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An electron transfer path connects subunits of a mycobacterial respiratory supercomplex

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We report a 3.5-Å resolution cryo-EM structure of a respiratory supercomplex isolated from *Mycobacterium smegmatis*. It comprises a complex III dimer flanked on either side by individual complex IV subunits. Complex III and IV associate such that electrons can be transferred from quinol in complex III to the oxygen reduction center in complex IV via a bridging cytochrome subunit. We observe a superoxide dismutase-like subunit at the periplasmic face, which may be responsible for detoxification of superoxide formed by complex III. The structure reveals features of an established drug target and provides a foundation for development of treatments for human tuberculosis.

In cellular respiration, chemical energy is extracted by coupling the oxidation of an energy source (e.g., sugars, fatty acids, amino acids) and the reduction of an electron acceptor (e.g., oxygen, sulfur, nitrate, sulfate) to synthesize adenosine triphosphate (ATP) which powers cellular reactions. In aerobic organisms, electrons are transferred from electron donors to oxygen, the terminal acceptor, through the electron transport chain (ETC) to pump protons across a membrane (cellular or mitochondrial). This creates a transmembrane proton gradient (proton motive force, PMF) that drives ATP synthesis (1). In ETCs, quinones and cytochromes are two types of electron carriers that shuttle electrons to and from large macromolecular structures embedded in the membrane. In the mitochondrial respiratory chain, four membrane oxidoreductases are involved in electron transfer: complex I (NADH:ubiquinone oxidoreductase, CI), complex II (succinate:ubiquinone oxidoreductase, CII), complex III (bc_1 -type ubiquinol:cytochrome *c* oxidoreductase, bc_1 -type CIII) and complex IV (aa_3 -type cytochrome *c* oxidase, aa_3 -type CIV). CIII oxidizes ubiquinol to ubiquinone and passes the electrons to soluble cytochrome *c* which then shuttles them to CIV, where oxygen is reduced to water (Fig. 1A). The

transmembrane PMF is generated by proton pumping in CI, CIII and CIV.

The situation is more complicated in prokaryotic respiratory chains (Fig. 1A) (2). A soluble cytochrome *c* pathway similar to that in mitochondria is common in Gram-negative bacteria. Variations include a membrane-anchored cytochrome *c* mediating electron transfer from CIII to CIV (3) and a caa_3 -type CIV with a fused cytochrome *c* domain (4). Gram-positive bacteria usually possess cytochrome *c* proteins that are anchored onto the membrane (5), or a fused cytochrome *c* domain (bcc -type CIII or caa_3 -type CIV) to mediate electron transfer between CIII and CIV (6, 7). *Mycobacteria* and other *Actinobacteria* such as *Corynebacterium glutamicum* are inherently devoid of a soluble cytochrome *c* in their genomes (8), but contain a bcc -type CIII with a di-heme cytochrome *c* domain fused to CIII (9, 10). Variations are also observed within CIII and CIV. Alternative Complex IIIs (ACIIIs) which are structurally unrelated to bc_1 -type CIII mediate quinol oxidation (11). Quinol oxidases couple quinol oxidation to oxygen reduction without the need for cytochrome *c* (12). Alternative oxidases (Aox) catalyze quinol oxidation/oxygen reduction without proton pumping (13).

Despite variation with ETCs, homologs or analogs of CI through to CIV are the most common components of respiratory chains in aerobic organisms. Structures of both prokaryotic and eukaryotic CI (14–16), CII (17, 18), CIII (19–21) and CIV (22–24) have been determined, elucidating the flow of electrons within these individual complexes. Structural information for the mitochondrial respirasome $\text{CI}_1\text{CIII}_2\text{CIV}_1$ and preliminary electron microscopic study of a $\text{CIII}_2\text{CIV}_2$ supercomplex from yeast have been reported (25, 26). However, cytochrome *c* is not visible in any of these structures. Thus, a complete pathway for electron flow is yet to be fully elucidated. Understanding the precise details of the structural assembly for a CIII–CIV supercomplex will greatly assist in this endeavor (27, 28). It has been reported recently that respiratory supercomplexes in situ have a conserved core of CI and a dimer of CIII but otherwise their stoichiometry and structure vary (29). Up to two copies of monomeric CIV were found associated with the CI_1CIII_2 assembly in bovine heart and the yeast *Yarrowia lipolytica*, but their positions varied (30). The conserved features of supercomplex assemblies such as CI_1CIII_2 and $\text{CIII}_2\text{CIV}_2$ suggest that these types of associations have important roles in respiratory electron transfer.

The *bcc*-type CIII from *Actinobacteria* has a di-heme *c* subunit (7). It has been suggested that one cytochrome *c* domain is the donor for the *aa*₃-type CIV and the other is the acceptor for the CIII Rieske Fe-S protein (31). In support of this concept of intra-supercomplex electron transfer, CIII–CIV supercomplexes have been isolated from *M. smegmatis*, *C. glutamicum*, and *M. tuberculosis* and shown to couple quinol oxidation to oxygen reduction without an external electron shuttle (9, 10, 32). Therefore, structural data for the *bcc-aa*₃ type CIII–CIV supercomplex (SC III–IV) can provide answers as to how CIII and CIV are coupled and how electrons are transferred from CIII to CIV.

Purification and characterization of SC III–IV

To isolate the SC III–IV in a functional form, we engineered the genome of *M. smegmatis* to incorporate a 10× His tag at the C terminus of the QcrB subunit of CIII, extracted and purified the CIII–CIV complex by Ni-NTA affinity chromatography and gel filtration. Gel filtration and Blue-Native PAGE showed a single peak and a single band, suggesting a highly-ordered supramolecular assembly (fig. S1, A and B). SDS-PAGE and mass spectrometry confirmed the presence of all the known components of CIII and CIV as well as several previously unknown components (fig. S1D and table S1). Native Orbitrap mass spectrometry gave a molecular weight of $873.4 \text{ kDa} \pm 10.4 \text{ Da}$ for the complex (fig. S1E). Both the electronic absorption spectrum and the electron paramagnetic resonance (EPR) spectrum showed peaks expected from the various hemes, copper centers and $[2\text{Fe-2S}]$ prosthetic groups

in CIII and CIV (fig. S1K). Since the *bcc-aa*₃ preparations from *M. smegmatis* are active with the more soluble menadiol (2-methyl-1,4-naphthoquinol) as substrate (33), the quinol:oxygen oxidoreductase activity of SC III–IV was assayed by measuring the rate of O_2 consumption in the presence of menadiol. SC III–IV oxidized menadiol and reduced O_2 with an apparent k_{cat} of $2.80 \pm 0.05 \text{ s}^{-1}$ for O_2 consumption or $11.20 \pm 0.20 \text{ e}^- \text{ s}^{-1}$ and a K_m of $120.70 \pm 3.72 \mu\text{M}$ for menadiol (fig. S2, A, B, and C). The k_{cat} value is comparable to the $65 \text{ e}^- \text{ s}^{-1}$ reported for this complex with 2,3-dimethyl-1,4-naphthoquinol (DMNQH_2) as the electron donor (9); the difference is likely due to DMNQH_2 being more reducing and a closer structural analog of the natural substrate menaquinol than menadiol. The data confirm that the purified sample is a functioning supercomplex containing CIII and CIV and capable of directly coupling quinol oxidation to oxygen reduction.

