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Breathing in thin air: structure, function and therapeutic opportunities of bd type oxidases

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Breathing in Thin Air

Structure, Function and Therapeutic Opportunities of
*bd*type Oxidases

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Breathing in Thin Air

Structure, Function and Therapeutic Opportunities of *bd*-type Oxidases

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Breathing in Thin Air

Structure, Function and Therapeutic Opportunities of
*bd*type Oxidases

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List of Abbreviations

Abbreviation	Definition
2-me	2-mercaptoethanol
ABC	ATP binding cassette
ACN	Acetonitrile
ADP	Adenosine diphosphate
AFIS	Aberration-free image shift
ATP	Adenosine triphosphate
AurD	Aurachin D
Auto-ox	Auto-oxidation
BDQ	Bedaquiline
Cryo-EM	Cryo electron microscopy
CTF	Contrast transfer function
CV	Cyclic voltammetry
CydADC	CydA -CydDC complex
Cyt <i>bd</i>	Cytochrome <i>bd</i>
DDA	Data-dependent acquisition
DDM	Dodecyl beta-d-maltoside
DMK	Demethylmenaquinone
<i>Ecbd</i>	<i>E. coli</i> cytochrome <i>bd</i>
EIS	Electrical impedance spectroscopy
EMDB	Electron Microscopy Data Bank
HDX	Hydrogen deuterium exchange
LC-MS	Liquid Chromatography – Mass Spectrometry
LMNG	Lauryl maltose neopentyl glycol
MDR	Multi drug resistant
MK	Menaquinone
<i>Mtbd</i>	<i>M. tuberculosis</i> cytochrome <i>bd</i>
NDH ₂	Type 2 NADH dehydrogenase

PA	Phosphatidic acid
PDB	Protein databank
PE	Phosphatidylethanolamine
PEP	Phosphoenolpyruvate
PG	Phosphatidylglycerol
PMF	Proton motive force
RMSD	Root mean square deviation
ROS	Reactive oxygen species
SAM	Self assembled monolayer
SEC	Size exclusion chromatography
SHE	Standard hydrogen electrode
SMF	Sodium motive force
TB	Tuberculosis
tBLM	Tethered bilayer lipid membrane
TCEP	Tris(2-carboxyethyl)phosphine
TDR	Total drug resistant
TEA	Triethylamine
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TMS	Tetramethylsilane
TSG	Template stripped gold
UQ	Ubiquinone
XDR	Extensively drug resistant

Chapter



General introduction

The antibiotic resistance crisis

Antibiotics have been among the most transformative achievements in modern medicine, offering powerful tools to combat a wide range of bacterial infections. However, the growing prevalence of antibiotic resistance represents one of the most urgent challenges to global health. The emergence and spread of multidrug-resistant (MDR), extensively drug-resistant (XDR), and totally drug-resistant (TDR) bacterial strains have rendered many conventional antibiotics ineffective. Recent projections estimate that the number of annual deaths associated with antibiotic resistant bacteria will increase from 4.7 million in 2021 to 8.2 million in 2050[1].

Among the most concerning pathogens is *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), which claims over one million lives annually. Approximately one-quarter of the global population is latently infected with *M. tuberculosis*, creating a substantial population for reactivation and drug resistance[2]. Drug-resistant TB strains have complicated treatment protocols, often necessitating prolonged regimens involving a cocktail of toxic and costly antibiotics[2]. Standard therapy spans 4–9 months of such antibiotic cocktail, yet up to 15% of patients exhibit treatment failure, requiring alternative intervention.

Alarmingly, *M. tuberculosis* acquires antibiotic resistance at an estimated rate of 5% per year, outpacing all other major bacterial pathogens[1]. Despite the World Health Organization's 2015 "End TB Strategy" targeting a 20% reduction in incidence by 2020, only an 11% decline was achieved within the seven years until 2022[2]. Without accelerated intervention, TB alone may account for over 31 million deaths between 2020 and 2050[3]. These trends underscore the urgent need for novel antimicrobials capable of overcoming drug-resistant *M. tuberculosis* and other pathogens. The antibiotic resistance crisis is compounded by a stagnation in antibiotic discovery. Most antibiotics in clinical use today were developed in the mid-20th century, and the discovery pipeline for new compounds remains sparse[4].

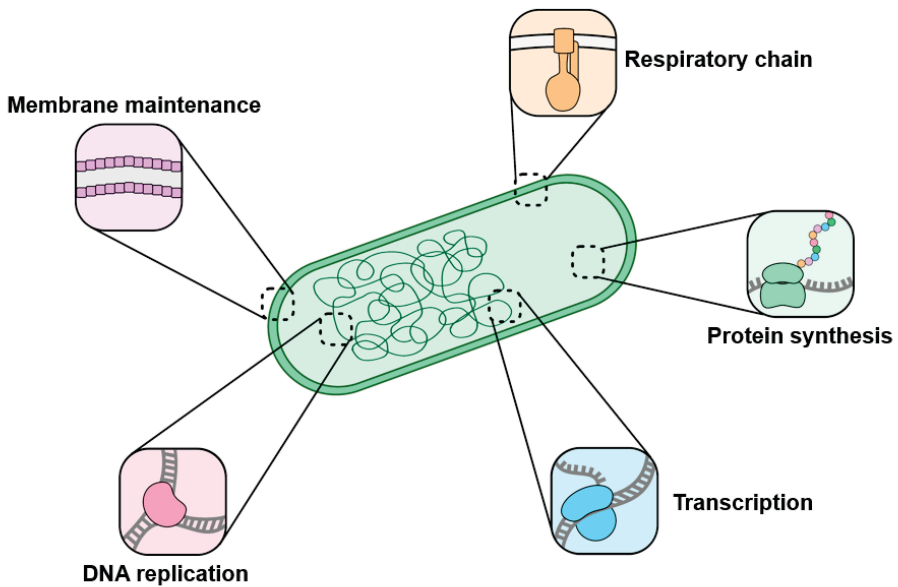


Figure 1.1: Overview of antibiotic classes. Antibiotics can impede membrane maintenance, DNA replication, transcription, protein synthesis, or the respiratory chain.

Traditional targets, such as cell wall synthesis, DNA replication, transcription, and protein translation (Fig. 1.1), have been extensively exploited, and resistance mechanisms against these pathways are now widespread[5]. To overcome this challenge, it is essential to identify new bacterial targets that can be therapeutically exploited.

How bacteria gain resistance

Antibiotic exposure imposes strong selective pressure, driving the evolution of resistant bacteria. Resistance arises from intrinsic bacterial traits, spontaneous genetic mutations, or horizontal gene transfer, enabling resistant subpopulations to thrive in antibiotic-rich environments. Four principal mechanisms underpin bacterial resistance[6]:

- *Drug Modification:* Bacteria produce enzymes that chemically inactivate antibiotics. For example, β -lactamases cleave the β -lactam ring, rendering drugs like cephamycins and carbapenems ineffective [7].
- *Reduced Intracellular Drug Concentration:* Resistance can arise through decreased drug uptake or increased efflux. Alterations in porins, e.g., replacement of OmpK35 with OmpK36, reduce β -lactam permeability, while efflux pumps such as Tet actively expel tetracycline from the cell [8].
- *Target Modification:* Mutations in antibiotic target genes can reduce drug binding. Proteins like TetO and TetM displace tetracycline from the ribosome, and mutations in DNA gyrase elevate fluoroquinolone MIC values [9].
- *Global Cellular Adaptations:* Regulatory systems such as LiaFSR modulate membrane responses to antibiotics like daptomycin, although the precise mechanisms remain under investigation [10].

A particularly resilient subset of bacteria, known as persister cells, exists in a metabolically inactive state, making them resilient to many conventional antibiotics. These cells reduce antibiotic susceptibility through low adenosine triphosphate (ATP) levels, which downregulate essential cellular processes [11]. Worryingly, current antibiotic development strategies often focus solely on actively replicating cells, overlooking the resilience of persister cells and rendering treatments less effective [12]. These persisters complicate treatment outcomes and highlight the importance of targeting pathways essential in both replicating and dormant states, such as the bacterial respiratory chain.

The *M. tuberculosis* respiratory chain

The *M. tuberculosis* respiratory chain is a series of membrane bound enzymes that are responsible for the majority of cellular energy generation in the form of ATP. This respiratory chain is a highly adaptable system, distinct from its mitochondrial counterpart in humans. It comprises multiple terminal oxidases and dehydrogenases with built-in redundancy that maintain energy production, redox balance, and survival under stress (Fig. 1.2). Targeting this system offers a unique opportunity to develop selective and potent antibiotics, particularly against non-replicating and persistent bacterial populations which rely on respiration to uphold their minimal metabolic activity which makes them resistant to conventional antibiotics.

The respiratory chain generates energy stored as a proton motive force (PMF) or sodium motive force (SMF), which drives ATP synthesis and solute transport. Novel inhibitors targeting the respiratory chain now represent over 30% of all new antimycobacterial drugs in clinical trials, and more than 65% of phase III candidates[13]. Most bactericidal compounds are thought to induce reactive oxygen species (ROS) formation by disrupting metabolic cycles and increasing energy demand[13–15].

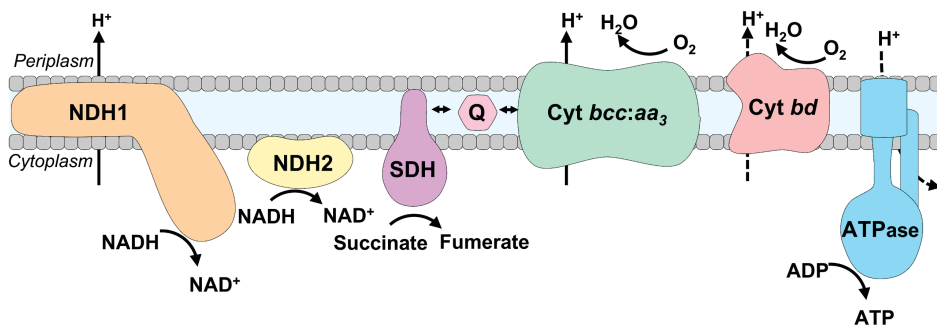


Figure 1.2: The respiratory chain of *M. tuberculosis*. Electrons enter the chain via the dehydrogenases and are shuttled to the terminal oxidases via the quinone pool. The proton motive force created is used the ATP synthase to regenerate ADP to ATP.

In contrast, bacteriostatic agents reduce oxygen consumption, pushing bacteria into a low-energy, non-replicative state[14]. Given the essentiality of various respiratory chain components in *M. tuberculosis* and various other bacteria, often coupled with their absence or divergence in humans, make this pathway a highly attractive drug target with drug development efforts undergoing for each of its members.

Quinones: the central electron carriers

Quinones act as a central electron carrier between the different enzymes of the respiratory chain. These carrier lipids molecules are readily reduced or oxidized to take up or release electrons at the target enzyme[16]. In its reduced form, quinol, the molecule has been dually protonated in conjunction with the uptake of two electrons. These electrons, together with the two protons, are subsequently released upon oxidation at the target enzyme (Fig. 1.3). Different types of quinols are used by different species under specific conditions[16,17]. Depending on the species, ubiquinone or menaquinone predominate, differing in their redox potentials and chemical structure[18]. While *M. tuberculosis* contains only menaquinone, other species like *E. coli* possess multiple quinones that are used under specific conditions. As such, ubiquinone supports aerobic respiration, whereas menaquinone predominates under anaerobic or microaerophilic conditions in *E. coli*.

Drug development efforts that target the quinone pool have resulted in the drug candidates SQ109 and Lysocin E. SQ109 inhibits the synthesis of new quinols by targeting the enzymes MenA and MenG[19], whereby the decrease in quinol concentration impairs the shuttling of electrons within the respiratory chain. Lysocin E on the other hand, binds directly to the menaquinone, blocking its redox cycling and collapsing the membrane potential[20]. Lysocin E has been shown to attenuate both *Mycobacterium smegmatis*[21] and *Staphylococcus aureus*[20] although their efficacy against clinically relevant *M. tuberculosis* strains and *in vivo* infection models warrants further evaluation.

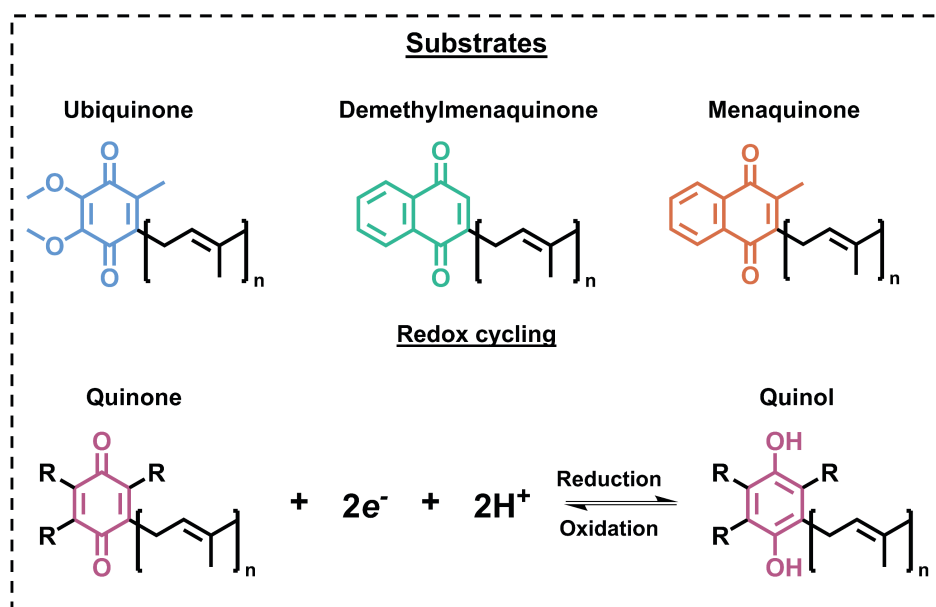


Figure 1.3: Quinone and their redox chemistry. Different quinone subtypes found across various bacterial species. All three quinone subtypes are found in *E. coli*, while *M. tuberculosis* is restricted to menaquinone. These quinones undergo reduction by the dehydrogenases to become quinol, followed by oxidation at the terminal oxidases back to quinone.

Respiratory dehydrogenases

NADH dehydrogenases

The NADH dehydrogenases (NDH) function in the reduction of the quinone pool, coupled to the oxidation of NADH to NAD^+ [13]. NDH exists in two subtypes, the proton pumping NDH-1 (Nuo) and the non-proton pumping NDH-2 (Ndh and NdhA)[22]. In *M. tuberculosis*, Nuo and NdhA are individually dispensable, while Ndh deletion causes growth defects[22,23]. Conversely, the inhibition of all three NADH dehydrogenases seems to be an effective antimycobacterial strategy[24]. This need for simultaneous inhibition underscores the functional redundancy in the respiratory chain of *M. tuberculosis*.

To study the potential of pharmaceutical intervention of the NADH dehydrogenases, two types of drugs have been repurposed; phenothiazines, and riminophenazines[13], although phenothiazines likely have a multi-target mechanism including a decrease in PMF [25–28]. Of these drug subtypes, Thioridazine[29,30] and Clofazimine[31] have been deemed most effective. Thioridazine is proposed to reinstate antibiotic susceptibility of bacteria by inhibiting the overexpressed efflux pumps that cause resistance[13], while Clofazimine competes with menaquinone as an electron acceptor, generating ROS that kill the bacteria[32]. Additional research is needed to fully understand the mechanism of Thioridazine[13], and decrease the side effect of Clofazimine[33] before they can become an important tool in the battle against antibiotic resistance.

Succinate dehydrogenase

Similar to the NADH dehydrogenases, succinate dehydrogenases (SDH) function to reduce quinones to quinols. SDH links the tricarboxylic acid cycle to respiration, oxidizing succinate to fumarate while reducing the quinone pool[34]. This enzyme holds similar pharmaceutical potential as the NADH dehydrogenases to inhibit the reduction of the quinone pool, effectively shutting down the electron injection into the respiratory chain. Drug development efforts, however, have been sparse, as hesitance is in place due to the presence of human counterparts to this enzyme[13]. The off-target effect of the potential antibiotic on the human counterpart enzyme could have detrimental effects on human energy generation, thereby killing our own cells. Although concerns about homology to human SDH have tempered development efforts, selective inhibitors targeting divergent bacterial residues hold promise[35].

One of the more promising succinate dehydrogenase inhibitors is Siccanin[36], a quinone mimicking molecule. It should be noted, however, that the Siccanin effectivity against most pathogens remains to be tested. Alternatively, 3-nitropropionate has been found as a potential inhibitor, however, little research has been conducted[37].

The terminal oxidases

Cytochrome *bcc:aa₃*

After reduction of the quinone pool by the dehydrogenases, the quinols shuttle the acquired electrons to the terminal oxidases. The primary terminal oxidase under aerobic conditions differs per species and consists of enzymes such as the cytochrome *bcc:aa₃* super complex in *M. tuberculosis*. These terminal oxidases oxidize quinols back to quinones and use the associated released electrons to reduce O₂ to H₂O. In addition to the chemical protons that are taken up in the cytosol and released in the periplasm during the turnover cycle, these terminal oxidases pump protons into the periplasm that add to the PMF. In total, cytochrome *bcc:aa₃* adds six protons to the PMF per quinol oxidized[38].

Early studies found that cytochrome *bcc:aa₃* is the primary terminal oxidase used and deemed it essential as no mutant could be obtained when they were deleted[39]. More recent studies, however, have shown that knockout strains can be obtained, although with severe growth defects, while glycerol is present [24].

Due to its prominent function in the respiratory chain, several drug development efforts have led to specific inhibitors. The most promising inhibitor is Q203, also called Telabec[40], and is currently in phase 2 clinical evaluation. This compound inhibits cytochrome *bcc-aa₃* through the binding of subunit QcrB, where the quinols bind for oxidation[41,42]. It has been reported however, that the use of Q203 alone is bacteriostatic in *M. tuberculosis*, highlighting the adaptability of the respiratory chain[43].

Additionally, the FDA-approved drug Lansoprazol has been found in a drug screen to be repurposed from the treatment of gastroesophageal reflux disease to the inhibition of the cytochrome *bcc:aa₃* complex[44]. It was shown that lansoprazole act as a prodrug that after conversion to lansoprazole sulfide binds to QcrB[44].

Cytochrome *bd*

An alternative terminal oxidase present in *M. tuberculosis*, and most other bacteria, is cytochrome *bd*. Cytochrome *bd* mainly functions as the alternative terminal oxidase once cytochrome *bcc:aa₃* is unable to function, increasing the adaptability of the respiratory chain. This happens once bacteria enter a hostile environment, such as low oxygen tensions and increased reactive oxygen species (ROS) concentrations[45]. Due to the higher oxygen affinity of cytochrome *bd*[46,47], it can reduce O₂ to H₂O under low oxygen tension to maintain the formation of the proton motive force required to generate ATP. Additionally, cytochrome *bd* acts as a defense against nitric oxide[48], hydrogen peroxide[49,50], ammonia[51], nitrite[52], peroxynitrite[53], cyanide[54,55], chromate[56], sulfide[56,57], as well as antibiotics[58–60]. Recent research has shown that cytochrome *bd* is an essential enzyme under infectious conditions, making it a promising target for pharmaceutical intervention[61]. Additionally, dual inhibition of cytochrome *bcc:aa₃* and cytochrome *bd* showed bactericidal against *M. tuberculosis*, further underlining its therapeutic value[43].

ATP synthase

The final protein in the respiratory chain required for ATP synthesis is the ATP synthase. This protein consumes the proton motive force created by the respiratory chain to turn ADP into ATP[62]. As this is the protein responsible for ATP synthesis, it has been a major target for drug discovery. Hence, the very first respiratory chain antibiotic that was approved by the FDA, bedaquiline (BDQ), also known as Sirturo, targets this synthase[63–65]. BDQ has shown its promise in the treatment of tuberculosis by its bactericidal activity against dormant cells[66], which most antibiotics cannot[13]. BDQ inhibits the ATP-synthase by binding to the C and ϵ subunit[67,68], rendering it inactive. Unfortunately, BDQ has a delayed bactericidal activity *in vitro* and *in vivo*[69], where it takes between 2 to 5 days to exterminate the target bacteria. Regardless, BDQ remains a potent antibiotic as seen by shortened treatment times in comparison to other TB antibiotics.

The success of respiratory chain antibiotics

The success of BDQ is highlighted in the NIX-TB trial, a clinical trial run in south Africa that recruited patients burdened with multi- or extreme- drug resistant tuberculosis, unresponsive to second line treatments. In this extremely difficult to treat patient group, an antibiotic mix of pretomanid, linezolid and bedaquiline resulted in patients reaching culture negative status within 16 weeks, curing 90% of the patient cohort[70].

Unfortunately, since the introduction of BDQ in 2012, the first cases of antibiotic resistance have been reported, rendering this treatment ineffective in these patients[71,72]. This resistance is caused by an efflux pump that actively removes BDQ from the bacterial cell, making it unable to bind to the ATP synthase. While many efforts are currently ongoing to avoid the effects of this efflux pump, either through direct inhibition or alteration of the bedaquiline structure, none of these have reached patient care.

A worrying study showed that the affinity of bedaquiline for the mycobacterial ATP-synthase is equally high for the human ATP-synthase[73]. Simultaneously this has not shown any detrimental side effects on intact cells, potentially due to relative selectivity to the *M. tuberculosis* ATP synthase due to either lower affinity or accessibility[74–76]. Regardless, it shows that careful design of these respiratory chain inhibitors can yield potent treatment options that prevent off target effects to the homologues human counterparts.

One of the more promising approaches of respiratory chain inhibition against *M. tuberculosis* is the dual inhibition of the two terminal oxidases[77], cytochrome *bcc:aa₃* and cytochrome *bd*, which have no human homology. While potent drug candidates are available against cytochrome *bcc:aa₃*, cytochrome *bd* lacks behind.

Cytochrome *bd* as a drug target

Cytochrome *bd* is the terminal oxidase which plays a critical role in cellular adaptation to both infection and stress conditions. This heme-containing enzyme, found predominantly in many gram-negative bacteria, is part of an alternative oxidase system that functions under low-oxygen or hypoxic conditions[47]. During infection, pathogens encounter fluctuating oxygen availability within the host, and cytochrome *bd* becomes essential for maintaining cellular energy production by facilitating efficient electron transfer in micro-aerobic environments[61,78]. Additionally, cytochrome *bd* is involved in the adaptation to oxidative stress, often present during host immune responses. This enzyme helps protect bacterial cells by reducing the production of reactive oxygen species (ROS), thus minimizing oxidative damage to cellular components[79]. Furthermore, cytochrome *bd* contributes to biofilm formation, a common feature in chronic infections, which enhances bacterial survival under adverse conditions[59]. Overall, cytochrome *bd*'s ability to sustain ATP synthesis, mitigate oxidative stress, and support biofilm development underscores its pivotal role in bacterial pathogenesis and survival during infection and stress.

The molecular structure of cytochrome *bd*

In the last few years, the protein structure of cytochrome *bd* from different species have been elucidated by cryogenic electron microscopy (Fig. 1.4A,B) [80-87]. The core structure of cytochrome *bd* consists of two subunits, CydA and CydB, each comprising of 9 transmembrane helices, and exist in a two-fold rotational pseudo-symmetric conformation[85].

CydA contains a hydrophilic periplasmic loop between the 6th and 7th trans membrane helix that had been proposed to bind the quinols for oxidation, termed the Q-loop. Despite this long-standing hypothesis, structural studies have yet to determine the structure of any cytochrome *bd* in the substrate-bound state[83]. Cytochrome *bd* oxidases have been phylogenetically classified in the qOR, OR-C and OR-N subfamilies[88]. The quinol binding *bd*

oxidases, qOR, are further subdivided in qOR1, qOR2, qOR3, and qOR4a, where qOR1 forms the predominant subfamily. These qOR *bd* oxidases can be further subdivided based on the length of the Q-loop. Firstly, the short Q loop variants, as found in *Geobacillus thermodenitrificans* [81], contain only the flexible N-terminal insert of the loop. The long Q-loop variants, as found in *E. coli*, however, are characterized by an additional rigid C terminal part of approximately 60 amino acids that seems to have a structural role[89]. Importantly, the Q-loop from mycobacterial cytochrome *bd*, such as *M. smegmatis* and *M. tuberculosis*, classically referred to as the short Q-loop family, contains an additional eight amino acid insert[82], which means they consist of neither the long or short Q-loop family (Fig. 1.4C). In addition, the N-terminal portion of the mycobacterial Q-loop contains a unique disulfide bond, reducing the characteristic flexibility found in other *bd* variants[82]. Furthermore, exchanging the Q-loops from different species renders cytochrome *bd* inactive, showing a crucial, species-specific role of the Q-loop layout[89,90].

The CydA subunit contains all three heme cofactors, *b*₅₅₈, *b*₅₉₅, and *d*, to transfer the electrons gained from quinol oxidation towards the heme *d* oxygen reduction site (Fig. 1.3D). These heme groups are orientated in a triangular fashion with iron-iron distances varying between 11 and 19 Å[83]. This distance significantly differs from the heme-copper oxidases, where the binuclear heme iron to copper distance is approximately 4 to 5 Å[85].

Despite the structural similarity between CydA and CydB, CydB does not contain any heme groups. Instead, CydB often carries a ubiquinone-8 or demethylmenaquinone-8 bound in the positions otherwise occupied by the heme groups[91]. Due to the position of these quinones, it is suggested that they serve a stabilizing function. Additionally, mutations in the quinone binding groove of CydB rendered the enzyme inactive[83]. In Mycobacteria, however, the quinone binding site is replaced by a series of tryptophan and phenylaniline residues that reconstitute the stabilizing abilities of the quinone[82,84].

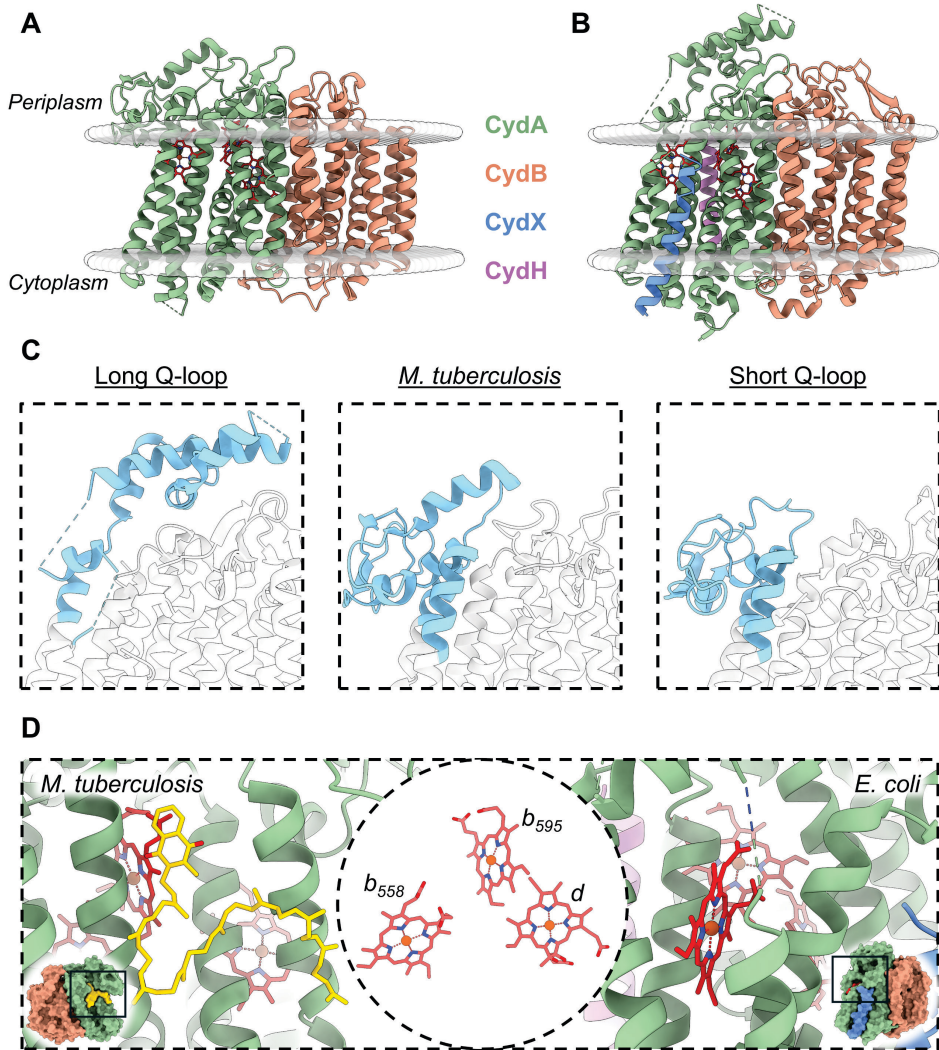


Figure 1.4: Structural features of the *bd* type oxidases. (A) Overview of the *M. tuberculosis* *bd* oxidase. (B) Overview of the *E. coli* *bd* oxidase. (C) structural differences between the different Q-loop families. (D) Proposed active sites in *M. tuberculosis* and *E. coli* cytochrome *bd*. The insert shows the triangular arrangement of the three heme groups.

Whereas the structure of cytochrome *bd* in *M. smegmatis* and *M. tuberculosis* is contained to the core components CydA and CydB[84], several different bacterial species have shown to have additional subunits. In *E. coli* for instance, the subunits CydX[83] (termed CydS in *G. thermodenitrificans* [81]) and CydH [83] (also termed CydY[86]) have been identified. Whereas CydX has been proposed to have a structural function[92], CydH is proposed to block a second, putative, oxygen channel reaching heme *b*₅₉₅[83]. It remains unclear whether cytochrome *bd* is otherwise functional and whether molecular oxygen can be reduced at two separate hemes in the absence of CydH. Potentially, however, oxygen can still be blocked from entering, regardless of CydH presence, by a conserved isoleucine residue[85]. Regardless, recent research has shown that cytochrome *bd* remain active despite knockout of CydH[93]. Interestingly, cytochrome *bd* from *M. tuberculosis* showed to have a bound menaquinone on the position of the oxygen channel that CydH covers in *E. coli* (Fig. 1.4D), which indicates a stabilizing function[90]. Conversely, cytochrome *bd* from *M. smegmatis* does not contain the bound menaquinol and has been proposed to have a second oxygen channel[84], although this is yet to be supported by biochemical analysis. The structure indicates that in mycobacteria, the open access to heme *b*₅₉₅ is used as an alternative electron pathway that bypasses heme *b*₅₅₈[82].

Maturation of cytochrome *bd* by the ABC transporter CydDC

The assembly and maturation of cytochrome *bd* oxidase requires the incorporation of the heme cofactors into the CydA subunit. The necessity of this step potentiates the indirect inhibition of cytochrome *bd* by impairing its maturation. This process is facilitated by the ATP-binding cassette (ABC) transporter CydDC, which exports heme and thiol compounds from the cytoplasm to the periplasm[94–96]. Genomically, the ABC transporter is located directly downstream of the cytochrome *bd* operon, providing concerted expression (Fig. 1.5A).

CydDC is composed of two subunits: CydD and CydC. It functions as a heterodimeric transporter that binds heme laterally from the membrane and translocates it across the inner membrane[94,97]. Structural studies have revealed the structural changes that CydDC undergoes during heme binding and transport, and highlight that mutations in CydD or CydC lead to defective cytochrome *bd* assembly, and thereby oxidase activity[94].

Similar phenotypes have been observed in *M. tuberculosis*, where CydDC is essential for cytochrome *bd* function and resistance to respiratory inhibitors[98]. The transporter also plays a role in maintaining redox balance by exporting glutathione and cysteine, which are important for oxidative stress tolerance[99].

How CydDC results in heme incorporation in CydA remains to be determined, although the current consensus points towards a presently unknown periplasmic partner protein that forms the missing link, shuttling the hemes between the two protein complexes (Fig. 1.5B). Given its central role in cytochrome *bd* maturation and stress resistance, CydDC represents a novel target for antimicrobial intervention. Inhibitors of CydDC could disrupt heme trafficking, thereby directly impairing cytochrome *bd* activity, and sensitizing bacteria to host defenses and antibiotics.

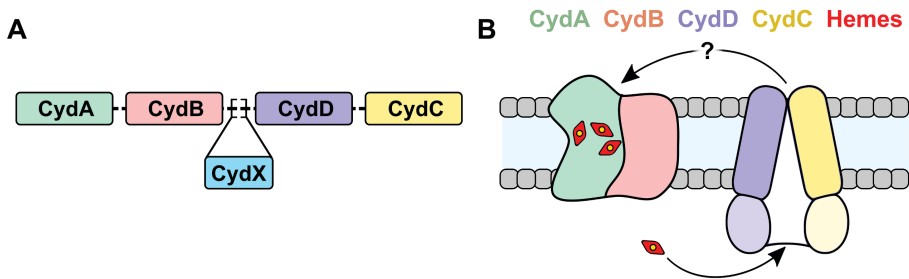


Figure 1.5: Heme maturation of cytochrome *bd* by the CydDC ABC transporter. (A) Genomic organization of the cytochrome *bd* - CydDC operon. CydX is only present in a subset of all species. (B) Cartoon of the proposed mechanism of heme incorporation into cytochrome *bd* by CydDC.

Drug discovery and inhibition strategies for cytochrome *bd*

Recent advances in structural biology and infection-adapted screening approaches have strengthened efforts to identify potent cytochrome *bd* inhibitors and accelerate their translation into clinical applications.

Several inhibitor classes have now established the druggability of cytochrome *bd*. Quinone analogs, such as Aurachin D derivatives, competitively bind the quinol oxidation site in CydA, preventing quinol binding and oxidation[100]. Synthetic molecules such as ND-011992 exhibit potent nanomolar activity against multiple species and demonstrate synergy with *bcc:aa₃* inhibitors, effectively collapsing respiratory flexibility[77,101]. Peptide-based inhibitors, exemplified by microcin J25[102,103], induces superoxide formation, offering an alternative scaffold distinct from classical small molecules (Fig. 1.6). Emerging approaches in drug discovery are addressing the challenges inherent in targeting the *bd* oxidase, which is dispensable under standard aerobic culture conditions[77]. Hypoxia-mimicking assays now replicate infection-relevant environments, selectively inducing *bd*-dependent respiration and enabling context-specific inhibitor screening[104,105]. High-throughput phenotypic platforms further integrate low-oxygen and nitric oxide stress to identify compounds active under host-like conditions[106].

Parallel advances in cryo-electron microscopy have yielded high-resolution structures of *bd* facilitating *in silico* docking and fragment-based drug design tailored to species-specific features of the Q-loop binding site[107,108]. Moreover, combination screens pairing *bd* inhibitors with *bcc:aa₃*-targeting agents have revealed synergistic interactions that accelerate bacterial killing and overcome tolerance[77,107,109].

