTCGCCAGGTCGTCCAGACCA CCGATCAGGAAGAACGCCTGC-3'. Strain GMC_MSM2, with the α subunits truncated after Ser 518 followed by a C-terminal 3×FLAG tag, was generated with oligonucleotide 5'-TGCGGACCTTGACGGATTCCTTCTCCAGGTCCTCGGGATCGAGGGCCTCGGCGTTCT CGGAGACGACCACGGTTTGTACCGTACACCACTGAGACCGCGGTGGTTGACCAGAC AAACCCGAGCTGCCGTCAGAGGCCTGGAAGCCCTTCTTGAATTCGTTGATGACCGAG ACCAGCTTCTCCTCGGCT-3'. Transformants were selected with hygromycin (50 μ g/ml) and correct insertion of the 3×FLAG sequence was confirmed by colony PCR.

Bacterial growth and protein purification

M. smegmatis was grown in Middlebrook 7H9 broth supplemented with 0.8 g/L NaCl, 2 g/L dextrose, 10 g/L tryptone, and 0.05% (v/v) Tween 80. Cells were harvested by centrifugation and resuspended in 4 mL/g lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM MgSO₄, 5 mM 6-aminocaproic acid, 5 mM benzamidine, 1 mM PMSF). The suspension was filtered through Miracloth (Millipore), before 3 to 4 passages through an EmulsiFlex-C3 High Pressure Homogenizer (Avestin) at 20 kpsi. Unbroken cells and debris were removed by centrifugation at 39,000 g for 30 min. Membranes were collected by centrifugation at 257,000 g for 90 min and

resuspended in buffer (50 mM Tris-HCl pH 7.4, 15% [v/v] glycerol, 5 mM MgSO₄, 150 mM NaCl, 5 mM 6-aminocaproic acid, 5 mM benzamidine, 1 mM PMSF) prior to storage at -80 °C. For protein purification, membranes were thawed and solubilized with 1% (w/v) dodecyl-β-D-maltoside (DDM) (Anatrace) and insoluble material was removed by centrifugation at 257,000 g for 60 min. Solubilized protein was then loaded onto 2 mL M2 affinity matrix (Sigma) previously equilibrated with wash buffer (50 mM Tris-HCl pH 7.4, 15% [v/v] glycerol, 150 mM NaCl, 5 mM 6-aminocaproic acid, 5 mM benzamidine, 0.05% DDM [w/v]) and the ATP synthase eluted with 150 µg/ml 3×FLAG peptide in wash buffer. The sample was concentrated and loaded onto a Superose 6 Increase 10/300 column (GE Healthcare) equilibrated with gel filtration buffer (50 mM Tris-HCl pH 7.4, 15% [v/v] glycerol, 150 mM NaCl, 0.05% DDM [w/v]). Eluted ATP synthase was pooled and concentrated to ~6 mg/ml and stored at -80 °C. ATP hydrolysis activity was measured as described previously (Kornberg and Pricer, 1951; Vasanthakumar et al., 2019) in ATPase buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10% [v/v] glycerol, 5 mM MgCl₂, 0.2 mM NADH, 2 mM ATP, 1 mM phosphoenol pyruvate, 3.2 units pyruvate kinase, 8 units lactate dehydrogenase, 0.05% [w/v] DDM). Inhibition with BDQ (AmBeed) was assayed at 2 µM.

CryoEM

Holey gold grids (Meyerson et al., 2014; Russo and Passmore, 2014) with a regular arrays of ~2 µm holes were nanofabricated as described previously (Marr et al., 2014). CryoEM specimens of ATP synthase without BDQ were prepared following removal of glycerol with a Zeba Spin desalting column (Thermo Fisher Scientific) with protein (2.5 µL) applied to grids that had been glow-discharged in air for 2 min. Grids were then blotted in a Vitrobot Mark III (Thermo Fisher Scientific) for 26 s at 4 °C and ~100% humidity before freezing in a liquid ethane/propane mixture (Tivol et al., 2008). Specimens of BDQ-saturated ATP synthase were prepared by mixing 1 µL of 2.5 mM BDQ in DMSO with 12 µL of 5 mg/ml ATP synthase and incubating on ice for 1 h. Immediately before freezing, glycerol was removed with a Zeba Spin desalting column equilibrated with buffer containing 0.2 mM BDQ (final DMSO concentration 2%). Specimens of ATP synthase with weakly-bound BDQ washed away were prepared by excluding BDQ from the final desalting step with the Zeba Spin column.

