The cryo-EM structure of the bd oxidase from *M. tuberculosis* reveals a unique structural framework and enables rational drug design to combat TB

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New drugs are urgently needed to combat the global TB epidemic. Targeting simultaneously multiple respiratory enzyme complexes of *Mycobacterium tuberculosis* is regarded as one of the most effective treatment options to shorten drug administration regimes, and reduce the opportunity for the emergence of drug resistance. During infection and proliferation, the cytochrome bd oxidase plays a crucial role for mycobacterial pathophysiology by maintaining aerobic respiration at limited oxygen concentrations. Here, we present the cryo-EM structure of the cytochrome bd oxidase from *M. tuberculosis* at 2.5 Å. In conjunction with atomistic molecular dynamics (MD) simulation studies we discovered a previously unknown MK-9-binding site, as well as a unique disulfide bond within the Q-loop domain that defines an inactive conformation of the canonical quinol oxidation site in Actinobacteria. Our detailed insights into the long-sought atomic framework of the cytochrome bd oxidase from *M. tuberculosis* will form the basis for the design of highly specific drugs to act on this enzyme.
Tuberculosis (TB) constitutes a major public health problem and is a leading cause of global mortality from infectious diseases. Even in 2020 more people died from TB than from COVID-19. The emergence of extensively drug-resistant (XDR) and totally drug-resistant strains threatens to return us to the pre-antibiotic era for this disease. This fact combined with the very long time required to treat TB has created an urgent need to develop novel and fast-acting drugs that are not vulnerable to existing drug resistance mechanisms. The ATP synthase inhibitor, Bedaquiline (Sirturo), which was approved by the FDA in 2012, was the first drug in 40 years to attack TB by a novel mechanism of action. This marked the starting point for exploration of the mycobacterial respiratory chain as a target space for new anti-biostatic agents. Targeting simultaneously multiple respiratory enzyme complexes in Mycobacterium tuberculosis is regarded as one of the most effective options to treat TB, shorten drug administration regimes, and reduce the opportunity for the emergence of drug resistance.

In this light, new lines of evidence point to a crucial role of the cytochrome bd oxidase for mycobacterial pathophysiology. This quinol-oxidizing terminal oxygen reductase is encoded by the cydAB gene cluster and is a central component of the branched respiratory chain of M. tuberculosis. The clinical relevance of the cytochrome bd oxidase is highlighted by deletion mutants that show dramatically increased susceptibility to the bcl2l13 supercomplex inhibitor. This synergistic lethal interaction between the two respiratory branches of M. tuberculosis demonstrates that cytochrome bd oxidase is a major target for combating multi- and extensive drug-resistant (MDR/XDR) tuberculosis infections.

Previous structural insights into the cytochrome bd oxidase family have been limited to the first reported structure from Geobacillus thermodenitrificans (G. th) at 3.8 Å resolution (PDB: 5DOQ) and the recently published cryo-EM structures of the prototypic enzyme from Escherichia coli (E. coli) at 2.7–3.3 Å resolution (PDB: 6RKO, 6RX4). While the core architecture of these two known bd oxidase structures remains similar, structural details on the level of side chains, arrangement and composition of prosthetic groups, and the presence of accessory subunits differ substantially. Structural divergence and mechanistic diversity observed between different bd oxidase homologs thus emphasize the need for detailed knowledge of the atomic structure of the bd oxidase from Mycobacterium tuberculosis, in order to provide a solid framework for structure-guided drug design. Here we present the cryo-EM structure of the bd oxidase from M. tuberculosis at 2.5 Å resolution which reveals a previously unknown MK-9 binding site at heme b595.

Results

General architecture and subunit composition. The cytochrome bd oxidase from M. tuberculosis (cyt. bdMtb) was recombinantly produced in a Mycobacterium smegmatis mc2 155 AcydBAB strain harboring a markerless deletion of its chromosomal cytochrome bd oxidase (AcydBAB). Cofactor assembly and oxygen reductase activity were validated via UV–Vis spectroscopy and oxygen consumption measurements (Supplementary Fig. 1a, 1b, 1c, 1d).

The three-dimensional structure of POPC nanodisc reconstituted cyt. bdMtb in the as isolated mixed-valence state was determined by single-particle electron cryo-microscopy (cryo-EM) to a resolution of 2.5 Å (Supplementary Fig. 1b).