Collectively, these advances exemplify the growing recognition of respiratory pathways as critical vulnerabilities in bacterial physiology. Targeting cytochrome *bd* not only exploits its infection-stage essentiality but also complements a broader shift toward respiratory chain inhibition as a strategy to eliminate both replicating and persistent bacterial populations.

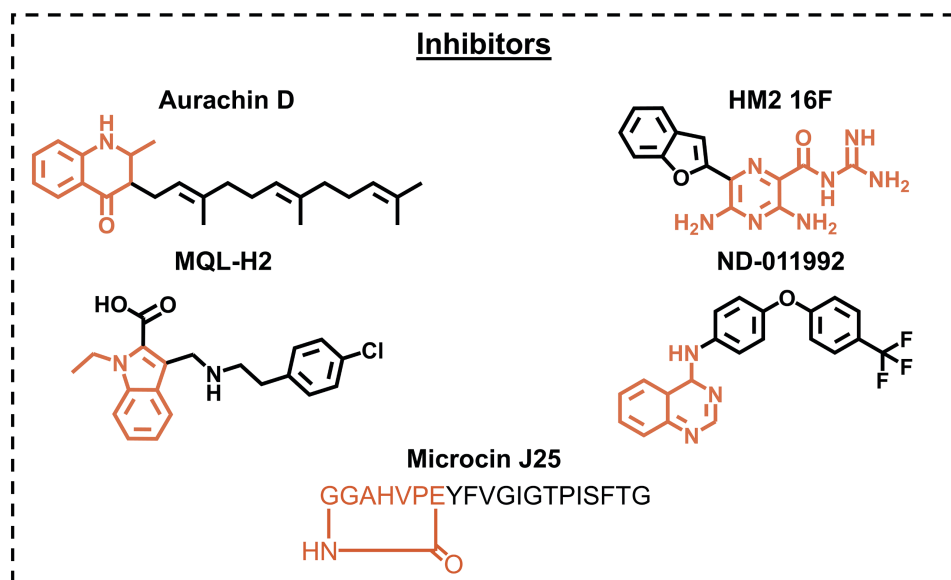


Figure 1.6: Overview of *bd*-type oxidases inhibitors, with the proposed interactive headgroup highlighted

By integrating structure-guided design, physiologically relevant screening, and rational combination therapies, *bd* inhibition represents a powerful addition to the emerging antimicrobial arsenal poised to address the escalating crisis of antibiotic resistance.

Outline of this thesis

The aim of this thesis is to study the molecular mechanism of cytochrome *bd*, with a specific aim of antibiotic development against *M. tuberculosis*. This chapter (**Chapter 1**) serves as a general introduction to the antibiotic resistance crisis and the respiratory chain as an antibiotic target space with an emphasis on the terminal oxidase cytochrome *bd*. **Chapter 2** describes the quinone substrate specificity of both *E. coli* and *M. tuberculosis* cytochrome *bd*. In addition, we show that *M. tuberculosis* contains a redox regulatory disulfide bond in the Q-loop, which allows activation upon disulfide oxidation. It is proposed that this disulfide bond allows enzyme-level regulation of cytochrome *bd* activity, providing a molecular basis for the quick respiratory rerouting found in *M. tuberculosis*. **Chapter 3** describes the molecular basis of quinone and inhibitor binding in *E. coli* cytochrome *bd*-I. We show that *E. coli* cytochrome *bd*-I exists in a dimeric form, stabilizing the Q-loop and allowing structural probing of the active site. This resulted in the first quinone bound structure of cytochrome *bd*, and showed that Aurachin D binding triggers structural rearrangement of the active site, closing the quinone binding pocket. **Chapter 4** describes a high throughput screening method to find cytochrome *bd* inhibitors. Using this method, over 10.000 compounds were screened resulting in a new fragment with a sub μM IC₅₀ value. This fragment showed to bind at the active site, thereby inhibiting cytochrome *bd* by competitively displacing the quinone. **Chapter 5** discusses the alternative hypothesis that heme transporter CydDC, essential for cytochrome *bd* maturation, incorporates heme by direct binding to the CydA subunit during cytochrome *bd* biogenesis. Lastly, **Chapter 6** provides the overall discussion about cytochrome *bd* and the contents of this thesis.

Chapter

2

Menaquinone-specific turnover by *Mycobacterium tuberculosis* cytochrome *bd* is redox regulated by the Q-loop disulfide bond

Cytochrome *bd* from *Mycobacterium tuberculosis* (*Mtbd*) is a menaquinol oxidase that has gained interest as an antibiotic target due to its importance in survival under infectious conditions. *Mtbd* contains a characteristic disulfide bond that has been hypothesized to allow for *Mtbd* activity regulation at the enzymatic level, possibly helping *M. tuberculosis* to rapidly adapt to the hostile environment of the phagosome. Here, the role of the disulfide bond and quinone specificity have been determined by reconstitution of a minimal respiratory chain and the single-particle cryo-EM structure in the disulfide-reduced form. *Mtbd* was shown to be specific for menaquinone, while regulation by reduction of the Q-loop disulfide bond decreased oxidase activity up to 90%. Structural analysis shows that a salt bridge unique to *Mtbd* keeps the Q-loop partially structured in its disulfide-reduced form, which could facilitate the rapid activation of *Mtbd* upon exposure to reactive oxygen species. We signify *Mtbd* as the first redox sensory terminal oxidase and propose that this helps *M. tuberculosis* in the defence against reactive oxygen species encountered during infection.

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Introduction

Cytochrome *bd* (cyt *bd*) is a terminal oxidase found in the respiratory chain across various bacterial phyla and couples the oxidation of quinols to the reduction of molecular oxygen to water[46,47,79]. The chemical protons released by quinol oxidation are separated from the proton uptake for oxygen reduction by the cytoplasmic membrane, generating a proton motive force required for ATP synthesis[79]. Compared to other terminal oxidases, cyt *bd* is distinguished by its resistance to inhibitors such as cyanide, high affinity for oxygen, and upregulation under microaerobic conditions[110].

Recent findings highlight the significance of cyt *bd* as a key survival factor for *Mycobacterium tuberculosis* during infection, particularly in the hostile granulomas where the bacteria reside[111]. Only upon inhibition of both terminal oxidases, Cyt *bcc:aa₃* and cyt *bd*, bactericidal effects are observed[43]. When *M. tuberculosis* cyt *bcc:aa₃* is inhibited and cyt *bd* (*Mtbd*) functions as the sole terminal oxidase, it can maintain a bacteriostatic state[43,77], and enhances resistance against numerous antibiotics, reactive oxygen species (ROS), and other toxic compounds[56–60,112]. This pivotal role during infection has identified *Mtbd* as a potential antibiotic target.

Despite this therapeutic potential, most of our current understanding of *Mtbd* at the molecular level is obtained from studies performed on homologous enzymes, such as *Corynebacterium glutamicum* cyt *bd* and the two *Escherichia coli* cyt *bd* isoforms, cyt *bd*-I (*Ecbd*) and cyt *bd*-II. Since these enzymes have been shown to have distinct structural features, number of subunits, and substrate binding pockets[80,82,83,91,113], caution is required when associating prior knowledge to *Mtbd*. An important characteristic feature of *Mtbd* is a disulfide bond constraining the N-terminal Q-loop domain near the quinone binding pocket[82]. It has been hypothesized that this disulfide bond provides a regulatory switch[82,114], and allows *M. tuberculosis* to instantly adapt to inhibition of its electron transport chain by rerouting electrons to *Mtbd*, suggesting regulation at the enzyme level in response to hostile conditions[31,115]. Redox-sensing disulfide bonds have been found in other proteins such as transcription factors, signalling enzymes, CO₂ reductases, and

peroxidases as a defence against oxidative stress[116,117]. However, whether such a regulatory function is conferred by the *Mtbd* Q-loop remains speculative, because its study has been hampered by the need for chemical reductants to reduce the quinone substrates in the standard activity assays for cyt *bd* oxidase. Given its significance as a potential antibiotic target, it is essential to uncover the main features of *Mtbd* enzyme kinetics.

Ecbd, *Mtbd*, and cyt *bd*s from other species use distinct quinone subtypes for turnover. The main bacterial quinone subtypes include menaquinone (MK), demethylmenaquinone (DMK), and ubiquinone (UQ), each differing in their quinone headgroup structure and corresponding redox potential (Fig. 2.1A)[18]. In addition, these quinones contain a hydrophobic isoprenoid tail made from a distinct number of isoprene units[118]. By convention, the number of isoprene units is indicated by the number (n) after the quinone, e.g. MK-n. *M. tuberculosis* primarily relies on MK-9[119], while *E. coli* demonstrates distinct functionalities for MK-8, DMK-8, and UQ-8[17,120]. Moreover, *E. coli* upregulates MK-8 during microaerobic conditions, coinciding with the upregulation of *Ecbd*, highlighting the adaptability of the *E. coli* quinone pool composition based on environmental requirements[18,121].

The interactions between quinones and cyt *bd* are intricate, as demonstrated by the distinct quinone binding sites in *Ecbd* and *Mtbd* (Fig. S2.1). While *Ecbd* has a proposed quinone binding pocket at the Q-loop of subunit CydA, transferring electrons to closely located heme *b*₅₅₈[83], *Mtbd* shows quinone binding at the back of CydA, which is hypothesized to transfer electrons directly to heme *b*₅₉₅, bypassing the initial heme *b*₅₅₈ (Fig. 2.1B)[82]. Additional structural quinone binding pockets are found in CydB, lacking participation in quinol oxidation[83,91]. The exact position of the *Ecbd* and *Mtbd* turnover sites remain to be determined.

Most studies make use of detergent solubilized cyt *bd* with water soluble quinone analogues. The use of detergent micelles, although convenient for studying cyt *bd* in solution, introduces an artificial environment that deviates from the native conditions.

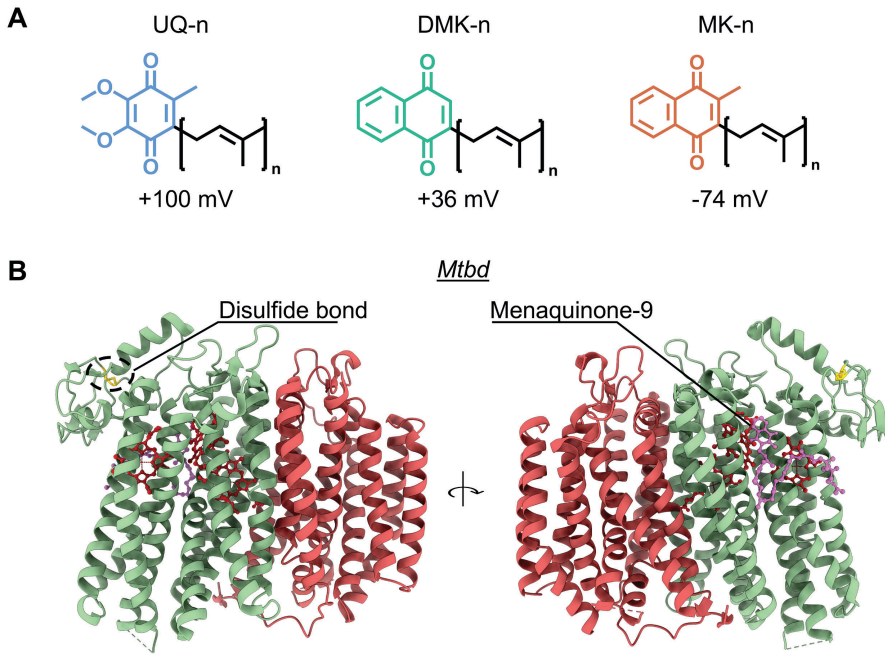


Figure 2.1. Overview of quinone subtypes and structural features of *Mtbd* (A) The structure of quinone analogues used in this study with the number of isoprenoid units (n) and their reduction potentials[18]. (B) Oxidized structure of *Mtbd*(PDB: 7NKZ)[82], CydA (Green), CydB (Red), indicating the Q-loop disulfide bond and MK-9 (Purple).

These detergent micelles can alter the conformation, partially unfold proteins, and strip away vital bound or annular lipids, leading to a loss of native mechanistic function and kinetic rates[122,123]. Additionally, using water soluble quinone analogues can significantly alter enzyme kinetics as the quinone isoprenoid tail has been shown to critically affect respiratory efficiency in *M. tuberculosis in vivo* [124]. The evaluation of cyt *bd* quinone interactions and disulfide regulation thus requires a native membrane environment and natural quinone substrates to mimic native conditions.

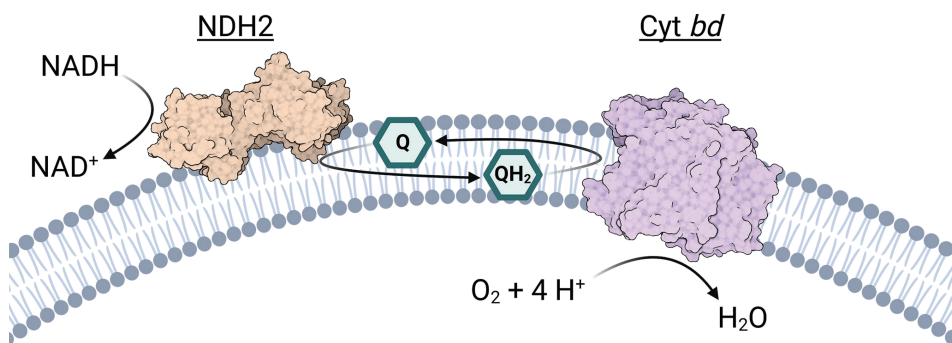


Figure 2.2. A minimal respiratory chain proteoliposomal system to interrogate specific interactions between quinones and *cyt bd*. This proteoliposomal system can also be used in detergent.

To study the specific interaction of *Ecbd* and *Mtbd* with the different quinones and interrogate the role of the *Mtbd* Q-loop disulfide bond, a minimal respiratory chain was constructed in which *cyt bd* is combined with a NADH-quinone oxidoreductase *Caldalkalibacillus thermarum* NDH-2 (Fig. 2.2). This approach was inspired by an approach in which complex 1 was studied using an alternative oxidase[125]. Enzyme kinetics reveal that water soluble quinone analogues lead to vast overestimations of *cyt bd* turnover rates, highlighting the need for precise experimental design. Furthermore, in contrast to *Ecbd*, *Mtbd* is subject to substrate inhibition by MK at physiologically relevant concentrations. Additionally, we signify *Mtbd* as the first terminal oxidase with a redox sensory disulfide bond. Cryo-EM analysis of the disulfide reduced *Mtbd* revealed a new interaction between Arg290 and Glu263 that might explain why *Mtbd* is readily reactivated upon reformation of the disulfide bond, for instance by exposure to H_2O_2 . We propose that redox regulation enables *Mtbd* to quickly adapt its activity to environmental redox pressures in defence against ROS encountered by *M. tuberculosis* during infection.

Results

Cyt bd oxygen consumption kinetics with water soluble quinone analogues

Ecbd, *Mtbd*, and NDH-2 were expressed and purified via affinity and size exclusion chromatography (Fig. S2.2). A minimal respiratory chain was constructed with an excess of NADH and NDH-2, such that the quinone pool remains fully reduced. Control studies with varying amounts of NDH₂ confirmed that cyt *bd* activity was limiting. The enzyme activity of *Ecbd* and *Mtbd*, comparing different quinone substrates, was determined by monitoring oxygen consumption in a Clark electrode. The activity was first measured in detergent conditions using water-soluble quinone analogues. Previous reports indicate that quinols, especially menaquinols, auto-oxidize under aerobic conditions in a concentration dependent manner[126]. To account and correct for the quinol (Q) auto-oxidation rate, the oxygen consumption was quantified for each assay before the addition of cyt *bd* (Fig. 2.3A). *Ecbd* displayed standard Michaelis-Menten kinetics with a 6-fold lower K_m for MK-1 compared to UQ-1 (Fig. 2.3B, Table 2.1). K_{cat} values for the three quinone analogues were similar (Table 2.1) ranging from 736 to 1033 Q/s (Table 2.1).

The kinetic profile of *Mtbd* was profoundly different than that of *Ecbd* (Fig. 2.3C). *Mtbd* exclusively turns over MK-1 and is subject to substrate inhibition. While substrate inhibition has been observed in other oxidases[91,127,128], its molecular mechanism remains undefined. Multivariate fits of the observed substrate inhibition profile indicated a large interdependency of parameters (K_m ; V_{max} ; inhibition constant, K_i) preventing the determination of the kinetic parameters for MK-1 oxidation in spite of the good fit (Fig. 2.3C). Importantly, *Mtbd* failed to initiate turnover with either DMK-1 or UQ-1. To investigate whether this inactivity resulted from a lack of substrate binding or a thermodynamic barrier limiting quinol oxidation, the effect of UQ-1 and DMK-1 on *Mtbd* activity was assessed. It was reasoned that DMK-1 and UQ-1 will present as competitive inhibitors if they bind to the same active-site

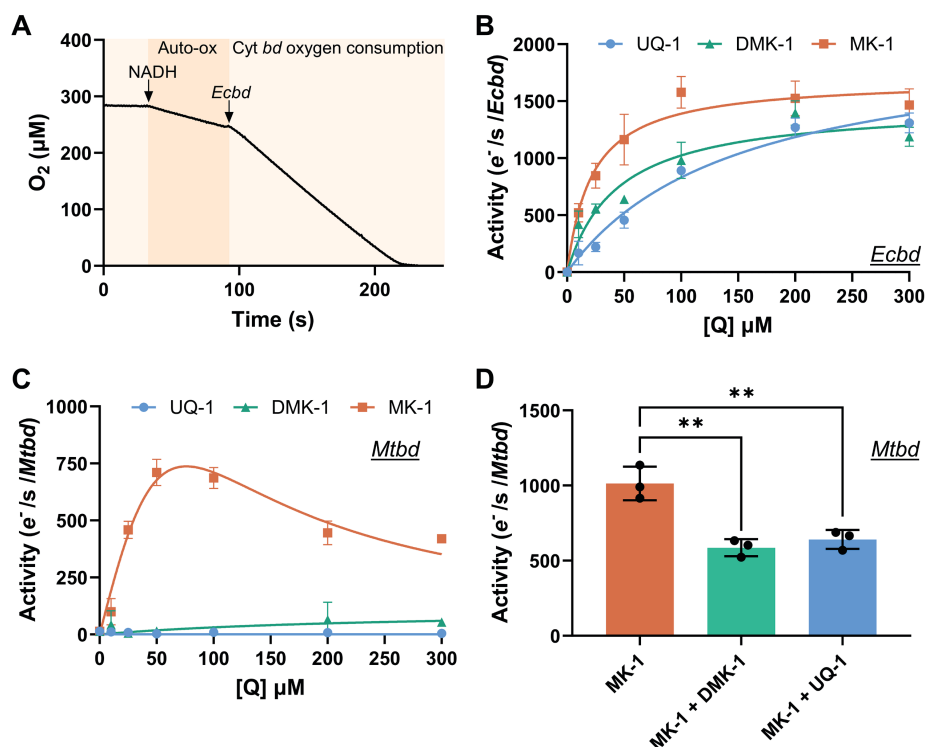


Figure 2.3. Oxygen consumption kinetics of detergent solubilized cyt *bd* with different quinone substrates. (A) Example of an oxygen consumption trace with MK-1 (50 μM) auto-oxidation (Auto-ox) and *Ecbd* oxygen consumption indicated. (B) *Ecbd* and (C) *Mtbdb* oxygen consumption kinetics using the assay described in the text at 20°C. Lines represent Michaelis-Menten fits without (*Ecbd*, see Table 1 for parameter values) or with (*Mtbdb*) substrate inhibition. (D) *Mtbdb* activity with 50 μM MK-1 in the absence or presence of either DMK-1 or UQ-1 (both 300 μM). Data is represented as the average and standard deviation of triplicate measurements of two different protein preparations ($n=3$). Significance is tested using an ANOVA with a Dunnett post-hoc test in GraphPad Prism. ** $p < 0.01$

pocket as MK-1. Upon addition of an excess DMK-1 or UQ-1, oxygen consumption activity was indeed significantly inhibited, suggesting that DMK-1 and UQ-1 bind to the active site, but are unable to be oxidized by *Mtbdb* (Fig. 2.3D). This inhibitory effect of DMK-1 was shown to be concentration dependent and indicates a lower overall affinity for DMK-1 than MK-1 (Fig. S2.3).

Table 2.1. Kinetic parameters for the oxidation of UQ-1, DMK-1, MK-1 by *Ecbd*^a

$K_{cat} (Q\ s^{-1})^b$			$K_m (\mu M)^c$			$K_{cat}/K_m (x10^{-6}\ M^{-1}\ s^{-1})^d$		
UQ-1	DMK-1	MK-1	UQ-1	DMK-1	MK-1	UQ-1	DMK-1	MK-1
1033 ±	736 ±	846 ±	149 ±	44 ±	22 ±	6.9 ±	16.7 ±	38.5 ±
85	51	38	26	10	4	1.3	4.0	7.2

^a K_{cat} values are represented in quinones per second ($2\ e^-/Q$). Oxygen consumption measurements were performed in 50 mM MOPS, 150 mM NaCl, 0.005% LMNG, pH 7.0 (n=3). Data represents best Michaelis-Menten fit values with the SEM. ^b UQ-1 vs DMK-1 *, ^c UQ-1 vs DMK **, UQ-1 vs MK-1 ***, DMK-1 vs MK-1 * ^d Errors represent the propagated SEM. * p < 0.05, ** p < 0.001, *** p < 0.0001

Ecbd and *Mtbd* oxygen consumption in proteoliposomes

To determine whether the observed substrate specificities in detergent are indicative of native conditions, the same principles were applied to a proteoliposomal system (Fig. 2.2) where cyt *bd* can be studied in a native-like lipid environment using the long isoprenoid quinones MK-9 and UQ-10.

Mtbd and *Ecbd* were reconstituted in liposomes containing 0.25% - 1% (molar; quinone/lipid) of the desired quinone (MK-9 or UQ-10) to achieve maximum catalytic rates. The *Mtbd* or *Ecbd* concentration after reconstitution was quantified using the Soret band of a detergent solubilized sample. Native quinone concentrations in bacterial membranes are estimated between 0.3 - 1.5% (see SI). Furthermore, 1% MK-9 or UQ-10 approximately coincides with a membrane concentration in the order of 10-15 mM (see SI), well above the K_m determined for *Ecbd*. We note however, that for the water soluble quinone analogues an unknown equilibrium exists between quinones in solution and quinones in detergent micelles, making the two systems difficult to directly compare. Consistent with the UQ-1/MK-1 kinetics, *Ecbd* showed a marginally higher catalytic rate with UQ-10 than MK-9 (Fig. 2.4A). Additionally, *Mtbd* displayed the same specificity for MK turnover, confirming the quinone analogue data (Fig. 2.4B). Substrate inhibition for *Mtbd* was confirmed by increasing the MK concentration from 0.25% to 1%, resulting in a significant decrease in enzyme activity.

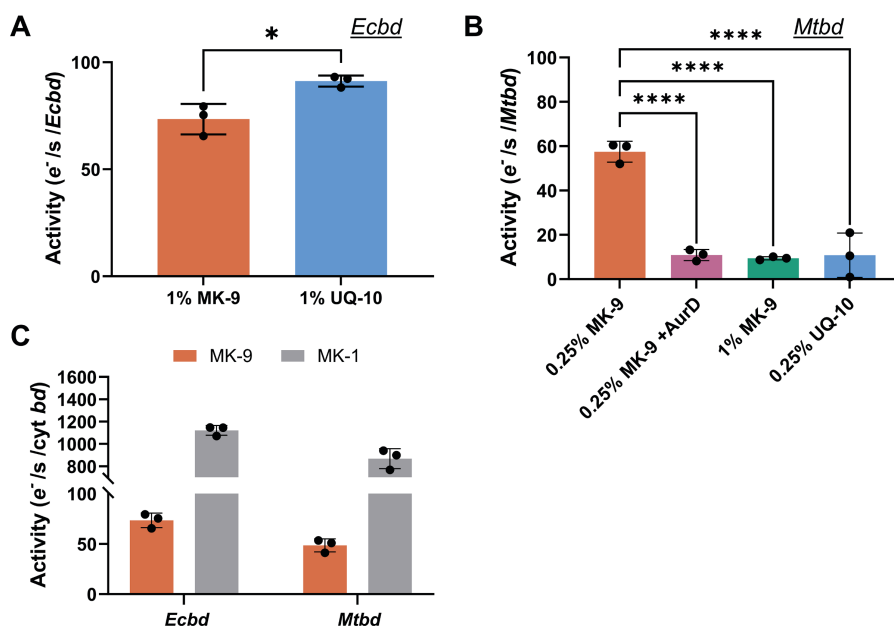


Figure 2.4. Quinone substrate specificity of *Ecbd* and *MtbD* in a native lipid environment. (A) Catalytic rates of *Ecbd* (20 nM) proteoliposomes containing 1% (molar; quinone/lipid) of either UQ-10 or MK-9. (B) Catalytic rates of *MtbD* (20 nM) with different concentrations of either UQ-10 or MK-9, and inhibited by AurD. (C) Reconstituted *Ecbd* and *MtbD* activity with native MK-9 (*Ecbd* 1% (molar; quinone/lipid) MK-9, *MtbD* 0.25% (molar; quinone/lipid) MK-9) versus MK-1 analog (50 μ M). Data is represented as the average and standard deviation from three individual proteoliposome reconstitutions. Significance is tested using an ANOVA with a Dunnett post-hoc test in GraphPad Prism. * $p < 0.05$, **** $p < 0.0001$

To study the effect of the quinone isoprenoid chain on cyt *bd* activity, *Ecbd* and *MtbD* were reconstituted in proteoliposomes, and turnover was measured with either membrane-embedded MK-9 or water-soluble MK-1. Surprisingly, the isoprenoid chain length of the quinone was shown to have a significant effect on the maximum catalytic rate of cyt *bd* (Fig. 2.4C). Utilizing the natural membranous quinones resulted in tenfold lower catalytic rates, emphasizing the role of the isoprenoid tail in substrate binding, via either diffusion or binding kinetics. This highlights that previously reported catalytic rates using quinone analogues can substantially overestimate the catalytic rates achieved *in vivo* [83,91,123].

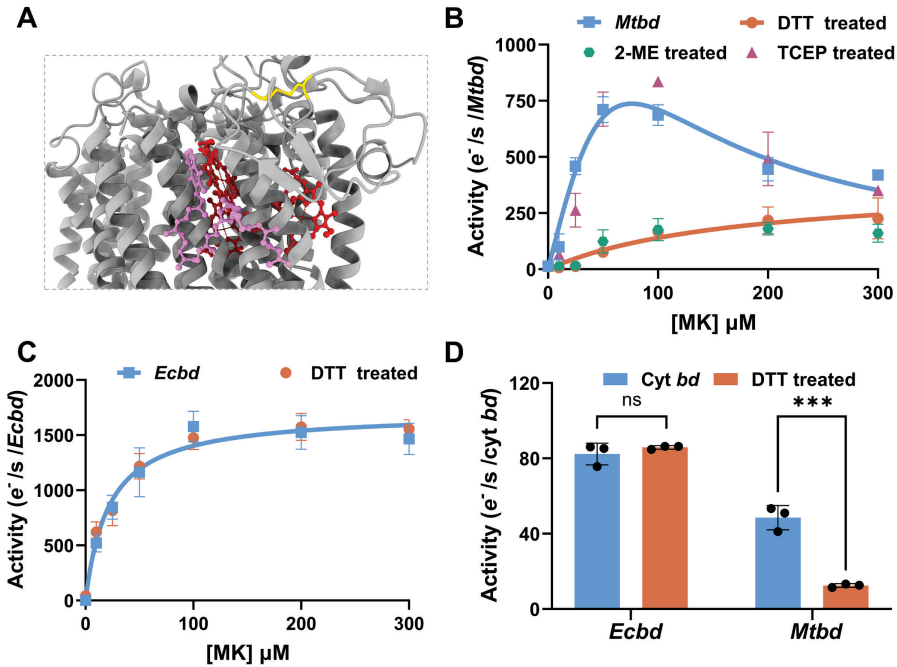


Figure 2.5. *MtbD* regulation by the Q-loop disulfide bond. (A) Cryo-EM structure of *MtbD* (PDB: 7NKZ)[82] with heme groups (red) MK-9 (blue) and unique Q-loop disulfide bond (yellow). The structure was analyzed using ChimeraX[129] (B) *MtbD* MK-1 kinetics in detergent micelles before and after treatment with TCEP (Purple), DTT(Orange), or 2-ME(Green). The reductants were maintained in the assay buffer to prevent the reformation of the disulfide bond. Significant inhibition of oxidase activity is observed after DTT or 2-ME treatment. (C) *Ecbd* MK-1 kinetics in detergent micelles before and after DTT treatment. No difference is observed. (D) Oxidase activity of proteoliposomes containing *MtbD* (0.25% MK-9) or *Ecbd* (1% MK-9) before and after DTT treatment. Significance is tested using an unpaired t-test in GraphPad Prism. *** $p < 0.01$

MtbD activity is regulated by a unique Q-loop disulfide bond

MtbD is characterized by a unique disulfide bond that was hypothesized to reduce the flexibility of the substrate binding Q-loop (Fig. 2.5A) and thereby confer a regulatory role for enzyme activity[82]. Previous studies examining this mechanism faced limitations, as they relied on chemical reductants such as DTT to reduce the quinone pool, which inadvertently affects the disulfide

bond itself. Using NDH-2 to enzymatically reduce the quinone pool enables the comparison of *Mtbd* activity before and after incubation with chemical reductants to reduce the disulfide bond and probe its effects on *Mtbd* activity.

Incubation of *Mtbd* with either DTT or 2-mercaptoethanol (2-ME) showed a significant reduction in oxidase activity (Fig. 2.5B). In addition to the decrease in *Mtbd* activity, the disulfide bond breakage eliminates MK substrate inhibition, strongly suggesting the reduction of the disulfide bond changes the structure or dynamics of the quinone binding pocket. Unexpectedly, preincubation with the reductant tris(2-carboxyethyl)phosphine (TCEP) did not decrease *Mtbd* activity, indicating that interactions with the disulfide bond might be hindered by surface accessibility or charge repulsion, as confirmed by non-reducing SDS-page (Fig. S2.4). To verify that the chemical reductants only impact the disulfide bond and no other aspects of *Mtbd*, the same measurements were performed with *Ecbd*, which lacks the Q-loop disulfide bond (Fig. 2.5C). This confirms that there is no difference in *Ecbd* activity after treatment with DTT and consolidates the conclusion that the decrease in the activity of *Mtbd* stems from the reduction of the Q-loop disulfide bond.

To ensure that the observed effects are not artifacts of the detergent environment, the measurements were repeated in our proteoliposomal system and corrected for liposomal MK-9 auto-oxidation (Fig. S2.4E). Again, the *Ecbd* activity remains unchanged by the DTT treatment, while the *Mtbd* activity decreased by approximately 75% (Fig. 2.5D).

Structural changes induced by the reduction of the Q-loop disulfide bond

To investigate the structural changes induced by the reducing conditions on *Mtbd*, single-particle cryo-electron microscopy (cryo-EM) analysis was performed. The reduced *Mtbd* structure was solved at a global resolution of 3.1 Å. This includes a 3–5 Å micelle, with the protein resolved at a resolution of 2.3–2.7 Å (Fig. S2.5). The reduced structure reveals notable structural changes in the Q-loop region and previously unstructured helix-connecting loops, as well as a

phosphatidylethanolamine (PE) lipid above the menaquinone and two cardiolipin molecules (Fig. 2.6A).

The PE lipid is positioned on top of the MK-9 binding pocket, stabilized via hydrogen bonding with Arg8^{CydA} (Fig. S2.6A). Interestingly, Arg8^{CydA} has also been suggested as one of the stabilizing interactions binding to the menaquinone headgroup[82]. One cardiolipin phospholipid is bound to CydB, located in a similar position as the cardiolipin in Cyt *bd*-II oxidase from *E. coli* (PDB: 7OY2)[91]. In addition, we assigned a second cardiolipin that is positioned superior to the first cardiolipin, occupying the same hydrophobic groove (Fig. S2.6B). Interestingly, mutations in this groove have been shown to affect *Mtbd* oxidase activity, further highlighting the stimulatory effect of cardiolipin[123,130].

The overall root-mean-square deviation (RMSD) of 0.39 Å between the oxidized (PDB: 7NKZ[82]) and the disulfide reduced structure indicates relatively minor global structural changes. However, increased local flexibility is observed for the *Mtbd* Q-loop residues 279 to 288, as evidenced by a weak electron density, suggesting a disordered conformation in the reduced state (Fig. 2.6B). To not bias our view of the Q-loop, we did not perform any micelle subtraction, as this could potentially remove electron density from the Q-loop region that is in close proximity to the micelle, leading to an inaccurate interpretation of the loop's structure and flexibility. Nevertheless, attempts to confidently model the Q-loop residues 279^{CydA} to 288^{CydA} were unsuccessful. The unstructured region within the Q-loop includes Cys285^{CydA}, which forms a disulfide bond with Cys266^{CydA} under oxidized conditions. The especially weak electron density compared to the entire N-terminal region of the Q-loop further confirms the breakage of this disulfide bond in the reduced structure.