Data collection

Cryo-EM images were collected at 300 kV with a Titan Krios G3 electron microscope equipped with a Falcon 4 camera (Thermo Fisher Scientific). Automated data collection was done with the *EPU* software package. For the drug-free dataset, 7,691 movies consisting of 30 exposure fractions were collected at a nominal magnification of 75,000 $\times$, corresponding to a calibrated pixel size of 1.03 Å. The camera exposure rate and the total exposure for the specimen were 5.0 e⁻/pix/s and ~ 45 e⁻/Å², respectively. For the BDQ-saturated dataset, 7,589 movies consisting of 29 exposure fractions were collected at the same magnification. The camera exposure rate and the total exposure of the specimen were 4.9 e⁻/pix/s and ~ 41 e⁻/Å², respectively. The BDQ-washed dataset consisted of 4,962 movies at the same conditions as the BDQ-saturated dataset.

Image analysis

Except where noted, image analysis was performed with *cryoSPARC* v2 (Punjani et al., 2017). Movie frames were aligned with *MotionCor2* (Zheng et al., 2017) using a 7 $\times$ 7 grid and contrast transfer function (CTF) parameters were estimated in patches. Templates for particle selection were generated by 2D classification of manually-selected particle images and datasets of particle images were extracted in 320 $\times$ 320 pixel boxes. This process provided 1,435,679 particle images for the drug-free condition, 1,825,672 particle images for the BDQ-saturated condition, and 1,865,961 particle images for the BDQ-washed condition. Datasets were cleaned with two rounds of 2D classification, reducing their size to 708,053, 797,936, and 608,800 particle images for the three conditions, respectively. After ab initio 3D classification and heterogeneous refinement, three classes corresponding to the main rotational states of the complex were identified in each dataset. Classes were refined with non-uniform refinement (Punjani et al., 2019), individual particle defocus parameters were estimated, and maps were refined again with non-uniform refinement. Image parameters were then converted to *Relion* 3.0 (Scheres, 2012) .star file format with the *pyem* package (DOI: [10.5281/zenodo.3576630](https://doi.org/10.5281/zenodo.3576630)) and beam-induced motion was corrected for individual particles in *Relion* (Zivanov et al., 2019). These images were imported back to *cryoSPARC* v2, CTF parameters were re-estimated, and non-uniform refinement and several rounds of ab initio 3D classification followed by heterogeneous refinement were performed to remove poorly behaved particle images from each class. A final round of non-uniform refinement produced 3D maps of the intact complex in the different

rotational states. For the drug-free condition the three classes contained 152,372, 46,960, and 120,424 particle images and reached 3.4 Å, 3.7 Å and 3.5 Å resolution, respectively. Masked local refinement of the F_O region with 319,756 particle images from all three classes yielded a map at 3.5 Å resolution. For the BDQ-saturated condition the three classes contained 112,421, 70,566, and 155,488 particle images and reached 3.3 Å, 3.4 Å and 3.2 Å resolution, respectively. Local refinement of the F_O region of State 3 yielded a map at 3.4 Å resolution. For the BDQ-washed condition the three rotational state classes contained 97,912, 69,407, and 127,165 particle images and reached 3.3 Å, 3.5 Å and 3.3 Å resolution, respectively. Masked local refinement of the F_O region with 294,484 particle images from all three classes yielded a map at 3.7 Å resolution. All the maps were locally sharpened with LocalDeblur (Ramírez-Aportela et al., 2020). For illustration purposes, composite maps of some of the rotational states were generated by combining the F₁ region of the intact maps with the F_O region of the maps from masked refinement using *UCSF Chimera* (Pettersen et al., 2004) and *relion_image_handler*.