The cyt. bdMtb is structurally characterized by the presence of a pseudo-symmetrical heterodimer formed by CydA and CydB (Fig. 1). Each subunit is composed of nine transmembrane helices arranged in two four-helix bundles along with an additional peripheral helix (Fig. 1b).

CydA subunit represents the catalytically active component of the enzyme complex, harboring the three prosthetic heme groups, and the quinol binding and oxidation domain (Q-loop) (Fig. 1b). Binding of small molecules to this domain can efficiently inhibit access of substrate quinols to their oxidation site and thus diminish oxygen reductase activity. Chromosomal deletion of the small CydX subunit encoded immediately downstream the cydB gene results in loss of active site high-spin heme groups of proteobacterial bd oxidases. Mycobacterial genomes do not encode for CydX-like accessory subunits in their bd oxidase operons. We took into account that such small subunits could be encoded at different loci of mycobacterial chromosomes and searched for homologous sequences. Our results indicate the absence of orphan genes that encode for known accessory bd oxidase subunits in mycobacteria. Despite the close structural similarity to the cytochrome bd oxidase from Escherichia coli (cyt. bdEcoli), the absence of a CydX subunit homolog neither affects cofactor assembly of cyt. bdMtb, nor does it influence cofactor composition as shown by our cryo-EM structure and spectroscopic data. The reduced-minus-oxidized UV-visible difference spectrum confirms the presence of hemes b595, b595, and d (Fig. 1c).

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The spectral properties of these hemes closely match those previously reported for the cytochrome bd oxidases of E. coli and Azotobacter vinelandii. While we cannot fully exclude the possibility of the existence of small accessory subunits of unknown sequence encoded by the genome of M. tuberculosis, we can confirm that the structural genes of the bd oxidase operon are sufficient for complete assembly of the canonical core dimer and the functional insertion of the prosthetic heme groups.

Solvent accessibility. Based on the structural framework of cyt. bdEcoli, we performed atomistic molecular dynamics (MD) simulations in a POPC lipid environment to probe solvent accessibility and understand the dynamic behavior of water within these channels. Our data show the existence of a major water-filled channel that starts as a shallow cavity on the cytoplasmic side with a diameter of 20 Å at the CydAB interface (Fig. 2). The cavity narrows to a 4 Å channel that runs perpendicular to the membrane plane between the inter-subunit four-helix bundle formed by TMH2/3.A and TMH2/3.B. This channel is aligned parallel to the quinone binding site of the oxidase and points towards the periplasmic face of the membrane.
Fig. 1 Cryo-EM structure of the bd oxidase from M. tuberculosis. a Surface representation of the bd oxidase cryo-EM density map at 2.5 Å resolution. The bd oxidase consists of a heterodimeric CydAB core dimer. The Q-loop of CydA comprises a mostly disordered QN and an ordered QC segment that are both exposed to the periplasm. b Cyt. bdMtbo contains 18 membrane-spanning helices. CydA and CydB form a pseudo-symmetric dimer. All heme cofactors (b558, b595, and heme d) are located within CydA. Dashed red circles indicate the positions of accessory transmembrane helices in other bd oxidase structures. c Redox-minus-oxidized difference UV-vis spectrum of purified cyt. bdMtbo. d Oxygen consumption rates from biological replicates (n = 7) of IMVs containing overproduced cytochrome bd oxidase. Control experiments were performed with IMVs from ΔcydAB cells containing an empty expression vector. Control data were obtained from biological triplicates (n = 3). All data are presented as mean values ± SD. e Triangular arrangement of the heme cofactors in CydA. f The oxygen conducting channel emerging from the membrane plane and running towards the active site. g Heme d sits in an amphipathic pocket where the hydrophilic proton channel and hydrophobic oxygen pathway converge at the dioxygen binding site. Distances between water molecules and conserved hydrophilic residues are shown. Color scheme: CydA, yellow; CydB, green; b-type hemes, purple; heme d, coral.

solvent-accessible channel is filled with water molecules that are abundant near the cytoplasmic surface and become less abundant as the channel extends towards Asp59.B. The hydrogen-bond network formed by Asp59.B, Glu106.A, Ser107.A, Ser139.A, and Glu396.A connects the solvent channel to the oxygen reduction site, which suggests a role in proton delivery (Fig. 1g). On the periplasmic side of the membrane, between TMHs 5 and 6, we identify an entry site for water molecules running horizontally towards heme b595. Within the space between the two b-type hemes our simulation suggests the existence of a string of solvent molecules connecting the propionate groups of heme b558 and the side chain of Glu396.A over a distance of 12 Å (Fig. 2, Supplementary Fig. 3a). The functional role of this solvent-filled region can be attributed to a “dielectric well”, similar to what has been described for the function of water molecules within the H-channel of A-type cytochrome c oxidases (CytOx)25. In this structural context solvent molecules could either facilitate charge compensation via protonation and deprotonation of carboxyl groups, or by reorientation of dipole charges in response to redox conditions of the prosthetic heme groups. Localization of most of the described waters molecules is consistent between structural and simulation data.