In contrast to the homologous cyt *bd* oxidases in *M. smegmatis* and *C. glutamicum*, where the entire N-terminal region of the Q-loop is disordered[80,84], the *Mtbd* Q-loop remains partially structured. Although *bd* oxidases from *M. smegmatis* and *C. glutamicum* contain the conserved cysteine residues, they do possess a disulfide bond under the conditions

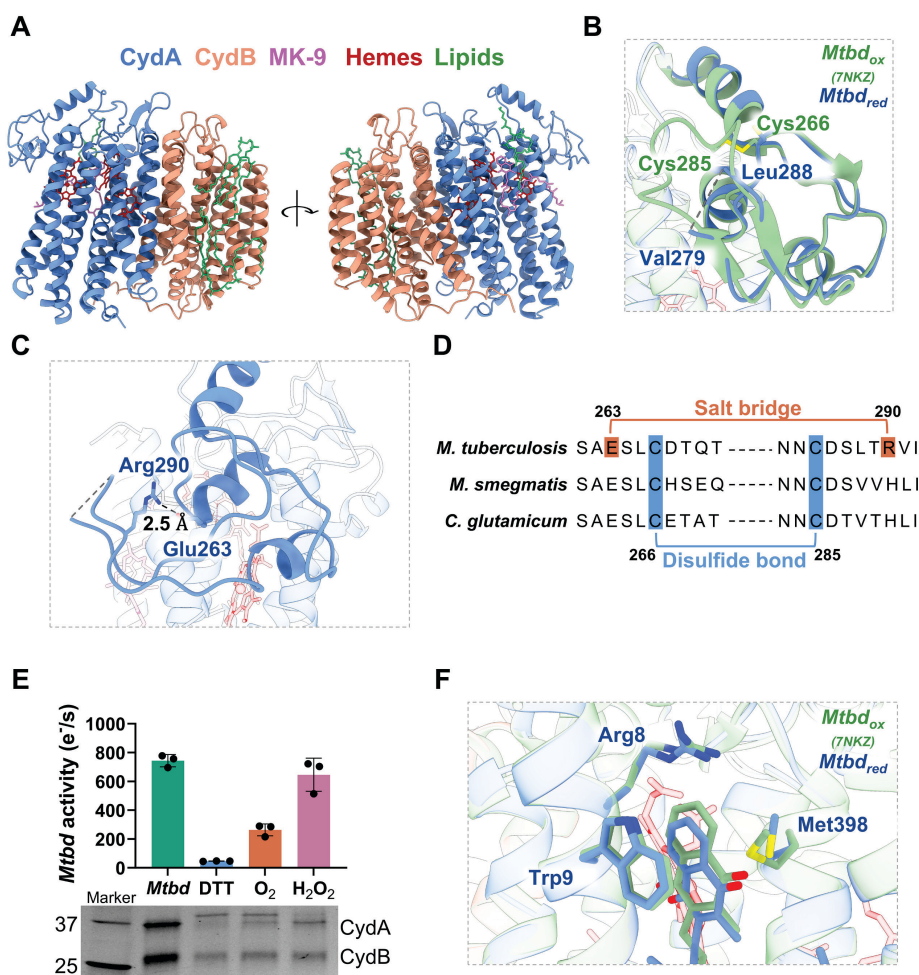


Figure 2.6. Structural changes induced by reduction of the *MtbD* Q-loop disulfide bond. (A) Cryo-EM structure of disulfide reduced *MtbD*. (B) Increased local flexibility in the Q-loop after disulfide reduction, resulting in a density loss from V279^{CydA} to L288^{CydA}. (C) A salt bridge between Arg290^{CydA} and Glu263^{CydA} stabilizes the N terminal part of the Q-loop (D) Sequence alignment of the *M. tuberculosis*, *M. smegmatis*, and *C. glutamicum* cyt *bd* Q-loop, showing the unique Arg290^{CydA} participating in the stabilizing salt bridge. (E) *MtbD* kinetics after disulfide reduction. Exposure to ambient oxygen or 50 μ M H₂O₂ for 30 min results in a regain of oxidase activity, together with a reformation of the bond. (F) Local changes in the MK9 binding pocket. Slight conformational changes are observed in Met398^{CydA} and MK-9.

tested[80,84]. This suggests that the *Mtbd* Q-loop possesses additional stabilizing interactions that help maintain a semi-structured conformation even after disulfide reduction. This could be attributed to the presence of Arg290^{CydA} in *Mtbd*, which forms a salt bridge with Glu263^{CydA}, thereby stabilizing the remaining Q-loop (Fig. 2.6C). Arg290^{CydA} is unique to *Mtbd*, as the homologous cyt *bd* oxidases in *M. smegmatis* and *C. glutamicum* possess a histidine in the corresponding position (Fig. 2.6D), and could explain the fully unstructured N-terminal Q-loop observed in these homologues.

The salt bridge might help to maintain spatial proximity between Cys266^{CydA} and Cys285^{CydA} and facilitate the eventual reformation of the disulfide bond, suggesting a potential regulatory mechanism for *Mtbd* activity. Indeed, exposure of the reduced *Mtbd* to ambient oxygen concentrations resulted in a ~30% recovery of activity, followed by near full recovery after exposure to 50 μ M H₂O₂. The activity regain was accompanied by reformation of the disulfide bond, as confirmed by non-reducing SDS-PAGE (Fig. 2.6E).

Despite the local changes in the Q-loop upon disulfide reduction, MK-9 remains tightly bound near Trp9. A minor shift of Met397^{CydA} is observed, increasing its distance to the MK-9 headgroup from 3.6 to 3.8 Å (Fig. 2.6F). Additionally, we observe a slight shift of MK-9 out of the binding pocket, increasing its distance to Trp9^{CydA} from 3.7 to 3.9 Å.

Importantly, however, the minor structural changes around MK-9 likely cannot account for the observed decrease in enzyme activity. We hypothesize that the observed MK-9 plays a structural role rather than serving as the substrate, which is supported by the cryo-EM density maps of *Mtbd* in the presence of the potent quinone-analogue inhibitors AurD[82] and AD3-11[100] (EMD-12532 and EMD-12533)[82]. Despite a vast molar excess of these inhibitors, the MK-9 density remains unaltered, suggesting that the inhibitors occupy a different binding pocket while *Mtbd* is fully inhibited[82]. This indicates the existence of a separate substrate binding site, potentially between heme *b*₅₅₈ and the Q-loop, as hypothesized in other cyt *bd* oxidases[83,91,113].

Discussion

Cyt *bd* is a critical part of the prokaryotic respiratory chain to maintain ATP regeneration under microaerobic conditions[79]. *Mtbd* has been highlighted for its essentiality under oxygen limiting conditions, and its related interest as an antibiotic target against *M. tuberculosis* [111]. Most current knowledge, however, comes from mechanistic studies on homologous enzymes such as *Ecbd* and *C. glutamicum* cyt *bd*, which have diverse structural features, such as a lack of the characteristic *Mtbd*Q-loop disulfide bond[80,82,83,113].

The minimal respiratory chain, both in detergent and liposomes, unambiguously indicates that *Ecbd* can oxidize UQ, MK, and DMK. The affinity of *Ecbd* for UQ-1 (K_m : $149 \pm 26 \mu\text{M}$) is in line with previously reported values[123,127,128,131,132], while the catalytic rate at $200 \mu\text{M}$ UQ-1 ($1268 \pm 81 e^- s^{-1}$), is higher than literature reported values ($889 \pm 30 e^- s^{-1}$)[83] and is attributed to the difference in the detergent environment and assay conditions. The fact we see activity with MK is in direct contrast with an earlier study that suggests that *Ecbd* cannot oxidize MK[91]. We note that in this earlier study, MK was reduced by DTT. Indeed, when we repeated the assay with DTT instead of NADH/NDH-2 to reduce MK, we did not observe any activity. Here, we propose that although DTT is a good reductant for UQ and hence a good reductant to measure oxygen-reducing activity by cyt *bd*, DTT might be a poor reductant and rate-limiting when the assay is performed with MK. The observation that *Ecbd* can oxidize MK and DMK is consistent with the *in vivo* upregulation of MK and DMK during microaerobic respiration, conditions that also lead to increased expression of *Ecbd*[17,121,133].

The catalytic rates of both *Ecbd* and *Mtbd* were approximately ten-fold lower when using the natural lipophilic quinones in comparison to their often-used water-soluble analogues. Similar effects have been shown before, where small differences in quinone isoprenoid units altered turnover rate[134,135]. This highlights the need for physiologically relevant assay conditions to obtain appropriate kinetic rates and inhibition values. The difference in activity when using the native quinones could be explained by a difference in binding or diffusion kinetics, as many of the quinone-bound oxidase structures have

shown that the quinone isoprenoid tails play a significant role in substrate-enzyme interactions[82,136], and longer chains might result in slower substrate binding and release[28].

Mtbd exclusively catalyzes MK oxidation, while competition experiments suggest that UQ and DMK can bind to the same active site. A similar phenomenon was shown for cyt *bd* from *C. glutamicum*, and cytochrome *bcc* from *M. tuberculosis*, which also lacked oxidase activity using UQ as a substrate[126,134]. We postulate that this lack of UQ-1 turnover in *C. glutamicum* cyt *bd* is caused by a thermodynamic barrier between two-electron oxidation of UQ (100 mV) and the single-electron acceptor heme *b* (102 mV)[80,137]. Similar suggestions have been made for mycobacterial cytochrome *bcc*, where the heme redox potentials are too low to allow the oxidation of UQ[126,138]. Although the heme potentials for *Mtbd* are unknown, we postulate that the same thermodynamic barrier applies here.

Mtbd was unable to oxidize DMK, which is similar in structure to MK and has a reduction potential that lies in between that of MK and UQ. In *M. tuberculosis*, DMK is a precursor in MK biosynthesis. Small molecule inhibitors of MenG, the enzyme that converts DMK into MK, were bactericidal in *M. tuberculosis* [139,140]. This substantiates the inability of *Mtbd* to turn over DMK and highlights the tight specificity between the quinone pool and *Mtbd*. Additionally, this emphasizes that inhibition of MK biosynthesis is a valid antibiotic strategy that targets the fundamental electron transfer steps between the respiratory chain components.

In contrast to our observation reported here, *Mtbd* expressed in *E. coli* has been reported to oxidize UQ when assayed in crude membrane extracts[135]. Potentially, the observed UQ oxidation was caused by interfering oxidases present in the membrane extracts, such as *E. coli* cyt *bd*-II. Further studies would be required to determine why the assay in crude *E. coli* membrane extracts gives rise to different results than *Mtbd* purified from *M. smegmatis*, as reported here.

Based on our structural analysis of *Mtbd*, we suggest that the observed MK-9 in the binding pocket adjacent to Trp9^{Cyda} confers a structural role rather than

acting as the substrate under the conditions tested. The published structure[82] and our structure of the disulfide reduced *Mtbd*, do not indicate an alternative quinone substrate binding site and it is therefore critical to uncover and prove the cyt *bd* quinone substrate binding pocket in the future. While the location of the *Mtbd* catalytic site remains speculative, studies on the homologues *Ecbd*[83] and *Ecbd-II*[87], have indicated binding at the N-terminal part of the Q-loop, stabilized by Asp239^{CydA} near heme *b*₅₅₈. Whether these results translate to *Mtbd*, remains unclear. The binding of a quinone substrate might be correlated to structural changes in which the disulfide bond has a more pronounced role, possibly explaining the role of the disulfide bond on the activity of *Mtbd*.

The *Mtbd* disulfide bond is unique among the currently studied cyt *bd* oxidases. Despite the conservation of these cysteine residues in *M. smegmatis* [84] and *C. glutamicum* [80] cyt *bd*, these proteins do not show a formed cysteine bond in their structures under the conditions tested. Here we show that the chemical reduction of the *Mtbd* disulfide bond results in a significant decrease in oxidase activity. Molecular dynamics studies previously suggested that reduction of the *Mtbd* disulfide bond greatly increases Q-loop flexibility[82]. In contrast, the structure of the disulfide reduced *Mtbd* indicates only minor structural changes outside of the Q-loop, while the Q-loop itself also remains mostly structured except for residues 279^{CydA} to 288^{CydA}. In the structure of the disulfide reduced state, the cysteines remain in relatively close proximity, possibly aided by a salt bridge between Arg290^{CydA} and Glu263^{CydA}, which could explain why the disulfide bond in *Mtbd* can fully (re)form upon exposure to hydrogen peroxide.

This redox sensory role of the Q-loop disulfide bond might contribute to a rapid adaptation of *M. tuberculosis* when exposed to the hostile conditions encountered during infection. We hypothesize that *Mtbd* is expressed and maintained in the disulfide reduced state, allowing for efficient respiration using the cytochrome *bcc:aa*₃ branch. This is in line with our results, showing that *Mtbd* maintains mostly reduced upon exposure to ambient oxygen. This primes *Mtbd* to be activated upon exposure to the ROS encountered during phagocytosis. Our hypothesis could also explain the instantaneous increase in

respiration by *M. tuberculosis* upon inhibition of the *bcc:aa₃* supercomplex[31], which is shown to also increase periplasmic ROS. The disulfide bond of *Mtbd* might be further regulated by the large network of proteins and small molecules controlling *M. tuberculosis* redox homeostasis [131,132]. How this regulatory mechanism would play into the larger metabolic response of *M. tuberculosis* to ROS remains unknown, especially in the rapid time frames *Mtbd* shows to be activated[115].

To our knowledge, this is the first proof of a terminal oxidase under the regulation of a redox sensing disulfide bond. Enzyme-level regulation of *Mtbd* with different redox pressures might be important for *M. tuberculosis* to reroute electrons from cytochrome *bcc:aa₃* to *Mtbd*, rapidly adapting to environmental changes. Potentially, this aids in the survival in ROS rich environments during infection[141,142], with the suggested role of *Mtbd* in hydrogen peroxide resistance[143]. In addition, *Ecbd* has been shown to actively detoxify the cellular environment from hydrogen peroxide[49] and peroxynitrite[53], indicating potential additional roles for *Mtbd* as a survival factor for *M. tuberculosis*. Future research should focus on the physiological role and regulation of the *Mtbd* disulfide bond and its influence on *M. tuberculosis* survival. Additional investigations of the interplay between *Mtbd* and environmental redox pressures, especially regarding its potential detoxifying role as seen with *Ecbd* [49], will offer valuable insights into the adaptive role of *Mtbd* in the defence against ROS and antibiotic compounds encountered during infection.

Experimental procedures

Expression and purification of E. coli cytochrome bd-I

The expression of *E. coli* cytochrome *bd-I* (*Ecbd*) was performed as previously described[90], with slight modifications. Briefly, MB43 cells[144,145] transformed with pET17b-CydABX-linkerstreptag were grown overnight in LB with 100 µg/mL ampicillin (250 RPM, 37°C). The culture was diluted to OD ~0.1 in LB ampicillin and grown to OD ~0.4. Expression was induced by the addition of

0.45 mM IPTG and carried out until OD ~2.0. Cells were harvested by centrifugation (6371 rcf, 20 min, 4°C) and resuspended in 50 mM MOPS, pH 7.4, 100 mM NaCl, cOmplete™ EDTA-free Protease Inhibitor (ROCHE), at 1 g wet cells per 5 mL buffer. Cells were disrupted by a single pass through a Stansted pressure cell homogeniser (270 MPa). Unbroken cells were pelleted and discarded by centrifugation (10.000 rcf, 20 min, 4°C). Crude membranes were isolated by ultra-centrifugation (200.000 rcf, 1h, 4°C) and resuspended to 10 mg/mL protein concentration in 50 mM MOPS, 100 mM NaCl, pH 7.4. Detergent extraction of the membrane proteins was performed by incubation with 0.5% Lauryl maltose neopentyl glycol (LMNG) for 1 hour at 4°C with gentle mixing. Insoluble material was pelleted and discarded by ultra-centrifugation (200.000 rcf, 30 min, 4°C) followed by application of the soluble fraction to a StrepTrap HP column (Cytiva) at 1 mL/min. To remove unbound proteins, the column was washed with 50 mM sodium phosphate, 300 mM NaCl, 0.005% LMNG, pH 8.0. Elution was performed by the addition of 50 mM sodium phosphate, 300 mM NaCl, 2.5 mM desthiobiotin, 0.005% LMNG, pH 8.0, after which purity was confirmed by SDS-page. Fractions containing pure *E. coli* cytochrome *bd*-I were pooled, concentrated, and stored at -80°C until further use.

Expression and purification M. tuberculosis cytochrome bd

M. tuberculosis cytochrome *bd* (*Mtbd*) expression and purification were performed as previously described with slight modifications [82]. *M. smegmatis* MC² 155 ΔCydAB[146] was transformed with the pLHCyd plasmid using electroporation with an Eppendorf eporator (2.5 kV). Positive transformants were selected by plating the cells on LB agar with 50 µg/mL hygromycin. A starter culture was inoculated in LB-hygromycin and grown for 72 hours (250 RPM, 37°C). The culture was diluted 1:100 and grown for an additional 72 hours (200 RPM, 37°C) until the cells were harvested (6371 rcf, 20 min, 4°C) and resuspended in a 5-fold volume of 50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 0.05% tween-80, cOmplete™ EDTA-free Protease Inhibitor. The cells were lysed by a double pass through a Stansted pressure cell homogeniser (270 MPa). Cell debris was pelleted and discarded by centrifugation at 10.000 rcf, 20

min at 4°C. The crude membranes were extracted by ultra-centrifugation (200.000 rcf, 1 hour, 4°C) and resuspended in 20 mM Tris-HCl, pH 7.4, 0.05% Tween-80, 10% glycerol to a total protein concentration of 10 mg/mL. The proteins were solubilized by the addition of 0.5% LMNG with gentle mixing (1h, 4°C). Insoluble material was pelleted by ultra-centrifugation (200.000 rcf, 30 min, 4°C) and discarded. The soluble fraction was incubated overnight at 4°C with Pierce™ Anti-DYKDDDDK Affinity Resin (Thermo Fisher) and purified according to the manufacturer's instructions. Briefly, the flow through was removed by centrifugation at 1000 rcf, followed by four washing steps with 10 CV of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.005% LMNG. The protein was eluted by the addition of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.005%LMNG, 1 mg/mL 3X-FLAG peptide (Genscript). Final purification was achieved by size exclusion chromatography (SEC) on a superdex 200 increase 10/200 column at 0.5 mL/min (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.005% LMNG). SEC peak fractions were evaluated on SDS-gel and pure *M. tuberculosis* cytochrome *bd* was pooled, concentrated, and stored at -80°C until further use.

Expression and purification of NDH-2

The gene for *Caldalkalibacillus thermarum* NDH-2 with an N-terminal hexahistidine tag was ordered from GeneArt and cloned in the pET28 vector between NcoI and XhoI, giving rise to the construct pET28-NDH-2_NtermHis. pET28-NDH-2_NtermHis was transformed into C41 (DE3) cells and plated on LB kanamycin to select positive transformants.

Expression and purification were performed based on the procedure from Heikal *et al.* with slight modifications[147]. Briefly, a streak of transformants was inoculated and grown overnight (250 RPM, 37°C). The overnight culture was diluted to -OD 0.1 in LB kanamycin and grown to -OD 0.5 before induction with 0.25 mM IPTG. Expression was carried out for 4 hours before cells were harvested (6371 rcf, 20 min, 4°C). The cells were resuspended in a 5-fold volume of 50 mM Tris-HCl, 5 mM MgCl₂, pH 8.0, and lysed by a single pass through a Stansted pressure cell homogeniser (270 MPa). Unbroken cells were pelleted by centrifugation (10.000 rcf, 20 min, 4°C) and discarded. Crude membrane

fractions were pelleted by ultra-centrifugation (200.000 rcf, 1h, 4°C) and resuspended at a 10 mg/mL total protein concentration in Tris-HCl, 150 mM NaCl, 20 mM Imidazole. Membrane proteins were extracted by treatment with 1% DDM for 1 hour at 4°C with gentle mixing. The membranes were removed by ultra-centrifugation (200.000 rcf, 30min, 4°C) followed by application of the soluble fraction to a HiTrap Nickel NTA column (Cytiva). The unbound proteins were washed from the column with washing buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 20 mM Imidazole, 0.02% DDM) followed by elution using stepwise addition of elution buffer (50 mM Tris-HCl, 150 mM NaCl, 500 mM Imidazole, 0.02% DDM). NDH-2 eluted at approximately 30% elution buffer, as confirmed by SDS-page gel and western blot. Final purification was achieved by size exclusion chromatography on a superdex increase 200 10/300 column (Cytiva) at 0.5 mL/min (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 5% glycerol, 0.02% DDM). Pure fractions were pooled, concentrated, and stored at -80°C until further use.

Cyt bd reconstitution in proteoliposomes

Lipids were purchased from Avanti Polar Lipids and used as received. A lipid mixture of POPE:POPG:CA (60:30:10 for *Ecbd* proteoliposomes, 30:60:10 for *Mtbd* proteoliposomes), enriched with the desired concentrations of ubiquinone-10 (UQ-10) (Sigma) or menaquinone-9 (MK-9) (Caymen chemical), was dried under a stream of nitrogen. Final traces of CHCl_3 were removed overnight under vacuum. The lipid film was rehydrated and resuspended to a final concentration of 10 mg/mL in 20 mM MOPS, 30 mM Na_2SO_4 , 100 mM KCl, pH 7.4, by vortexing. Cyt *bd* reconstitution was performed as described[148]. Briefly, cyt *bd* was added to the liposome solution at 1 w/w% protein/lipids and mixed for 30 min by inversion at RT. Insoluble materials were removed by centrifugation in an Eppendorf tabletop centrifuge (14.100 rcf, 5 min). The proteoliposome cyt *bd* concentration was determined by re-dissolving a sample in 2% octyl- β -glucoside, followed by quantification of the soret band with the corresponding extinction coefficient (*Ecbd*: $\epsilon_{417} 230 \text{ mM}^{-1} \text{ cm}^{-1}$ [55], *Mtbd*: $\epsilon_{414} 279 \text{ mM}^{-1} \text{ cm}^{-1}$). The latter extinction coefficient of the *Mtbd* soret band (414 nm) was determined from UV vis absorbance in relation to protein

concentrations determined by BCA assay from three different protein preparations. The extinction coefficient ($\epsilon_{414\text{nm}} = 279 \pm 13 \text{ mM}^{-1} \text{ cm}^{-1}$) was comparable to other *bd*-type oxidases[149].

Enzyme kinetics with water soluble quinone analogs

Oxygen consumption of LMNG solubilized cyt *bd* with quinone analogs was measured on an oxygraph (Hansatech Ltd.) system at 20°C. The quinone analogs, UQ-1 (Sigma), deoxylapachol (DMK-1, MedChem Express), or MK-1 (Santa Cruz Biotech) were added to the reaction chamber at the desired concentration in 50 mM MOPS pH 7.0, 150 mM NaCl, 0.005% LMNG. Quinone-mediated auto-oxidation was determined by enzymatic reduction of the quinones by *C. thermarum* NDH-2 (30 nM) after the addition of 1 mM NADH. Oxygen consumption was initiated by the addition of cyt *bd* (4 nM for *Ecbd*, 6.5 nM for *Mtbd*). The enzyme activity was measured by subtraction of the quinone auto-oxidation rate from the initial slope after cyt *bd* addition. The kinetics curves were fit and where possible, enzymatic parameters were determined using GraphPad Prism using either Michaelis Menten (eq.1) or substrate inhibition kinetics (eq.2).

$$V_i = K_{cat}[E][S]/K_m + [S]$$

$$V_i = K_{cat}[E][S]/K_m + [S] + [S]^2/K_i$$

Cyt *bd* oxygen consumption in proteoliposomes

Oxygen consumption was measured on an oxygraph system at 20°C. Prior to the measurement, cytochrome *bd* proteoliposomes were diluted to the desired concentration (20 nM cyt *bd*) in 50 mM MOPS, 150 mM NaCl, pH 7.0. The proteoliposomes were incubated for 30 min at room temperature with 100 nM NDH-2 to complete the proteoliposomal system. Oxygen consumption was

initiated by the addition of 1 mM NADH. The oxygen consumption rate was quantified using the initial slope after NADH addition and corrected for auto-oxidation from *cyt bd* free liposomes.

Cyt bd oxygen consumption after treatment with reductants

The effect of *Mtbd* Q-loop disulfide bond reduction was studied by 30 minutes of preincubation with chemical reductants (10 mM TCEP, 10 mM DTT or 100 mM 2-ME). When required, the reductant was removed by spin filtration before exposure to either ambient oxygen concentration or 50 μ M H₂O₂ for 30 min. The samples were diluted to the concentrations used previously in the LMNG solubilized or proteoliposomal measurements. The buffer was supplemented with the respective chemical reductant to maintain the reductive environment during the measurement (1 mM TCEP (10 mM showed auto-oxidation)), 10 mM DTT, 100 mM 2-ME). The measurements were performed as mentioned above.

Non-reducing SDS-page

The effects of environmental redox potentials on the *Mtbd* disulfide bond were determined by non-reducing SDS page, omitting reductant from the loading buffer. *Mtbd* was exposed to the desired amount of reductant (10 mM DTT) for 1 hour, before removal by spin filtration. If required, *Mtbd* was exposed to ambient oxygen concentrations or 50 μ M H₂O₂ for 30 min before denaturation in SDS-page buffer without additional reductant. Shifts in the *Mtbd* CydA protein band were determined by non-reducing SDS-page gel.

Cryo-EM sample preparation and data collection

C-flat R1.2/1.3-Cu 300 mesh grids (Electron Microscopy Sciences) were freshly glow discharged with PELCO easiGlow device at 15 mA for 90 s. LMNG solubilized *Mtbd* was pretreated with 4 mM DTT for 1 hour to break the Q-loop disulfide bond, followed by the application of 4 μ l sample (2.5 mg/ml) on the grid. Sample blotting was performed for 3 seconds, at 20 blot force using a Vitrobot IV device (ThermoFisher Scientific) operating at 4°C and 100% relative humidity, directly before plunge freezing into liquid ethane.

A total of 14,078 movies were collected on a Titan Krios G1 (ThermoFisher Scientific) operating at 300 kV, equipped with a Gatan K3 detector and BioQuantum energy filter with a slit width of 20 eV. Movies were acquired in electron counting mode using aberration-free image-shift (AFIS) in EPU (ThermoFisher Scientific). A total dose of 100 e/ $\text{\AA}^2$ with 100 frames, at 105,000 $\times$ magnification with a calibrated pixel size of 0.836 $\text{\AA}$ and a defocus range of -1.0 to -2.0 μ m.

Cryo-EM data analysis

CryoSPARC live[150] was used for on-the-fly processing of data with motion correction using Patch Motion Correction, CTF estimation using Patch CTF estimation, and particle picking based on Blob-picker using minimum/maximum diameters and filtering based on NCC score and Power score. Curated micrographs were imported into the regular version of CryoSPARC (4.3.1) for further processing. After importing filtered micrographs and 2D class templates from the live session, Template-picker was used to fine-tune the picking parameters. Multiple rounds of 2D classification were run to clean junk particles. An Unsupervised *ab-initio* map was built and the map and extracted particles were taken further to multiple rounds of heterogeneous refinement. Only the best particles along with all volumes were taken to further heterogeneous refinement jobs until particles became constant in the best class. Particles from the best class were taken to non-uniform refinement jobs for global and local CTF refinements along with other high-order

aberration corrections[151]. Movies were taken to another motion correction job to remove the last 50 frames, in order to check for any particle damage from relatively higher electron dose in the later frames. These movies and particles from last refinement job were used to re-extract with the original box size. This was further taken to several more rounds of non-uniform refinements. Data processing was finished by achieving a high-resolution sub-atomic map of 2.96 Å. During the data processing, LMNG micelle wasn't subtracted from the maps, so as not to create any signal biasedness between detergent micelle and flexible parts of the protein.

Model Building

Model building was done using *Mtbd* model from 7NKZ and fitting main chains using ARP/wARP server. After the main chain fitting, all side chains were manually checked and fitted and missing loops and termini were added in Coot software (version 0.9), and missing residues from loops, N- and C-termini were added. Cardiolipins and phosphatidylethanolamine (PE) were added to the designated density in *Mtbd* Models and the corresponding maps were assessed and analyzed in ChimeraX (version 1.7.1).

Supplementary information

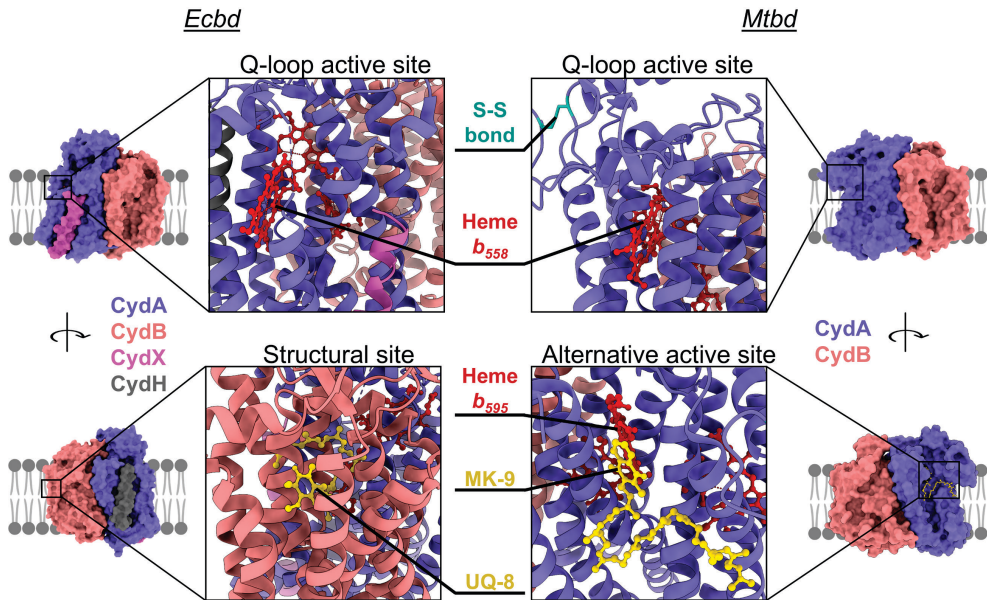


Figure S2.1. Cryo EM structures of *Ecbd* (PDB: 6RKO) and *Mtbd* (PDB: 7NKZ) with active and structural sites indicated.

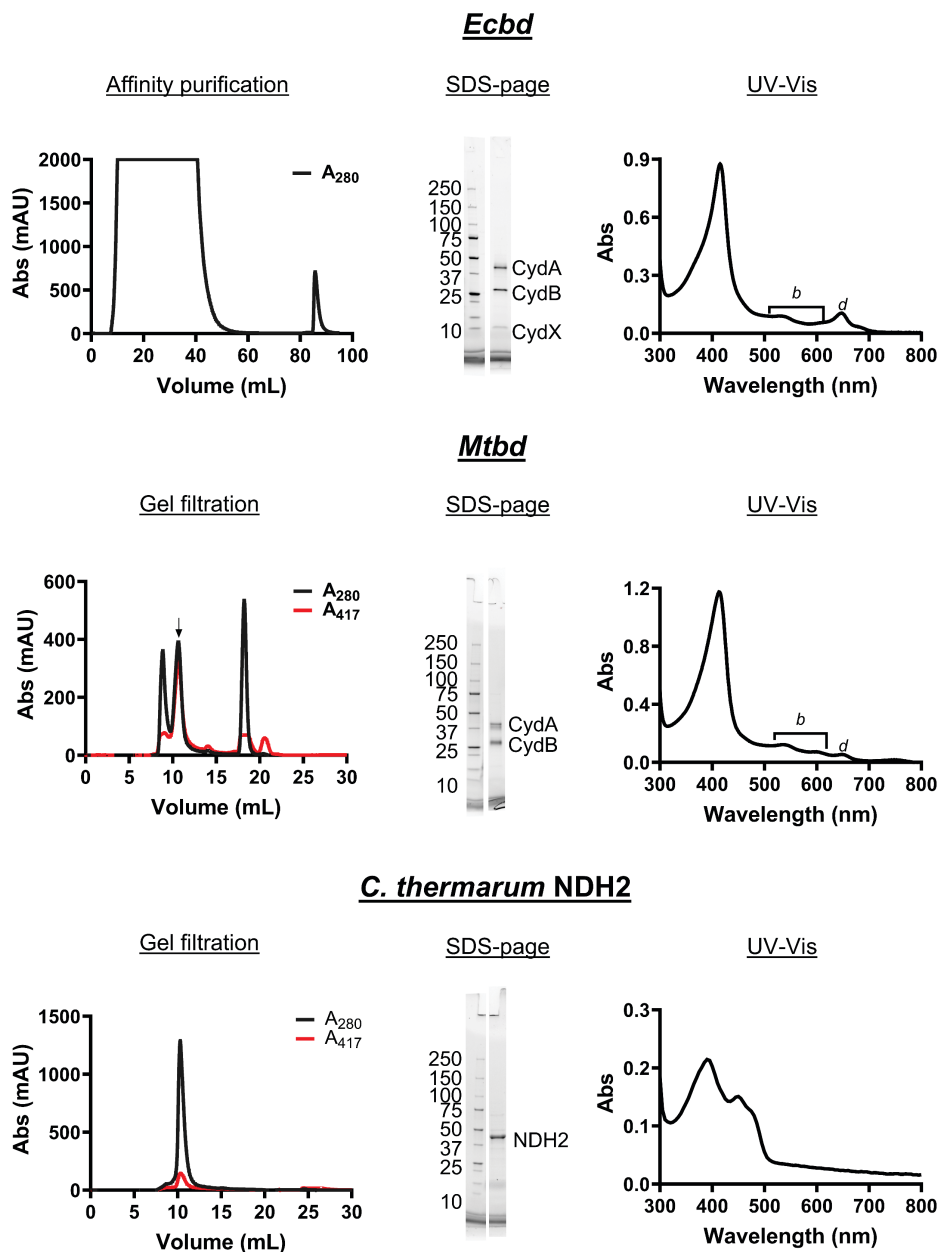


Figure S2.2. Purification, SDS-Page (TGX stain), and UV-Vis spectra of the isolated *Ec*bd, *Mt*bd and *C. thermarum*NDH₂.

Breathing in Thin Air

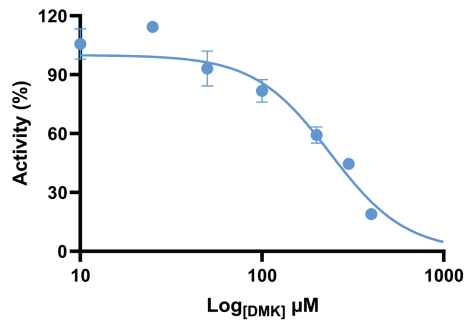


Figure S2.3. Inhibition of *Mtbd* (50 μM MK-1) by increasing DMK-1 concentration.

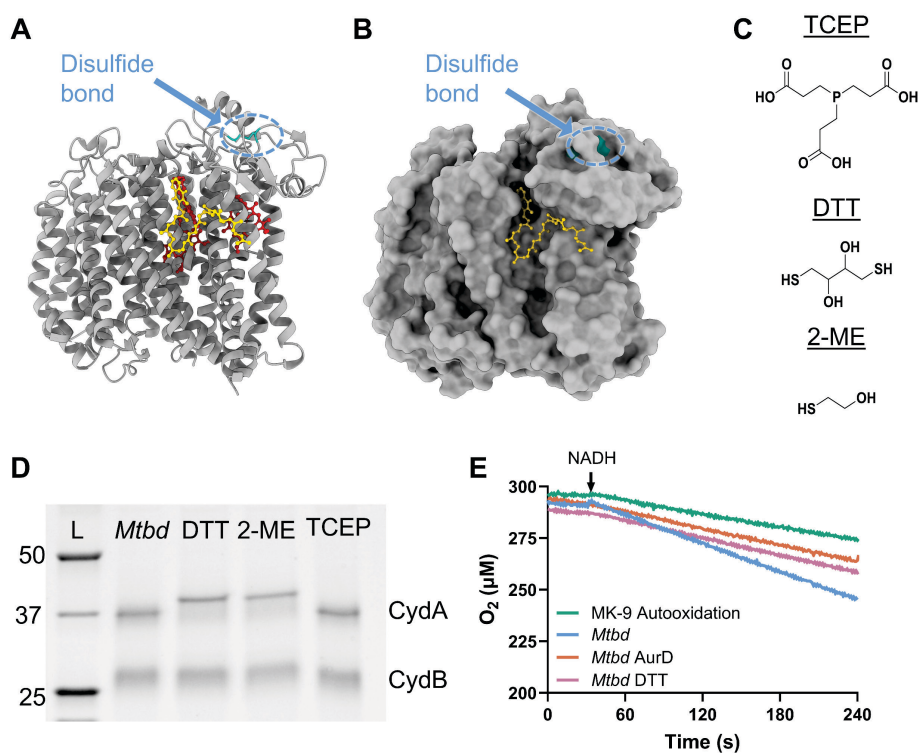


Figure S2.4: Accessibility of the *MtbD* disulfide bond. Cartoon (**A**) and surface (**B**) representation of *MtbD* with hemes (Red), MK-9 (yellow) and disulfide bond (blue). (**C**) Chemical structure of the reductants used in this study. (**D**) Non-reducing SDS page (TGX stain) of *MtbD* with prior treatment with different reductants. (**E**) Representative oxygraph traces of *MtbD* liposomes (10 nM) before and after DTT treatment and inhibited by AurD, and MK-9 autooxidation trace of *cyt bd* free liposomes.