Atomic model building and refinement

The State 1 map for the BDQ-free specimen and the State 3 map for the BDQ-saturated specimen had the highest overall resolutions and were used for building atomic models. To model the F₁ region, the crystal structure of the *M. smegmatis* F₁-ATPase containing subunits α₃β₃γε (PDB 6FOC) (Zhang et al., 2019) was fit rigidly into the maps with *UCSF Chimera* (Pettersen et al., 2004). Manual adjustment and building of the missing regions was performed in *Coot* (Emsley and Cowtan, 2004). To model the F_O region, the crystal structure of the *M. phlei* c ring in complex with BDQ (PDB 4V1F) (Preiss et al., 2015) was rigidly fit into the maps of the F_O regions from focused refinement. Manual adjustment of the model and building of subunit a and the membrane-embedded regions of subunits b-δ and b' were done in *Coot*. F₁ and F_O models were then modified in *ISOLDE* (Croll, 2018) to improve dihedral angles and rotamer fitting before being refined in *Phenix* (Adams et al., 2010) with Ramachandran, rotamer and secondary structure restraints. One of the three N-terminal α helices from subunit α in the BDQ-free model was modeled as polyalanine (Table S4). Model quality was evaluated with *Molprobit* (Chen et al., 2010) and *EMRinger* (Barad et al., 2015). Full models of the BDQ-saturated rotational state 3 and BDQ-free rotational state 1 were prepared by rigid fitting of the F₁ and F_O models into the maps with *UCSF Chimera*, manual adjustment of the connecting

regions with *Coot*, and optimization of geometry with *ISOLDE*. Backbone models of the BDQ-saturated States 1 and 2 were built by flexible fitting of the F₁ region of the BDQ-saturated rotational state 3 model into the corresponding maps with *ISOLDE*. The F₁ regions were then combined with the ab'b- δ subcomplex and c ring from the BDQ-saturated rotational state 3 model fit separately into the maps as rigid bodies. Backbone models of the BDQ-free States 2 and 3 were built from the BDQ-free State 1 model in the same way. Figures and videos were generated with UCSF Chimera (Pettersen et al., 2004) and ChimeraX (Goddard et al., 2018).

Bibliography

- Abrahams, J.P., Leslie, A.G., Lutter, R., and Walker, J.E. (1994). Structure at 2.8 Å resolution of F₁-ATPase from bovine heart mitochondria. *Nature* **370**, 621–628.
- Adams, P.D., Afonine, P. V., Bunkóczi, G., Chen, V.B., Davis, I.W., Echols, N., Headd, J.J., Hung, L.W., Kapral, G.J., Grosse-Kunstleve, R.W., et al. (2010). PHENIX: A comprehensive Python-based system for macromolecular structure solution. *Acta Cryst. D66*, 213–221.
- Andries, K., Verhasselt, P., Guillemont, J., Gohlmann, H.W.H., Neefs, J.-M., Winkler, H., Gestel, J. Van, Timmerman, P., Zhu, M., Lee, E., et al. (2005). A diarylquinoline drug active on the ATP synthase of *Mycobacterium tuberculosis*. *Science* (80-.). **307**, 223–227.
- Barad, B.A., Echols, N., Wang, R.Y.R., Cheng, Y., Dimaio, F., Adams, P.D., and Fraser, J.S. (2015). EMRinger: Side chain-directed model and map validation for 3D cryo-electron microscopy. *Nat. Methods* **12**, 943–946.
- Chen, V.B., Arendall, W.B., Headd, J.J., Keedy, D.A., Immormino, R.M., Kapral, G.J., Murray, L.W., Richardson, J.S., and Richardson, D.C. (2010). MolProbity: all-atom structure validation for macromolecular crystallography. *Acta Cryst D66*, 12–21.
- Cingolani, G., and Duncan, T.M. (2011). Structure of the ATP synthase catalytic complex (F₁) from *Escherichia coli* in an autoinhibited conformation. *Nat. Struct. Mol. Biol.* **18**, 701–707.
- Conradie, F., Diacon, A.H., Ngubane, N., Howell, P., Everitt, D., Crook, A.M., Mendel, C.M., Egizi, E., Moreira, J., Timm, J., et al. (2020). Treatment of Highly Drug-Resistant Pulmonary Tuberculosis. *N. Engl. J. Med.* **382**, 893–902.
- Croll, T.I. (2018). ISOLDE: A physically realistic environment for model building into low-resolution electron-density maps. *Acta Crystallogr. Sect. D Struct. Biol.* **74**, 519–530.
- Emsley, P., and Cowtan, K. (2004). Coot: Model-building tools for molecular graphics. *Acta Cryst D60*, 2126–2132.
- Gajadeera, C.S., and Weber, J. (2013). *Escherichia coli* F₁F_o-ATP Synthase with a b/delta Fusion Protein Allows Analysis of the Function of the Individual b Subunits. *J. Biol. Chem.* **288**, 26441–26447.
- Goddard, T.D., Huang, C.C., Meng, E.C., Pettersen, E.F., Couch, G.S., Morris, J.H., and Ferrin, T.E. (2018). UCSF ChimeraX: Meeting modern challenges in visualization and analysis. *Protein Sci.* **27**, 14–25.
- Guillemont, J., Meyer, C., Poncelet, A., Bourdrez, X., and Andries, K. (2011). Diarylquinolines, synthesis pathways and quantitative structure-activity relationship studies leading to the discovery of TMC207. *Future Med. Chem.* **3**, 1345–1360.
- Guo, H., Bueler, S.A., and Rubinstein, J.L. (2017). Atomic model for the dimeric FO region of