The mycobacterial bd oxidase exhibits a unique Q-loop architecture. Cytochrome bd oxidases are classified into either a short (S) or a long (L) subfamily depending on the length of the Q-loop domain20. Members of the L-subfamily exhibit a C-terminal insertion in their Q-loop which adopts a helix-turn-helix fold located at the periplasmic surface of CydA. This C-terminal half of the Q-loop is essential for folding and stability of the CydA subunits of the L-subfamily bd oxidases26,27. Several homologs, including the one from M. tuberculosis, cannot be clearly assigned to either of these two subfamilies (Supplementary Fig. 6). Our cryo-EM structure provides structural insight into this unclassified group of cytochrome bd oxidases. In the structure of cyt. bdMtbo the well-conserved N-terminal segment of the
Q-loop (QN) is completely resolved and comprises residues Pro256.A to Val309.A (Figs. 1b, 3a). This segment emerges between TMH6 and TMH7 of CydA and is characterized by two short helical motifs (Qh1, Qh2) and a large disordered loop region (Fig. 3a). The Qh1 helix plays a critical role in coordinating propionate A of heme b$_{558}$ and substrate quinols$^{16}$. The C-terminal stretch (residues Thr310.A to Asn333.A) of the mycobacterial Q-loop (Qc) contains a short horizontal helix Qh3 which is exposed to the periplasm (Fig. 3a). It does not span the entire periplasmic surface of the CydA subunit as does the structurally similar but sequence unrelated horizontal helix of cyt. bd$_{Ecoli}$. In the mycobacterial enzyme this helix covers the periplasmic surface of TMHs 6 and 7 and extends only up to the large periplasmic loop connecting TMHs 8 and 9 (PL8) (Fig. 3a). At this interface, a hydrophobic cluster is located which is composed of aromatic residues Tyr321.A, Phe325.A and Tyr330.A of Qh3, and the conserved Pro401.A, Trp402.A and Pro406.A residues from PL8 (Fig. 3a). The aromatic residues Phe325.A and Tyr330.A are known to be critical for the activity of mycobacterial cytochrome bd$_{oxid}$ oxides$^{28}$. The location of these residues suggests that they play a structural role by stabilizing the Qh3-PL8 interaction instead of being involved in substrate recognition or redox chemistry of the oxidase. The C-terminal half of PL8, which represents a short polypeptide insertion unique for the mycobacterial bd$_{oxid}$ oxides, forms a series of backbone hydrogen bonds with the N-terminal segment of PL8 (Supplementary Figs. 6, 7). Analysis of atomic interactions at the Qc–PL8 interface showed that nine hydrogen bonds of PL8 (segments 399–418) were preserved during simulation runs. Further, our data showed that π–π interactions between Tyr321.A, Phe325.A, Tyr330.A of Qh3 and Trp402.A of PL8, hydrophobic interaction between Phe325.A and Pro401.A as well as the cation-π interaction between Phe325.A and Arg415.A remained stable for the entire duration of the runs. Overall, our data demonstrate that this stabilizing network contributes to the rigidity of PL8 important for maintaining the interaction with Qh3. Our structural findings confirm that the Q-loop architecture of the mycobacterial cytochrome bd$_{oxid}$ oxides resembles that of the large Q-loop variants. Although the length of the horizontal Qh3 helix is shorter compared to the analogous helix of L-subfamily members, its placement and interaction pattern with the CydA surface suggest that it has a similar stabilizing effect (Supplementary Fig. 4b)$^{28}$.

A distinguishing feature of the mycobacterial Q-loop is the presence of a disulfide bond between Cys266.A and Cys385.A within the QN region exposed towards the periplasmic space (Fig. 3b). Our simulations demonstrate that the disulfide bond rigidifies the disordered Q-loop segment and reduces flexibility between QN and Qh1 (Fig. 3c). This structural property is in stark contrast to the QN domain of cyt. bd$_{Ecoli}$ which does not contain a disulfide at this position (Fig. 3d)$^{13,14}$. For this enzyme, two independent structural studies confirmed that even in the presence of bound inhibitors the characterized binding site located at Q$_{N}$-loop remains highly flexible and does not adopt a distinct conformation$^{13,14,16}$. The dynamic nature of the proteobacterial QN is most likely required for a transient binding of substrate quinols in order to maintain continuous electron flow and oxygen consumption of the high-turnover bd$_{oxid}$ oxidase.