Calculation of *E. coli* membrane quinone content

Variables	Value	Ref
UQ content <i>E. coli</i>	1.5×10^3 nmol/g dry cell weight	[16]
	3.6×10^2 nmol/g dry cell weight	[18]
<i>E. coli</i> dry weight	3.0×10^{-13} gram per cell	[152]
Number of lipids per <i>E. coli</i> cell	2.2×10^7 lipids	[152]
Avogadro's number	6.022×10^{23}	

Calculation

Number of lipid per cell: $2.2 \times 10^7 / 6.02 \times 10^{23} = 3.6 \times 10^{-17}$ mol/cell

Number of lipids in 1 gram cells: 3.6×10^{-17} mol/cell $\times (1/3.0 \times 10^{-13}$ g/cell) = 1.2×10^5 nmol/g

% UQ in membrane[16]: $= 1.5 \times 10^3$ nmol/g / 1.2×10^5 nmol/g $\times 100\%$ = 1.23%

% UQ in membrane[18]: $= 3.6 \times 10^2$ nmol/g / 1.2×10^5 nmol/g $\times 100\%$ = 0.30%

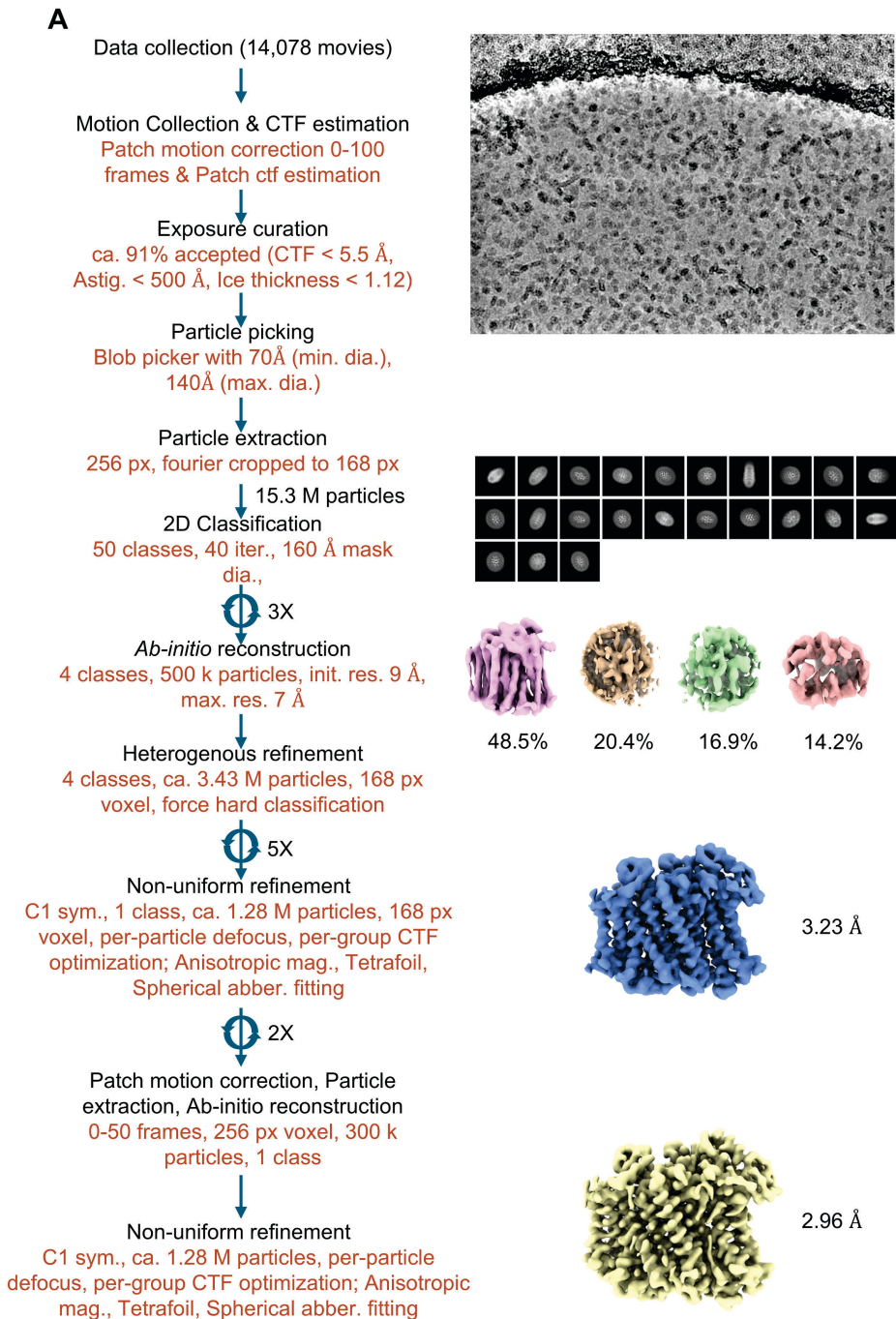
Assuming a surface area of POPC to be $65 \text{ \AA}^2$ (MW = 760 g/mol) and a lipid bilayer thickness of 4 nm, 1% (w/w) UQ-10 (MW = 865 g/mol) is equivalent to 11 mM

Volume of 1 mol POPC bilayer:

$(6.022 \times 10^{23} \times 0.65 \text{ nm}^2 \times 4 \text{ nm}) / 2 = 7.8 \times 10^{23} \text{ nm}^3 = 0.8 \text{ L}$

1% (w/w) UQ-10 of 1 mol POPC is $= 0.01 \times 760 \text{ g} = 7.6 \text{ g}$ or $(7.6 \text{ g} / 865 \text{ g/mol}) = 8.8 \text{ mmol}$

$8.8 \text{ mmol} / 0.8 \text{ L} = 11 \text{ mM}$



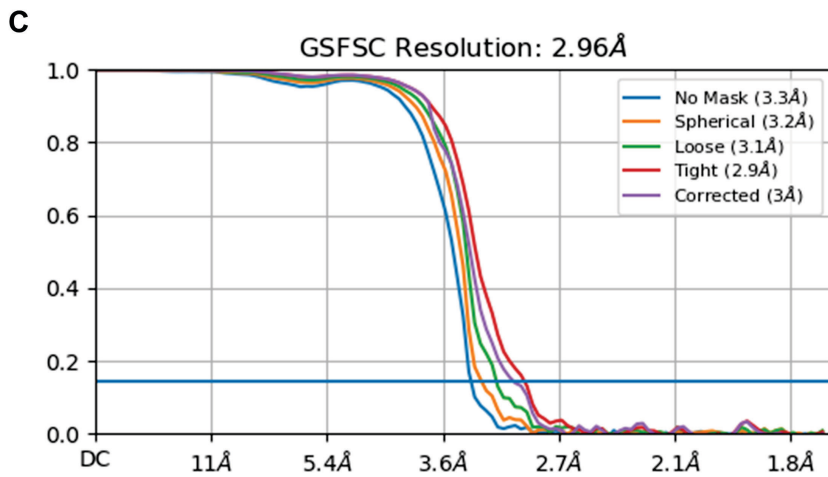
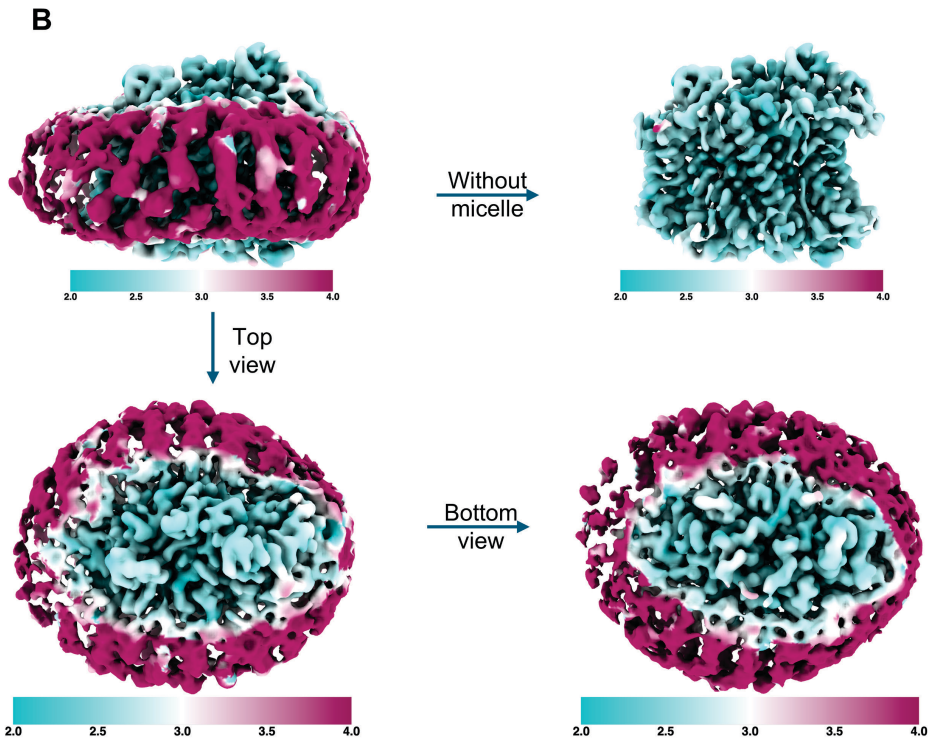


Figure S2.1: CryoSPARC cryoEM data processing scheme. (A) on the left side starting from importing movies, motion correction, ctf estimation for pre-processing steps along with curating exposures based on ctf resolution, astigmatism, and relative ice thickness. This was followed by particle-picking using blob picking based on particle diameter, inspecting picked particles, extracting and Fourier-cropping particle box to a smaller size of 168 pixels, to save processing time. Approximately 15.3 million picked particles were subjected to 3 rounds of 2D classification, followed by ab-initio reconstruction and taking all volumes and selected particles for 3 rounds of heterogeneous refinement to refine and sort particles into best 3D classes simultaneously. The best particle class and volume were further taken for 3 rounds of non-uniform refinements. A total of ca. 1.28 million particles yielded in a 3.32 Å reconstruction. From 100 total frames used in all processing until this step, the last 50 frames were cut off to process only the first 50 frames to check map quality and resolution improvements. Also, particles were re-extracted to the full box size of 256 pixels to check for any further resolution improvements. 3 more rounds of non-uniform refinements to optimize per-group ctf estimation, per-particle defocus, and fit spherical aberration, tetrafoil, anisotropic magnification, were performed before finalizing the cryo-EM map of 2.96 Å. On the right side of the scheme, there is an exemplary micrograph to show particle distribution. This is followed by selected 2D classes and then 4 ab-initio classes with increasing to decreasing order of particle distribution. Only the single highest class with 48.5 % particles was taken for further processing after careful inspection of all classes in ChimeraX. (B) Local resolution estimations between the resolution of 2 Å (Teal) to 4 Å (Bordeaux). The first 3 figures counterclockwise show estimates along with LMNG detergent micelle from the side view (top left), top view (bottom left), bottom view (bottom right), and the fourth figure on the top right shows estimates at a threshold of 0.0637 to hide LMNG micelle and show local resolution for just protein parts. (C) FSC curve of the final reconstruction at a resolution of 2.96 Å from gold-standard FSC of 0.143.

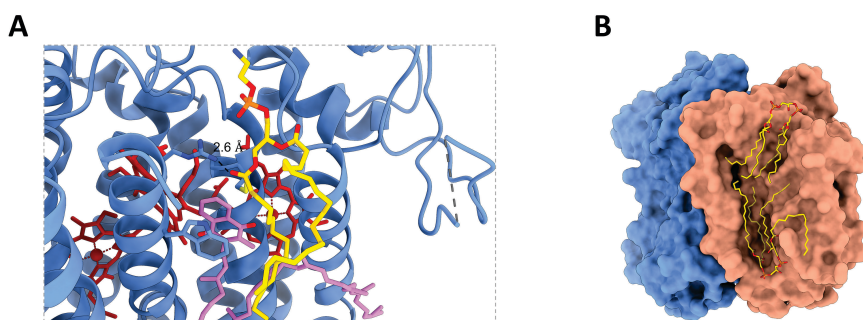


Figure S2.2: Lipids resolved in the *MtbD* structure. (A) a PE lipid complementing the MK-9 binding pocket via hydrogen bonding to Arg8. (B) two cardiolipin molecules bound in the CydB hydrophobic groove.

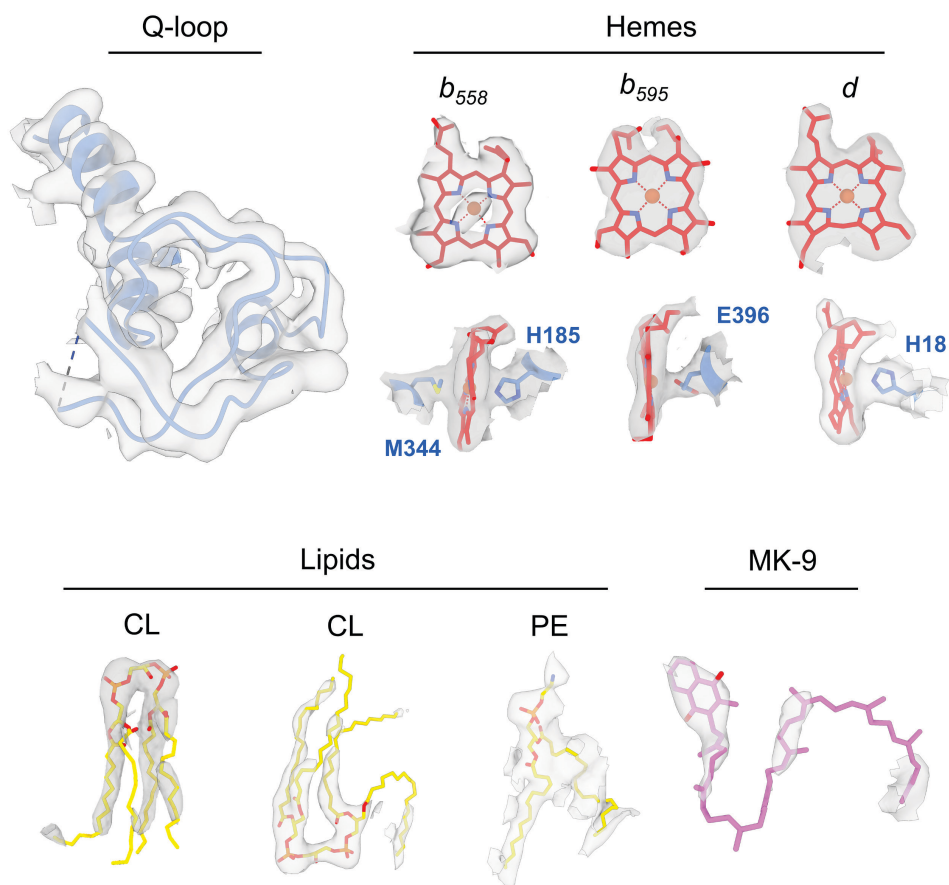


Figure S2.3: Density features of the disulfide reduced *MtbD* structure.

Chapter

3

Visualizing the mechanism of quinol oxidation and inhibition of a *bd*-type oxidase using cryo-EM

Cytochrome *bd* is a prokaryotic terminal oxidase recognized as an antibiotic target against various pathogens. Despite its critical role in respiration, failure to capture the mechanism of quinol oxidation and inhibition prohibits structure guided drug discovery. Here, we present cryo-electron microscopy structures of *Escherichia coli* cytochrome *bd*-I in monomeric and dimeric forms, in several quinone and inhibitor-bound states. We identify a dynamic Q-loop lid that undergoes a disorder-to-order transition upon substrate binding to the dimer, completing the active site and enabling catalysis. Structure-guided mutagenesis confirms Tyr243^{CydA} and Arg298^{CydA} as conserved catalytic residues unique to long Q-loop oxidases, highlighting evolutionary divergence from other subfamilies. Inhibition by Aurachin D triggers refolding of the active site, occluding substrate access via an Asp239^{CydA}-mediated mechanism. The structural and mechanistic insights presented here establish a comprehensive framework, opening paths for drug discovery against *bd* oxidases.

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Introduction

The bacterial respiratory chain has emerged as a promising target for next-generation antibiotics due to its essential role in ATP generation[153]. Disruption of this pathway has proven effective in eliminating pathogens resistant to conventional therapeutics, as demonstrated by the clinical success of bedaquiline, an ATP synthase inhibitor approved for the treatment of multidrug-resistant tuberculosis[63]. Among the terminal oxidases, cytochrome *bd* (cyt *bd*) is characterized by an exceptionally high oxygen affinity and ability to confer resistance to oxidative stress and tolerance against diverse antibiotics [46–48,56,59,154]. This enables pathogens to sustain respiration under hostile conditions encountered during infection. Notably, cyt *bd* is absent in eukaryotes, making it an attractive target for selective antimicrobial development against pathogens such as *Mycobacterium tuberculosis* [77], *Salmonella enterica* [155], pathogenic *Escherichia coli* [78], and *Staphylococcus aureus* [156].

Cyt *bd* catalyzes the reduction of molecular oxygen to water using electrons from the quinone pool. During this turnover, cyt *bd* releases the chemical protons associated with quinol oxidation to the periplasm while capturing cytoplasmic protons for oxygen reduction, thereby contributing four protons per oxygen reduced to the proton motive force required for ATP synthesis (Fig. 3.1A,B). The canonical enzyme architecture comprises two core subunits, CydA and CydB, and in some species, auxiliary subunits such as CydS, CydX, and CydH[81,83]. CydA harbors the three heme cofactors, *b*₅₅₈, *b*₅₉₅, and *d*, as well as the putative quinone binding site near the Q-loop, a flexible periplasmic region adjacent to heme *b*₅₅₈[83]. The cyt *bd* oxidase family of enzymes has been phylogenetically classified into qOR, OR-C and OR-N subfamilies. The cyt *bd* oxidases with a quinol binding site in CydA belong to the qOR subfamily, which are further subdivided into qOR1, qOR2, qOR3 and qOR4a[88], where qOR1 forms the major cluster. The qOR1 family can be further subdivided into long and short Q-loop subfamilies based on the presence of a C-terminal Q-loop extension found in the proteobacterial clade, though the functional implications of the phylogenetic divergence remain unresolved[89,90].

E. coli *cyt bd-I* (*Ecbd*), representative of the qOR1 long Q-loop subfamily, consist of the four subunits CydA, CydB, CydX and CydH. The available substrate pool in *E. coli* consists of three quinone subtypes, ubiquinone (UQ), demethylmenaquinone (DMK) and menaquinone (MK). The composition of this quinone pool adapts based on the environmental oxygen concentration, switching from predominantly UQ to MK under micro-aerobic conditions when *Ecbd* is expressed [18]. Despite extensive characterization [83,86,91], structural and mechanistic insights into the quinol oxidation and inhibition mechanism of *cyt bd* have remained elusive.

To gain insights into quinone turnover and inhibition of *bd*-type oxidases, we have resolved cryo-EM structures of *Ecbd* in its unbound, MK bound and inhibitor bound state. We identified both monomeric and dimeric forms of *Ecbd*, with the latter exhibiting increased catalytic activity. The dimeric form revealed three discrete quinone binding sites per protomer. In the quinol oxidation site, located near heme *b*₅₅₈, MK was observed to occupy a cavity formed by a disorder-to-order transition of the Q-loop lid. Unexpectedly, the quinol oxidation site undergoes a structural rearrangement upon binding of the inhibitor Aurachin D, occluding the quinone from binding to its pocket. Utilizing these structures, site directed mutagenesis, enzyme kinetics, and sequence analysis we propose the catalytic cycle of *Ecbd*, and underline critical structural features required for quinol turnover and inhibition.

Results

Purification and structure determination of Ecbd

Ecbd was expressed in MB43 cells and purified using affinity chromatography followed by gel filtration using the detergent Lauryl Maltose Neopentyl Glycol (LMNG). To gain insights into the turnover mechanism of *Ecbd*, we determined cryo-EM structures in LMNG and observed heterogeneity on the grid. 3D classification of the as-isolated sample resulted in the structures without MK (I, unbound) and with MK (II, bound). Within the MK bound structures, open and closed states were observed (II, bound^{open}; III, bound^{closed}). To further improve

the resolution of state (III), *Ecbd* was reconstituted into phosphatidylcholine (PC) nanodiscs doped with 5% MK-9 (product). Last, incubation of *Ecbd* with AurD in LMNG resulted in the inhibitor bound state (IV). The different structures and samples conditions are shown in Fig. 3.1C, Table S3.1, and Fig. S3.1-4.

Our cryo-EM analysis identified previously uncharacterized dimeric forms of *Ecbd*, potentially due to the mild solubilization with LMNG, which mirrored the dimeric structure of the homolog *E. coli* *cyt bd-II*[87]. The dimeric states of *Ecbd* (I), (II), (III), and (IV) were resolved at 2.2-2.5 Å overall resolution. Additionally, on the same grids, the canonical monomeric architecture for sample (I), (II), and (IV) were resolved at 2.4-2.6 Å overall resolution (Fig. S3.5). However, all three monomeric structures show an identical state, with no additional ligand binding being observed other than a structural quinone in CydB. Consistent with previous reports[83] *Ecbd* adopts a heterotetrameric architecture that consists of the subunits CydA, CydB, CydX and CydH (Fig. 3.1D). CydA forms the catalytic core, harboring the three heme cofactors (b_{558} , b_{595} , and d) and the putative quinone-binding Q-loop required for oxidase activity (Fig. 3.1B). In contrast to previous reports, our high resolution cryo-EM map indicates that the heme d hydroxychlorin γ -spirolactone exists in the trans rather than the cis orientation (Fig. S3.6). This trans orientation is further confirmed by prior NMR and IR analysis of the isolated heme[157], and positions the spirolactone group opposite the bound dioxygen molecule. Close examination of prior *cyt bd* density maps indicates the possibility of a more general trans heme d orientation within the *bd*-oxidase family, although unambiguous assignment of the chirality remains challenging due to limitations in resolution.

The *Ecbd* dimer consists of 20 transmembrane helices existing in a symmetrical architecture around the CydX dimer interface (Fig. 3.1E). CydX forms hydrophobic contacts with CydA helix 1, 5, and 6 to connect the two protomers together. To confirm the physiological relevance of both monomer and dimeric states, *Ecbd* was extracted from the membrane using styrene-maleic acid lipid particles (SMALPs). Cryo-EM imaging followed by 2D classification showed clear populations consisting of dimeric and monomeric particles, indicating the existence of both oligomeric states *in vivo* (Fig. S3.7).

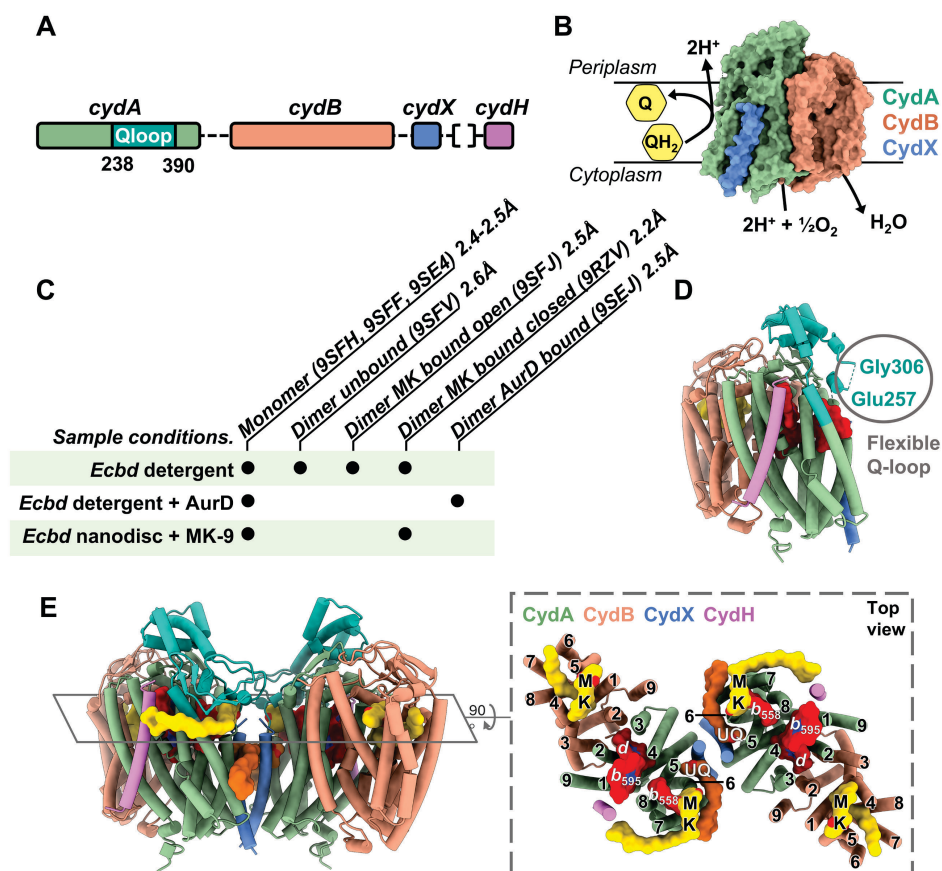


Fig. 3.1. Overview of the structures and function of *Ecbd*. (A) Genomic organization of the *Ecbd* subunits. Subunit CydH originates from a different operon than the other *Ecbd* subunits. (B) Schematic oxidase activity of *Ecbd*. (C) Overview of the different sample conditions used to yield the presented monomer and dimeric states presented, including their state and resolution. PDB 9RZV was resolved from the nanodisc sample. (D) Structural overview of the *Ecbd* monomer. CydA is shown in green, with the Q-loop in cyan, CydB in orange, CydX in blue, CydH in purple. (E) Structural overview of the *Ecbd* dimer in the MK bound state with the Q-loop highlighted. MK is shown yellow and UQ in dark orange. The right shows a cross section of the *Ecbd* dimer with a total of 40 transmembrane helices, highlighting all identified MK (yellow) and UQ (dark orange), as well as heme groups (red).

Both oligomeric states contained tightly bound phospholipids at the protein periphery and dimer interface (Fig. S3.8). Lipids lacking discernible headgroups were modeled as phosphatidic acid, suggesting either headgroup flexibility or phospholipid heterogeneity.

Quinone binding and electron transfer in the *Ecbd* dimer

The dimer interface of *Ecbd* is formed by CydX in conjunction with coordinated phosphatidylglycerol (PG) lipids (Fig. 3.2A,B). These PG lipids also interact with the CydA Q-loop via hydrogen bonding between Arg298^{CydA} and the lipid carbonyl group. The homodimeric CydX–CydX interface is stabilized by a methionine–aromatic motif (Met1^{CydX}–Trp2^{CydX}) and a leucine zipper along the helical axis (Fig. 3.2B). Within the dimer, each *Ecbd* protomer contains three distinct quinone-binding pockets. The primary quinone binding site, located beneath the Q-loop, accommodates a well-resolved MK headgroup (Fig. 3.2C). This shallow, hydrophobic cavity is positioned 6.7 Å from the heme *b*₅₅₈ iron center, enabling fast electron transfer. The MK isoprenoid tail is only partially resolved, indicating its flexibility during quinone binding and turnover. The MK headgroup is stabilized by π – π stacking with Tyr243^{CydA} (4.5 Å) and hydrogen bonding with Arg298^{CydA} (3.4 Å), both of which facilitate semiquinone stabilization and proton transfer during catalysis. This site is strongly implicated as the quinol oxidation center, as mutagenesis of either Tyr243^{CydA} or Arg298^{CydA} to alanine (Y243A, R298A) abolished enzymatic activity with both MK and UQ in *Ecbd* monomers, confirming its role as the principal quinol oxidation site (Fig. 3.2F). The arrangement of the three hemes in CydA follows a triangular topology, as previously described[83], with the MK-bound structure facilitating mapping of the full electron transfer route from the quinol oxidation site to the oxygen reduction center (Fig. 3.3A). Although no proton release pathway could be identified unambiguously, the protons released during quinol oxidation could transfer to the periplasm via the closely positioned Asp239^{CydA} and Glu240^{CydA} pair at the membrane interface, or transfer via a series of structured water molecules to the conserved Lys252^{CydA} (Fig. S3.9). The associated electrons are transferred sequentially from this MK site via heme *b*₅₅₈ to *b*₅₉₅ (14.9 Å) and finally to heme *d* (11.2 Å), where molecular oxygen reduction occurs in two cycles of quinol oxidation (Fig. 3.3A).

A second quinone binding site is located at the CydA–CydX interface and contains a co-purified ubiquinone molecule (Fig. 3.2D).

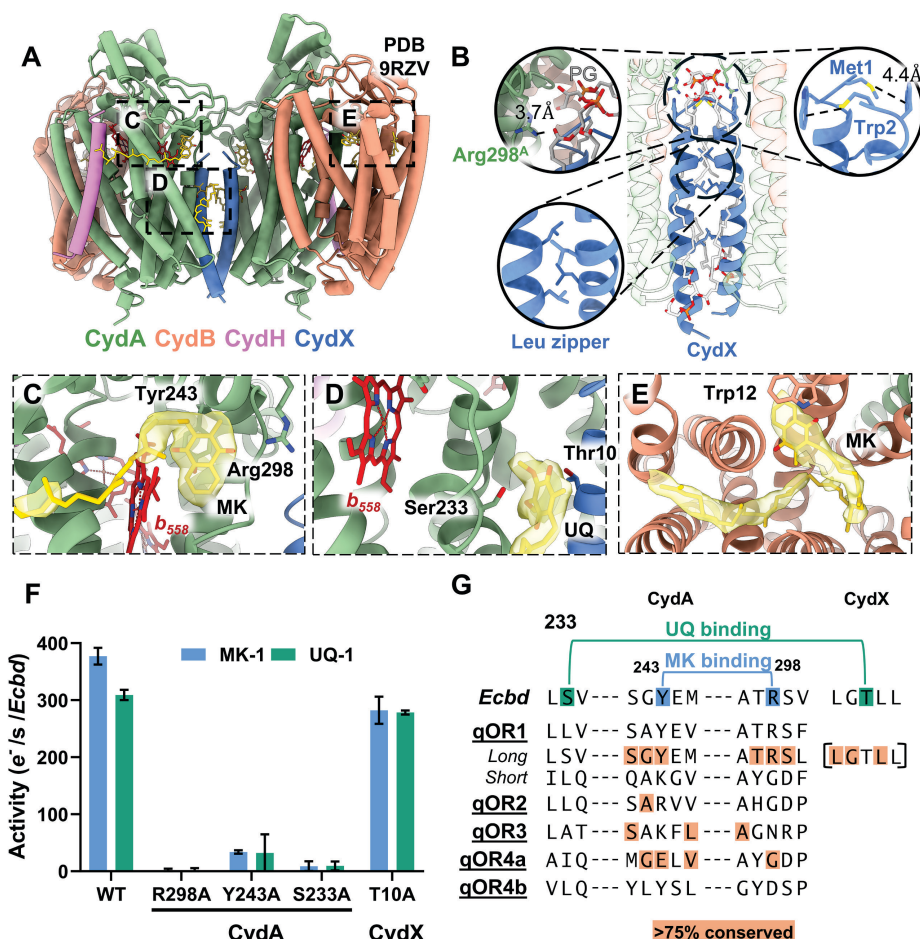


Fig. 3.2. The *Ecbd* dimer contains three distinct quinone binding sites per protomer (A) Overview of the *Ecbd* dimer and the quinone binding pockets. (B) The *Ecbd* dimer interface is formed CydX via Met-Trp interactions, and a leucine zipper. Additionally, a PG phospholipid forms a hydrogen bond with Arg298^{CydA} further stabilizing the interface. (C) The Q binding site near heme *b*₅₅₈, showing a bound MK stabilized by Tyr243^{CydA} and Arg298^{CydA}. (D) The UQ binding site wedged between subunit CydA and CydX. The UQ is stabilized by interactions with Ser233^{CydA} and Thr10^{CydX}. (E) The quinone binding pocket in CydB with a bound MK, stabilized by π - π stacking with Trp12^{CydB}. The quinone headgroup is situated outside of electron transfer distance from the heme chain. (F) Oxidase activity of monomeric *Ecbd* WT and quinone binding site mutants (N=3). (G) Consensus sequence alignment of the qOR1-4 subfamilies as defined by Murali *et al.*[88] The qOR1 family was subdivided in long- and short-Q-loop cyt *bd* variants indicating conservation of Y243^{CydA} and R298^{CydA} in the long Q-loop family (extended sequence variation in table S3.2). The CydX subunit is only present in a subset of qOR1-long Q-loop family. The consensus sequence and conservation of CydX defined by Allen *et al.*[158]

This UQ is coordinated via a hydrogen-bonding network involving Ser233^{CydA} and Thr10^{CydX} of the same protomer. Positioned 14 Å from the heme *b*₅₅₈ iron center and 13.2 Å from the primary MK site, this second site lies within electron transfer distance, with potential for both direct electron transfer and indirect electron transfer into the heme chain. However, although mutation of S233^{CydA} to alanine in the second quinone binding pocket (S233A) in CydA also fully inactivates *Ecbd*, mutation of T10^{CydX} to alanine (T10A) remains active (Fig. 3.2F). Although the activity of T10A^{CydX} strongly suggests the second quinone binding site is not a quinol oxidation site, we further investigate the abolished activity of the S233A^{CydA} mutant. Notably, analysis of the heme spectra reveals distinct alterations among the mutants. Both R298A^{CydA} and Y243A^{CydA} exhibit a partial decrease in heme *d* content or reduction, whereas in the S233A^{CydA} mutant heme *d* is completely absent. In addition, heme *b*₅₅₈ in S233A is significantly shifted, indicating pronounced changes in the heme environment (Fig. S3.10). These observations suggest that S233^{CydA} plays a primarily structural role rather than a direct role in ubiquinol oxidation. Thus, while this site is capable of binding quinones tightly, retaining UQ even after incubation with excess MK, it likely does not function as a site for quinol oxidation.