mitochondrial ATP synthase. *Science* (80-.). 358, 936–940.

Guo, H., Suzuki, T., and Rubinstein, J.L. (2019). Structure of a bacterial ATP synthase. *Elife* 8, e43128.

Haagsma, A.C., Abdillahi-Ibrahim, R., Wagner, M.J., Krab, K., Vergauwen, K., Guillemont, J., Andries, K., Lill, H., Koul, A., and Bald, D. (2009). Selectivity of TMC207 towards Mycobacterial ATP synthase compared with that towards the eukaryotic homologue. *Antimicrob. Agents Chemother.* 53, 1290–1292.

Haagsma, A.C., Podasca, I., Koul, A., Andries, K., Guillemont, J., Lill, H., and Bald, D. (2011). Probing the Interaction of the Diarylquinoline TMC207 with Its Target Mycobacterial ATP Synthase. *PLoS One* 6, e23575.

Hahn, A., Vonck, J., Mills, D.J., Meier, T., and Kuhlbrandt, W. (2018). Structure, mechanism, and regulation of the chloroplast ATP synthase. *Science* (80-.). 360, eaat4318.

Junge, W., Lill, H., and Engelbrecht, S. (1997). ATP synthase: an electrochemical transducer with rotary mechanics. *Trends Biochem. Sci.* 22, 420–423.

Klusck, N., Murphy, B.J., Mills, D.J., Yildiz, O., and Kuhlbrandt, W. (2017). Structural basis of proton translocation and force generation in mitochondrial ATP synthase. *Elife* 6, e33274.

Kornberg, A., and Pricer, W.E. (1951). Enzymatic phosphorylation of adenosine and 2,6-diaminopurine riboside. *J. Biol. Chem.* 193, 481–496.

Kortagere, S., Ekins, S., and Welsh, W.J. (2008). Halogenated ligands and their interactions with amino acids : Implications for structure–activity and structure–toxicity relationships. *J. Mol. Graph. Model.* 27, 170–177.

Koul, A., Dendouga, N., Vergauwen, K., Molenberghs, B., Vranckx, L., Willebrords, R., Ristic, Z., Lill, H., Dorange, I., Guillemont, J., et al. (2007). Diarylquinolines target subunit c of mycobacterial ATP synthase. *Nat. Chem. Biol.* 3, 323–324.

Koul, A., Vranckx, L., Dendouga, N., Balemans, W., Van Den Wyngaert, I., Vergauwen, K., Göhlmann, H.W.H., Willebrords, R., Poncelet, A., Guillemont, J., et al. (2008). Diarylquinolines are bactericidal for dormant mycobacteria as a result of disturbed ATP homeostasis. *J. Biol. Chem.* 283, 25273–25280.

Kundu, S., Biukovic, G., Grüber, G., and Dick, T. (2016). Bedaquiline targets the ϵ subunit of mycobacterial F-ATP synthase. *Antimicrob. Agents Chemother.* 60, 6977–6979.

Lounis, N., Guillemont, J., Veziris, N., Koul, A., Jarlier, V., and Andries, K. (2010). R207910 (TMC207): A new antibiotic for the treatment of tuberculosis. *N. Med. Mal. Infect.* 40, 383–390.

Lu, P., Lill, H., and Bald, D. (2014). ATP synthase in mycobacteria: Special features and implications for a function as drug target. *Biochim. Biophys. Acta - Bioenerg.* 1837, 1208–1218.

Marr, C.R., Benlekbi, S., and Rubinstein, J.L. (2014). Fabrication of carbon films with approximately 500 nm holes for cryo-EM with a direct detector device. *J Struct Biol* 185, 42–47.