Overall architecture of SC III–IV

The structure of SC III–IV was determined by cryo-EM to an overall resolution of $3.5 \text{ \AA}$ (Fig. 1B; table S2; fig. S3, A to G; and movie S1). The dimensions of the supercomplex are $200 \times 70 \times 120 \text{ \AA}$, with a linear dimeric $\text{CIV}_1\text{-CIII}_2\text{-CIV}_1$ arrangement where individual CIVs flank the central CIII dimer on either side (Fig. 1, B and C, and Movie 1). This C_2 symmetrized linear architecture is completely different from those previously reported for respiratory supercomplexes (fig. S4). CIII is comprised of canonical three subunits as a homodimer (Fig. 2A and fig. S5A). In addition to the four known subunits of *M. smegmatis* CIV, two subunits were observed that match two of the newly identified proteins, CtaI and CtaJ (Fig. 2B and fig. S3H), showing a similar topology and binding schema to subunit Va and IV in mitochondrial CIV (fig. S5J).

There is extra density within the interface between CIII and CIV as well as at the top of the CIII dimer (Fig. 1B). The density within the interface could be modeled by two proteins, LpqE and PRSAF1 (prokaryotic respiratory supercomplex association factor 1) (Fig. 1C and Movie 1). LpqE was found to be a N-terminal triacylated lipoprotein (Fig. 4F), with a N-acylated-S-diacylated modification of Cys24 within the lipobox ($-^{21}\text{Lxx}^{24}\text{C}-$) sequence (34). On the periplasmic side the density on top of the CIII dimer consisted of a peptide fragment linked with a region of bulk density which could be visualized in a low-pass filtered map. The peptide fragment was modeled with residues Cys21–Pro45 of the N-terminal sequence of superoxide dismutase SodC of *M. smegmatis*. The EM map indicated side chain modifications, including triacylation at Cys21 that was part of a lipobox ($-^{18}\text{Lxx}^{21}\text{C}-$) sequence (Fig. 4E) and possible glycosylation sites (fig. S3H). Native mass spectrometry further identified that SodC is a component of SC III–IV (fig. S1F). The stoichiometry of SodC was confirmed as a dimer through the collisional

dissociation of the SodC complex. Extensive glycosylation and copper-binding of SodC was observed in the mass spectrum (fig. S1F). The bulk density was therefore fitted with a dimer of SodC (Fig. 1, B and C, and fig. S3F). SC III–IV possesses superoxide dismutase activity, with a specific activity of 132.56 ± 12.57 IU/mg-SOD, assuming 100% occupancy (fig. S2E). SodC association with SC III–IV was also confirmed by isolation of the supercomplex by Ni-affinity chromatography when a His-tag was introduced only to the C terminus of SodC and not any of the CIII and CIV subunits. The SodC-tagged form of the complex showed a higher specific activity of 957.36 ± 23.34 IU/mg-SOD but still lower than the 1000–6000 IU/mg-SOD typically observed with soluble SOD enzymes. It also indicates an ~14% (SodC)₂ occupancy in the purified QcrB-tagged SC III–IV particles for cryo-EM study. Thus, it appears that there might be some dissociation of SodC from SC III–IV during detergent solubilization. It is also possible that the SOD occupancy is growth regulated since an up-regulation of *sodC* (the gene encoding the SOD here) in response to phagocytosis by human macrophages has been reported (35). Further work on the role of SOD in association with SC III–IV is in progress.

All the prosthetic groups predicted from the canonical CIII and CIV subunits were clearly resolved and found to be coordinated with conserved canonical residues (figs. S3J and S8). The menaquinone (MK) molecules were observed at the quinone binding sites in CIII (fig. S7, A and B). The calculated molar ratio between iron atoms and copper atoms in the final model is 1.6, in excellent agreement with the value of 1.5 determined by atomic absorption spectroscopy (fig. S1C). SodC from *M. tb* does not contain zinc (36), and atomic absorption spectroscopy analysis showed that zinc was absent from SC III–IV (fig. S1C). We were in total able to build 34 phospholipids (fig. S3I) and ten MK molecules (fig. S7) in SC III–IV. The total molecular mass of the model, including the new identified subunits, is ~760 kDa which is lower than the $873.4 \text{ kDa} \pm 10.4 \text{ Da}$ determined by native Orbitrap mass spectrometry (fig. S1E). This difference may be accountable on the basis of contributions from the detergents/lipids and the possibility of the presence of additional unidentified subunits.

Structure of CIII and CIV in the supercomplex

The cryo-EM map clearly shows QcrA, QcrB and QcrC of CIII in a dimeric form with all their prosthetic groups visualized (Fig. 2A; fig. S3, H and J; and Movie 2). QcrA has a “U” shaped structure within its N-terminal domain while the equivalent subunit in *bc*₁-type CIII has only one TMH (equivalent to QcrA-TMH3) (Fig. 2A and fig. S5A). The region linking the two arms is located near the cytoplasmic side. The C-terminal domain of QcrA-TMH3 is on the periplasmic side and holds the [2Fe-2S] cluster. Importantly, QcrA here also has a

roof-like structure on the periplasmic side which is involved in the dimerization of CIII (Fig. 2A) while the *bc*₁-type CIIIs do not have this feature. The heme *b*_H and heme *b*_L cofactors are bound within four TMHs in QcrB (Fig. 2A). The N-terminal periplasmic portion of QcrC can be divided into two heme-containing cytochrome *c* domains D1 and D2 (equivalent to the *c*₁ domain in *bc*₁ complex) (Fig. 2A and fig. S5N). These two domains are in close contact with and face each other in an anti-parallel orientation. The D2 domain interacts extensively with QcrA and QcrB, while the additional D1 domain protrudes out of the core of CIII and is involved in direct intra-supercomplex electron transfer (see below). Overall, although the *bcc*-type CIII in the supercomplex shares a similar dimeric association as the bacterial *bc*₁-type CIII and mitochondrial CIII, the structural details are markedly different (fig. S5B).