In order to characterize the binding of competitive quinol-like inhibitors to the mycobacterial Q-loop, we determined cyt. bd$_{Mcbb}$ cryo-EM structures in the presence of Aurachin D and AD3-11 at 3.3 Å and 2.7 Å resolution, respectively (Supplementary Fig. 1d). Even with a molar excess of 50–100:1 of these highly potent inhibitors, we were not able to identify any density consistent with bound quinolone derivatives at the conserved and previously characterized binding site located at Q$_{N}$. This observation agrees with data from our MD simulations showing that both reduced and oxidized MK-9 do not form specific interactions at the QN domain and migrates into the membrane space (Supplementary Fig. 10). We conclude that the confined conformation of the QN-region sterically hinders an interaction between substrate molecules and the highly conserved Glu263.A which is involved in quinol binding and oxidation in immediate proximity to heme b$_{558}$$^{16}$.

**Identification of a MK-9 binding pocket.** In proximity to the disulfide bond, within the cleft formed between TMH 1 and 9 we identified a bound menaquinone-9 (MK-9) molecule (Fig. 4a). LC-MS analysis confirmed that this co-purified MK-9 group is in the oxidized state (Supplementary Fig. 8). The local environment that forms this previously unknown MK-9 binding pocket is composed of the heme b$_{558}$ porphyrin scaffold, Arg8.A and Trp9.A of TMH1, and Met397.A of TMH9 (Fig. 4a). The Trp9.A side chain represents the main contributor to the cavity formation and shields the MK-9 head group from the surrounding membrane environment. Its aromatic plane is aligned parallel to the naphthoquinone head group of MK-9 with geometry and distance consistent with stacking π–π interactions (Fig. 4a). On the distal side of the naphthoquinone group Met397.A is in close distance to form stabilizing van-der-Waals interactions (Fig. 4a). Remarkably, this quinone binding site is occupied by the CydH subunit in cyt. bd$_{Ecoli}$. We characterized the binding dynamics of the
Fig. 3 Q-loop architecture of cyt. bdMtb. a Left: The mycobacterial Q-loop is organized in a mostly disordered QN (blue) and a rigid, well-ordered QC segment (cyan). QN starts at Pro256.A and contains the horizontal helix Qh1 with the conserved residues Lys258.A and Glu263.A that covers heme b558, and a second small helix Qh2 comprising residues Tyr296.A to Ala303.A. The QC segment is exposed to the periplasm and consists of the third Q-loop helix Qh3 that covers the surface of the TMHs 6 and 7. Right: Van-der-Waals interaction cluster between Qh3 and the periplasmic loop 8 (PL8) of CydA. b Top view of a superposition of CydA snapshots obtained from MD simulations performed in presence and absence of the Cys266.A-Cys285.A disulfide bond. Positions of cysteine residues are indicated. The red dashed circle indicates the location of the intact disulfide bond. CydA (oxidized), yellow; CydA (reduced), violet. c Top: average root mean square fluctuation plot of CydA residues in MD simulations for 2 × 750 ns with reduced and oxidized disulfide bond (Cys266.A-Cys285.A) of the QN-loop. Middle: Data plots of Cα-Cα distance distribution between disulfide-forming cysteines during MD simulations. Bottom: Distribution of QN displacement in Z direction during MD simulations. Yellow data plots indicate a simulation setup with intact disulfide bond. Purple data points indicate a simulation setup with a reduced disulfide bond. d Sequence alignment of representative cytochrome bd oxidases.
identified MK-9 molecule in the reduced and oxidized state via MD simulations (Fig. 4b, c). In the oxidized setup, MK-9 remained bound to its specific pocket by either forming π–π interactions with Trp9.A (Fig. 4b—snapshot 1) or heme b595 (Fig. 4b—snapshot 2). Oxidized MK-9 intermittently forms H-bonds with both Arg8.A and Trp9.A (10–25% of the simulation time). In the reduced state, the menaquinol head group shows increased dynamics and transiently adopts different binding positions between TMH1 and 9 while the interaction with Trp9.A increased dynamics and transiently adopts different binding time). In the reduced state, the menaquinol head group shows increased dynamics and transiently adopts different binding positions between TMH1 and 9 while the interaction with Trp9.A increased dynamics and transiently adopts different binding time). In the reduced state, the menaquinol head group shows increased dynamics and transiently adopts different binding positions between TMH1 and 9 while the interaction with Trp9.A increased dynamics and transiently adopts different binding time).