McNeil, M.B., Ryburn, H.W., Harold, L.K., Tirados, J.F., and Cook, G.M. (2020). Transcriptional inhibition of the F1F0-type ATP synthase has bactericidal consequences on the viability of mycobacteria. *Antimicrob. Agents Chemother.* *In press*.

Médecins Sans Frontières (2019). MSF Press Release.

Meyerson, J.R., Rao, P., Kumar, J., Chittori, S., Banerjee, S., Pierson, J., Mayer, M.L., and Subramaniam, S. (2014). Self-assembled monolayers improve protein distribution on holey carbon cryo-EM supports. *Sci. Rep.* 4.

Murphy, K.C., Nelson, S.J., Nambi, S., Papavinasundaram, K., Baer, C.E., and Sassetti, C.M. (2018). ORBIT: a New Paradigm for Genetic Engineering of Mycobacterial Chromosomes. *MBio* 9, e01467-18.

Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C., and Ferrin, T.E. (2004). UCSF Chimera - A visualization system for exploratory research and analysis. *J. Comput. Chem.* 25, 1605–1612.

Preiss, L., Langer, J.D., Yildiz, Ö., Eckhardt-Strelau, L., Guillemont, J.E.G., Koul, A., and Meier, T. (2015). Structure of the mycobacterial ATP synthase Fo rotor ring in complex with the anti-TB drug bedaquiline. *Sci. Adv.* 1, e1500106.

Punjani, A., Rubinstein, J.L., Fleet, D.J., and Brubaker, M.A. (2017). cryoSPARC: Algorithms for rapid unsupervised cryo-EM structure determination. *Nat. Methods* 14, 290–296.

Punjani, A., Zhang, H., and Fleet, D.J. (2019). Non-uniform refinement: Adaptive regularization improves single particle cryo-EM reconstruction. *BioRxiv* 2019.12.15.877092.

Ragunathan, P., Sielaff, H., Sundararaman, L., Biukovic, G., Sony, M., Manimekalai, M.S.S., Singh, D., Kundu, S., Wohland, T., Frasc, W., et al. (2017). The uniqueness of subunit alpha of mycobacterial F-ATP synthases: An evolutionary variant for niche adaptation. *J. Bioenerg. Biomembr.* 292, 11262–11279.

Ramírez-Aportela, E., Vilas, J.L., Glukhova, A., Melero, R., Conesa, P., Martínez, M., Maluenda, D., Mota, J., Jiménez, A., Vargas, J., et al. (2020). Automatic local resolution-based sharpening of cryo-EM maps. *Bioinformatics* 36, 765–772.

Russo, C.J., and Passmore, L.A. (2014). Ultrastable gold substrates for electron cryomicroscopy. *Science* (80-.). 346, 1377–1380.

Scheres, S.H.W. (2012). RELION: implementation of a Bayesian approach to cryo-EM structure determination. *J Struct Biol* 180, 519–530.

Shirakihara, Y., Shiratori, A., Tanikawa, H., Nakasako, M., Yoshida, M., and Suzuki, T. (2015). Structure of a thermophilic F1-ATPase inhibited by an epsilon-subunit: deeper insight into the epsilon-inhibition mechanism. *FEBS J.* 282, 2895–2913.

Sirimulla, S., Bailey, J.B., Vegesna, R., and Narayan, M. (2013). Halogen Interactions in Protein – Ligand Complexes: Implications of Halogen Bonding for Rational Drug Design. *J. Chem. Inf. Model.* 53, 2781–2791.

Sobti, M., Smits, C., Wong, A.S.W., Ishmukhametov, R., Stock, D., Sandin, S., and Stewart, A.G. (2016). Cryo-EM structures of the autoinhibited E. coli ATP synthase in three rotational states. *Elife* 5, e21598.

Sobti, M., Walshe, J.L., Wu, D., Ishmukhametov, R., Zeng, Y.C., Robinson, C. V, Berry, R.M., and Stewart, A.G. (2020). Cryo-EM structures provide insight into how E. coli F1Fo ATP synthase accommodates symmetry mismatch. *Nat. Commun.* 11.

Tivol, W.F., Briegel, A., and Jensen, G.J. (2008). An improved cryogen for plunge freezing. *Microsc. Microanal.* 14, 375–379.

Vasanthakumar, T., Bueler, S.A., Wu, D., Beilsten-Edmands, V., and Robinson, C. V (2019). Structural comparison of the vacuolar and Golgi V- ATPases from *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U. S. A.* 116, 7272–7277.