CIV in SC III–IV belongs to the type A heme-copper oxidase (HCO) family (Fig. 2B and Movie 3) (37). The central cavity of the barrel-like arrangement of the 12 TMHs in the CtaD subunit holds heme *a*, heme *a*₃ and Cu_B. The C-terminal hydrophilic β barrel domain of CtaC holds the two Cu_A ions. The four protons required for oxygen reduction by heme *a*₃:Cu_B in CIVs are transferred to the catalytic center through two pathways denoted D and K (38). Due to the limited resolution, water molecules are not observed in our model. However, structure comparison revealed that the D and K pathways are conserved in SC III–IV (fig. S5, K and L).

Interaction between CIII and CIV and the contribution of association subunits and lipids

The linear form of SC III–IV arises from the dimerization of CIII which is mediated by contacts between subunits QcrA and QcrB (Fig. 2A and fig. S6, A to D). There are extensive contacts between CIII and CIV on both the cytoplasmic and periplasmic sides of the membrane (Fig. 3). Of the three association subunits LpqE, PRSAF1 and SOD in SC III–IV, both LpqE and PRSAF1 form numerous contacts with CIII and CIV, suggesting an important role for both in supercomplex stability (Fig. 3B and fig. S6, E to G). SOD utilizes its lipid-modified N-terminal fragment to associate with CIII (Fig. 4E) and forms a dimer on the periplasmic side of the CIII dimer. There are no direct interactions between the main body of the SOD dimer and CIII or CIV. This might allow a flexible orientation for this subunit which could be important for efficient clearance of ROS generated by side reactions when electrons are transferred from CIII to CIV (see below).

Phospholipids, especially cardiolipin (CL), are known to contribute to both the assembly and stability of respiratory complexes and supercomplexes (39). In the structure of SC III–IV, phospholipids are identified in the transmembrane space of CIII and CIV and the interface between CIII and CIV (Fig. 4). Of particular note are the four CL molecules in the

large groove between CIII and CIV (Fig. 4D); the sixteen fatty acid chains fill most of the space in the groove and may play an important role in stabilizing the supercomplex. The N-terminal modified lipid tails of the lipoproteins SOD and LpqE mediate inter-subunit hydrophobic interactions and contribute to the stability of the supercomplex (Fig. 4, E and F). These lipid modifications described here are similar to those observed in a recently described structure of alternative complex III (17).

Quinone and quinone binding pockets

Quinone binding sites of respiratory complexes are of great interest because they are part of the Q-cycle hypothesis. They have varied sequences and specificities between species, and are often the sites for inhibitor binding and thus are important for drug discovery. We have identified the two quinone binding sites (Q_P and Q_N) in SC III–IV (Fig. 5). The quinol oxidation site (Q_P site) responsible for menaquinol (MKH₂) oxidation is near heme b_L while the quinone reduction site (Q_N site) responsible for menaquinone (MK) reduction is close to heme b_H .

The Q_P site near heme b_L is at the center of an inverted triangle structure and surrounded by helices (Fig. 5A). Residues at this site are not conserved compared to the bc_1 complex (Fig. 5C). The typical “PEWY” motif in the bc_1 complex is replaced by “PDFY” (Fig. 5C). One MK molecule is identified at this site with its naphthoquinone ring surrounded mainly by hydrophobic residues. The edge-to-edge distance from MK to heme b_L is 16 Å. Thus, we speculate that the endogenous electron donor MKH₂ would bind closer to heme b_L to facilitate electron transfer and what we observe here might be a representation of the oxidized product as it leaves the Q_P site. Furthermore, there are no observed hydrogen bonds to the carbonyl groups of MK (Fig. 5A). It is known that proton abstraction from MKH₂ is coupled to electron donation to CIII. Thus, hydrogen bonds are needed between the binding residues and the hydroxyl group of MKH₂ to help deprotonate the substrate. Hence MKH₂ should bind deeper inside the pocket, close to polar residues such as Q_{crB} Tyr159, Q_{crB} Thr308, Q_{crB} Asp309 (Fig. 5A). Structural superposition with the inhibitor bound bc_1 complex (40) shows that the MK at the Q_P site binds deeper into the pocket in the bc_1 complex than in this SC III–IV complex.

By contrast, the head group of MK at the Q_N site is bound in a similar fashion to ubiquinone in the structures of bc_1 complexes (Fig. 5B). As found for the Q_P site, these residues are not conserved compared to the bc_1 complexes from bacteria to eukaryotes (Fig. 5C). In particular, the carbonyl groups of ubiquinone are coordinated by the conserved His and Asp amongst bc_1 complexes; these residues are substituted by Q_{crB} Trp231 and Q_{crB} Ser261 in the *bcc*-type CIII of *M. smegmatis*. The carbonyl groups of MK in SC III–IV interact with the side

chains of Q_{crB} Tyr48 and Q_{crB} Ser261 that may supply protons for MK reduction. The MK head group is ideally placed for electron transfer, being within 5 Å of the A-edge of heme b_H .

Besides finding quinone molecules at the Q sites, we also observed map signals for another three possible MK/MKH₂ molecules in the supercomplex (fig. S7, C to E). However, further experiments are needed to clarify the identity and function of these molecules.

The new family of candidate anti-mycobacterials, the imidazo[1,2-a]pyridines (IP) represented by Q203 operate by competing with menaquinone for binding at the Q_P site of CIII of *M. tuberculosis* (41). Sequence alignments indicate a high similarity between the Q_P sites of CIIIs from *M. tuberculosis* and *M. smegmatis* (Fig. 5C), thus suggesting Q203 would also have a similar binding mechanism and a similar effect on the activity of *M. smegmatis* CIII. Indeed, recent studies of the anti-mycobacterial activity of Q203 on *M. tuberculosis* and *M. smegmatis* demonstrated that Q203 targets the *bcc* complex in both with similar affinity (42). We investigated the in vitro inhibition of *M. smegmatis* SC III–IV by Q203 by the menadiol/oxygen oxidoreductase activity assay and compared the effect with a hybrid supercomplex of *M. tuberculosis* *bcc*-CIII and *M. smegmatis* *aa*₃-CIV. Q203 showed inhibition of menadiol-induced oxygen consumption with IC₅₀ values of 0.84 ± 0.22 μM and 0.61 ± 0.16 μM for SC III–IV and the hybrid supercomplex, respectively (fig. S2D).