Discussion

Given the two remarkable structural features identified in the cyt. bdMtb structure namely the disulfide within the Q-loop and the occurrence of the MK-9-binding site near the location of the b595 heme, the question arises whether their existence is exclusively conserved among mycobacterial cytochrome bd oxidases. If functional association imposes selective constraints, then these two structural properties would evolve in a correlated fashion. In this regard, a multiple sequence alignment was generated using 561 CydA sequences from the manually curated representative genome list (Seed) of the protein family database (Pfam). We found that 94 CydA sequences (16.8%) exhibited a conserved tryptophan residue in TMH1 at this position (Supplementary Fig. 9a). More than half of these sequences (n = 58) belong to the phylum of Actinobacteria that includes the important pathogens from genera of Mycobacteria, Nocardia, and Corynebacteria. Among the total number of analyzed sequences only 30 orthologs (5.7%) exhibited the two cysteine residues participating in disulfide bond formation within the Q-loop domain thus appears to have a strong selective pressure for the Trp9.A residue, and consequently infers the existence of the identified MK-9 binding site.

Judging from its peripheral location and close distance to heme b595, it is unlikely that the MK-9 molecule acts solely as a structural component contributing to stability of the polypeptide framework. In cyt. bdMtb, the existence of the MK-9 binding site in immediate proximity of heme b595 strongly suggests a function as a quinol oxidation domain compensating for the inability of the mycobacterial Q-loop to bind substrate. Membrane quinols

Fig. 4 Allosteric MK-9 binding site of cyt. bdMtb. a Left: Side view closeup of the MK-9 binding pocket composed of residues from TMH1, TMH9, and the heme b595 porphyrin scaffold. Right: Front view closeup of the MK-9 binding pocket composed of residues from TMH1, TMH9, and the heme b595 porphyrin scaffold. Van-der-Waals interaction distances are indicated. b Representative snapshots of different MK-9 binding modes during 2 × 750 ns MD simulations in reduced and oxidized states. For simplicity, hydrocarbon chains of MK-9 molecules are not shown. c Distance histograms between MK-9 and heme b595 (Top), and MK-9 and Trp9.A (Bottom) during MD simulations. Blue data plots indicate a simulation setup with reduced MK-9. Green data plots indicate a simulation setup with oxidized MK-9. Oxidized MK-9, green; reduced MK-9, blue; heme b595, purple; Trp9.A, yellow.
provides the long-sought atomic framework for the design of colors.

differentially regulated by breaking or forming the disulphide bond in the QN raises the question of whether the activity of the bd oxidase in \textit{M. tuberculosis} can be differentially regulated by breaking or forming the disulphide bond through which the canonical electron transfer pathway would be re-activated.

The molecular structure of the bd oxidase from \textit{M. tuberculosis} provides the long-sought atomic framework for the design of highly specific drugs to target this respiratory enzyme. It may be possible to design inhibitors that prevent the interaction between the Q$_{C}$ domain and PL8 and thus interfere with the assembly of a functional enzyme. An alternative target is the membrane-embedded entry site of the oxygen-conducting O-channel that would be targeted by hydrophobic compounds to block the channel and abolish oxygen uptake. The newly identified secondary MK-9 binding site represents another highly specific target site. The latter however requires more in-depth insights about the detailed mechanistic origin of this unique domain.