Vik, S., and Antonio, B.J. (1994). A mechanism of proton translocation by F1F0 ATP synthases suggested by double mutants of the a subunit. *J Biol Chem* 269, 30364-9.

Wellington, S., and Hung, D.T. (2018). The Expanding Diversity of Mycobacterium tuberculosis Drug Targets. *ACS Infect. Dis.* 4, 696–714.

World Health Organization (2019a). Global Tuberculosis Report 2019 (Geneva: World Health Organization).

World Health Organization (2019b). The Selection and Use of Essential Medicines (Geneva: World Health Organization).

536 Zhang, A.T., Montgomery, M.G., Leslie, A.G.W., Cook, G.M., and Walker, J.E. (2019). The
537 structure of the catalytic domain of the ATP synthase from *Mycobacterium smegmatis* is a target
538 for developing antitubercular drugs. *Proc. Natl. Acad. Sci. U. S. A.* *116*, 4206–4211.
539 Zhao, J., Benlekhir, S., and Rubinstein, J.L. (2015). Electron cryomicroscopy observation of
540 rotational states in a eukaryotic V-ATPase. *Nature* *521*, 241–245.
541 Zheng, S.Q., Palovcak, E., Armache, J.-P., Verba, K.A., Cheng, Y., and Agard, D.A. (2017).
542 MotionCor2 : anisotropic correction of beam-induced motion for improved cryo-electron
543 microscopy. *Nat. Methods* *14*, 331–332.
544 Zhou, A., Rohou, A., Schep, D.G., Bason, J.V.J., Montgomery, M.G., Walker, J.E., Grigorieff,
545 N., and Rubinstein, J.L. (2015). Structure and conformational states of the bovine mitochondrial
546 ATP synthase by cryo-EM. *Elife* *4*, e10180.
547 Zivanov, J., Nakane, T., and Scheres, S.H.W. (2019). A Bayesian approach to beam-induced
548 motion correction in cryo-EM single-particle analysis. *IUCrJ* *6*, 5–17.
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Figure 1. Structure of the mycobacterial ATP synthase. **A**, Composite cryoEM density map (left) and atomic model (right). Scale bar, 25 Å. The red oval indicates that subunit ε is in a non-inhibitory ‘down’ conformation. **B**, Interaction of subunit b’ and b-δ with subunit a in the membrane. **C**, Cross section through the F₁ region of the model showing nucleotide content consistent with an uninhibited enzyme. **D**, Close-up view of nucleotide content in the catalytic sites.

Figure 2. Autoinhibition of ATP hydrolysis. **A**, ATP hydrolysis is inhibited in intact mycobacterial ATP synthase, but inhibition is released by truncation of the α extension at Ser518. ATP hydrolysis in the enzyme with truncated α extension is inhibited ~60% by addition of BDQ. Error bars, $\pm$ SD (N=6, two biological replicates with three technical replicates each). **B**, Each rotational state shows one α extension bound to the γ subunit. **C**, The α extension interacts with subunit γ by forming a two-stranded parallel β sheet. Ser518 of subunit α is indicated with a red arrow. **D**, Rotation in the ATP hydrolysis direction is inhibited by binding of the α extension but rotation in the ATP synthesis direction releases the α extension (see also Video 1).

Figure 3. Inhibition of mycobacterial ATP synthase by BDQ. **A**, Structure of BDQ. **B**, In saturating conditions, BDQ binds seven sites in the mycobacterial ATP synthase: five ‘c-only’ sites (yellow), a leading site (pink), and a lagging site (blue). The direction of proton flow and c ring rotation are indicated (purple arrow). **C**, Atomic model (left) and surface representation (right) of the lagging (top), c-only (middle), and leading (bottom) BDQ binding sites. The red arrows indicate movement of residues upon BDQ binding (F69 in subunit c and F221 in subunit a), with their position in the drug-free structures shown in transparent light blue.