Prosthetic groups and implication for direct electron transfer

The prosthetic groups of CIII (heme b_H/b_L , [2Fe-2S] clusters and heme c_{D1}/c_{D2}) and CIV (Cu_A, Cu_B and heme a/a_3) are clearly identified from the cryo-EM map (Fig. 2, A and B, and fig. S3J). The redox centers in CIII and CIV are within distances that allow long-range electron transfer (Fig. 6A). Both heme b_L and heme b_H are found in Q_{crB} CIII (figs. S3J and S8A). The edge-to-edge distance between the two heme groups is 12 Å, allowing rapid inter-heme electron transfer. The shortest distance from b_L to MK at the Q_P site and from b_H to MK at the Q_N site are 16 Å and 5 Å, respectively (Fig. 6A). Previous studies have proposed that CIIIs from different species adopt a dimeric architecture and form a H-shaped electron transfer system that distributes electrons between four quinone oxidation-reduction sites within the CIII dimer (43). Consistent with this hypothesis, upon dimerization of *M. smegmatis* CIII, the two b_L heme groups from the CIII monomers are 14 Å apart (Fig. 6A), allowing electron tunneling between the two hemes.

The heme c_{D1} -containing D1 domain of Q_{crC} CIII protrudes into the periplasm and interacts with the Cu_A-containing periplasmic domain of CtaCIV (Fig. 6A). The edge-to-edge distance from heme c_{D1} to the Cu_A center is 12 Å. At the interface between these two domains there is a gating residue

CtaC Trp138 on the Cu_A binding loop. The distance between CtaC Trp138 and the Cu_A center and that between CtaC Trp138 and heme c_{D1} are approximately equal. It has been proposed, based on mutagenesis studies, that the corresponding Trp121 (44) of *P. denitrificans* CIV is the electron entry site from cytochrome *c*. CIVs from *B. taurus* also possesses a tryptophan residue at the equivalent location (45). Thus, the heme c_{D1}-containing D1 domain of QcrC_{CIII} here interacts with CIV on a similar electron entry site as in other respiratory complexes.

Within CIV, the heme *a* group is just below the Cu_A center and the heme *a*₃ group is beside heme *a* (Fig. 6A). Cu_B is coordinated to three conserved His residues. The space between the iron of heme *a*₃ and Cu_B is the catalytic center for reduction of oxygen. In proximity to propionate groups of heme *a*₃, a Cu_C ion was modeled according to the cryo-EM map (fig. S3J). In other reported CIV structures the equivalent density at this site is occupied by a water molecule (46) or a Mg²⁺ ion (47). However, we did not detect magnesium in our AAS analysis (fig. S1C). The edge-to-edge distance between Cu_C and the Cu_A center and that between Cu_C and heme *a*₃ are 12 Å and 9 Å, respectively.

From the positions of the prosthetic groups in *M. smegmatis* SC CIII₂CIV₂SOD₂ and the redox center separations, it is possible to trace an uninterrupted pathway for the flow of electrons within the supercomplex starting from the electron donor at the Q_P site in CIII to the final site of oxygen reduction in CIV (Fig. 6B). Menaquinol (MKH₂) from the Q-pool binds at the Q_P site near heme b_L and transfers one electron to the [2Fe-2S] cluster and the other to heme b_L which passes the electron via heme b_H to a menaquinone (MK) bound at the Q_N site, generating the highly reactive intermediate menaquinone semiquinone (MK•). The reduced [2Fe-2S] cluster is within electron tunneling distance to transfer an electron to heme c_{D2} which rapidly passes the electron to heme c_{D1}. A second MKH₂ then binds at the Q_P site and repeats the process. The MK• at the Q_N site is fully reduced to MKH₂ and released to the Q-pool. This completes the Q cycle. An electron path between CIIIs in the dimer is also possible through tunneling between the adjacent b_L heme groups, albeit with low efficiency (43). The reduced [2Fe-2S] cluster again transfers the other electron to heme c_{D2}/c_{D1}. Once heme c_{D1} is reduced, two Cu_A ions of CtaC_{CIV} accept the electron through contacts forged with the D1 domain of QcrC_{CIII}. At this point, an electron is transferred from CIII to CIV. Within CIV, the electron is transferred through heme *a* (or possibly the Cu_C center) and finally reaches the terminal heme *a*₃:Cu_B reaction center for oxygen reduction. As a consequence of the electron transfer, protons are translocated to the periplasm, forming a transmembrane PMF. Throughout the entire pathway from the Q_P site to the terminal oxygen reduction center, electrons tunnel between prosthetic groups that are all buried inside this integral complex.

Quinone reduction at the Q_N site to complete the Q cycle can be bypassed/short-circuited if both electrons from MKH₂ oxidation at the Q_P site are transferred to the [2Fe-2S] cluster and then to CIV for oxygen reduction. Hence, competent energy transduction requires that electron transfer from the Q_P site has to be bifurcated between reduction of the [2Fe-2S] cluster and heme b_L. In essence, the pathway for transferring electrons from the [2Fe-2S] cluster eventually to CIV has to be sufficiently slow for electron transfer to the Q_N site for quinone reduction to occur. In the bc₁ complex the [2Fe-2S] cluster domain cycles between “*b*” and “*c*₁” states. In the *b*-state the [2Fe-2S] cluster is close to the Q_P site to accept an electron from quinol oxidation but too far away (26 Å) (fig. S5E) to transfer an electron to the c₁ heme at an appreciable rate. The cluster domain undergoes a “head displacement” conformation change to the c₁-state where the [2Fe-2S] cluster moves to within 11 Å of the c₁ heme to facilitate electron transfer. This head displacement or “gating” step occurs with a rate constant of 6 × 10⁴ s⁻¹ (48).

We cannot rule out the possibility that SC III-IV could adopt a different conformation and cycle between states similar to the *b* and c₁ states of bc₁ complexes. However, the dimer of QcrA is held firmly in place by the periplasmic roof-like structure and further surrounded by QcrB and QcrC, which might limit the space for the potential conformational change (fig. S5D). Structural superposition shows that the position of the [2Fe-2S] in QcrA is similar to that of bc₁-type CIII in the *b* state, i.e., rapid cluster reduction by MKH₂ can occur. The [2Fe-2S] cluster is at a distance of 16 Å from heme c_{D2}. We used this distance, the reported midpoint potentials of the prosthetic groups (10), and a reorganization energy (λ) of 0.7 eV to calculate a rate constant of electron transfer of 5.6 × 10² s⁻¹ from [2Fe-2S] to heme c_{D2} (49). This is much slower than the head displacement gating step in bc₁ complexes (6 × 10⁴ s⁻¹). We conclude that short-circuiting in SC III-IV is likely to be rate-limited by slow electron transfer between the [2Fe-2S] cluster and heme *c* made possible by positioning of the chain of redox centers, rather than by conformational changes as found in bc₁ complexes.