A third quinone site, typically occupied by UQ in CydB[83,91], was found to contain MK in the MK-incubated structure (Fig. 3.2E). Although the density for this MK is less defined, indicating either heterogeneity or flexible binding, the headgroup appears repositioned near Trp12^{CydB}, in contrast to as isolated structures. Despite this rearrangement, the MK headgroup remains 30 Å from the heme *d* center and lacks nearby residues to stabilize a semiquinone, indicating a structural rather than catalytic role. Functional assays confirmed that quinone exchange at this site does not affect enzymatic activity, precluding any allosteric effects (Fig. S3.11).

Sequence alignment across the *bd*-type oxidases family revealed conservation of the Tyr243^{CydA} or Arg298^{CydA} residues exclusively within the qOR1-long Q-loop subfamily (Fig. 3.2G, Table S3.2), indicating an evolutionary divergence in quinol oxidation site architecture between short and long Q-loop variants.

Functional role of the quinone pool in *Ecbd* regulation

While *Ecbd* is active with both MK and UQ[159], we aimed to assess if the content of the quinone pool provides any regulation on *Ecbd* activity. However, classical solution-based assays fail to distinguish between MK and UQ when simultaneously present. To assess this putative allosteric regulation by the available quinone pool, we employed a tethered bilayer lipid membrane (tBLM) electrochemical system, which can distinguish UQ and MK based on their redox potentials. This system enables direct electron transfer from a gold electrode to membrane-embedded quinones (Fig. 3.3C)[160], and has previously been used to study a variety of respiratory enzymes[28,148,161]. To form the tBLM, *Ecbd* was reconstituted in liposomes doped with either MK-9 or UQ-10 as native-like substrates.

Formation of the tBLM was confirmed by a characteristic drop in surface capacitance[162]. Reduction of the quinone pool and subsequent quinol oxidation by *Ecbd* was monitored via cyclic voltammetry (CV). CV measurements revealed catalytic currents for both the UQ-10 and MK-9 *Ecbd* samples with identical onset potentials and similar catalytic currents, indicating that the co-purified UQ in the MK sample is redox-active and capable of supporting *Ecbd* turnover (Fig. 3.3D). Whether this activity originates directly from the CydA-CydX site or reflects quinone exchange at the primary quinol oxidation site remains to be determined. We conclude that *Ecbd* is promiscuous regarding the quinol pool and not allosterically regulated by either UQ or MK.

Proton and oxygen delivery to the oxygen reduction center

Following the reduction of heme *d*, molecular oxygen and protons need to be guided towards the oxygen reduction site to complete the catalytic cycle. Using Mole 2.5[163] we identified two distinct channels converging at the heme *d* site, previously deemed the proton and oxygen channels (Fig. 3.3B)[83]. The oxygen channel (O-channel) originates between helices 1 and 9 of CydB and runs parallel to the membrane. The channel is lined with hydrophobic residues to facilitate the preferential entry of molecular oxygen. Near heme *d*, the

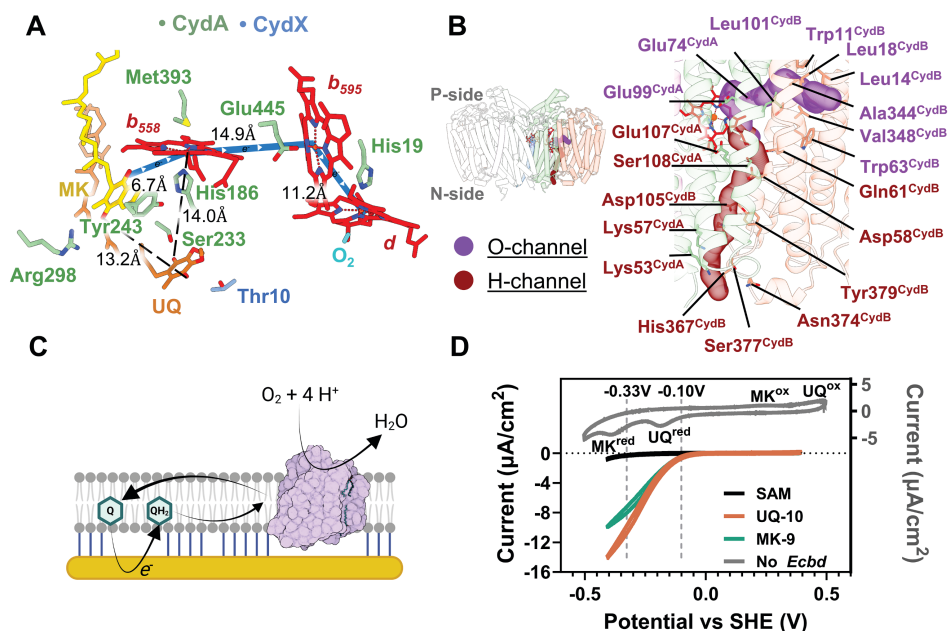


Fig. 3.3. The electron transfer, proton, and oxygen pathways (A) Proposed electron transfer routes from the MK (yellow) depicted in dark blue. Distances from UQ (orange) to MK and heme b_{558} are indicated. (B) Residues lining of the O- and H-channel leading towards the heme d oxygen reduction center. (C) Overview of the tBLM electrochemical system, where the quinone pool is directly reduced at the gold electrode to initiate *Ecbd* turnover. (D) Cyclic voltammograms of the tBLM system. Inset in grey shows a model membrane containing both MK (1 mol%) and UQ (1 mol%) without *Ecbd* (100 mV/s scan rate). The reduction and oxidation peaks of UQ and MK are indicated. The SAM negative control (black), *Ecbd* tBLMs containing only UQ-10 (Orange), or MK-9 (Green) (10 mV/s scan rate) showing the same onset potential at the reduction of UQ.

channel is terminated by two glutamate residues providing hydrogen bonding during oxygen reduction.

The H-channel originates at the CydA–CydB interface and extends perpendicularly from the cytoplasmic side toward heme d , and is lined with titratable residues forming a putative proton wire. Our predictions using PROPKA of residue pKa suggest proton transfer involving Lys53^{CydA} (pKa 9.01), Lys57^{CydA} (pKa 7.45), Asp105^{CydB} (pKa 4.53), Asp58^{CydB} (pKa 8.14), Glu107^{CydA} (pKa 9.47), and Glu74^{CydA} (pKa 11.07). The high pKa of Lys53^{CydA} allows for fast proton extraction from the cytoplasm to increase turnover rates. Among the channel

residues, Asp105^{CydB} emerges as the most acidic and has been previously implicated as critical in proton transfer[164]. Notably, Glu107^{CydA}, positioned 7.8 Å from the heme *d* iron center, exhibits a pKa consistent with the proposed terminal proton donor identified by Janczak *et al.*[164] and is highly conserved across CydA homologues (>90% sequence conservation)[88]. These findings support a model in which Lys53^{CydA} initially extracts protons from the cytoplasm, after which Glu107^{CydA} serves as the primary proton donor for oxygen reduction, completing the proton delivery pathway.

Closing of the Q-loop lid completes the quinol oxidation site and enhances catalytic efficiency

Despite exposure to high concentrations of MK, no density is observed near the Q-loop in monomeric *Ecbd*, indicating persistent conformational flexibility. This precludes reliable modeling of residues Glu239–Gly306^{CydA} in the *Ecbd* monomer. Structural comparison between monomeric and dimeric *Ecbd* revealed a rotation of Trp2^{CydX} upon dimerization, facilitating hydrogen bonding with Asp239^{CydA} (Fig. 3.4A,B). This interaction anchors the Q-loop segment spanning Asp239–Asp247^{CydA}, positioning Tyr243^{CydA} optimally for quinol capture. Together, this suggests that the dimeric state is more conducive to MK binding and, hence, MK oxidation.

Ecbd monomers and dimers were separated using gel filtration to probe the activity of the individual states. After isolation in LMNG, the monomeric and dimeric *Ecbd* remained in their oligomeric state as shown by SEC-MALS. This indicates that after isolation there is no re-equilibration between monomer and dimer, even after incubation with various lipids or quinones, enabling kinetic evaluation of the distinct monomeric and dimeric *Ecbd* (Fig. S3.12). Catalytic activity was assessed using a coupled assay in which quinones were reduced by an excess of NADH dehydrogenase[159]. The oxygen consumption rate was determined using an oxygraph and recalculated into *Ecbd* activity at 4 electrons per oxygen reduced. The observed structural stabilization in the *Ecbd* dimer correlates with a twofold increase in catalytic turnover rate relative to the monomer, without a substantial change in substrate K_m (Fig. 3.4C).

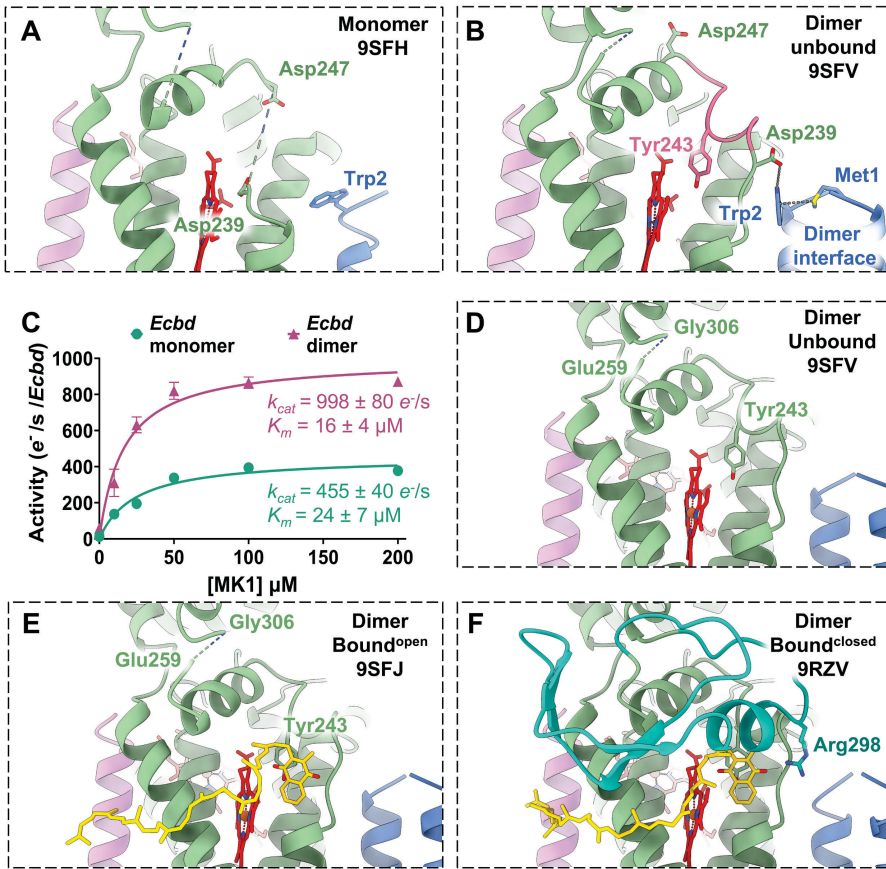


Fig. 3.4. Q-loop stabilization increases the turnover rate in the *Ecbd* dimer (A) Selected view of the quinone binding pocket in the *Ecbd* monomer (B) and in the *Ecbd* dimer. The pink stretch is stabilized by hydrogen bonding between Asp239^{CydA} and Trp2^{Cydx}, positioning Tyr243^{CydA} for quinol capture. (C) Michaelis-Menten kinetics of the *Ecbd* monomer and dimer ($N=3$). (D) unbound *Ecbd* dimer. (E) *Ecbd* dimer in the MK bound^{open} state showing initial interaction with the quinone. (F) *Ecbd* dimer in the bound^{closed} state showing closing of the Q-loop lid. CydA is represented in green, with the Q-loop lid in cyan. CydH is represented in violet, with CydX in blue.

This behavior could be explained if quinol dissociation is much faster than quinol oxidation in both monomer and dimer, while quinol oxidation proceeds slower in the monomer. Under this assumption, K_m would approximate the quinol dissociation constant (K_d), which would seem to be unchanged in dimer versus monomer. Alternatively, if quinol dissociation occurs on slower or

similar timescale as its oxidation, K_m could remain unchanged if both the substrate binding rate and catalytic turn-over are slower in the monomer. As transient Q-loop remodeling is required in the monomer to position Y243^{CydA} for quinol capture, it is possible if not likely that this conformational transition is rate-limiting in the monomer. In contrast, the dimeric enzyme maintains a partially-formed, catalytically competent quinol oxidation site. We propose that this results in a higher rate of substrate binding and catalysis for the *Ecbd* dimer, which together improve catalytic turnover without substantially changing K_m . We cannot exclude that the catalytic rate of the dimer might be limited by product release. While this would explain why quinone binding is only observed in the dimer, the Michaelis-Menten kinetics cannot unambiguously determine the rate limiting step.

Although the Asp239–Asp247^{CydA} segment is stabilized in the dimer, the remainder of the Q-loop remains disordered in the unbound state (I) (Fig. 3.4D). This precludes modelling of the amino acid stretch between Glu259^{CydA} and Gly306^{CydA}, hereafter referred to as the Q-loop lid. This flexible region remains unstructured even after initial MK engagement by Tyr243^{CydA} (MK bound^{open}, II) (Fig. 3.4E). Only in this MK bound state can the Q-loop lid undergo a structural rearrangement to stabilize the fold, enabling confident modelling of side chains and revealing the complete quinol oxidation site architecture (MK bound^{closed}, III). The Q-loop lid wraps around the quinone binding site before looping back onto the main body of CydA, mirroring the fold found in *Ecbd-II* and *M. tuberculosis* cyt *bd* (Fig. S3.13)[82,87]. The rearrangement of the Q-loop lid allows for hydrogen bonding between Arg298^{CydA} and the quinone headgroup, completing the quinol oxidation site for turnover (Fig. 3.4F).

Inhibition by Aurachin D triggers active site refolding

AurD, a MK analog derived from *Stigmatella aurantiaca*, is a potent and specific inhibitor of cyt *bd* oxidases[100]. To elucidate its mechanism of action, we resolved cryo-EM structures of both monomeric and dimeric *Ecbd* in the presence of AurD. While no density for AurD was observed in the monomer, the

dimeric structure revealed clear binding near the quinol oxidation site (inhibitor bound, IV) which allows for unambiguous modeling of the AurD binding pose (Fig. 3.5A). Surprisingly, AurD does not bind in the same pocket as MK, but induces a remarkable conformational change in helix 6, converting it from a loop into an α -helix. This conformational change closes the quinone binding pocket, effectively blocking substrate access. Close examination of the refolding process indicates that Asp239^{CydA} forms a hydrogen bond to the AurD amine group and hereby pulls the loop inwards to stabilize the helical conformation (Fig. 3.5B). The AurD headgroup is further stabilized by hydrophobic interactions with Gly242^{CydA}, Tyr243^{CydA}, Leu253^{CydA}, Glu257^{CydA}, Phe269^{CydA}, Ile295^{CydA}, Ala296^{CydA}, Phe298^{CydA}, Phe390^{CydA}, Arg391^{CydA}, Val394^{CydA}, Ala395^{CydA}, and heme *b*₅₅₈ (Fig. 3.5C). These hydrophobic interactions in the AurD binding pocket are conserved across the *bd* oxidase families, while Asp239^{CydA} is mainly conserved in the qOR1-long Q-loop family (Fig. 3.5D, Table S3). Interestingly, AurD binding occurs deeper into the binding pocket compared to the binding in *Ecbd-II*[87] (Fig. S3.14).

To confirm Asp239^{CydA} as the main effector residue for inhibition with AurD we performed mutagenesis (D239A^{CydA}). Although Asp239^{CydA} lies outside the quinol oxidation site in the MK-bound state, its mutation to alanine abolishes enzymatic activity, resulting in the inability to support bacterial growth in the MB43 strain lacking all terminal oxidases in the genome (Fig. 3.5E, Fig. S3.15), consistent with prior reports[165]. We postulate that inactivity of the *Ecbd* D239A^{CydA} mutant results from the impairment to form the hydrogen bond with Trp2^{CydX} (Fig. 3.3B), effectively closing the MK binding pocket as seen in the AurD bound state. In contrast, substitution with glycine partly preserves activity while significantly reducing AurD sensitivity (Fig. 3.5E,F), confirming Asp239^{CydA} as a key determinant of inhibitor binding and efficacy, as seen in *Ecbd-II*[87].

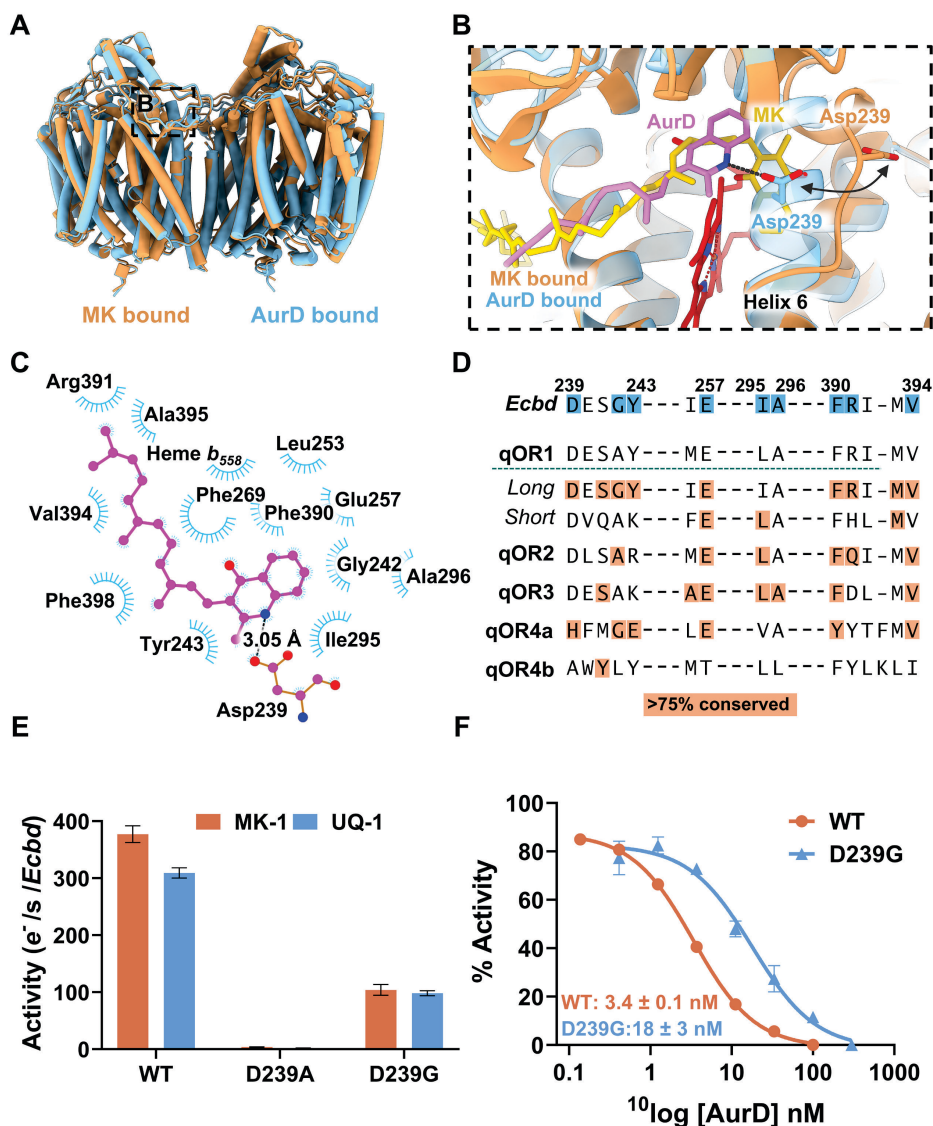


Fig. 3.5. Inhibitory mechanism of Aurachin D. (A) Overview of the *Ecbd* dimer in the MK and AurD bound state. (B) Refolding of the *Ecbd* active site by AurD by the attraction of Asp239^{CydA}. (C) Two dimensional representations of the interactions in the AurD binding pocket. (D) Conservation of the AurD binding pocket residues. (extended sequence variation in table S3.3) (E) Activity of the monomeric *Ecbd*/WT and D239A^{CydA} and D239G^{CydA} mutants (N=3). (F) IC₅₀ of AurD against the monomeric *Ecbd* WT and D239G^{CydA} mutant (N=3), P<0.001.

Discussion

The structural and mechanistic insights presented here establish a comprehensive framework for understanding quinone turnover, electron transfer, and inhibition in *bd*-type oxidases. Prior structural studies of cyt *bd* oxidases have predominantly captured the enzyme in a monomeric state, with no resolved structures of the quinol oxidation site. A notable exception is the dimeric structure of *E. coli* cyt *bd*-II bound to AurD, resolved in amphipols at 3.0 Å resolution[87]. However, the physiological relevance of this dimeric state remained uncertain, as it was speculated to be an artifact of amphipol reconstitution. Using a cryo-EM approach we were able to resolve the dimeric state of *Ecbd* in detergent and nanodiscs, and show it as a native state *in vivo* by SMALP isolation and the presence of native *E. coli* lipids at the dimer interface. The CydX dimer interface is well conserved, indicating common dimerization of cyt *bd* in the species containing the CydX subunit[158]. Interestingly, CydX is solely found within a sub-population of the qOR1-long Q-loop family, showing further divergence within cyt *bd* oxidases[158]. Similar, but not homologous, single-helical subunits are found in short Q-loop *bd* oxidases, such as CydS in *G. thermodenitrificans* cyt *bd* [81]. CydS, however, does not seem to facilitate dimer formation in AlphaFold prediction (Fig. S3.14). In addition, CydS has a profoundly different sequence than CydX, indicating a role in stabilization rather than dimerization.

Functionally, the dimeric form of *Ecbd* exhibits significantly enhanced catalytic activity compared to the monomer, suggesting that dimerization is physiologically important for its function. In the monomeric state, the Q-loop remains disordered despite quinone availability, suggesting that formation of the quinol oxidation site is transient and rate-limiting. This is further supported by previous studies showing that the deletion of the dimer-forming subunit CydX compromises *Ecbd* expression, stability, and activity[92,166]. We hypothesize that dimerization could serve as a regulatory mechanism, enabling *Ecbd* to become catalytically enhanced under microaerobic conditions when it is upregulated to assist the more energetically efficient

cytochrome *bo₃*. This dynamic switch may allow *E. coli* to balance respiratory efficiency with adaptability to stress.

The *Ecbdd* dimer shows quinone (product) binding in a shallow peripheral pocket that stabilizes only the headgroup, consistent with rapid substrate exchange from the quinone pool. This primary quinol oxidation site, located beneath the Q-loop lid near heme *b₅₅₈*, is stabilized by CydA residues Tyr243^{CydA} and Arg298^{CydA}, both uniquely conserved in qOR1-long Q-loop *bd* oxidases (Fig. 3.2G). This conservation underscores a mechanistic divergence from the other subfamilies. Moreover, Tyr243^{CydA} and Arg298^{CydA} are distinctly different from the previously indicated quinol oxidation site residues, Lys252^{CydA}, Glu257^{CydA}, and Glu280^{CydA}[113]. These latter residues stabilize the Q-loop in the MK bound form by forming hydrogen bonding with the main body of CydA, which could explain the drop in activity upon mutation[113].

The Q-loop undergoes a disorder-to-order transition upon quinone binding, completing the quinol oxidation site and enabling catalysis. This large structural rearrangement during the catalytic cycle represents substantial energetic investments to form the catalytically active state. Such large structural rearrangement during catalysis has previously been indicated to prevent irreversible binding of the substrate while presenting a binding pocket highly optimized for the transition state[167]. This means that the closed Q-loop state could represent an intermediate specifically stabilized by the semi-quinone. The binding energy associated with this state will release upon quinone formation, expelling the quinone from the active site to improve turnover rates. This mechanistic adaptation might allow cyt *bd* to maintain high catalytic activity and low K_m , necessary to uphold membrane potential despite displacing less protons than cytochrome *bo₃* or other pumping terminal oxidases[167].

In addition to the primary site, we identified a secondary ubiquinone-binding pocket at the CydA-CydX interface. This site is specific for UQ and mutagenesis suggests it contributes to structural stabilization of the dimer rather than direct catalysis. Additionally, neither the evolution of the long Q-loop, nor the addition of the CydX subunit seem critical for ubiquinol turnover, as neither the

long Q-loop nor a CydX subunit is found in *Pseudomonas aeruginosa* which can utilize ubiquinol[168] (Fig. S3.14). We hypothesize that oxidation of ubiquinol substrates in *Ecbd* is catalyzed at the same menaquinol oxidation site identified in this study. Furthermore, we did not observe any substrate preference or allosteric modulation in the presence of both UQ and MK (Fig. 3.3D).

A particularly notable finding is the dual role of Asp239^{CydA}. This residue is essential for both quinol oxidation site stabilization and inhibition by AurD. Despite the structural similarity between MK and AurD, AurD binding induces a remarkable refolding of helix 6 into an α -helix, preventing substrate access. Interestingly, this α -helical conformation mirrors that observed in all solved structures of short Q-loop *bd* oxidases (Fig. S3.16)[80,82,84]. Despite the sequence and structural conservation of the binding pocket in *M. tuberculosis* cyt *bd*, no densities have been observed for AurD in the presence of vast molar excesses[82]. To probe a similar binding poses of AurD in short Q-loop *bd* oxidases, we performed docking in the *M. tuberculosis* cyt *bd* Q-loop. Despite the conservation of the pocket, no similar binding pose was observed (Fig. S3.17). This raises the possibility that short Q-loop *bd* oxidases undergo structural rearrangement of the Q-loop lid upon turnover, as shown here for *Ecbd*. This conformational change might be present only transiently, or require additional binding partners, prohibiting direct structural interrogation. Alternatively, we cannot exclude that short Q-loop *bd* oxidases have evolved a fundamentally distinct catalytic mechanism, and therefore inhibition with AurD, explaining why no quinone or AurD binding in these enzymes has been observed[80-82,84].

The series of cryo-EM structures presented here enables us to propose a global overview of the entire quinol oxidation mechanism (Fig. 3.6). Even though our quinone bound structures represent the product rather than the substrate, we postulate that the minor difference in substrate (quinol) and product (quinone), do not result in major structural changes and therefore represent either state. We cannot exclude, however, that the equilibria between the different states change depending on the binding of either quinone or quinol. The catalytic cycle starts in the unbound state, in which the Q-loop remains flexible, and Tyr243^{CydA} is positioned for quinol capture (Fig. 3.6A). After quinol

binding (Fig. 3.6B), the Q-loop undergoes a conformational transition that closes the lid and completes the quinol oxidation site by forming a hydrogen bond between Arg298^{Cy^dA} and the quinol head (Fig. 3.6C). Proton-coupled electron transfer proceeds with electrons relayed through the heme chain to the oxygen reduction site at heme *d* (Fig. 3.6D), after which the Q-loop opens and the quinone is released (Fig. 3.6E). A second sequence of quinol oxidation occurs, during which O₂ is fully reduced to two H₂O, completing the *Ecbd* turnover cycle.

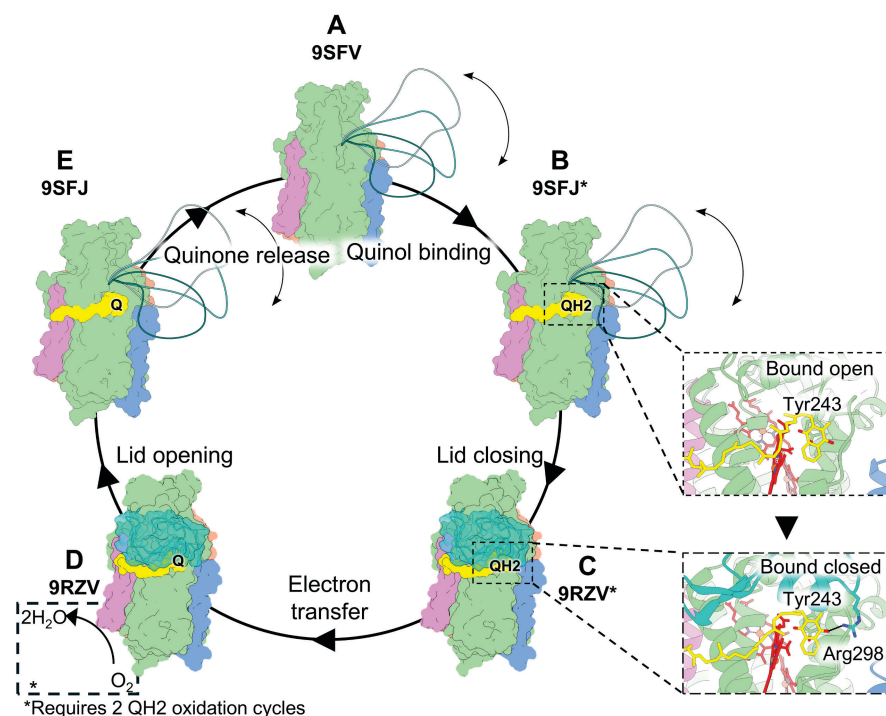


Fig. 3.6. Schematic overview of the proposed turnover mechanism of *Ecbd*. (A–D) Overall schematic view of *Ecbd* turnover. States with an asterisk are presumed to be structurally the same in the quinol and quinone bound states. (A) The unbound *Ecbd* dimer has a flexible Q-loop lid and sits ready for quinol binding. (B) The *Ecbd* dimer undergoes quinol binding. (C) From the quinol bound state (C1), the Q-loop lid closes, finishing the formation of the quinol oxidation site by coordinating Arg298^{Cy^dA} to the quinol head (C2). (D) Electron transfer occurs via the heme chain onto molecular oxygen. (E) The Q-loop lid opens to release the quinone and start another catalytic cycle.

Together, these insights provide a detailed molecular blueprint of *Ecbd* function and establish a foundation for rational drug design targeting *bd*-type oxidases. Given the essential role of cyt *bd* in the survival of several clinically relevant pathogens, these findings have broad implications for the development of next-generation antibiotics.

Experimental procedures

Structure guided mutagenesis

Structure guided *Ecbd* mutagenesis was performed on the pET17b-CydABX-linkerstreptag[92] plasmid using Whole Plasmid Synthesis or Gibson assembly (See Table S4 for primers). Mutants were confirmed using Sanger sequencing.

Ecbd purification

The expression of *E. coli* cytochrome *bd*-I (*Ecbd*) was performed as stated previously[90]. Briefly, MB43 cells[144,145] transformed with pET17b-CydABX-linkerstreptag or MB43ΔCydA[90] transformed with the mutant plasmids were grown overnight in LB with 100 µg/mL ampicillin (250 RPM, 37°C). The WT culture was diluted to OD ~0.1 and grown to OD ~0.4 before induction with 0.45 mM IPTG. At OD 2.0 the cells were harvested by centrifugation (6371 rcf, 20 min, 4°C) and resuspended in 50 mM MOPS, pH 7.4, 100 mM NaCl, cOmplete™ EDTA-free Protease Inhibitor (ROCHE), at 5 mL per 1 gram of wet cells. The *Ecbd* mutants were expressed overnight and otherwise followed the same procedure. The cells were lysed by a passing through a Stansted pressure cell homogeniser (270 MPa). Cell debris were pelleted by centrifugation (10.000 rcf, 20 min, 4°C). Crude membranes were isolated by ultra-centrifugation (200.000 rcf, 1h, 4°C) followed by resuspension in 50 mM MOPS, 100 mM NaCl, pH 7.4 (10 mg/mL total protein). Solubilization of the *Ecbd* was performed by incubation with 0.5% Lauryl maltose neopentyl glycol (LMNG) for 1 hour at 4°C with gentle mixing. Insoluble material was pelleted by ultra-centrifugation (200.000 rcf, 30 min, 4°C) followed by application of the soluble fraction to a StrepTrap HP column (Cytiva) at 2 mL/min. The column was washed with 50 mM sodium

phosphate, 300 mM NaCl, 0.005% LMNG, pH 8.0 to remove unbound proteins. *Ecbd* elution was performed by addition of 50 mM sodium phosphate, 300 mM NaCl, 2.5 mM desthiobiotin, 0.005% LMNG, pH 8.0, after which purity was confirmed by SDS-page. If needed, *Ecbd* dimers and monomers were separated using size exclusion chromatography (SEC) on a Superdex increase 200 10/300 column (Cytiva) at 0.5 mL/min (50 mM sodium phosphate, 300 mM NaCl, 0.005% LMNG, pH 8.0). The defined fractions were pooled, concentrated, and stored at -80°C until further use.

NDH2 purification

NDH2 was required to reduce the quinone pool in order to measure *Ecbd* activity. Expression and purification of NDH2 were performed as previously described[159]. Briefly, C41 (DE3) cells, transformed with pET28-NDH-2_NtermHis, were grown overnight LB Kanamycin (250 RPM, 37°C). The culture was diluted 20-fold and grown to -OD 0.5 before induction with 0.25 mM IPTG. NDH2 expression was maintained for 4 hours at 37°C before harvesting by centrifugation (6371 rcf, 20 min, 4°C). The cells were resuspended in a 5 mL of 50 mM Tris-HCl, 5 mM MgCl₂, pH 8.0 per 1 gram of cells, and disrupted by passing through a Stansted pressure cell homogeniser (270 MPa). Cell debris were pelleted by centrifugation (10.000 rcf, 20 min, 4°C) before harvesting of the crude membranes by ultra-centrifugation (200.000 rcf, 1h, 4°C). The membranes were resuspended in Tris-HCl, 150 mM NaCl, 20 mM Imidazole (10 mg/mL total protein). NDH2 was extracted by treatment with 1% DDM for 1 hour at 4°C with gentle mixing. The remaining insoluble material were removed by ultra-centrifugation (200.000 rcf, 30min, 4°C) before application of the soluble fraction on a HiTrap Nickel NTA column (Cytiva). The unbound proteins were washed from the column with 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 20 mM Imidazole, 0.02% DDM. NDH2 was eluted by addition of 30% elution buffer (50 mM Tris-HCl, 150 mM NaCl, 500 mM Imidazole, 0.02% DDM). Final purification was achieved by gel filtration on a Superdex increase 200 10/300 column (Cytiva) at 0.5 mL/min (50 mM Tris-HCl, 500 mM NaCl, 5% glycerol, 0.02% DDM). Pure NDH2 fractions were pooled, concentrated, and stored at -80°C until further use.