Figure 4. Large scale conformational changes and high-affinity binding of BDQ. **A**, Shown for rotational state 1, addition of BDQ induces rotation of the rotor to form drug binding sites. **B**, The induced conformational changes from BDQ binding corresponds to a 28° rotation in the ATP synthase direction for rotational state 1, 25° in the ATP synthase direction for rotational state 2, and 8° in the ATP hydrolysis direction for rotational state 3. **C**, Comparison to the BDQ-saturated (*top*) and BDQ-free (*bottom*) structures shows that removal of weakly-bound BDQ by washing (*middle*) leads to loss of density at the c-only sites, indicating a lower affinity for these sites. **D**, Map densities for F69 in the c-only sites of the washed complex resemble the BDQ-free state (*red arrow*) while in the leading- and lagging-sites they match the drug-bound state. Density for Y68 and F74 support the map resolution in all of the sites. (see also Video 2).

Fig. S1. Workflow for cryoEM image analysis. Example micrograph (A) and 2D class average images (B) for BDQ-saturated *M. smegmatis* ATP synthase. Example particles are circled in white. Workflow for obtaining maps of the three rotational states (C) and F_O region (D). Summary of workflows for the BDQ-free (E) and BDQ-washed (F) datasets.

Fig. S2. CryoEM map validation. Corrected Fourier shell correlation curves after gold-standard refinement, orientation distribution plots, and local resolution maps are shown for maps of the entire complex from the BDQ-free dataset (A), BDQ-saturated dataset (B), BDQ-washed dataset (C), as well as F_0 region maps from focused refinement (D).

Fig. S3. Examples of model-in-map fit to demonstrate map quality. Examples are shown from each subunit in the BDQ-free model (A) and BDQ-saturated model (B).

Fig. S4. Comparison of the subunit a model from *M. smegmatis*, *Bacillus* PS3, and *E. coli*.
A, The *Bacillus* PS3 structure is in grey and the *M. smegmatis* structure is in green. **B**, The *E. coli* structure is in purple and the *M. smegmatis* structure is in green.

Fig. S5. The N-terminal domain of the δ region of the b- δ fusion protein (*right side, top*) is substantially larger than the N-terminal domain of *Bacillus* PS3 δ subunit (*right side, middle*), composed of ~270 residues, rather than ~170 residues. The extra sequence contributes five additional α helices to the six found in the *Bacillus* PS3 δ subunit for a total of eleven α helices in the N-terminal domain of b- δ (Fig. 1B, *bottom*). *M. smegmatis* b- δ and *Bacillus* PS3 δ also form different interactions with the N-terminal sequences of the α subunits that help attach the peripheral stalk to F₁ region (*right side, red*).

Fig. S6. Two conformations of the α -extension are seen in rotational state 2. Density for the α -extension in its predominant conformation is colored pink while the alternative conformation is colored red.

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— saturated BDQ model — washed BDQ map

Fig. S7. The BDQ-washed structures are in the BDQ-saturated conformation. Fit of the BDQ-saturated protein models in the BDQ-washed maps shows that the two preparations adopt the same conformation.

Table S1. EM statistics

	BDQ-free	BDQ-saturated	BDQ-washed
Data Collection			
Electron Microscope	Titan Krios G3		
Camera	Falcon 4		
Mode	Counting		
Voltage (kV)	300		
Nominal Magnification	75,000×		
Calibrated physical pixel size (Å)	1.03		
Exposure time (s)	9.6	8.9	8.9
Total exposure (e ⁻ /Å ²)	45	41	41
Exposure rate (e ⁻ /pixel/s)	5	4.9	4.9
Number of frames	30	29	29
Defocus range (μm)	0.8-2.3	0.6-3.0	0.5-4.0
Image Processing			
Micrograph motion correction software	MotionCor2		
CTF estimation software	cryoSPARC v2		
Particle selection software	cryoSPARC v2		
3D classification and refinement software	cryoSPARC v2		
Particle motion correction software	Relion 3		
Number of micrographs	7,691	7,589	4,962
Number of particle images selected	1,435,679	1,825,672	1,865,961
Number of particle images after cleanup	708,053	797,936	608,800
Number of particle images in final maps	319,756	338,475	294,484
Symmetry	C1		
Model building			
Modeling software	Coot, ISOLDE		
Refinement software	Phenix		

Table S2. Map statistics

Map	Resolution (Å, FSC =0.143)	Applied B-factors (Å ²)	Number of particle images
BDQ-free state 1	3.4	-114.6	152,372
BDQ-free state 2	3.7	-109.8	46,960
BDQ-free state 3	3.5	-94.7	120,424
BDQ-saturated state 1	3.3	-103.9	112,421
BDQ-saturated state 2	3.4	-100.5	70,566
BDQ-saturated state 3	3.2	-110.8	155,488
BDQ-washed state 1	3.3	-109.5	97,912
BDQ-washed state 2	3.5	-112.3	69,407
BDQ-washed state 3	3.3	-104.9	127,165
BDQ-free focused Fo	3.5	-96.6	319,758
BDQ-saturated focused Fo	3.4	-79.0	155,488
BDQ-washed focused Fo	3.7	-90.7	294,484