Role of SOD association

In aerobic organisms, the respiratory ETC not only generates the energy needed to fuel biological functions but is also a major source of intracellular ROS that can cause damage to cellular structures and components (50). Complex III is one of the major sites of ROS production (51). While ROS are emerging as important elements in the bacterial response to lethal stress (52), it is well documented that they can disturb respiratory activity via oxidative damage of ETC complexes which are in turn protected by the ROS scavengers, SOD and catalase (53–55). Up-regulation of *M. tuberculosis* *sodC* (the gene encoding the SOD here) in response to phagocytosis by

human macrophages has been observed (35). This Mycobacterial Cu, Zn superoxide dismutase SodC was identified as a membrane-bound enzyme and proposed to protect specific membrane-associated targets from oxy-radical damage, thus facilitating mycobacterial intracellular growth (35). A null *sodC* mutant of *M. tuberculosis* was shown to be readily killed by externally generated superoxide and by activated macrophages producing oxidative bursts (56). In this work, we found that catalytically active SodC is an integral part of a respiratory supercomplex $\text{CIII}_2\text{CIV}_2\text{SOD}_2$. SodC could serve to scavenge ROS generated locally or by other ETC complexes, as well as ROS released by the immune response of the host. Its recruitment has the potential to make this critical respiratory machinery a robust system even under the high oxidative stress inside macrophages. Immunoblotting of *Caenorhabditis elegans* respiratory supercomplexes separated by BN-PAGE showed that mitochondrial superoxide dismutase-2 (mtSOD-2) is associated with the respirasome $\text{CI-CIII}_2\text{-CIV}$, suggesting that the mtSOD might also provide similar local protection against ROS damage (57).

Conclusions

The cryo-EM structure of a CIII-CIV respiratory supercomplex from *M. smegmatis* has revealed a complete intracomplex electron transfer pathway from quinol oxidation in CIII to oxygen reduction in CIV, a new mechanism for bifurcating electron transfer to ensure completion of the Q cycle for energy transduction, and the association of a superoxide dismutase that can provide protection against oxidative damage by reactive oxygen species. The structure of the quinone binding sites also provides a framework for structure-based anti-mycobacterial drug discovery.

Materials and Methods

Bacterial Strain

A *M. tuberculosis*-like highly hydrogen peroxide-resistant *M. smegmatis* mutant strain, mc² 51 (58), was used in this study. The draft genome sequence data can be obtained through the GenBank accession no. JAJD000000000.1. The code for the 10× His tag was introduced at the C terminus of the QcrB or SodC genome loci through homologous recombination. This modification allowed the metal affinity purification step. The presence of the histidine tag was confirmed by PCR and Western blot.

M. smegmatis culture and membrane isolation

Culturing of the cells and membrane isolation were performed as described in previously published protocols, with some modifications (9, 32). The cells were grown in LB media supplemented with hygromycin (50 µg/mL), carbenicillin (20 µg/mL) and Tween 80 (1 mL/L), at 37°C with shaking to maintain oxygenation. A total of 10× 1 L bacteria solutions

were cultured until the OD₆₀₀ reached ~1.5 and then harvested by centrifugation for 30 min at 4,000 rpm. The obtained bacteria were resuspended in buffer A (20 mM MOPS, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM PMSF). Cell lysis was achieved by three passes through a high pressure cell disrupter at 4°C and 1,200 bar. The lysate was centrifuged at 14,000 rpm for 10 min to remove cell debris and non-lysed cells. The resulting supernatant was centrifuged at 36,900 rpm for 1 hour in a Ti45 rotor (Beckman). The membrane pellets were harvested and stored at -80°C.

Supercomplex purification

Membranes were thawed and homogenized in buffer A. Respiratory supercomplexes were extracted from the membrane by 1% (w/v) digitonin for three hours with slow stirring at 4°C. Insoluble materials were removed by centrifugation at 18,000 rpm for 30 min at 4°C. The supernatant was loaded onto a Ni-NTA column followed by gravity feeding three column volumes of buffer A through the resin. The resin was further washed by buffer B (20 mM MOPS, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.1% (w/v) digitonin, 50 mM imidazole). The protein was eluted with buffer C (20 mM MOPS, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.1% (v/w) digitonin, 500 mM imidazole). Eluted protein was concentrated using a 100-kDa cut-off centrifugal concentrator (Millipore) and this sample was then loaded onto a Superose 6 (10/300 GL, GE Healthcare) column equilibrated in a buffer containing 20 mM MOPS, pH 7.4, 100 mM NaCl, 1 mM EDTA and 0.1% (v/w) digitonin. The peak fractions (elution volume between 12.25 mL and 13 mL) were pooled and concentrated to 5.5 mg mL⁻¹ by using a 100-kDa cutoff centrifugal concentrator.

Characterization of the respiratory supercomplex $\text{CIII}_2\text{CIV}_2\text{SOD}_2$

The supercomplex was characterized by optical spectroscopy, mass spectrometry (MS) and 3,3'-diaminobenzidine (DAB) staining. To identify the heme groups, selected fractions were analyzed by recording spectra from 250 to 700 nm before and after reduction with dithionite according to previously described methods (9, 32). To detect the protein components, the concentrated supercomplex sample was subjected to MS analysis at the National Center for Protein Science (Shanghai, China). The protein sample was analyzed by Blue Native PAGE (BN-PAGE) and then a separate strip of the gel was stained with DAB (59-61).

Pyridine haemochrome assay

For denaturing redox difference spectra samples were taken up in 20% (v/v) pyridine, 0.1 M NaOH. Redox difference spectra were recorded in the range 500-620 nm using potassium ferricyanide for oxidation and sodium dithionite for reduction. Potassium ferricyanide (250 µM) was added to the

sample and the spectrum of this oxidized form was recorded. Sodium dithionite (5 mM) was then added to this sample, the solution was mixed well and the spectrum was scanned repeatedly until it remained unchanged, giving the spectrum of the reduced form. The heme *a*, *b* and *c* contents were determined from the reduced-minus-oxidized difference spectrum using the following extinction coefficients $\Delta \epsilon_{587-620} = 21.7 \text{ mM}^{-1} \cdot \text{cm}^{-1}$, $\Delta \epsilon_{557-540} = 23.98 \text{ mM}^{-1} \cdot \text{cm}^{-1}$, $\Delta \epsilon_{550-535} = 23.97 \text{ mM}^{-1} \cdot \text{cm}^{-1}$.