**Methods**

**Generation of the cydABDC expression construct for cytochrome bd oxidase production.** To create the expression construct for production of cytochrome bd oxidase, the \textit{cydABDC} operon from \textit{Mycobacterium tuberculosis} was cloned into the episomal expression vector pYUB28h\cite{30}. For affinity purification, the 3’ end of cydA gene (C-terminus of CydB) was modified with a TEV cleavage site sequence followed by a FLAG affinity tag sequence. Initially, the \textit{cydABDC} operon was amplified from \textit{M. tuberculosis} genomic DNA using the primer pair cydAB_fw (5’-TAAAGAGGA-GATATACCAGAATGCTGCGACCATTTCCGGTG-3’) and cydAB_rv1 (5’-GCCAGACCGGTTGAGGATGT-3’). Following this, two rounds of PCR amplification were performed with the \textit{cydABDC} PCR product using different reverse primers in combination with \textit{cydAB} fw. Firstly, the TEV cleavage site was added with cydAB_rv (5’-GCCCTGAAATAACAGGTTCCCAGGGCGGGCCGGTG-3’) and then the FLAG tag was added with cydAB_rv3 (5’-GCCCTGGTCTACCTATCTGGTGCACTTTGATTAGCCGCTGAAATAACAGGTTCCCAGGGCGGGCCGGTG-3’) to the final \textit{cydAB} PCR product. The cydDC portion of the primer pair cydDC fw (5’-CGATAAGTGAGACCAAGCAGCCGCGGGAGGCA-3’) and cydDC rv (5’-TGAGTTCTGGCAGGCTGGGTG-3’) was re-activated.

**Methods**

**Production of cytochrome bd oxidase from \textit{M. tuberculosis}.** The cytochrome bd oxidase from \textit{M. tuberculosis} (M. tb) was produced in \textit{Mycobacterium smegmatis} mc2 155 ΔcydAΔ cells transformed with the plHcyd plasmid carrying genes of the cytochrome bd oxidase from \textit{M. tb} with a C-terminal FLAG-tag modification at cydB, the CydDC assembly factor of the cytochrome bd oxidase from \textit{M. tb}, and a hygromycin resistance cassette. \textit{Mycobacteria} were grown in LB Lennox media (10 g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl) with 0.05% w/v Tween 80 and 50 µg/ml hygromycin B added before use. Single aliquots of electrocompetent cells were transformed with 1 µl DNA and 260 µl of 10% ice-cold glycerol. Electro-poration was carried out in 0.2 cm cuvettes using a Bio-Rad Gene Pulser XL according to standard conditions with appropriate annealing temperatures and extension times.

**Methods**

**Production of cytochrome bd oxidase in lipid nanodiscs.** Cytochrome bd oxidase was produced in 20 mM Tris-HCl (pH 7.4), 0.05% Tween 80 and 10% glycerol. Positive transformants were selected by plating cells on LB agar containing 50 µg/ml hygromycin B. Prior to freezing the protein concentration of the isolated membrane fraction was adjusted to 10 mg/ml using Bradford reagent.

**Methods**

**Production and purification of MSP1D1.** MSP1D1 production was carried out according to published protocols\cite{31}. In brief, BL21Gold (DE3) cells transformed with pMSP1D1 were grown in LB-Kan medium (50 µg/ml kanamycin) at 37 °C until OD$_{600}$ of 0.6 was reached before MSP1D1 production was induced by addition of IPTG to a final concentration of 1 mM. Growth was carried out for 4 h before cell harvest. MSP1D1 was purified according to standard conditions described earlier\cite{31} and stored in a buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.02% DDM.

**Reconstitution of bd oxidase in lipid nanodiscs.** Cytochrome bd oxidase was reconstituted into lipid nanodiscs according to protocols by Ritchie et al.\cite{31}. In brief, POPC, MSP1D1, and bd oxidase were mixed in a molar ratio of 400:1:6 and incubated at 4 °C for 1 h. Detergent was removed from the mixture by sequential addition of 4 ×0.2 mg dried Bio-Beads every 2 h. Finally, the mixture was incubated at 4 °C for additional 12 h while shaking. Bio-Beads were removed via filtration and the reconstituted bd oxidase particles were separated from empty nanodiscs via SEC on a Superdex 200 10/300 Increase column using a buffer containing 20 mM Na-HEPES (pH 7.5) and 100 mM NaCl. Nanodisc reconstituted samples were used.
for downstream cryo-EM experiments. Aurachin D was added to the detergent-solubilized protein solution before reconstitution at a final concentration of 500 mM. DAD-11 was added to the detergent DDM solubilized protein solution before reconstitution at a final concentration of 1 mM. Inhibitor concentration was maintained throughout the reconstitution procedure and until grid preparation.

**Sample vitrification.** Quantifoil R1/2/1.3 copper grids (mesh 200) were washed in chloroform and subsequently glow discharged twice with a PELECO easiGlowlow electron-beam device at 15 mA for 45 s. A volume of 4 µl sample (1.5 mg/ml) was applied on a grid immediately before plunge freezing. Samples were vitrified at 4 °C, 100% humidity, and a blott force of 20 using a Vitrobot IV device (Thermo Fisher). Blotting was carried out for 4 s before plunge freezing in liquid ethane.