Table S3. Atomic model statistics

	BDQ-free state 1	BDQ-saturated state 3	BDQ-free masked Fo	BDQ-saturated masked Fo
Number of residues	4,830	4830	1022	1017
Ligand	3 Mg-ATP 1 Mg-ADP 1 Pi	3 Mg-ATP 1 Mg-ADP 1 Pi 7 BDQ	-	7 BDQ
Map-model CC_mask	0.69	0.71	0.65	0.68
RMS bond length (Å)	0.007	0.007	0.007	0.008
RMS bond angle (°)	1.027	1.055	0.642	0.736
Clash score	3.15	3.75	3.73	5.86
Ramachandran				
Favoured (%)	98.36	97.80	98.09	97.58
Allowed (%)	1.51	2.05	1.91	2.42
Outliers (%)	0.13	0.15	0.00	0.00
Rotamer outliers (%)	0.14	0.14	0.45	0.15
C ^β outliers	0.00	0.00	0.00	0.00
CaBLAM outliers (%)	0.98	0.87	0.41	0.62
Average B factors (Å²)				
Protein	67.94	83.37	22.48	63.71
Ligands	68.92	69.99	-	65.71
MolProbity score	1.11	1.21	1.16	1.41
EMRinger score	2.60	3.16	2.65	3.40

Table S4. Residues included in atomic models. F_O atomic models contain the same residues as the F_O region in the corresponding models of the full structure.

Chain ID (subunit)	BDQ-free state 1		BDQ-saturated state 3
	Atomic model	Poly alanine	Atomic model
A (α_1)	5-516, 533-545	-	7-520
B (α_2)	11-22, 28-520	-	8-22, 28-520
C (α_3)	29-520	11 residues at the N terminus	9-22, 29-515, 533-546
D (β_1)	8-471	-	8-471
E (β_2)	8-471	-	8-471
F (β_3)	8-471	-	8-471
G (γ)	4-164, 178-213, 222-303	-	4-164, 178-213, 222-303
H (ϵ)	3-119	-	3-119
a (a)	31-113, 123-246	-	31-113, 123-246
b (b')	20-164	-	24-164
d (b- δ)	1-165, 172-255, 260-285, 288-331, 337-444	-	1-157, 169-331, 337-444
1-9 (c ₁ -c ₉)	5-85	-	5-85

Table S5. Deposited maps and models

Map	EMDB code	PDB ID	Model type
BDQ-free state 1	EMD-22311	7JG5	atomic
BDQ-free state 2	EMD-22312	7JG6	backbone
BDQ-free state 3	EMD-22313	7JG7	backbone
BDQ-saturated state 1	EMD-22314	7JG8	backbone
BDQ-saturated state 2	EMD-22315	7JG9	atomic
BDQ-saturated state 3	EMD-22316	7JGA	backbone
BDQ-washed state 1	EMD-22317	-	-
BDQ-washed state 2	EMD-22318	-	-
BDQ-washed state 3	EMD-22319	-	-
BDQ-free focused F _O	EMD-22320	7JGB	atomic
BDQ-saturated focused F _O	EMD-22321	7JGC	atomic
BDQ-washed focused F _O	EMD-22322	-	-

Video 1. Release of the inhibitory α extension during ATP synthesis. The video shows interpolation between the different rotational states of the enzyme, including the alternative conformation of rotational state 2, which suggests the sequence of events that occur during release of the α -extension during ATP synthesis and binding of the α -extension to subunit γ to inhibit ATP hydrolysis. **Video may be viewed as a loop.**

Video 2. Binding of BDQ to the mycobacterial ATP synthase. The video shows interpolation between the conformation of the ATP synthase in the BDQ-free and BDQ-bound states. BDQ in the leading, c-only, and lagging binding sites are colored pink, yellow, and blue, respectively. Phe69 in subunit c and Phe221 in subunit a, which undergo large conformational changes upon drug binding, are shown as space-filling models. **Video may be viewed as a loop.**