Native mass spectrometry of supercomplex

Purified supercomplex was buffer exchanged into 200 mM ammonium acetate buffer pH 7.5 containing DDM at 2 times critical micelle concentration. The sample was immediately introduced into a modified Q-Exactive mass spectrometer (Thermo) and ions were transferred into the Higher-energy collisional dissociation (HCD) cell following a gentle voltage gradient (injection flatapole, inter-flatapole lens, bent flatapole, transfer multipole: 7.9, 6.94, 5.9, 4 V respectively). The capillary voltage was maintained at 1.2-1.4 eV at temperature of 200°C. The optimized acceleration voltage for intact supercomplex is 150 V and 100-120 V in the source and HCD cell, respectively. SodC subunits were dissociated from the complex with high acceleration voltage (250-300 V) in the source and the collisional dissociation of dimeric SodC was performed with the voltage ramp from 0-150 V in HCD cells. Backing pressure was maintained at $\sim 1.20 \times 10^{-9}$ mbar and data was analyzed using Xcalibur 2.2 SP1.48.

Independent lipidomics analysis 1

Co-purified lipids from supercomplex were extracted by chloroform/methanol (2:1, v/v) and lyophilized and re-dissolved in 60% acetonitrile (ACN). For LC-MS/MS analysis, the extracted lipids were loaded into a 5- μL sample loop using an autosampler by a full-loop method with an overfill factor of 1.4, and then transferred to a 1- μL injection loop using a loading pump at a flow rate of 5 $\mu\text{L}/\text{min}$ with 70% solution A and 30% solution B. The lipids in the injection loop were injected onto a C18 column (Acclaim PepMap 100, C18, 75 $\mu\text{m} \times 15 \text{ cm}$, Thermo Fisher Scientific) using a nanopump at a flow rate of 300 nL/min, 30% solution B. The lipids were separated on the C18 column at 40°C by a gradient starting from 30% solution B. After 10 min, solution B was ramped to 65% over 1 min, then 80% over 6 min, before being held at 80% for 10 min, then ramped to 99% over 6 min and held for 7 min. (Solution A: (ACN: H₂O (60:40), 10 mM ammonium formate, 0.1% formic acid) and solution B (IPA: ACN (90:10), 10 mM ammonium formate, 0.1% formic acid)). The column eluent was delivered via a dynamic nanospray source to a hybrid LTQ Orbitrap mass spectrometer (Thermo Scientific). Typical MS conditions were: spray voltage (1.8 kV) and capillary temperature (175°C). The LTQ-Orbitrap XL was operated in negative ion mode and in data-dependent acquisition with one MS

scan followed by five MS/MS scans. Survey full-scan MS spectra were acquired in the orbitrap (*m/z* 350 – 2,000) with a resolution of 60,000. Collision-induced dissociation (CID) fragmentation in the linear ion trap was performed for the five most intense ions at an automatic gain control target of 30,000 and a normalized collision energy of 38% at an activation of *q* = 0.25 and an activation time of 30 ms.

Independent lipidomics analysis 2

Homogenized membranes and protein crystals were extracted with 800 μL of chloroform:methanol:water (1:1:0.1) in glass vials. After shaking the samples vigorously at 1500 rpm for 30 min at 4°C, 350 μL of water was added to break the phases. Samples were centrifuged and the lower organic phase was transferred to new glass vials. The remaining membrane and/or protein pellets were re-extracted with 400 μL of chloroform. The combined extracts were dried using a SpeedVac (Genevac, UK). Samples were stored at –80°C until mass spectrometric analysis.

Liquid chromatography-mass spectrometric (LCMS) analyses were carried out on an Exion UPLC coupled with a SciexQTRAP 6500 Plus mass spectrometer. Analysis of phospholipids and free mycolic acids were carried out using normal-phase LCMS as described previously (62, 63). Levels of menaquinone species were quantified using a reverse-phase LCMS method (64).

Quinone reduction

Menadiol was prepared as previously described, with minor modifications (65). To prepare reduced menadiol, 3.4 mg 2-methyl-1,4-naphthoquinone (Sigma M5625) was dissolved in 1 mL N₂-saturated anhydrous cyclohexane to yield a 20 mM solution. The solution was mixed with 5 mL N₂-saturated 1 M sodium dithionite solution (in H₂O) and shaken vigorously. After phase separation, the organic phase containing the reduced menadiol was removed and transferred to a 15 mL centrifuge tube (all steps performed under a stream of N₂). The cyclohexane was evaporated under an N₂ stream, while the sample was kept at 40°C in a water bath. Subsequently, the reduced quinol was dissolved in N₂-saturated, acidified ethanol (ethanol with 10 mM HCl), aliquoted, flash frozen in liquid nitrogen, and stored at –20°C.

Oxygen consumption assay

Since the *bcc:aa₃* preparations from *M. smegmatis* are active with menadiol (2-methyl-1,4-naphthoquinol) as substrate and electron donor, which has better solubility in water than menaquinol (33), the supercomplex was routinely characterized by its menadiol:O₂ oxidoreductase activity. Rates of oxygen consumption were determined using a Clark-type oxygen electrode (Hansatech) in a magnetically stirred chamber at 25°C in 20 mM MOPS pH 7.4, 100 mM NaCl, 0.005% LMNG,

5 mM DTT, following a method previously described, with minor modifications (33). The rate of menadiol autoxidation was measured in parallel as a reference and subtracted from the supercomplex-dependent rate.

Enzyme kinetics of supercomplex CIII₂CIV₂SOD₂

The Michaelis-Menten curves were obtained by measuring the initial supercomplex-dependent oxygen consumption rates as a function of menadiol concentration. The initial rate data were fitted to the Michaelis-Menten equation using a non-linear fitting program (GraphPad Prism 6.0).

In vitro inhibition of the supercomplex activity by Q203

The supercomplexes were pre-incubated for 10 min with inhibitors, menadiol was added as electron donor to a final concentration of 200 μ M and the oxygen respiration was measured for 3 min. Data were normalized relative to solvent (Ethanol) control for full activity and to a sample with 160 μ M Antimycin A for complete inhibition. The supercomplex-dependent initial rates were used to generate an inhibition curve and determine the IC₅₀ value.