**Cryo-EM imaging recording.** A total of 12070 movies were recorded in Energy-Filtered Transmission Electron Microscopy mode using a Titan Krios G3i microscope operated at 300 kV (Thermo Fisher). Electron-optical alignments were performed using automated strategies of EPU 2.9. Micrographs were recorded using automatic dose fractionation movies were recorded at an electron exposure of 0.837 Å. Dose fractionation movies were recorded at an electron dose of 500 µM. AD3-11 was added to the detergent DDM solubilized protein solution at 15 mA for 45 s. A volume of 4 µl sample (1.5 mg/ml) was applied on a grid and subsequently glow discharged twice with a PELCO easiGlow device optimized using the FFTK toolkit48 based on the heme scope operated at 300 kV (Thermo Fisher). Electron-optical alignments were carried out for 4 s before plunge freezing in liquid ethane.

**Cryo-EM image processing.** MotionCor2 was used to correct for beam-induced motion. HR image processing was used to estimate the contrast transfer function (CTF) parameters and perform correction steps33. Images with estimated poor resolution (<4 Å) and severe astigmatism (>300) were removed at this step. Particles were picked by cryOLO34. A total of ca. 15 M particles were picked and used for all further processing steps. Initial model building, 3D classification, CTF refinement, Bayesian polishing, NDI1D volume subtraction, and final map reconstructions were performed with RELION-3.135. A detailed overview of our processing workflow is found in SI.

**Model building and refinement.** The atomic model of the cytochrome bd oxidase was built in Coot (version 0.8.9) initially using the pdb model of the cytochrome bd-I oxidase from E. coli (6RKO) as reference structure36. After manual backbone tracing and docking of side chains in the respective map densities, we performed real-space reparametrization (version 1.14)37. Final refinement results were visually inspected and corrected if needed. The final model was validated by the MolProbity online server38. Map-to-model cross validation was performed in Phenix (version 1.14). FSC0.5 was used as cutoff to define resolution. A summary of the model parameters and the corresponding cryo-EM map statistics is found in Supplementary Table 1. The final model was visualized using ChimeraX39.

**Analysis of tunnels and interior cavities.** Tunnels and interior cavities were mapped with MOLE 2.5 (bottleneck radius: 1.4 Å, bottleneck tolerance 3 Å, origin radius 5 Å, surface radius 8 Å, probe radius 5 Å, interior threshold 1.1 Å)40, considering densities for non-proteinogenic cofactors. Several variations in the overall morphology of the tunnels and interior cavities were observed, indicating a high degree of flexibility or plasticity in this region.

**Structural alignments.** The overall folds of the mcytocadial bd oxidase subunits CydA and CydB were compared with corresponding subunit architectures of the available cytochrome bd oxidase structures from E. coli (6RKO, 6RXA) and G.sh (5DOQ). The DALI protein database comparison sever was used for performing structural alignments and calculating Ca RMSD values, and Z-scores41.

**Molecular dynamics simulation.** The cyt. bd08a structure was embedded in a bilayer composed of POPC using CHARMM-GUI42. All systems were hydrated with 150 mM NaCl electrolyte. The all-atom CHARMM36m force field was used for lipids, ions, and cofactors, and protein with TIP3P water43–45. The heme b and O2 parameters were taken from the CHARMM36m, while heme d parameters were optimized using the FTKTK toolkit46 based on the heme b parameters. MK-9 was parameterized in both reduced and oxidized forms using the FFTK toolkit. MD trajectories were analyzed using Visual Molecular Dynamics software49 and MDAnalysis package50. All simulations were performed using GROMACS 2019.651. Starting systems were energy-minimized for 5000 steepest descent steps and equilibrated initially for 500 ps of MD in a canonical (NVT) ensemble and later for 7.5 ns in an isothermal–isobaric (NPT) ensemble under periodic boundary conditions. During equilibration, the restraints on the positions of non-hydrogen protein atoms of initially 4000 kJ mol⁻¹ nm² were gradually released. Particle-mesh Ewald summation52 with cubic interpolation and a 0.12-nm grid spacing was used to treat long-range electrostatic interactions. The time step was initially 1 fs, and was increased to 2 fs during the NPT equilibration. The LINCS algorithm was used to fix all bond lengths53. Constant temperature was set initially with a Berendsen thermostat, combined with a coupling constant of 1.0 ps. A semi-isotropic Berendsen barostat was used to set a pressure of 1 bar12. During production runs, the Berendsen thermostat and barostat were replaced by a Nosé–Hoover thermostat and a Parrinello–Rahman barostat53–55. Analysis was carried out on unconstrained simulations. Simulations of oxidized and reduced states of MK-9 and reduced Q-loop disulfide were performed for 2 x 750 ns.