MSP1D1 expression and purification

MSP1D1 expression was performed as described before[169]. Briefly, BL21(DE3) pLysS transformed with pET28a containing the MSP1D1 gene[169] were grown in terrific broth at 37°C, 200 RPM until OD ~2.0. The cultures were cooled to 30°C before induction with 1 mM IPTG. The cells were induced for 5 hours, followed by cell harvesting (10.000 rcf, 20 min, 4°C). The cells were resuspended in 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 1% Triton X-100. The cells were lysed by a single pass through a Stansted pressure cell homogeniser (270 MPa). Debris and membrane fractions were pelleted by ultra-centrifugation (200.000 rcf, 1h, 4°C). The supernatant was applied to a HisTrap (Cytiva) column followed by extensive washing with buffer containing 1% (w/v) Triton X-100 and 50 mM Na-cholate. Finally, the MSP1D1 was eluted using 50 mM Tris, 300 mM NaCl, 300 mM Imidazole. The MSP1D1 peak fractions were concentrated and further purified on a HiLoad 16/100 75 pg column (Cytiva) using 50 mM Tris-HCl, pH7.5, 200 mM NaCl. The peak fractions were concentrated to 10 mg/mL and stored at -80°C until further use.

Ecbd reconstitution in nanodiscs

Ecbd nanodiscs were assembled as described previously with slight modifications[83]. Briefly, the POPC:MK-9 mixture was dissolved in 20 mM HEPES (pH 7.4), 150 mM NaCl 100 mM Na-cholate by sonication. Following, MSP1D1, POPC, MK-9 and *Ecbd* were mixed at the molar ratio of 20:760:40:1 and incubated for 1h on ice. The detergent was removed by the stepwise addition of 5% w/v SM-2 biobeads every two hours, totaling 15% w/v before overnight incubation at 4°C while mixing. The biobeads were removed by filtration before application of the nanodisc mixture on a Superdex increase 200 10/300 column (Cytiva) at 0.5 mL/min (20 mM HEPES, 150 mM NaCl, pH 7.4). *Ecbd* nanodisc peak fractions were concentrated and used for further analysis and grid preparation.

SEC-MALS analysis

The *Ecbd* in detergent or nanodiscs were characterized using a SEC-MALS system comprised of a miniDAWN® TREOS®, Optilab differential refractometer (Wyatt technology) and 1260 Infinity II multiple wavelength absorbance detector (Agilent). The nanodisc composition was determined using the protein conjugate method in Astra software (Version 8) after defining the dn/dc_{protein} , dn/dc_{lipids} and ε_{417nm} , protein. *Ecbd* monomer and dimer equilibria were determined after incubation with lipids, quinones or Aurachin D.

Ecbd oxygen consumption

Oxygen consumption of *Ecbd* was measured on an oxygraph (Hansatech Ltd.) system at 20°C [159]. The quinone (MK-1 or UQ-1) was added to the reaction chamber at the indicated concentration in 50 mM MOPS, 150 mM NaCl, (0.005 LMNG if needed) pH 7.0. The enzymatic quinone reduction with *C. thermarum* NDH-2 (30 nM) was initiated by the addition of 1 mM NADH, followed by the determination of the quinone autooxidation (background). Oxygen consumption was initiated by the addition of *Ecbd* (4 nM). The enzyme activity was measured by subtraction of the quinone autooxidation rate from the initial slope after *Ecbd* addition. In case of IC₅₀ determination, the desired amount of AurD was added after *Ecbd* addition. The slope after AurD addition was divided by the slope before AurD addition to determine the inhibition rate. For direct kinetic comparison of *Ecbd* with its mutants, monomeric *Ecbd* was used.

Ecbd reconstitution in proteoliposomes for electrochemistry

Lipids dissolved in chloroform were purchased from Avanti Polar Lipids. A lipid mixture of POPE:POPG:CA 60:30:10, enriched with the 1 mol% of ubiquinone-10 (UQ-10) (Sigma) or menaquinone-9 (MK-9) (Caymen chemical), was dried under a stream of nitrogen followed by overnight incubation under vacuum. The lipids were rehydrated to a final concentration of 10 mg/mL (20 mM MOPS, 30 mM

Na_2SO_4 , 100 mM KCl, pH 7.4) and extruded to 200 nm using an Avanti extruder. *Ecbd* reconstitution was performed as previously described[148]. LMNG solubilized *Ecbd* was added to the liposome solution at 1 w/w% protein/lipids and mixed for 30 min by inversion at RT. Insoluble materials were removed by centrifugation in an Eppendorf tabletop centrifuge (14100 rcf, 5 min). The reconstituted *Ecbd* concentration was determined by re-dissolving a sample in 2% octyl- β -glucoside, followed by quantification of the Soret band ($\epsilon_{417} 230 \text{ mM}^{-1} \text{ cm}^{-1}$).

Electrode preparation and electrochemistry in a tBLM system

Template-stripped gold (TSG) electrodes were produced in-house by evaporating a 150 nm thick 99.99% pure Au layer (Thessco) on a cleaned atomically smooth silicon wafer using a Plassys MEB600SL E-beam Evaporator operating at 10^{-8} mbar. The gold deposition was monitored using a piezoelectric quartz crystal at 6.0 MHz. Glass slides (1.2 cm^2) were glued onto the gold layer with Epo-Tek 377 two-component glue and heated to 120°C for 2 hours for curing. Functionalization of the TSG electrodes with a self-assembled monolayer (SAM) was achieved by overnight incubation of freshly stripped electrodes in a mixture of 0.11 mM EO_3 -cholesteryl[170], 0.89 mM 6-mercaptohexanol (Sigma) dissolved in 2-propanol. Prior to measurement, the functionalized TSG electrodes were washed with 2-propanol and thoroughly dried under a stream of nitrogen. The TSG electrodes were placed into a custom polyether ether ketone (PEEK) sample holder with a Viton® O-ring seal ($A = 0.29 \text{ cm}^2$). The sample holder was placed into a custom electrochemical cell and submerged in the electrolyte solution (20 mM MOPS, 30 mM Na_2SO_4 , pH 7.4). The electrochemical setup was completed by the addition of a Pt counter electrode, a saturated double junction Ag/AgCl (sat. KCl) reference electrode (Radiometer analytical) and placing it in a steel Faraday cage. All potentials are given versus standard hydrogen electrode (SHE) using 0.199 mV vs SHE for the Ag/AgCl reference electrode. The electrochemical measurement was performed using an Autolab (Eco Chemie) electrochemical analyzer equipped

with a PGSTAT30 potentiostat and a FRA2 frequency analyzer. Impedance spectroscopy (EIS) was used to confirm that the SAM consisted of ~50% EO3-cholesterol, as described previously[171]. The electrolyte solution was purged with 95% Ar, 5% O₂ to remove most oxygen. Cyclic voltammograms were taken at 10 mV/s by holding the potential at 0.4 V vs SHE for 5 seconds and scanning between 0.4 and -0.4 V vs SHE. Background cyclic voltammograms of the SAM were taken before the addition of the tethered bilayer lipid membrane (tBLM) system to the electrode. The tBLM was created by the addition of 10 mM CaCl₂ and *Ecbd* proteoliposomes with 1 w/w% quinone (UQ-10 or MK-9) to a concentration of 0.5 mg/mL. The formation of the tBLM was followed by impedance spectroscopy and deemed finished once the signal stabilized (~1 hour). The catalytic activity of *Ecbd* was measured using cyclic voltammetry.

Cryo-EM sample preparation and data collection

Quantifoil UltraAUfoil R1.2/1.3 grids (mesh 300) were glow discharged with a PELCO easiGlow device at 15 mA for 90s on the foil side and 60 seconds on the other. *Ecbd* nanodiscs or in LMNG micelles (4 μ L, 1.5 mg/ml), supplemented 100 with μ M AurD if needed, were applied to the grid, followed by blotting at 4°C, 100% humidity, 20 blot force for 4 seconds using a Vitrobot IV device (Thermo Fisher) immediately before plunge freezing in liquid ethane. Blot time for *Ecbd* in LMNG was increased to 6 seconds.

Grids were either imaged on a Titan Krios G1 (ThermoFisher Scientific) operating at 300 kV, equipped with a Gatan K3 detector and BioQuantum energy filter with a slit width of 20 eV, or a Titan Glacios (ThermoFisher Scientific) operating at 200 kV with a Falcon 4i detector and selectris energy filter with a slit width of 20 eV. Movies were gathered in electron counting mode using aberration-free image-shift (AFIS) in EPU (ThermoFisher Scientific). A total dose of 100 e/ $\text{\AA}^2$ with 100 frames, at 105,000 $\times$ magnification with a calibrated pixel size of 0.836 $\text{\AA}$ (Krios), or a magnification of 130,000 $\times$ with a calibrated pixel size of 0.880 $\text{\AA}$ (Glacios), at and a defocus range of -0.8 to -2.0 μ m.

Cryo-EM data analysis

Data processing was performed in CryoSPARC. Image preprocessing was performed in CryoSPARC live[150] using Patch Motion Correction and Patch CTF estimation. The processed micrographs were exported to CryoSPARC for further processing. Initial particle picking was performed with blob picking, followed by template generation for both the monomer and dimer particles. Template particle picking was performed for the monomer and dimer particles followed by multiple rounds of 2D classification to remove junk particles. Three volume unsupervised *ab-initio* model generation was performed to generate initial and bait volumes for further particle cleanup. Multiple rounds of heterogenous refinement were performed until the best class with clean particles became consistent. This clean particle stack was moved to Non-uniform refinement[151] for local CTF refinements and higher order aberration fitting. The movies were reprocessed in patch-based motion correction to remove the last 50 frames to account for accumulated beam damage. The particles were subjected to reference based motion correction[172], followed by another round of Non uniform refinement before assessment of heterogeneity using 3D classification. Dimer particles in the unbound dataset were split into the unbound and a bound-open class. The local resolution was improved using local refinement masking a single *Ecbd* protomer in the *Ecbd* dimer.

Model building and analysis

Model building was started from pdb 6RKO for the *Ecbd* monomer and an AlphaFold[173] prediction for the dimer. Model building was performed in Coot software[174] (version 0.9.8.1) followed by real space refinement in Phenix[175] (version 1.21.1-5286). Models and their corresponding maps were assessed and analyzed in ChimeraX[176] (version 1.7.1). Interior cavities and tunnels were analyzed using MOLEonline[163] (5 probe, 1 interior threshold, 5 origin radius, 8 surface over radius, 1.1 bottleneck radius, 3 bottleneck tolerance). The pKa of the titratable residues along the proton channel were estimated using PROPKA. Ligand binding pockets were visualized using LigPlot+[177].

Sequence alignment

For analysis of conserved residues, the multiple sequence alignment of Muralli *et al.*[88] was utilized, with additional division of qOR1 into the long and short Q-loop subfamilies. The sequence alignment was visualized using jalview, and reported as the consensus sequence with residues colored according to conservation >75%. The consensus sequence for CydX was taken from Allen *et al.*[158].

UV-Vis spectroscopy

Heme reduction in the *Ecbd* WT and mutants was analysed using an oxidized minus reduced spectrum. *Ecbd* was oxidized with 0.25 mM hexacyanoferrate in 50 mM NaPi, 300 mM NaCl, 0.005% LMNG, pH 8 and reduced by adding a few grains of sodium dithionite. Specific heme reduction was found by subtracting the oxidized from the reduced spectrum. The spectra were normalized to the Soret band at 417 nm of the respective oxidized spectra.

Supplementary information

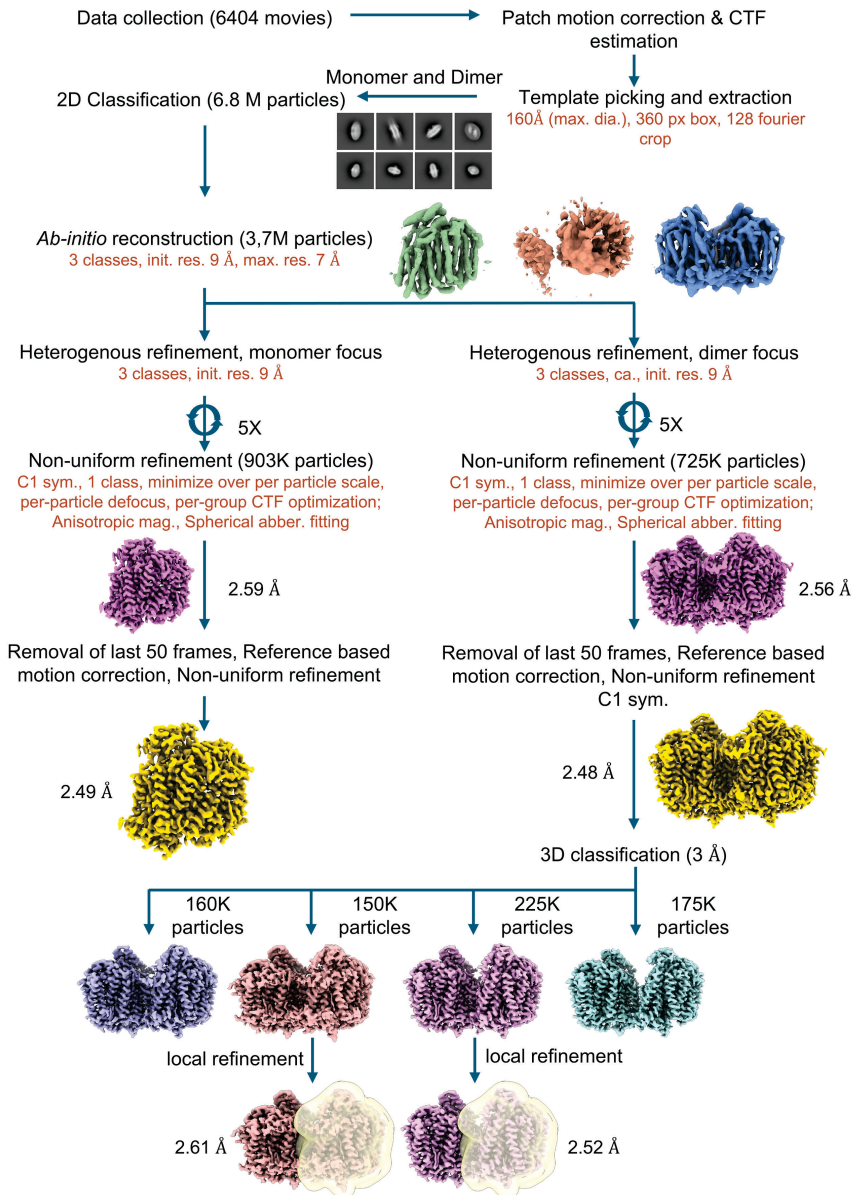


Figure S3.1: Cryo EM processing of the *Ecbd* unbound and bound^{open} structures (9SE4, 9SFV, 9SFJ). Templates for template picking were generated using the MK-bound dataset (in nanodiscs). The same templates were used for processing of all other datasets.

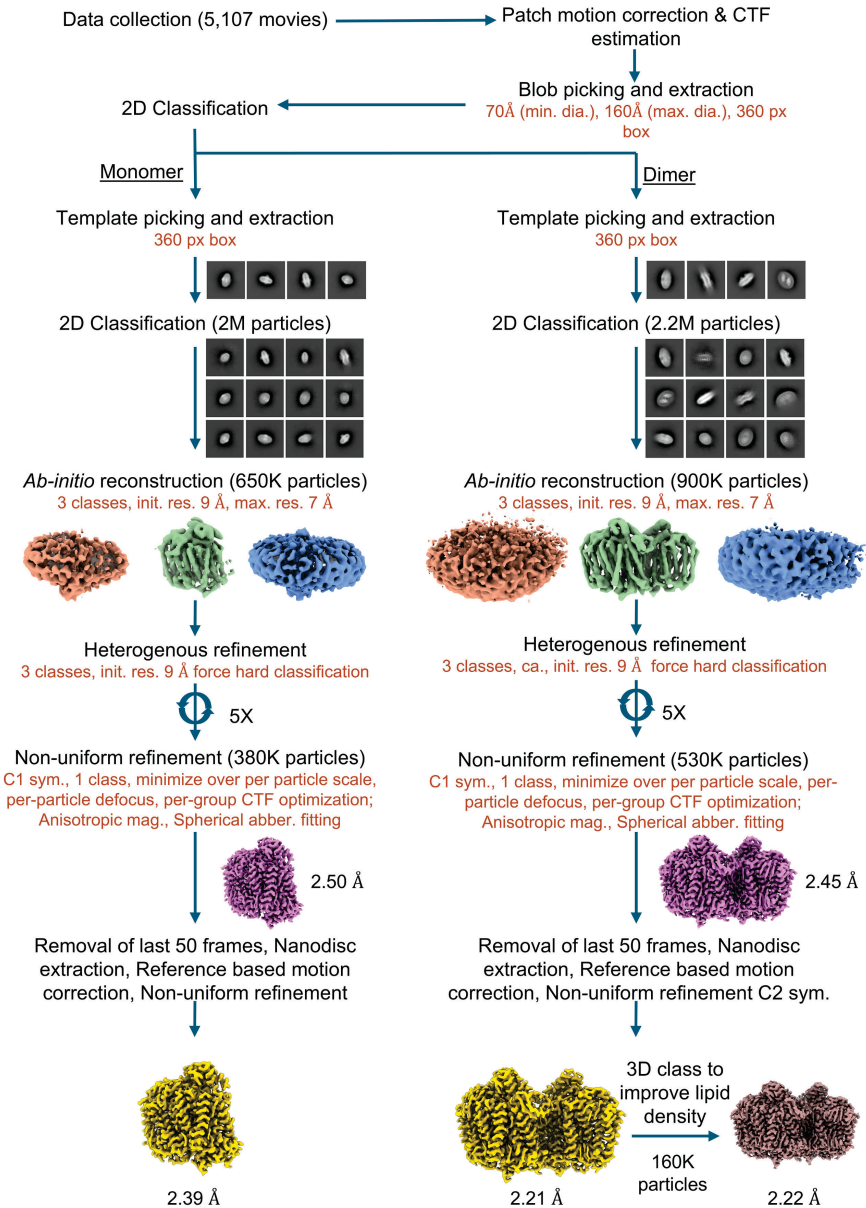


Figure S3.2: Cryo-EM processing of the *Ecbd* MK bound^{closed} structure (9SFF, 9RZV). Templates for template picking were generated using the MK-bound dataset (in nanodiscs) as shown in Fig. S1. The same templates were used for processing of all other datasets.

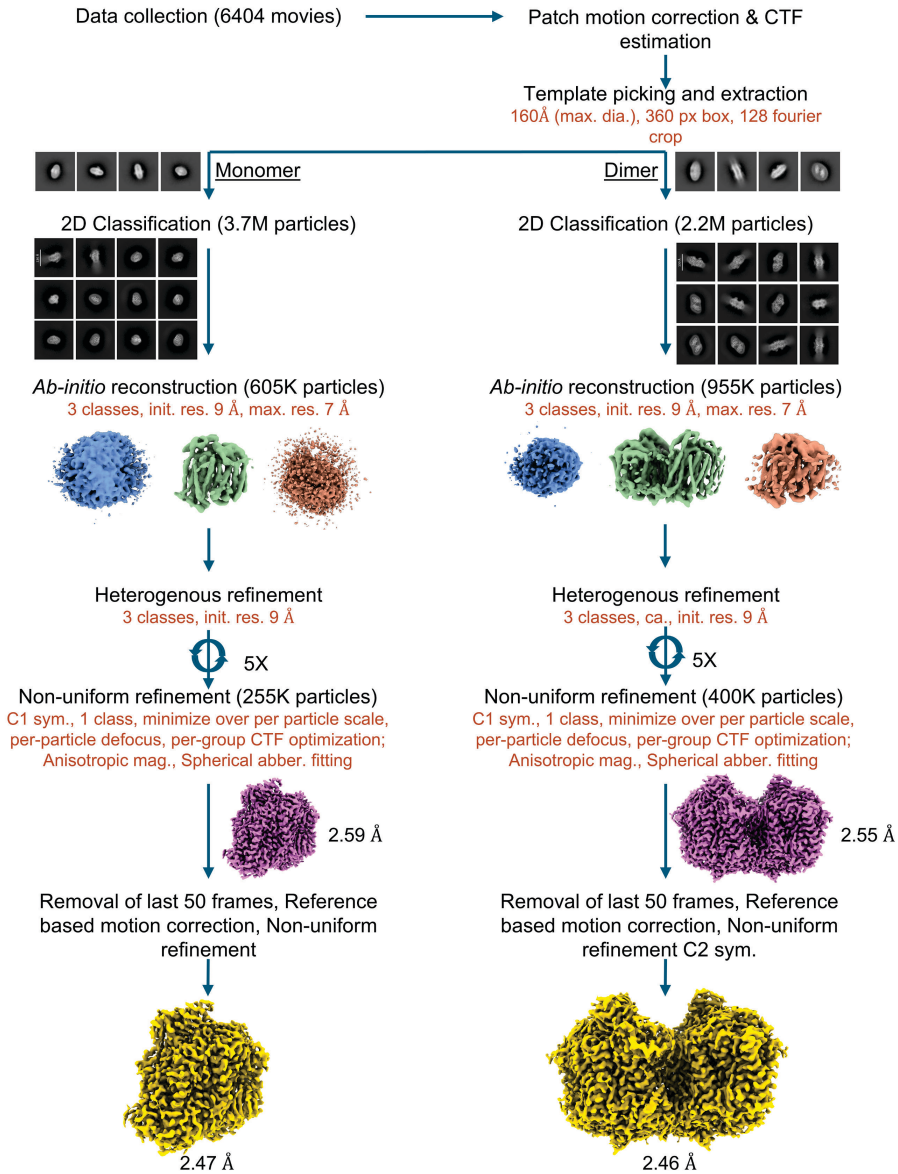


Figure S3.3. Cryo-EM processing of the Aurachin D bound *Ecbd* structures (9SFH, 9SEJ). Templates for template picking were generated using the MK-bound dataset (in nanodiscs) as shown in Fig. S1. The same templates were used for processing of all other datasets.

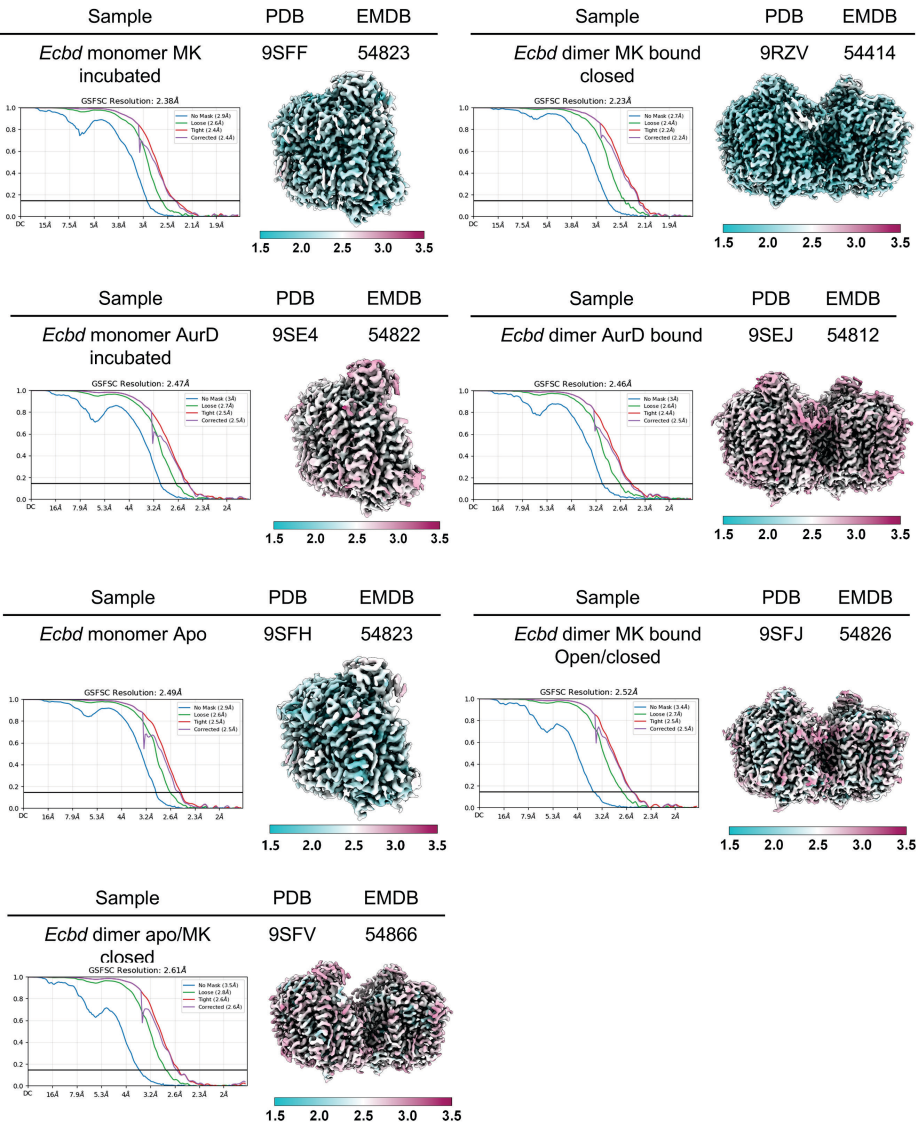


Figure S3.4: GSFSC curves and local resolution estimates

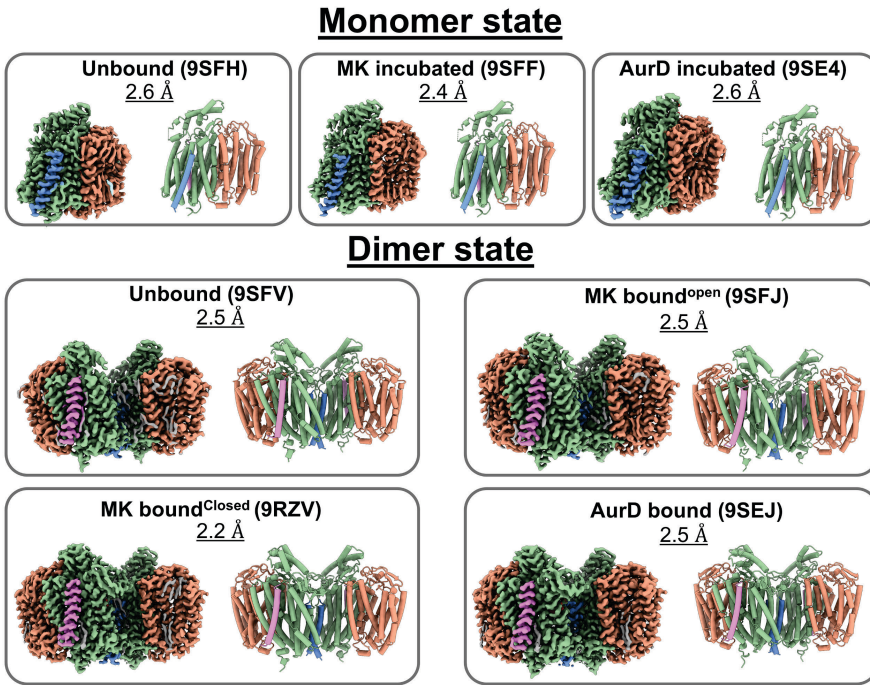


Figure S3.5. Overview of cryo-EM structures solved in this study

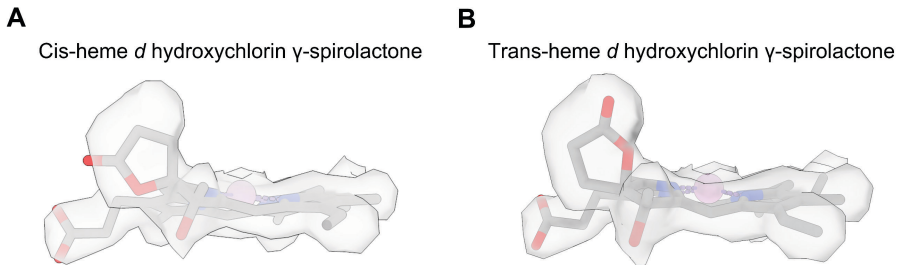


Figure S3.6. Fitting of the heme density with (A) cis heme *d* hydroxychlorin γ -spirolactone or (B) trans heme *d* hydroxychlorin γ -spirolactone.

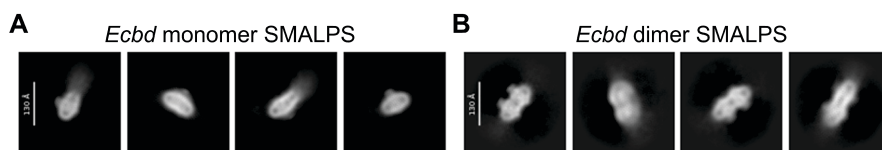


Figure S3.7. 2D classes of *Ecbd* after SMALP isolation (A) 2D classes of the *Ecbd* monomer (B) 2D classes of the *Ecbd* dimer showing of both oligomeric states *in vivo*.

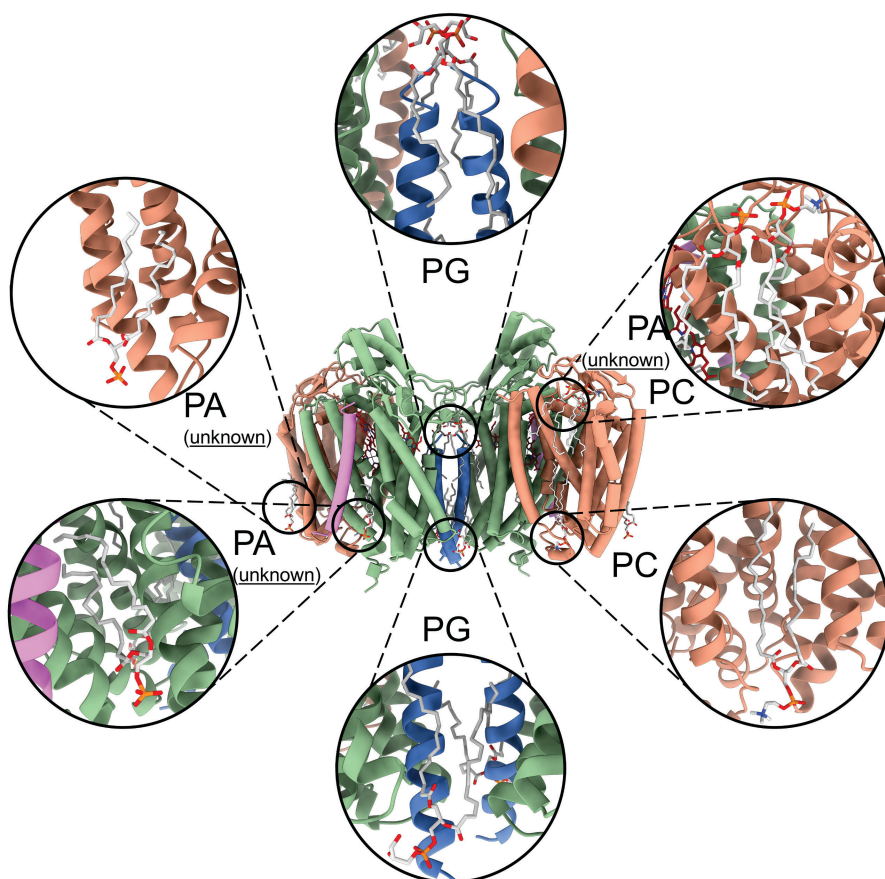


Figure S3.8. Lipids bound peripherally and to the interface of the *Ecbd* dimer. Lipids with unclear headgroup densities are modelled as phosphatidic acid (PA).

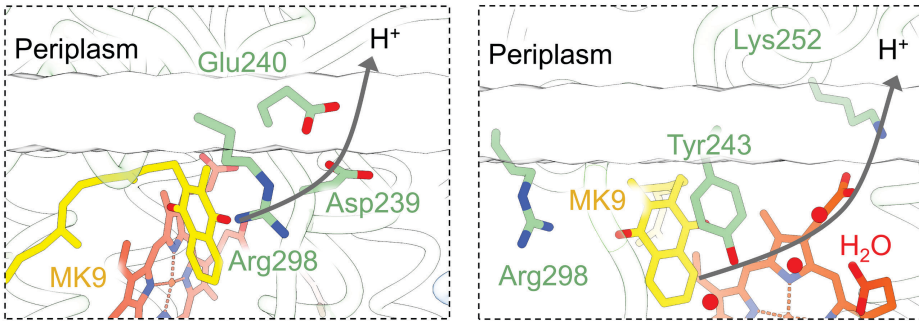


Figure S3.9. Putative proton transfer routes from the quinol oxidation site towards the periplasm. One route transfers past the Asp239 Glu240 pair near the quinol oxidation site. The other route transfers via structures water molecules towards Lys252 at the membrane interface.

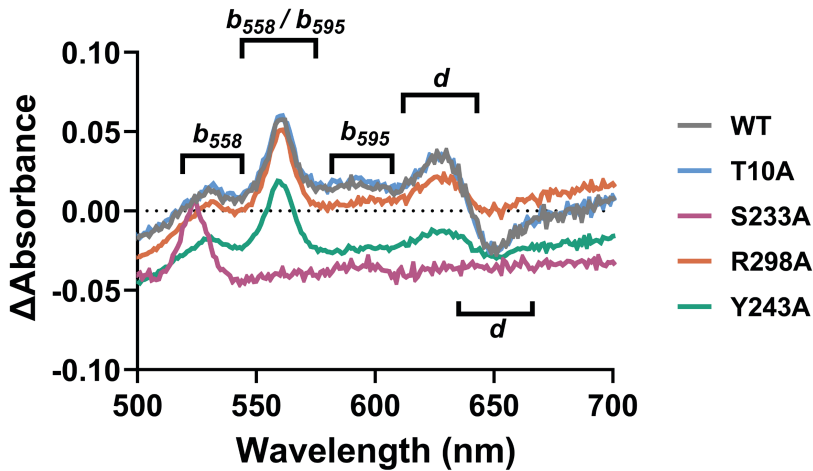


Figure S3.10. Reduced minus oxidized spectra of the *Ecbd*/WT and the Y234A, R298A, T10A, S233A mutants. The data was normalized to the Soret band (415 nm) of the oxidized spectrum.

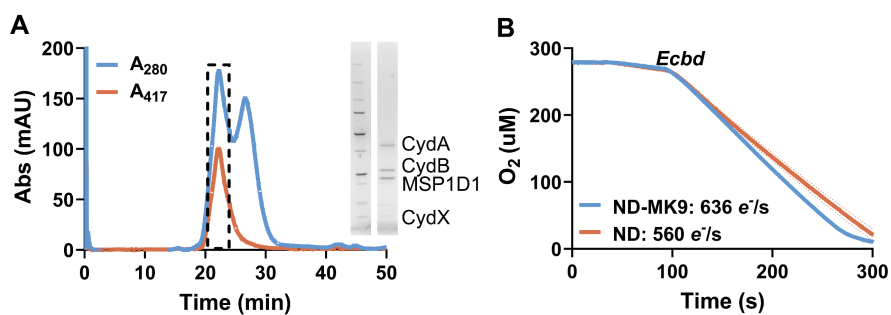


Figure S3.11. *Ecbd* reconstitution in nanodiscs. (A) Size exclusion of *Ecbd* nanodiscs. (B) Activity of *Ecbd* nanodiscs with either MK or UQ.