Determination of SOD Activity in the supercomplex

The superoxide dismutase (SOD) activity was measured using a SOD Assay Kit-WST (19160; Sigma-Aldrich) following the kit instruction. Since the absorption maximum of WST-1 formazan is 450 nm and it is proportional to the amount of superoxide anion, the SOD activity (inhibition rate %) as an inhibition activity can be quantified by measuring the decrease in the color development at 450 nm. The SOD activity of the complex purifications and a reference SOD sample with a known specific activity in IU/mg (international unit per milligram) from bovine erythrocytes (Catalog number S5395; Sigma) were both determined in this assay. Based on the linear relationship between 1/(inhibition rate) and 1/(enzyme activity), the IC₅₀ (50% inhibition activity) of the complex and bovine SOD can be determined. By comparing the IC₅₀ of each, the specific SOD activity of the complex purification was determined and normalized into international unit per milligram. To be noted, the IU here is refer to the MF unit (McCord-Fridovich unit) which is determined by measuring the reduction of cytochrome *c* by the xanthine oxidase/hypoxanthine system at 550 nm and is the international unit for SOD activity (EC 1.15.1.1) measurement (66). To avoid the potential interference from the cytochrome *c* domains of QcrC subunit, McCord-Fridovich test was not chosen here.

EPR spectroscopy

Low temperature EPR spectra were recorded on a Bruker X-band (9.4 GHz) EMX plus 10/12 spectrometer equipped

with an Oxford Instrument EPR 910 liquid Helium continuous-flow cryostat. A cylindrical resonator (ER4119hs TE011) was used for data collection. 100 μ L of supercomplex solution at 10 μ M was transferred to an EPR tube (Wilmad 707-SQ-250M Quartz EPR sample tube) and rapidly frozen in liquid nitrogen before inserting into the resonator. For the reduced sample, sodium dithionite (100mM) was added and mixed with the supercomplex to give them spectrum of the reduced form. Specific parameters for EPR experiments were summarized here as: temperature 10 K, microwave power 2.0 mW, microwave frequency 9.39 GHz, modulation amplitude 0.9 mT, modulation frequency 100 kHz, time constant 163.84 ms, and scan rate 3.125 mT/s. Multiple scans were accumulated to obtain a good S/N ratio.

Cryo-electron microscopy analysis

Uranyl acetate (1% w/v) was used for negative staining. 5 μ L of the supercomplex sample at concentration of 0.05 mg mL⁻¹ was applied to glow discharged copper grids supported by a thin layer of carbon film (Zhongjingkeyi Technology Co. Ltd) for one minute and stored at room temperature. Images were taken on an FEI Tecnai Spirit microscope operating at 120 kV. This data allowed initial model building.

Aliquots (4 μ L) of freshly purified supercomplex at a concentration of 0.5 mg mL⁻¹ were applied to glow-discharged holey carbon grids (Quantifoil Au R1.2/1.3). The glow discharge was followed the standard receipts of H₂ and O₂ mixture in Gatan Solarus 950 for 1 min. Grids were blotted for 2.5 s and flash-frozen in liquid ethane cooled by liquid nitrogen using an FEI Mark IV Vitrobot operated at 8°C and 100% humidity. Images were taken using an FEI Titan Krios electron microscope operating at 300 kV with a Gatan K2 Summit detector at a nominal magnification of 18,000 $\times$. Images were recorded in super-resolution mode and binned to a pixel size of 1.35 Å. Automated single-particle data acquisition was performed with SerialEM (67). Defocus values varied from 1.3 to 2.7 μ m. 32 frames per stack were collected with a total exposure time of 11.4 s. The dose rate was set to $\sim$ 8 e⁻/pixel/s and the total dose was $\sim$ 50 e⁻/Å².

Image processing

We first generated a low-resolution reconstruction of the supercomplex from 53 micrographs of the negative-stained sample. Particle picking was performed with the EMAN2 (68) subroutine e2boxer.py in an interactive boxing mode, yielding 25,287 particles. Reference-free classification was performed with e2refine2d.py, generating 36 good classes from a total of 80 classes. The reconstruction model was generated by the good classes using e2initialmodel.py, which served as an initial model for the subsequent cryo-EM image processing.

For Cryo-EM image processing, a total of 7,600 good micrographs were manually selected from 8,200 original micrographs. All processing steps were performed using Relion 1.4 or Relion 2.0 (69–72). A diagram of the procedures for data processing is presented in fig. S3C. At the whole-image level motion-corrected stacks were produced and binned 2 fold by MotionCorr (73) to produce motion-corrected stacks. Further motion correction and dose weighting was performed by MotionCorr2 (74) to average the output stacks. The contrast transfer function parameters of each image were estimated using Gctf (75). A total of ~1,294,000 particles were automatically picked using Gautomatch (<http://www.mrc-lmb.cam.ac.uk/kzhang/Gautomatch/>). The particles were extracted using a 336² pixel box and sorted by two rounds of reference-free 2D classification, resulting in ~844,000 particles selected from good 2D classes. These were subjected to 3D classification with the initial model that was low-pass filtered to 60 Å. The 3D classification was performed several times with different K values resulting in ~400,000 particles of good quality. The relative orientation of the QcrC subunit was variable, producing 3D classes with two different states. After 3D classification, the sets of particles within class-I and class-II were re-extracted and re-centered using a 292² pixel box and subjected to the final refinement, respectively. The resolution of class-I particles was then corrected using the “high-resolution noise substitution” method (76) and estimated as 3.9 Å according to the gold-standard FSC (Fourier shell correlation) 0.143 criterion. The pixel size was also calibrated to 1.30 Å/pixel provided by the Center for Biological Imaging (CBI), Institute of Biophysics, Chinese Academy of Science. Then the resolution of the class-II particles was estimated to be 4.9 Å. Another 3D classification with class-I particles was performed by applying C₂ symmetry, resulting in ~202,000 particles with good symmetry. The particles with C₂ symmetry were further refined to yield a final reconstruction with the resolution of 3.5 Å according to the gold-standard FSC 0.143 criterion. Prior to visualization, all density maps were corrected for the modulation transfer function (MTF) of the detector, and then sharpened by applying a negative B-factor (77) that was estimated using automated procedures. Local resolution variations were estimated using ResMap (78). The orientation distribution of the particles used in the final reconstruction was calculated using RELION 2.0.

Model building and refinement

All the subunits were initially built as poly-alanine chains, except for the residues that coordinate the prosthetic groups. Then residue assignment was performed, which was guided largely by secondary structure analysis carried out using PSIPRED (79) and Phyre2 (80).

It is noteworthy that the sequence of PRSAF1 in the model has an extra 16 amino acids before the N terminus of WP_003893930.1 but it is consistent with the revised complete ORF in the genome of *M. smegmatis* str. MC² 51 (genome locus tag AD56_RS0106885 added with the immediately preceding 64 bp). Model building was performed using Coot 0.8 (81) and further refined using the real space method in Phenix (82), except for the SOD catalytic domain (47Ala-236Gly) which was homology modeled (83) based on the structure of SodC from *M. tuberculosis* (PDB Code: 1PZS) (36) and fitted into the map with the aid of the “Fit in Map” feature of Chimera (84). The completeness of the final resulting model was summarized in table S3.