**Mass spectrometry.** Menaquinone-9 was identified in purified cyt. bd08a samples by high-resolution mass spectrometry using a Shimadzu LCMS-9030 instrument (Shimadzu Scientific Instruments Inc.). A volume of 2 µl of purified cyt. bd08a was applied onto a C18 Shimadzu 2.0 x 50 mm Shim-pack XR-ODSIII column (1.6 µm) connected to a Shimadzu Nexera X2 HPLC system. The HPLC run was performed using 100 % methanol for isocratic elution. ESI source operating conditions: nebulizer gas 3 L/min, dry gas 10 L/min, interface voltage 3.00 kV, interface temperature 300 °C. Mass spectra were processed using LabSolutions Insight Explore LCMS software (Version 5.96). Oxidized menaquinone-9 was identified, as adducts, at a retention time of 37 min using a high-resolution fit. M+ H at 785.6271 Da (predicted 785.6231 Da), M+ Na+ at 809.6239 Da (predicted 809.6207 Da) M+ K+ 825.5962 Da (predicted 825.5946 Da).

**UV–Vis absorption spectroscopy.** Heme cofactors of the cytochrome bd oxidase were analyzed using ultraviolet-visible (UV–Vis) absorption spectroscopy. Spectra shown in this work were recorded with a Lambda 35 UV–Vis spectrometer from Perkin Elmer (Waltham, USA) using quartz micro-cuvettes from Hellma (Mülheim, Germany) with an optical path length of 10 mm. A sample volume of 400 µl was used for each measurement. UV–Vis data were collected in a spectral range between 380 nm and 700 nm with a scanning speed of 120 nm/min, data intervals of 0.2 nm, and an optical slit width of 1 nm. Samples were reduced by addition of sodium dithionite, and oxidized by addition of potassium ferrocyanide.

**Oxygen consumption in inverted membranes vesicles (IMVs).** IMVs of the indicated strains were prepared using a cell lysis protocol as described in the purification section. Oxygen consumption was measured using an Oroboros O2k fluoropertor as previously described51. Briefly, IMVs were resuspended in buffer (50 mM Tris, 100 mM KCl, 5 mM MgCl2, pH 7.5) and 100 mM NADH was used to initiate respiration. For Aurachin D titrations, 500 mM TB47 (an inhibitor of cytochrome bc0 complex)52 was added prior to the experiment to eliminate the contribution of the HCO type terminal oxidase to oxygen consumption. Membranes were used at a protein concentration 12.5 g/mL. Stepwise titrations were performed using the Oroboros O2k TIP2k automatic injection micropump, with rates measured at 120 s intervals between each injection. Data are normalized to protein concentrations that were estimated by BCA assay (Thermo), using a BSA standard. Addition of the bc0-cytochrome supercomplex inhibitor TB47 allowed us to measure specific oxygen consumption rates of the cytochrome bd oxidase of IMV containing overproduced cytochrome bd oxidase. For negative control measurements, we used the IMVs of a Mycobacterium smegmatis mc2 155 AgyaABDC strain transformed with an empty pYUB28B vector. IC50 values were determined from logistic regression fits using Origin Lab Pro 9.5 (Additive GmbH, Germany). Samples were measured as biological replicates (n = 7) and control data was obtained from biological triplicates (n = 3).

**Multiple sequence alignments and taxonomy analyses.** For conservation analysis of structural properties, we retrieved 361 CydA sequences from the manually curated representative genome list (Seed) of the protein family database (PFam). Multiple sequence alignment was performed using Clustal Omega52. After manual curation, phylogenetic trees of orthologs containing either Trp9.A or the cysteine pair in the QC-loop domain were generated using NCBI Common Tree and visualized by FigTree (Version 1.4.4).

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The Cryo-EM maps of the cytochrome bd oxidase from M. tuberculosis are deposited at the Electron Microscopy Data Bank under accession numbers EMD-12451, EMD-12532, EMD-12533. The model of the cytochrome bd oxidase structure was submitted to the PDB with accession number 7NKZ. All other data are presented in the main text or Supplementary Information. Source data are provided with this paper.

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