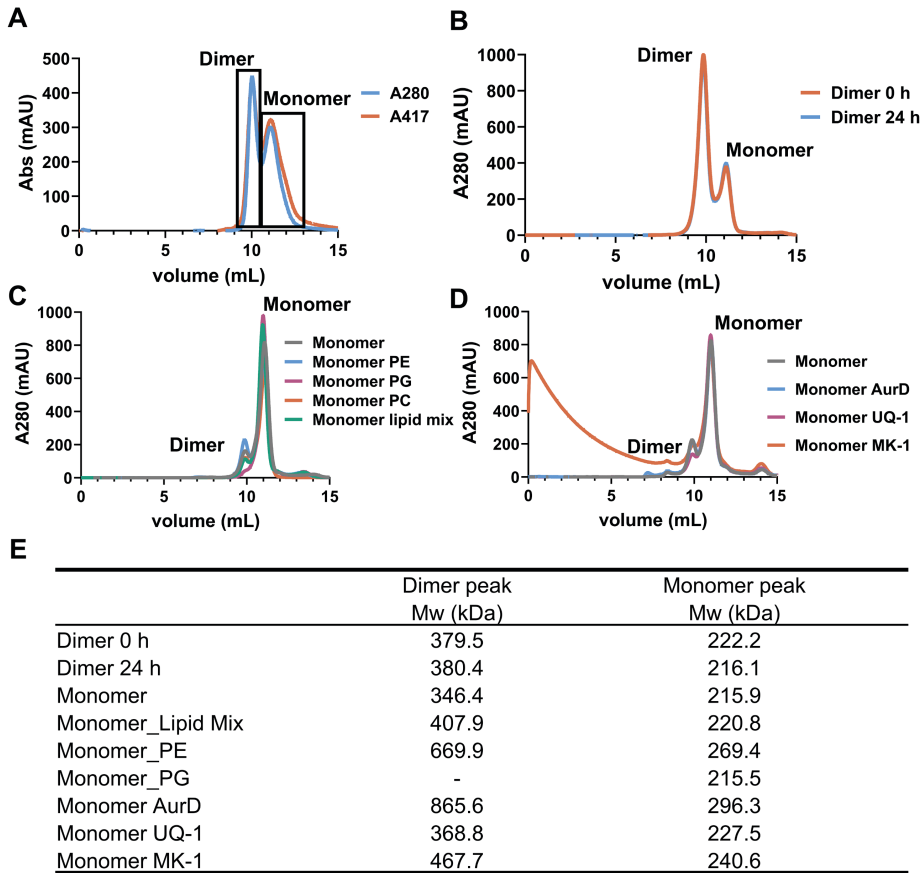


Figure S3.12. *Ecbd* reconstitution in nanodiscs. (A) SEC of the Strep purified *Ecbd* in LMNG on a superdex 200 column, enriching monomer and dimer *Ecbd* samples. (B) Stability of the SEC-purified *Ecbd* dimer (boxed in panel (A)) in detergent on SEC MALS. (C) SEC-MALS traces of SEC-purified *Ecbd* monomers (boxed in panel (A)) showing stable oligomeric state upon incubates with lipids or (D) incubation with quinones and Aurachin D (AurD). The addition of MK results in a decreasing baseline due to its absorption at 280 nm. (E) Mass determination by SEC MALS of the monomeric and dimeric *Ecbd* peaks.

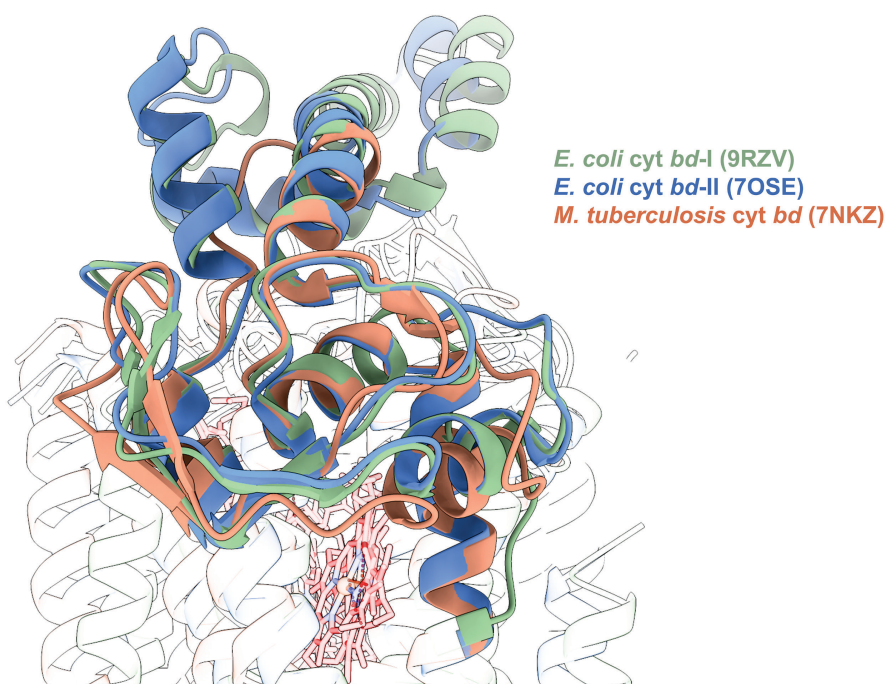


Figure S3.13. Overlay of the Q-loop fold found in the *E. coli* cyt *bd*-I MK bound state (9RZV), *E. coli* cyt *bd*-II AurD bound state (7OSE), and *M. tuberculosis* cyt *bd* (7NKZ).

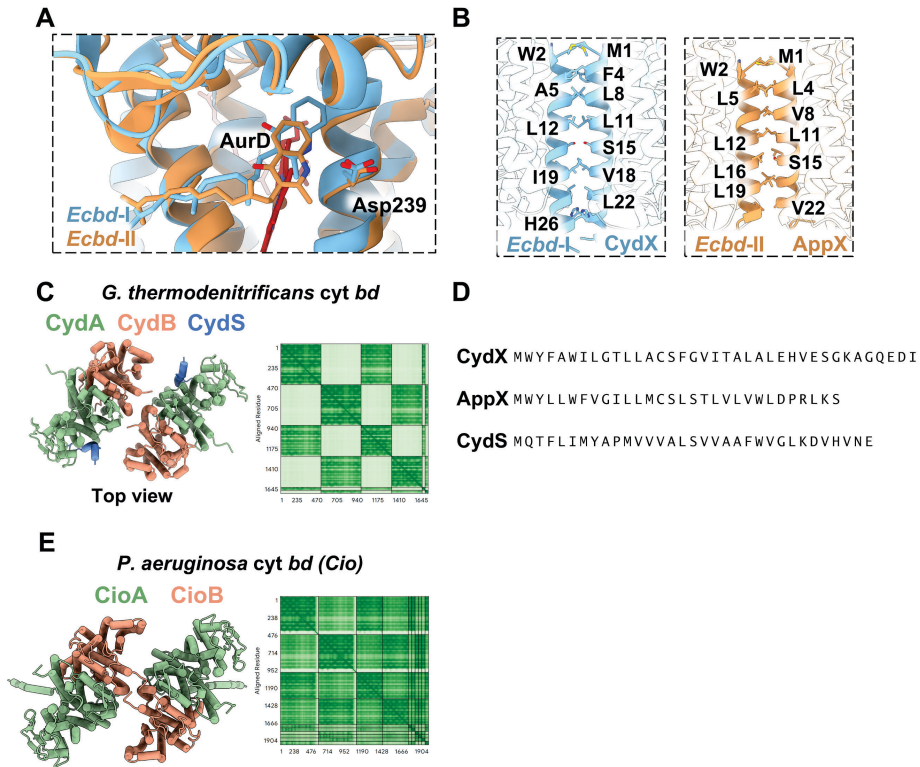


Figure S3.14. Comparison of AurD binding and the dimer interface (A) binding of AurD to *Ecbd-I* and *Ecbd-II* (70SE) (B) Dimer interface of *Ecbd-I* CydX and *Ecbd-II* AppX (C) Predicted *G. thermodenitrificans* *cyt bd* dimer using AlphaFold 3, indicating Cyd S does not stabilize dimer formation. (D) sequence comparison of *E. coli* CydX, AppX and *G. thermodenitrificans* CydS. (E) Predicted *P. aeruginosa* *cyt bd* dimer using AlphaFold 3, indicating dimer formation formation via interactions of CydB.

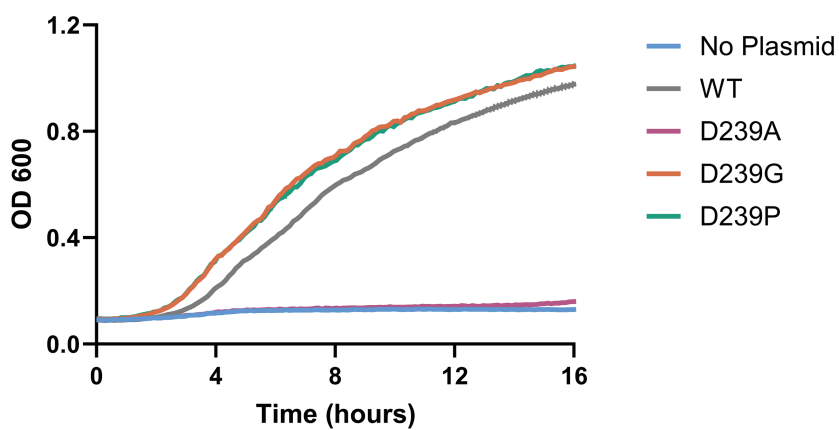


Figure S3.15. Growth curves of the MB43 Δ cydA knockout strain supplemented with *Ecbd*/WT or its D239^{CydA} mutants

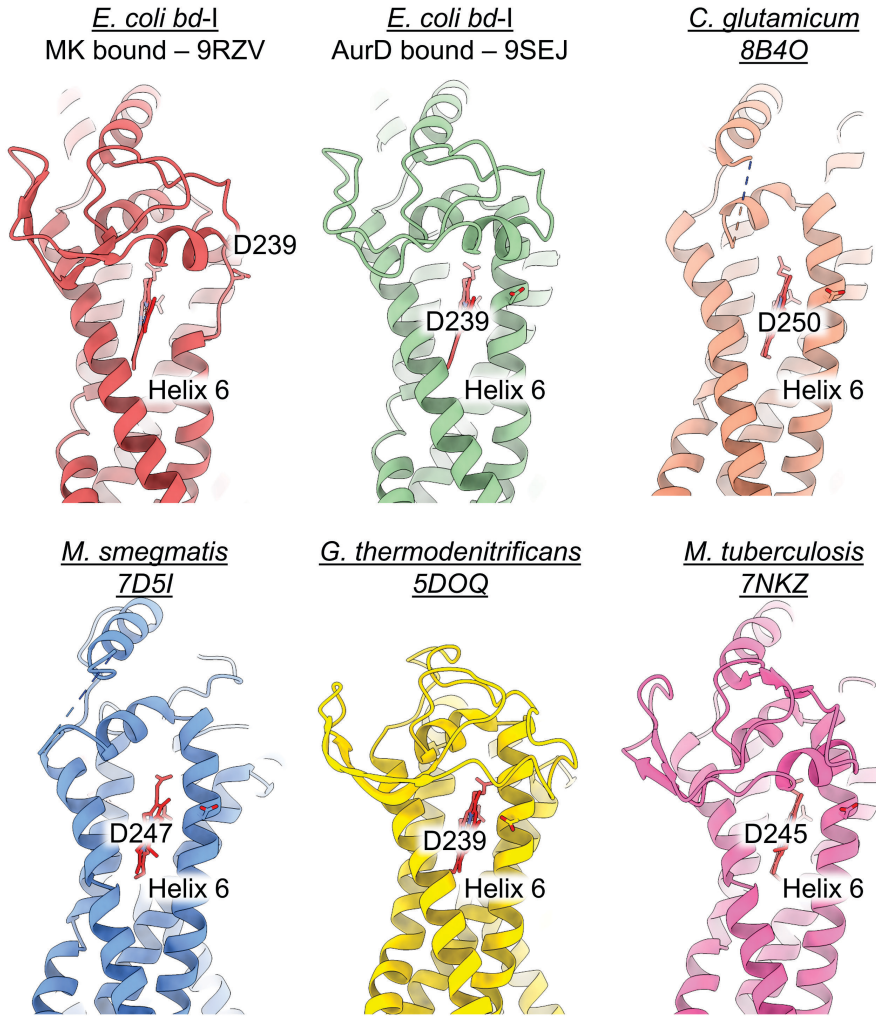


Figure S3.16. Overview of the helical states of cyt *bd* oxidases. The short Q-loop *bd* oxidases represent the helical fold of helix 6, as in the AurD inhibited state of *E. coli* cytochrome *bd*-I

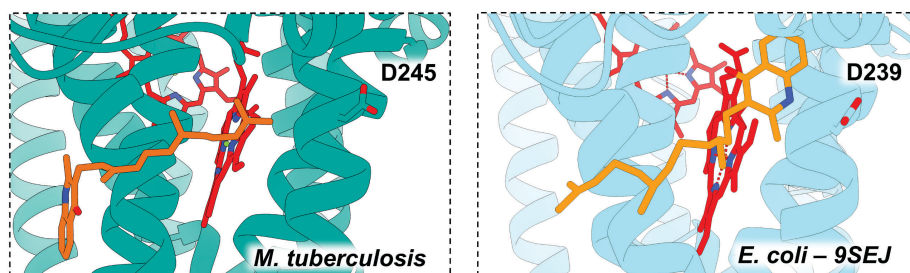


Figure S3.17. Comparison of the predicted binding pose of Aurachin D in *M. tuberculosis* cytochrome *bd* using AutoDock Vina[178,179] via Swissdock[180,181], with the cryo-EM structure of *Ecbd* bound to Aurachin D (9SEJ).

Table S3.1. Cryo-EM data collection, refinement and validation statistics

	<i>Ecdb</i> monomer Unbound (EMDB-54823) (PDB 9SFH)	<i>Ecdb</i> monomer MK incubated (EMDB-54822) (PDB 9SFF)	<i>Ecdb</i> monomer AurD incubated (EMDB-54801) (PDB 9SE4)	<i>Ecdb</i> dimer MK bound Closed (EMDB-54414) (PDB 9RZV)	<i>Ecdb</i> dimer MK bound-open/closed (EMDB-54826) (PDB 9SFJ)	<i>Ecdb</i> dimer unbound/MK closed (EMDB-54866) (PDB 9SFV)	<i>Ecdb</i> dimer AurD (EMDB-54812) (PDB 9SEJ)
Data collection and processing							
Magnification	130,000	105,000	130,000	105,000	130,000	130,000	130,000
Voltage (kV)	200	300	200	300	200	200	200
Electron exposure (e-/Å ²)	100	100	100	100	100	100	100
Defocus range (μm)	-0.8 - -2.0	-1.0 - -2.2	-0.8 - -2.0	-1.0 - -2.2	-0.8 - -2.0	-0.8 - -2.0	-0.8 - -2.0
Pixel size (Å)	0.880	0.836	0.880	0.836	0.880	0.880	0.880
Symmetry imposed	C1	C1	C1	C2	C1	C1	C2
Initial particle images (no.)	6,873,607	2,979,789	3,721,318	3,289,126	6,873,607	6,873,607	2,009,440
Final particle images (no.)	764,760	380,310	399,575	530,094	224,083	152,474	255,127
Map resolution (Å)	2.49	2.39	2.47	2.23	2.52	2.61	2.47
FSC threshold	0.143	0.143	0.143	0.143	0.143	0.143	0.143
Map resolution range (Å)	2.46-2.6	2.39-2.51	2.47-2.56	2.21-2.28	2.52-2.58	2.5-2.56	2.45-2.51
Refinement							
Initial model used (PDB code)	6RKO			AlphaFold			

Model resolution (Å)	2.6	2.4	2.5	2.2	2.4	2.5	2.3
FSC threshold	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Map sharpening B factor (Å ²)	-60	-60	-60	-40	-60	-60	-60
Model composition							
Non-hydrogen atoms	7492	7492	7492	16307	15464	15412	15975
Protein residues	892	892	892	1902	1902	1858	1902
Ligands	2 HEM, 1 A1JN4, 3 LPP, 1 UQ8, 1 OXY	2 HEM, 1 A1JN4, 3 LPP, 1 MQ9, 2 POV, 1 OXY	2 HEM, 1 A1JN4, 3 LPP, 1 UQ8, 1 OXY	4 HEM, 2 A1JN4, 6 LPP, 4 MQ9, 4 PGT, 4 POV, 2 UQ8, 2 OXY	4 HEM, 2 A1JN4, 6 LPP, 2 MQ8, 4 UQ8, 2 OXY	4 HEM, 2 A1JN4, 6 LPP, 1 MQ8, 4 UQ8, 2 OXY	4 HEM, 2 A1JN4, 4 LPP, 2 oNI, 2 PGT, 4 POV, 4 UQ8, 2 OXY
Water	32	32	32	77	10	10	98
Bfactors (Å²)							
Protein	68.40	79.60	63.40	53.92	97.41	124.62	78.28
Ligand	52.36	87.37	50.36	62.80	100.32	122.41	77.50
R.m.s. deviations							
Bond lengths (Å)	0.003	0.003	0.003	0.004	0.004	0.004	0.004
Bond angles (°)	0.805	0.703	0.806	0.749	1.460	1.140	1.47
Validation							
MolProbity score	1.60	1.39	1.58	1.31	1.40	1.32	1.37
Clashscore	7.79	5.68	7.51	2.01	5.05	2.29	4.28
Poor rotamers (%)	1.5	0.55	1.64	0.26	1.32	1.12	0.58
Ramachandran plot							
Favored (%)	97.85	97.84	97.96	99.26	97.83	97.61	97.93

Allowed (%)	2.04	2.16	2.04	0.96	2.17	2.39	1.96
Disallowed (%)	0.11	0	0	0.05	0	0	0.11

Table S3.2. Mutagenesis primers

Primer	Sequence
<i>Ec</i> bd.AS233A Fw	atggctgctgttctggctgttattgttctgggt
<i>Ec</i> bd.AS233A Rv	accagaacaataacagccagaacagcagccat
<i>Ec</i> bd.AD239A Fw	tctgttattgttctgggtgccgaatccggctacgaaatgggc
<i>Ec</i> bd.AD239A Rv	gcccatttcgtagccggattcggcaccagaacaataacaga
<i>Ec</i> bd.AD239G Fw	tctgttattgttctgggtggcgaatccggctacgaaatgggc
<i>Ec</i> bd.AD239G Rv	gcccatttcgtagccggattcggcaccagaacaataacaga
<i>Ec</i> bd.AD239P Fw	attgttctgggtccagaatccggctac
<i>Ec</i> bd.AD239P Rv	gtagccggattctggaccagaacaat
<i>Ec</i> bd.AY243A Fw	ctgggtgacgaatccggcgccgaaatgggcgacgtgcag
<i>Ec</i> bd.AY243A Rv	ctgcacgtgcccatctcggcgccggattcgtcaccag
<i>Ec</i> bd.AR298A Fw	ctgggcatcattgcaacggcatccgtggataccccggtt
<i>Ec</i> bd.AR298A Rv	aaccggggtatccacggatgccgttgcaatgatgccag
<i>Ec</i> bd.XT10A Fw	gcatggattctgggagctcttcttgccctgttcg
<i>Ec</i> bd.XT10A Rv	cgaacaggcaagaagagctcccagaatccatgc
<i>Ec</i> bd.Gibson Fwd	gcccgaagggaagctgagtt
<i>Ec</i> bd.Gibson Rev	aactcagcttccttcgggc

Chapter

4

Discovery of a novel chemotype targeting *Mycobacterium tuberculosis* cytochrome *bd* through rapid screening and structural elucidation

Antibiotic resistance in *Mycobacterium tuberculosis* is a pressing global health challenge demanding new therapeutic strategies. The bacterial respiratory chain comprises promising antibacterial targets, with dual inhibition of the terminal oxidases cytochrome *bcc:aa₃* and cytochrome *bd* (cyt *bd*) showing bactericidal activity. While *bcc:aa₃* inhibitors such as Q203 have advanced clinically, cyt *bd* remains underexplored due to difficulties in assigning activity of the purified enzyme and structurally resolving the quinol substrate binding site. Here, we report a rapid *in vitro* screening platform for cyt *bd* inhibitors by engineering a minimal respiratory system that couples the activity of cyt *bd* to that of a type 2 NADH dehydrogenase. This coupled assay enables spectroscopic monitoring of NADH oxidation as a proxy for cyt *bd* activity, allowing rapid screening of over 10,000 compounds. Screening identified WSL017, a fragment with low micromolar potency against both *M. tuberculosis* and *E. coli* cyt *bd*. Kinetic and structural analyses revealed competitive inhibition at the quinol-binding site, providing the first structural insights into cyt *bd* inhibition by a non-quinone scaffold. WSL017 displayed growth inhibition of *M. tuberculosis* H37ra, corroborating oxidase inhibition as a promising therapeutic strategy. This work establishes a pipeline for cyt *bd* inhibitor discovery and highlights new opportunities for structure-guided drug development against cytochrome *bd* oxidases.

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Introduction

Antibiotic resistance is an escalating global health crisis, projected to cause 8.2 million deaths annually by 2050[1]. Among resistant pathogens, *Mycobacterium tuberculosis* poses a particularly urgent challenge as it gains antibiotic resistance at the highest pace of all major pathogens[1]. Tuberculosis (TB) treatment currently requires a prolonged multi-drug regimen lasting up to nine months, imposing significant burden on cost, toxicity, and patient compliance[182]. A major breakthrough in TB treatment was marked in 2012 with the approval of bedaquiline, shortening treatment duration to six months by targeting the ATP synthase[63]. However, resistance to bedaquiline has already emerged, driven by an efflux mechanism that expels the drug from the bacterial cell[71]. These developments underscore the urgent need for next-generation TB antibiotics in order to decrease treatment duration and maintain potent treatment options.

The bacterial respiratory chain offers a compelling target for drug discovery due to its central role in energy metabolism and pathogen survival[183] (Fig. 4.1). Q203, an inhibitor of the terminal oxidase cytochrome *bcc:aa₃*, has successfully completed phase IIa clinical trials[184]. Yet its bacteriostatic activity reflects metabolic compensation by the alternative terminal oxidase, cytochrome *bd* (cyt *bd*), which sustains electron flow under stress conditions[77,185]. This respiratory flexibility highlights the necessity of dual inhibition of cytochrome *bcc:aa₃* and *bd* to achieve bactericidal effects[186]. Despite its pivotal role, cyt *bd* remains therapeutically underexplored, with no inhibitors currently in clinical development.

Cyt *bd* is distinguished by its high oxygen affinity, enabling respiration under hypoxic conditions typical of infection[46,47]. Beyond energy generation, cyt *bd* mitigates reactive oxygen species, promotes biofilm formation, and enhances persistence, functions that collectively aid in virulence and antibiotic tolerance[48,51,53,56–59,78,112,154,187]. Studies confirm its essentiality during infection and its ability to compensate for inhibition of other respiratory complexes[185].

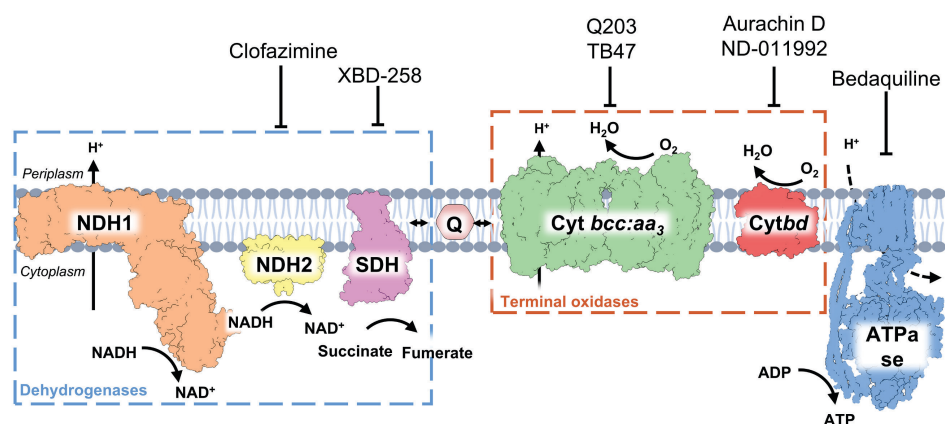


Figure 4.1. The *M. tuberculosis* respiratory chain and its inhibitors. The dehydrogenases are highlighted in the blue box, and the terminal oxidases in the orange box. Dual inhibition of the two terminal oxidases is highlighted as potent antibiotic strategy. Inhibitors are annotated based on Adolph, C. et al.[188] and Saha, P. et al.[189]. The membrane was generated using Biorender.

Strikingly, *cyt bd* knockout strains exhibit hypersensitivity to bedaquiline, reinforcing its synergistic potential with other respiratory enzymes[190]. Importantly, *cyt bd* is absent in eukaryotes, making it an attractive target for selective antimicrobial strategies.

Despite this promise, progress on developing *cyt bd* inhibitors has been limited. Existing inhibitors, such as the quinone analog Aurachin D (AurD), exhibit potent inhibition but suffer from cytotoxicity due to structural similarity to ubiquinone, the central respiratory substrate used in mitochondria[191]. Few synthetic *cyt bd* inhibitors have been reported and often mimic the AurD architecture, potentially maintaining its cytotoxic effects[101,114,135]. Furthermore, structural insights remain restricted to AurD bound to *E. coli* *cyt bd* (*Ecbd*) [87,192]. Expanding the toolbox of *cyt bd* inhibitors and elucidating inhibitor binding modes are critical next steps for structure-guided drug design against the *bd* oxidases.

Thus far, conventional screening approaches have fallen short in populating the drug development pipeline due to technical challenges. *In vivo* assays in *M.*

tuberculosis are hampered by slow growth rates and the need for dual oxidase inhibition to achieve bactericidal effects. Additionally, *in silico* approaches are constrained by uncertainty surrounding the *M. tuberculosis* cyt *bd* (*Mtbd*) active site architecture. Substrate and inhibitor bound structures could still not be obtained, even when applying vast molar excess of these ligands, affecting guided docking efforts[82]. Lastly, current *in vitro* methods studying purified *Mtbd* activity measure its oxygen consumption rate on an Oxygraph system and lack the throughput for large-scale screening.

To overcome these barriers, we developed a rapid *in vitro* screening method for cyt *bd* inhibitor discovery. By engineering a minimal respiratory system incorporating *Caldalkalibacillus thermarum* type 2 NADH dehydrogenase (NDH₂) and *Mtbd* (Fig. 4.2A), we enable spectroscopic monitoring of NADH oxidation as a proxy for *Mtbd* activity. Using this assay, a library of fragments harboring covalently reactive groups (warheads) was screened. This platform facilitated screening of over 10,000 compounds and yielded a potent inhibitor fragment, which was validated to bind in a non-covalent fashion. Subsequent kinetic and structural characterization of this fragment revealed new opportunities for targeting *bd* oxidases and advancing next-generation TB therapeutics. enzymes[190].

Results

Development of a rapid screening method against Mtbd

To enable *in vitro* examination of *Mtbd*, the enzyme was heterologously expressed in *M. smegmatis* and purified *via* 3xFLAG affinity chromatography followed by gel filtration (Fig. S4.1). Probing of *Mtbd* oxygen consumption was achieved by our previously reported minimal respiratory chain system using excess *C. thermarum* NDH₂ as a quinone reductase, enabling the monitoring of *Mtbd* activity without affecting its regulatory disulfide bond[159] (Fig. 4.2A). To improve experimental throughput of this system, we hypothesized that the NADH oxidation rate is representative of *Mtbd* activity, provided *Mtbd* activity

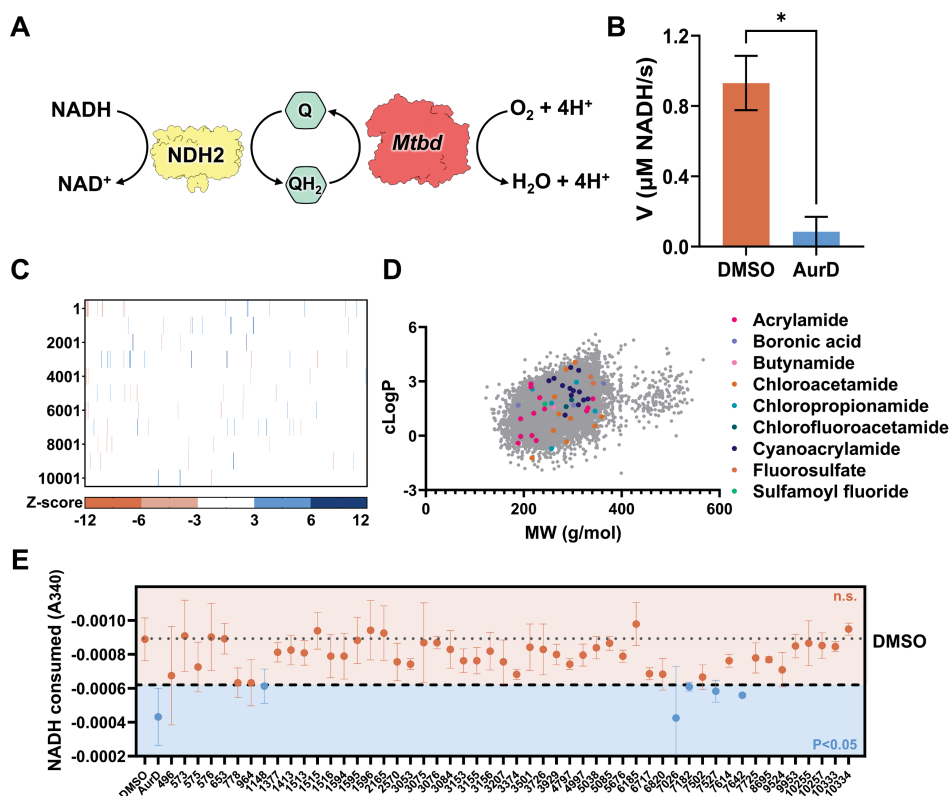


Figure 4.2. Rapid inhibitor screening strategy against *MtbD*. (A) Overview of the minimal respiratory chain used for inhibitor screening, enabling monitoring of NADH oxidation as a proxy of *MtbD* activity. (B) NADH oxidation rate of the coupled enzyme system showing the inhibitory effect of AurD (1 nM *MtbD*, 60 nM NDH2, 50 μ M menaquinone-1 (MK-1), 250 μ M NADH in 50 mM MES pH 6.5, 150 mM NaCl, 0.005% LMNG). (C) Heatmap of the inhibitory and stimulatory activity of the full library screened against *MtbD* at a concentration of 3 μ M. (D) Hit distribution across the chemical library. Hit compounds are colored by their chemotype. All non-hits are shown in grey. (E) Rescreening of the inhibitor hits in *MtbD* proteoliposomes (POPE:POPG:Cardiolipin; 30:60:10 molar ratio), probing effective diffusion of the inhibitor into the membrane to target *MtbD* (6 nM *MtbD*, 60 nM NDH2, 50 μ M MK-1, 250 μ M NADH, 50 mM MES pH 6.5, 150 mM NaCl, 3 μ M Inhibitor). All compound numbers are those of the combined ENL library.

is rate limiting, allowing for spectroscopic activity measurements in plate-based assays. As proof of concept of this system, molar excess of NDH2 was used to maintain the quinone pool reduced and ensure rate limitation by *MtbD*.

The NADH oxidation reaction rate of our minimal respiratory chain was almost completely inhibited upon addition of the known *Mtbd* inhibitor Aurachin D (AurD) (Fig. 4.2B), validating this approach for inhibitor screening. Importantly, this spectroscopic assay enables high-throughput plate-based monitoring, in contrast to Oxygraph measurements that quantify *Mtbd* oxygen consumption[159].

To discover new chemical scaffolds for cyt *bd* inhibitors, we screened a library of fragments with covalent warheads comprising 10,570 compounds that span diverse sizes and have hydrophobicity properties suitable for membrane-embedded targets. The library consists solely of fragments with covalent warheads in order to increase the chance of finding initial hits. Screening in the coupled assay at 3 μ M compound concentration yielded 52 primary inhibitor hits (0.49% hit rate) (Fig. 4.2C, Table S4.1), and 43 stimulator hits. For the inhibitors, 48 hits were confirmed upon triplicate testing, while none of the stimulators showed significant effects. Inhibitor hits were broadly distributed across size, cLogP, and chemotype (Fig. 4.2D). To ensure activity in a native-like membrane environment, hits were re-screened in proteoliposomes reconstituted with our minimal respiratory chain system. Five compounds retained significant inhibitory activity at 3 μ M concentration (Fig. 4.2E). Among these, a chloroacetamide derivative, compound ENL1148 (Z57044282), exhibited a clear dose-response relationship and was prioritized for further characterization.