For the phospholipids, the results of three independent mass spectrometry analyses confirmed that the majority variants of lipids in the purified sample are PE, PI and CL (fig. S1, L and N). No PC or other branching phospholipids was detected. Since most of the phospholipids are localized through the neighboring peptides and are located in the transmembrane space which falls in the 3.2–3.5 Å high local resolution range, the CL molecules were readily identified based on the four alkyl groups and the PI and PE molecules were distinguishable through the significant difference in their “head” group size. A CL molecular was also distinguishable from two PI or PE molecules through the different distances between the two phosphatidic acid moieties. Finally, all the identified and assigned lipid backbones were modeled according to the EM potential map with necessary truncations and the results are summarized and illustrated against the context of the covering map in fig. S3I.

All the figures were created using PyMOL (85) or UCSF Chimera (84).

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SUPPLEMENTARY MATERIALS

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Fig. 1. Respiration in *Actinomycetes* and overall architecture of the Mycobacterial respiratory machine CIII₂CIV₂SOD₂. (A) The respiratory electron transfer chain in *Actinomycetes* (left) and the five major prokaryotic cytochrome c pathway variants with the organization schemes in representative organisms (right). The *M. smegmatis* cytochrome c pathway has its cytochrome c fused with complex III, forming a *bcc*-type complex III that interacts with the *aa*₃-type complex IV to assemble into a CIII–CIV supercomplex. MK=menaquinone/menaquinol. (B) Overall architecture of the *bcc*-*aa*₃ type respiratory CIII–CIV supercomplex from *M. smegmatis*. The cryo-EM map of the supercomplex shows a linear two-fold dimerized form of CIV₁–CIII₂–CIV₁ with dimensions 200 × 70 × 120 Å. CIII is colored in orange, CIV in magenta and the association factors, PRSAF1 in green, LpqE in blue and SOD in grey. (C) Cartoon representation of the side view of the supercomplex (top) and a cross sectional view (bottom). The menaquinone/menaquinol (MK) is presented as bright green colored solid spheres and the phospholipids as yellow sticks. In the cross-sectional view (bottom), the boundaries of CIII, CIV and the association factor PRSAF1 are depicted with dashed lines in color (orange for CIII, magenta for CIV and green for PRSAF1).

Fig. 2. Structure of CIII₂ and CIV from *M. smegmatis*. (A) Overall structure of the CIII dimer (left) and the spatial location (right) of prosthetic groups. QcrA, QcrB and QcrC are colored pink, blue and gold, respectively. The two-fold symmetry of the dimer is depicted by the black axis. The zoom-in view shows the heme c binding domains (D1 and D2) of QcrC. The heme groups (b_H , b_L , c_{D1} , and c_{D2}) and [2Fe-2S] clusters are shown as spheres and menaquinone/menaquinol (MK) are shown as sticks. The regions of ridge roof, ceiling junction, and base plate of CIII dimer are marked with dashed ellipses. (B) Overall structure of CIV (left) and the spatial location (right) of prosthetic groups. CtaC, CtaD, CtaE, CtaF, CtaI and CtaJ are colored in magenta, dark green, yellowish brown, cyan, brown and violet, respectively. Prosthetic groups are shown as spheres.

Fig. 3. Interaction between CIII and CIV and roles of association subunits. (A) A section profile of the interaction interface between CIII and CIV. The left/right images show the surface of CIII/CIV and the bound structural segments from CIV/CIII subunits and the association subunits, LpqE and PRSAF1. (B) Interactions between the association subunits (PRSAF1, LpqE and SOD) and the subunits of CIII and CIV. The corresponding subunits of CIII and CIV are shown in surface representation and colored as indicated. The association subunits are shown in ribbon and colored as indicated. The catalytic domain of SOD was fitted in map and shown as a dimer (SOD₂). The lipid modification of SOD is shown in yellow sticks (see Fig. 4E for detail).

Fig. 4. Important roles of phospholipids in the stability and assembly of the supercomplex. (A) The distribution of phospholipids in the membrane region (middle, view from periplasmic side) at the junction between the CIII monomers (left) and the interfaces between CIII and CIV (right). (B) A PE molecule mediates the interaction between PRSAF1 (TMH2) and CIV (CtaC and CtaD). (C) A PI molecule binds to the interface between $Q_{cr}C$ TMH1 and $C_{ta}F$ TMH4. (D) Four CL molecules are bound in the groove between the TM regions of CIII and CIV. (E) The N-terminal lipid modification of SOD at Cys21 binds to $Q_{cr}B$ via hydrophobic interactions. (F) The N-terminal lipid modification of LpqE at Cys24 mediates the interactions between CtaD and PRSAF1.

Fig. 5. Structures of the menaquinone (MK)/menaquinol (MKH₂) binding sites in *M. smegmatis* CIII. (A) The Q_P binding site. (B) The Q_N binding site. The residues potentially involved in the binding of MK/MKH₂ are shown with side chains in a stick representation. The MK molecules are colored in green. The heme and [2Fe-2S] groups are shown in spheres and labeled accordingly. (C) Sequence alignment for the Q_P site and Q_N site in QcrB with other *Actinobacteria* and *Homo sapiens* ETC systems. The red and green dots indicate whether *Homo sapiens* shares a common conservative site with *Actinobacteria* (red) or not (green).

Fig. 6. The complete electron transfer pathway in the *bcc-aa₃* type respiratory CIII/CIV supercomplex. (A) The prosthetic groups in the supercomplex are shown in sticks or spheres and labeled accordingly with the corresponding midpoint potentials shown in brackets. These values are based on the measurement of that of the *bcc-aa₃* type supercomplex from *C. glutamicum* (10). The edge-to-edge distances between adjacent prosthetic groups are shown in black dashed lines with the numbers in the brackets representing the center-to-center distances. (B) A schematic diagram showing the entire electron transfer pathway from CIII to CIV and the relevant proton translocations in CIII and CIV. The potential role of associated SOD for the clearance of ROS is also proposed.

Movie 1. The overall architecture of the supercomplex. Cartoon representation of the supercomplex. The menaquinone/menaquinol (MK) is presented as bright green colored solid spheres and the phospholipids as yellow sticks.

Movie 2. The composition and structure of CIII dimer. Cartoon representation of the complex III. The menaquinone/menaquinol (MK) is presented as bright green colored solid spheres and the phospholipids as yellow sticks.

Movie 3. The composition and structure of CIV. Cartoon representation of the complex IV. The phospholipids are shown as yellow sticks.