Inhibitor ENL1148 is competitive and acts through a non-covalent mechanism

Despite its covalent warhead, washout and time-based inhibition assays indicated that ENL1148 does not inhibit *Mtbd* covalently. To confirm this, we synthesized a non-covalent analog lacking the chloroacetamide warhead, yielding compound WSL017 (Fig. 4.3A, Fig. S4.2, S4.3). Further kinetic evaluation of the inhibitors was performed measuring the cyt *bd* oxygen consumption rate

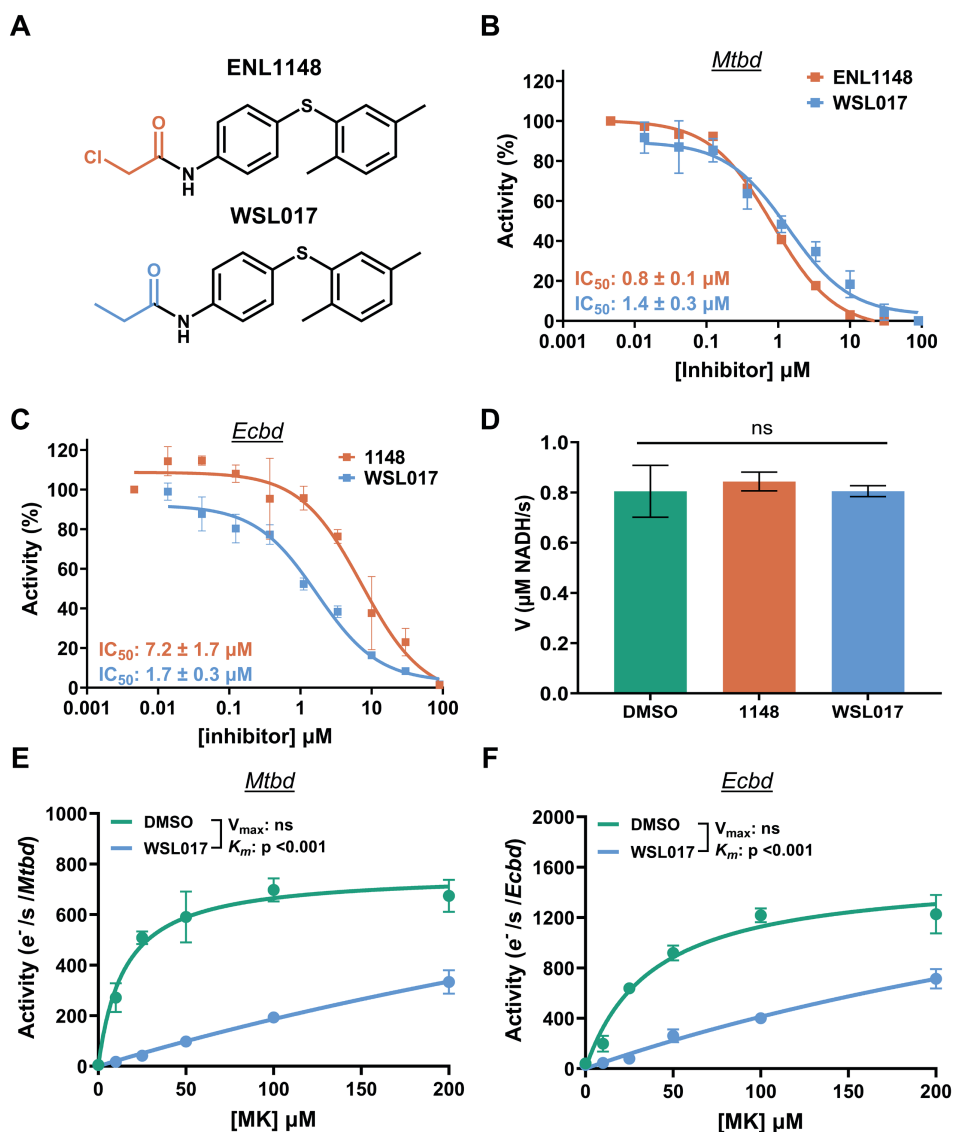


Figure 4.3. Inhibitor kinetics of ENL1148 and WSL017. (A) Inhibitor structure of ENL1148 and WSL017, highlighting the removal of the warhead. (B) IC_{50} determination on the Oxygraph system against *Mtbd* proteoliposomes (POPE:POPG:Cardiolipin ; 30:60:10 molar ratio) and (C) against *Ecdb* proteoliposomes (POPE:POPG:Cardiolipin ; 60:30:10 molar ratio). (D) Neither inhibitor affects NDH₂ activity (100 nM NDH₂, 200 μM menadione, 150 μM NADH). (E) Inhibitor competition kinetics against *Mtbd* proteoliposomes and (F) *Ecdb* proteoliposomes. Assays were performed using 6 nM cyt *bd*, 60 nM NDH₂, 50 μM MK-1, 50 mM MES pH 6.5, 150 mM NaCl.

on an Oxygraph system. Both ENL1148 and WSL017 displayed low micromolar IC_{50} values against *Mtbd* and *Ecbd* in our proteoliposomal oxygen consumption assays (Fig. 4.3B,C), demonstrating potent activity for a small fragment. To ensure that the observed inhibition was acting directly on the *bd* oxidase, the activity of NDH2 was measured in a separate assay. Neither ENL1148 nor WSL017 showed inhibition of NDH2 activity (Fig. 4.3D), confirming the direct inhibition of *Ecbd* and *Mtbd*. Further experiments were performed using the non-covalent compound WSL017. Kinetic analysis of WSL017 revealed competitive inhibition of *Mtbd* and *Ecbd*, significantly increasing K_m without a significant effect on V_{max} (Fig. 4.3E,F). These data suggest that WSL017 binds at the quinol-binding site, displacing the natural quinol substrate in both *Mtbd* and *Ecbd*.

Cryo-EM reveals the binding mode of Wsl017 in the Ecbd active site

To confirm the binding site of WSL017, we conducted cryogenic electron microscopy (cryo-EM) on *Mtbd*. *Mtbd* was plunge frozen in the presence of WSL017, and under turnover conditions in the presence of WSL017 and was solved between 2.9 Å and 3.2 Å resolution. The overall *Mtbd* structure showed to be identical to the one reported before [82], consisting of the subunits CydA and CydB. Regretfully, none of these conditions showed a density that could be assigned to WSL017. Neither did the resulting density maps show displacement of the menaquinone (MK) bound at Trp9^{CydA} (Fig. S4.4). Similar observations have been made before, even after incubation with vast molar excesses of the canonical inhibitor AurD [82]. The lack of ligand binding, whether substrate or inhibitor, to *Mtbd* hinders drug development and remains subject to further study.

To still gain insights into the binding mode of WSL017 to *bd* oxidases, we performed cryo-EM of *Ecbd* in the presence of WSL017. The structure of *Ecbd* was solved in both a monomeric and dimeric state at 2.64 Å and 2.58 Å, respectively (Fig. S4.5-S4.7). The *Ecbd* structures resemble previously reported structures, consisting of the subunits CydA (including the quinol binding Q-loop), CydB, CydX, and CydH, and the characteristic triangular heme

arrangement containing heme b_{558} , b_{595} and d [83,192]. As seen before, the flexible Q-loop lid (Glu257^{CydA} to Gly306^{CydA}) in the monomer state of *Ecbd* prohibits assignment of any ligand in the quinol oxidation site [192]. This flexible quinol oxidation site in *bd* oxidases prohibited structure guided drug discovery until recent structural advances showed a dimeric state of *Ecbd* [87,192].

In the dimeric state of *Ecbd*, the Q-loop is stabilized and allows for modelling of the active site architecture. We previously reported binding of menaquinone and AurD in this dimeric *Ecbd* state, revealing Y243^{CydA} and R298^{CydA} as the main catalytic residues [192]. Under turnover conditions, the Q-loop lid (Glu257^{CydA}-Gly306^{CydA}) undergoes a disorder-to-order transition to enable quinol oxidation.

Here, WSL017 was shown to occupy the same quinol oxidation site as menaquinone in the substrate bound state (Fig. 4.4A). WSL017 is bound in a shallow pocket under the Q-loop lid typically unoccupied without added ligands. In this pocket, binding of WSL017 is stabilized by the sulfur moiety interacting with Y243^{CydA} (Fig. 4.4B), that typically forms interactions by π - π stacking with the menaquinone headgroup during substrate binding (Fig. 4.4C). This pocket is characterized by hydrophobic interactions with CydA Val236, Leu237, Gly238, Glu240, Tyr243, Phe269, Ile295, Ala296, Arg298, Phe390, and Heme b_{558} . These hydrophobic interactions maintain the Q-loop in the closed state, impairing the disorder-to-order transitions typically seen during quinol turnover [192]. We have previously shown that the mutation of Y243A^{CydA} abolished enzyme activity [192], prohibiting the interrogation of the effect of this mutation on inhibitor resistance. Interestingly, the amide side chain of WSL017 makes few interactions and points out of the pocket, explaining why the removal of the covalent warhead of inhibitor ENL1148 had no effect on inhibition.

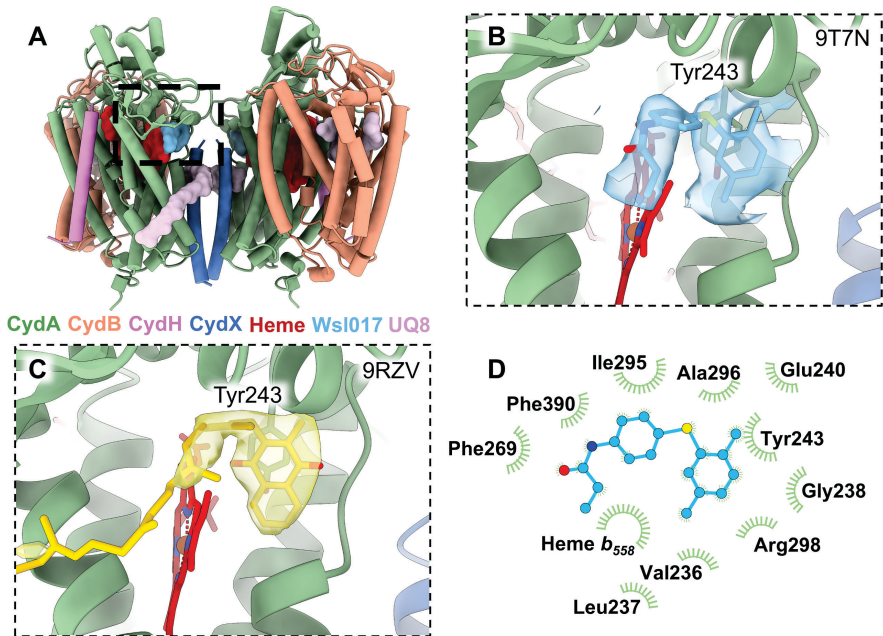


Figure 4.4. Structural features of *Ecbd* bound to WSL017. (A) Cryo-EM structure of the *Ecbd* dimer bound to WSL017. (B) Density of the active site pocket highlighting WSL017 and its density feature interacting with Tyr243^{CydA}. (C) Density of the previously solved active site pocket highlighting a bound menaquinone interacting with Tyr243^{CydA} (PDB 9RZV)[192]. (D) Schematic representation of the WSL017 binding pocket within subunit CydA quinol oxidation site as analyzed by Ligplot[177]. Hydrophobic interactions are indicated by spokes semi circles.

WSL017 inhibits growth of *M. tuberculosis* H37ra

To validate the inhibitor strategy with WSL017 in cells, we performed growth inhibition assays on the attenuated *M. tuberculosis* H37ra, representative of the virulent *M. tuberculosis* H37rv[193]. To determine inhibition, H37ra was exposed to WSL017 and/or Q203 and incubated at 37°C for 7 days. Due to the low cell densities, growth was determined using Alamar blue, allowing for fluorescent capture of the metabolic activity of remaining cells[194]. Upon exposure to 25 μ M WSL017, H37ra showed significant growth impairment, although not

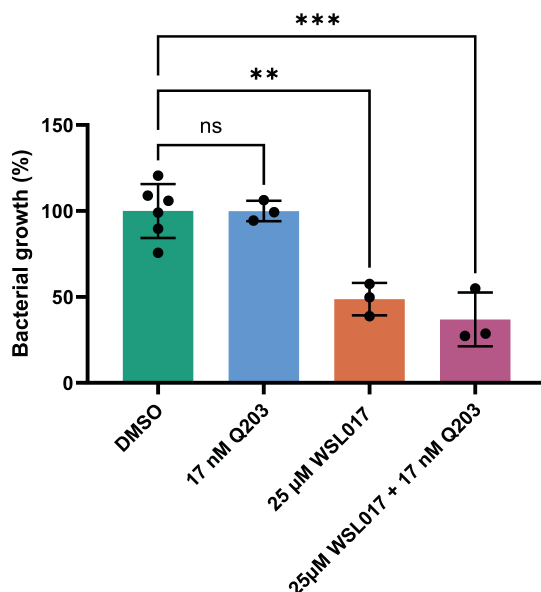


Figure 4.5. In cellulo inhibition of *M. tuberculosis* H37ra growth by the *bd* oxidase inhibitor WSL017 and *bcc:aa₃* inhibitor Q203.

achieving complete growth inhibition (Fig. 4.5). Exposure to the Cyt *bcc:aa₃* inhibitor Q203 at 17 nM showed no significant effects, while supplementation of WSL017 with 17 nM Q203 showed marginally, but not significantly reduced growth compared to WSL017 alone.

Discussion

Tuberculosis is a major health threat, gaining resistance at a rate outpacing other human pathogens[1]. Especially for these resistant cases, the respiratory chain of *M. tuberculosis* has been shown as key weakness for antibiotic intervention[13]. The combinatory inhibition of the two terminal oxidases, cytochrome *bcc:aa₃* and *Mtbd*, holds major potential for treating tubercular infections[77]. Here, we present a spectroscopic assay platform that allows for

rapid kinetic screening for *Mtbd* inhibitors. Using this engineered minimal respiratory chain, we identified the inhibitor WSL017, showing low μM potency against both *Mtbd* and *Ecbd*. Notably, this assay can be broadly adapted to target other respiratory enzymes, including NADH dehydrogenases and quinol oxidases from diverse pathogens such as *Staphylococcus aureus* and *Listeria monocytogenes*, expanding its utility beyond the work presented here[183].

It is important to note that in the results presented here, *Mtbd* was assayed in liposomes and did not display substrate inhibition kinetics at the MK-1 concentrations tested, which allowed for the determination of WSL017 binding competitively to the *Mtbd* quinol oxidation site. This is in contrast with our previous report, where *Mtbd* is subject to substrate inhibition at high MK-1 concentrations when assayed in detergent[159]. We hypothesize that this difference stems from distinct local substrate concentrations at the *Mtbd* active site due to altered diffusion of the quinone into the liposome or detergent micelle.

Mechanistic and structural analyses revealed that WSL017 acts as a competitive inhibitor, displacing quinol at the cyt *bd* active site. Cryo-EM of *Ecbd* provided the first structural insights into *bd* oxidase inhibition by a non-quinone scaffold, highlighting a hydrophobic pocket that stabilizes WSL017 and offers opportunities for rational optimization. Optimization might utilize π - π stacking to Phe269^{CydA} and Phe390^{CydA}, together with hydrogen bonding to Arg298^{CydA} and the conserved Glu240^{CydA}. Especially interactions with Glu240^{CydA}, Tyr243^{CydA} and Arg298^{CydA} will prove valuable due to the conservation and essentiality for oxidase activity in *Ecbd*[192]. Interestingly, the inhibitor binding pocket here is not conserved in *Mtbd*, indicating that WSL017 binding is mediated by different interactions or occurs at a different pocket (Fig. S4.8). Although no ligand density was observed in our *Mtbd* maps, these findings underscore the feasibility of targeting *bd* oxidases with novel chemotypes.

WSL017 shares structural similarity with previously reported *bd* oxidase inhibitors ND-011992[101] and CK-2-63[135], but lacks the AurD headgroup implicated in cytotoxic side effects (Fig. S4.9)[191]. Surprisingly, the absence of

this moiety in WSL017 indicates that it is not required for binding to the cyt *bd* active site. Unfortunately, no structure has been solved to date with either CK-2-63 or ND-011992 bound to *Ecbd*, or any other *bd*oxidase, preventing structural comparison to WSL017. Since WSL017 is similar to these two inhibitors, but without the AurD type headgroup, it will be interesting to see, how each inhibitor feature contributes interactions in the *Ecbd* binding pocket.

Excluding the AurD quinolone moiety from the next generation of cyt *bd* inhibitors may reduce cross-reactivity with human respiratory enzymes, improving selectivity. Furthermore, unlike AurD, which induces active-site refolding in *Ecbd* CydA helix 6 by hydrogen bonding with Asp239^{CydA}[192], WSL017 inhibits *Ecbd* without triggering such conformational changes, showing a distinct competitive mechanism of action (Fig. S4.10). WSL017 binds *via* predominately hydrophobic interactions within the main body of CydA and the Q-loop lid, maintaining the Q-loop lid in a closed state. We hypothesize that this prohibits the disorder-to-order transitions characteristic of *Ecbd* quinol oxidation, impairing release of WSL017. This indicates that the structural changes induced by AurD are not required for inhibition of cyt *bd* and opens a new chemical space for cyt *bd* inhibitor development.

Despite its low μM IC_{50} value *in vitro*, WSL017 did not yield complete growth inhibition *in cellulo*. In the H37ra strain assayed here, WSL017 reached growth inhibition of approximately 50%, despite complementation with the cytochrome *bcc:aa₃* inhibitor Q203. This is in stark contrast with inhibitors such as ND-011992 and CK-2-63, which are bactericidal in combination with Q203[77,135]. This indicates that WSL017 requires further optimization to reach improved affinity or local concentrations in the mycobacterial inner membrane to better inhibit the *bd*oxidase.

Future work should focus on optimizing WSL017 to enhance potency and studying its specificity, as well as evaluating its activity against *bd*oxidases in other clinically relevant pathogens. Given the demonstrated synergy between *bd* and *bcc:aa₃* inhibition[77], such compounds could form the basis of combination therapies that shorten treatment duration and overcome resistance in tuberculosis and beyond.

Experimental procedures

General chemistry

All reagents and solvents were purchased from commercial sources and were of analytical grade (TCI, Sigma-Aldrich and BroadPharm®). All moisture sensitive reactions were performed under inert atmosphere. Solvents were dried using 4 Å molecular sieves prior to use when anhydrous conditions were required. Water used in reactions was always demineralized. Analytical Thin-layer Chromatography (TLC) was routinely performed to monitor the progression of a reaction and was conducted on Merck Silica gel 60 F254 plates. Compounds on the TLC plates were visualized by UV irradiation (λ 254) and/or spraying with potassium permanganate solution (K_2CO_3 (40 g), $KMnO_4$ (6 g), and H_2O (600 mL)) followed by heating as appropriate. Purification by flash column chromatography was performed using Screening Devices B.V. silica gel 60 (40–63 μm , pore diameter of 60 Å).

Solutions were concentrated using a Heidolph laborata W8 4000 efficient rotary evaporator with a Laboport vacuum pump. Analytical purity was determined with Liquid Chromatography-Mass Spectrometry (LC-MS) using a Finnigan LCQ Advantage MAX apparatus with electrospray ionization (ESI), equipped with a Phenomenex Gemini 3 μm NX-C18 110 Å column (50x4.6mm), measuring absorbance at 254 nm using a Waters 2998 PDA UV detector and the m/z ratio by using an Acquity Single Quad (Q1) detector. Injection was with the Finnigan Surveyor Autosampler Plus and pumped through the column with the Finnigan Surveyor LC pump plus to be analyzed with the Finnigan Surveyor PDA plus detector. Samples were analyzed using eluent gradient 10%→90% ACN in MiliQ water (+0.1% TFA (v/v)).

1H and ^{13}C Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker AV 400 (400/100 MHz) spectrometer at ambient temperature using $CDCl_3$ as solvent. Chemical shifts (δ) are referenced in parts per million (ppm) with tetramethylsilane (TMS) or $CDCl_3$ resonance as the internal standard peak (δ 0.00 for 1H (TMS), δ 77.16 for ^{13}C ($CDCl_3$)). Multiplicity is reported as s = singlet,

d = doublet, dd = doublet of doublet, t = triplet, q = quartet, m = multiplet. Coupling-constants (*J*) are reported in Hertz (Hz).

Synthesis of

N-(4-((2,5-dimethylphenyl)thio)phenyl)propionamide (WSL017)

4-((2,5-Dimethylphenyl)thio)aniline (50.0 mg, 218 μ mol, 1.0 eq.) and TEA (151.7 μ L, 1.1 mmol, 5.0 eq.) were dissolved in THF (1 mL) and the solution was cooled to 0 °C. Propionyl chloride (38.1 μ L, 436 μ mol, 2.0 eq.) was added dropwise, and the reaction mixture was stirred overnight at rt. After TLC analysis indicated completion of the reaction, sat. $\text{NH}_4\text{Cl}_{(\text{aq})}$ (10 mL) was added. The mixture was diluted with EtOAc and the organic and aqueous layers were separated. The organic layer was washed with sat. $\text{NH}_4\text{Cl}_{(\text{aq})}$, brine, dried over MgSO_4 , filtered and concentrated *in vacuo*. The crude product was purified with column flash chromatography (0→25% EtOAc in pentane) to yield Wsl017 as a white solid (55.1 mg, 193 μ mol, 89%).

$^1\text{H NMR}$ (400 MHz, CDCl_3) δ 7.48 – 7.44 (m, 2H), 7.39 (s, 1H), 7.22 – 7.16 (m, 2H), 7.10 (d, *J* = 7.7 Hz, 1H), 7.03 (s, 1H), 6.98 (d, *J* = 7.7 Hz, 1H), 2.38 (q, *J* = 7.6 Hz, 2H), 2.31 (s, 3H), 2.23 (s, 3H), 1.23 (t, *J* = 7.6 Hz, 3H).

$^{13}\text{C NMR}$ (101 MHz, CDCl_3) δ 172.23, 136.91, 136.41, 136.12, 134.24, 132.56, 131.38, 130.70, 130.49, 128.46, 120.63, 30.85, 20.97, 20.13, 9.78.

LC-MS (ESI, 10% → 90% ACN in MiliQ water (+ 0.1% TFA (v/v)): t_R = 7.86 min.

HRMS calculated for $\text{C}_{17}\text{H}_{19}\text{NOS} + \text{H}^+$: 286.12601, found 286.12621.

HRMS calculated for $\text{C}_{17}\text{H}_{19}\text{NOS} + \text{Na}$: 308.10796, found 308.10822.

M. tuberculosis cytochrome *bd* (*Mtbd*) expression and purification

Mtbd was expressed and purified as described before [159]. *M. smegmatis* MC² 155 ΔCydAB transformed with the plasmid pLHCyd, were grown in LB-hygromycin for 72 hours at 250 RPM and 37°C. This culture was then diluted 1:100 and grown for an additional 72 hours at 200 RPM and 37°C. Cells were harvested by centrifugation (6371 rcf, 20 min, 4°C) and resuspended in 5 mL of buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 0.05% Tween-80, and cOmplete™ EDTA-free Protease Inhibitor per gram of cells. Cell lysis was performed by two passes through a Stansted pressure cell homogenizer at 270 MPa. Debris was removed by centrifugation (10,000 rcf, 20 min, 4°C), and crude membranes were collected by ultracentrifugation (200,000 rcf, 1 h, 4°C). Membranes were resuspended in 20 mM Tris-HCl (pH 7.4), 0.05% Tween-80, and 10% glycerol to a final protein concentration of 10 mg/mL. Solubilization was achieved by adding 0.5% Lauryl Maltose Neopentyl Glycol (LMNG) and gently mixing for 1 hour at 4°C. Insoluble material was removed by ultracentrifugation (200,000 rcf, 30 min, 4°C). The soluble fraction was incubated overnight at 4°C with Pierce™ Anti-DYKDDDDK Affinity Resin (Thermo Fisher) and purified according to the manufacturer's protocol. Flow-through was removed by centrifugation (1000 rcf), followed by four washes with 10 column volumes of buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.005% LMNG. *Mtbd* was eluted using buffer composed of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.02% DDM, and 1 mg/mL 3X-FLAG peptide (Genscript). Final purification was performed by size-exclusion chromatography on a Superdex 200 Increase 10/200 column at 0.5 mL/min using buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.005% LMNG. SEC peak fractions were analyzed by SDS-PAGE, and pure *Mtbd* was pooled, concentrated, and stored at -80°C until use.

E. coli cytochrome *bd* expression and purification

Ecbd was expressed and purified as previously described[159]. MB43 cells transformed with pET17b-CydABX-linkerstreptag, were cultured overnight in LB medium containing 100 µg/mL ampicillin (250 RPM and 37°C). The culture was diluted to an OD of ~0.1 and grown to OD ~0.4 before induction with 0.45 mM IPTG. Cells were harvested at OD 2.0 by centrifugation (6371 rcf, 20 min, 4°C) and resuspended in buffer containing 50 mM MOPS (pH 7.4), 100 mM NaCl, and cOmplete™ EDTA-free Protease Inhibitor (ROCHE) at a ratio of 5 mL per gram of wet cell pellet. Cell lysis was performed using a Stansted pressure cell homogenizer at 270 MPa. Cell debris was removed by centrifugation (10,000 rcf, 20 min, 4°C), and crude membranes were isolated by ultracentrifugation (200,000 rcf, 1 h, 4°C). Membranes were resuspended in 50 mM MOPS, 100 mM NaCl, pH 7.4, to a final protein concentration of 10 mg/mL before solubilization of *Ecbd* was achieved by incubating with 0.5% LMNG for 1 hour at 4°C with gentle mixing. Insoluble material was removed by ultracentrifugation (200,000 rcf, 30 min, 4°C), and the supernatant was loaded onto a StrepTrap HP column (Cytiva) at a flow rate of 2 mL/min. The column was washed with buffer containing 50 mM sodium phosphate, 300 mM NaCl, 0.005% LMNG, pH 8.0, to remove unbound proteins. *Ecbd* was eluted using buffer composed of 50 mM sodium phosphate, 300 mM NaCl, 2.5 mM desthiobiotin, and 0.005% LMNG, pH 8.0. Peak fractions were pooled, concentrated, and stored at -80°C until further use.

NDH2 expression and purification

To enable measurement of Cyt *bd* activity, NDH₂ was required to reduce the quinone pool. Expression and purification of NDH₂ followed previously established methods[159]. In brief, C41 (DE3) cells transformed with pET28-NDH-2_NtermHis were cultured overnight in LB medium supplemented with kanamycin at 250 RPM and 37°C. The overnight culture was diluted 1:20 and grown to an optical density of ~0.5 before induction with 0.25 mM IPTG. NDH₂ expression proceeded for 4 hours at 37°C, after which cells were harvested by centrifugation (6371 rcf, 20 min, 4°C). Cell pellets were resuspended in 50 mM

Tris-HCl, 5 mM MgCl₂, pH 8.0 (5 mL per gram of cells) and lysed using a Stansted pressure cell homogenizer at 270 MPa. Cell debris was removed by centrifugation (10,000 rcf, 20 min, 4°C), and crude membranes were collected by ultracentrifugation (200,000 rcf, 1 h, 4°C). Membranes were resuspended in buffer containing 50 mM Tris-HCl, 150 mM NaCl, and 20 mM imidazole at a concentration of 10 mg/mL total protein. NDH₂ was solubilized by incubating the membrane suspension with 1% DDM for 1 hour at 4°C with gentle mixing. Insoluble material was removed by ultracentrifugation (200,000 rcf, 30 min, 4°C), and the supernatant was loaded onto a HiTrap Nickel NTA column (Cytiva). Unbound proteins were washed with buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 20 mM imidazole, and 0.02% DDM. NDH₂ was eluted using 30% elution buffer (50 mM Tris-HCl, 150 mM NaCl, 500 mM imidazole, 0.02% DDM). Final purification was performed by size-exclusion chromatography on a Superdex Increase 200 10/300 column (Cytiva) at a flow rate of 0.5 mL/min using buffer containing 50 mM Tris-HCl, 500 mM NaCl, 5% glycerol, and 0.02% DDM. Purified NDH₂ fractions were pooled, concentrated, and stored at -80°C until use.

High throughput inhibitor screening

Inhibitor screens were performed in 384 well format containing the following reaction solution: 1 nM *Mtbd*, 60 nM NDH₂, 50 μM menaquinone-1 in 50 mM MES pH 6.5, 150 mM NaCl, 0.005% LMNG, and 3 μM of the inhibitor candidate. A surplus of NDH₂ (60 nM) was added to ensure rate limitation by *Mtbd*. The reaction was initiated by the addition of 250 μM NADH. The NADH oxidation rates were monitored at 340 nm in a FLUOstar Omega plate-reader (BMG LABTECH). Each plate contained multiple 1 μM Aurachin D positive control to ensure assay validity. The sample activity for each well was determined by the initial slope of NADH oxidation. Each inhibitor was ascribed a Z-score according to the following equation, where X is the observed reaction rate, μ is the plate mean, and σ is the plate standard deviation.

$$Z = \frac{X - \mu}{\sigma}$$

Primary hits were identified with a Z-score < -3.0 (stimulator) or > 3.0 (inhibitor). Hit validation was performed in triplicate, where the inhibitors were compared to the DMSO control. Hits were deemed significant ($p < 0.05$) after ANOVA with Dunetts *post hoc* analysis.

Cyt *bd* proteoliposomes reconstitution

Cyt *bd* was reconstituted in proteoliposomes based on the method by Godoy-hernandez *et al.*[148]. Lipids were obtained from Avanti Polar Lipids and used as received. Lipid mixtures were prepared using POPE:POPG:cardiolipin (CA) in ratios of 60:30:10 for *Ecbd* proteoliposomes and 30:60:10 for *Mtbd* proteoliposomes[195]. These mixtures were dried under a stream of nitrogen. Residual chloroform was removed overnight by vacuum desiccation. The resulting lipid film was rehydrated to a final concentration of 10 mg/mL lipids in buffer containing 20 mM MOPS, 30 mM Na_2SO_4 , and 100 mM KCl at pH 7.4, using vortexing to ensure thorough resuspension. Cyt *bd* in LMNG (0.005%) was added to the liposome suspension at a protein-to-lipid ratio of 1% (w/w) and mixed by inversion for 30 minutes at room temperature. Insoluble material was removed by centrifugation using an Eppendorf tabletop centrifuge (14,100 rcf, 5 min).

To determine the concentration of cyt *bd* in the proteoliposomes, a sample was solubilized in 2% octyl- β -glucoside and analyzed by UV-visible spectroscopy. Quantification was based on the solet band using the appropriate extinction coefficients: *Ecbd* at 417 nm ($\epsilon = 230 \text{ mM}^{-1} \text{ cm}^{-1}$)[55] and *Mtbd* at 414 nm ($\epsilon = 279 \text{ mM}^{-1} \text{ cm}^{-1}$)[159].

Hit confirmation and characterization in proteoliposomes

Confirmed hits were further culled by screening for inhibition in *Mtbd* proteoliposomes, ensuring the inhibitors can diffuse into the membrane to reach the binding site. Proteoliposomes were assayed in 6 nM *Mtbd* or *Ecbd*, 60 nM NDH₂, 50 μ M menaquinone-1 in 50 mM MES pH 6.5, 150 mM NaCl, and 3 μ M of the inhibitor hits. Hit confirmation was performed by measuring the NADH oxidation rate on a FLUOstar system (250 μ M NADH). The data was analyzed by ANOVA with Dunetts post hoc test (Graphpad Prism). Significance was set at $p < 0.05$. The IC₅₀ value and inhibitor competition was determined using the *Mtbd* or *Ecbd* oxygen consumption rate on an Oxygraph system (500 μ M NADH) (Hansatech).

NDH2 activity counterscreen

Hits were counter screened against NDH₂ to ensure the observed inhibitory effect is caused by *Mtbd*. Briefly, NADH oxidation was monitored in a solution of 100 nM NDH₂ with 200 μ M menadione in 50 mM MES pH 6.5, 150 mM NaCl. NDH₂ turnover was initiated by addition of 150 μ M NADH, followed by rate determination. The data was analyzed by ANOVA with Dunetts post hoc test (Graphpad Prism). Significance was set at $p < 0.05$.

Growth inhibition in *M. tuberculosis* H37ra

Inhibitor growth inhibition was determined using the *M. tuberculosis* H37ra strain. Cells were grown in 7H9 media supplemented with ADS (0.5% BSA, 0.75% dextrose, and 0.08% NaCl). The cells were diluted to OD 0.1 prior to exposure to the desired inhibitor concentration. Cells were exposed to Q203 or Wsl017. To determine synergistic effects between cyt *bcc:aa₃* and the cyt *bd* inhibitors, cells were exposed to a combination of Q203 and Wsl017. Sterile media and DMSO controls were included to ensure assay validity. Cell growth was quantified by addition of Alamar blue dye followed by incubation for 6-8 hours at 37°C. Alamar blue fluorescence was determined by fluorescence (530 ex, 590

em.) and normalized to the DMSO control to measure cell growth to calculate the percentage of cell growth.

Cryo-EM sample preparation and data collection

UltrAUfoil R1.2/1.3-Cu 300 mesh grids were freshly glow discharged at 15 mA for 45 s followed by 15 mA and 90 S on the foil side. LMNG solubilized *Ecbd* was incubated with 100 μ M Wslo17, followed by the application of 4 μ l sample (1.5 mg/ml) on the grid. Sample blotting was performed for 6 seconds, at 20 blot force using a vitrobot IV device (Thermo Fischer) operating at 4°C and 100% humidity, directly before plunge freezing.

A total of 8645 movies were collected on a Titan Glacios (Thermo Fisher) operating at 200 KeV, equipped with a Falcon 4i detector and selectris energy filter with a slit width of 20 eV. Movies were acquired using a total dose of 100 $e^-/\text{\AA}^2$ with 100 frames, at 130,000 $\times$ magnification with a calibrated pixel size of 0.880 $\text{\AA}$ and a defocus range of -1.0 to -2.0 μ m.

Cryo-EM data analysis

Data processing was carried out in CryoSPARC. Image preprocessing was performed in CryoSPARC Live[150] using Patch Motion Correction and Patch CTF estimation. Initial particle picking was conducted with blob picking, followed by template generation for both monomeric and dimeric particles. The processed micrographs and templates were then exported to CryoSPARC for downstream analysis. Template-based particle picking was applied to both monomer and dimer datasets, followed by multiple rounds of 2D classification to remove junk particles. Three-volume unsupervised *ab-initio* reconstruction was performed to generate initial and bait volumes for further particle cleanup. Successive rounds of heterogeneous refinement were carried out until a consistent class containing clean particles was obtained. This refined particle stack was subjected to Non-uniform refinement[151], including local CTF refinement and higher-order aberration fitting. To mitigate beam damage, movies were reprocessed using patch-based motion correction with the final

50 frames excluded. Particles were then subjected to reference-based motion correction^[172], followed by an additional round of Non-uniform refinement to achieve the final reconstructions.

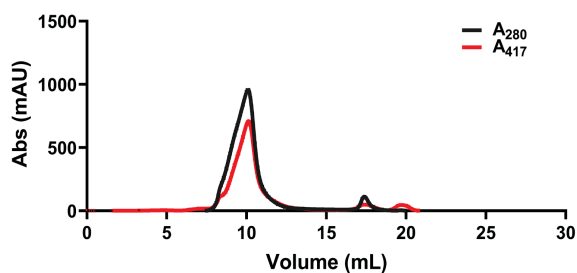
Data availability

The cryo-EM map is deposited at the Electron Microscopy Data Bank under accession code: EMD-55645. Atomic model of *Ecbd* bound to WSL017 has been deposited to the Protein Data Bank under accession number: 9T7N. All other data are presented in the main text or supplementary information.

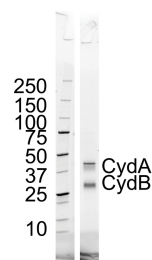
Supplementary information

Mtbd

Gel filtration

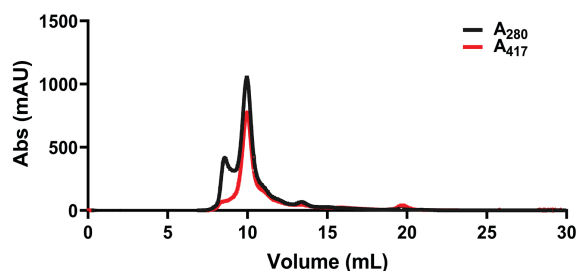


SDS-page

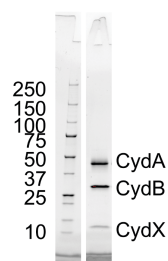


Ecbd

Gel filtration

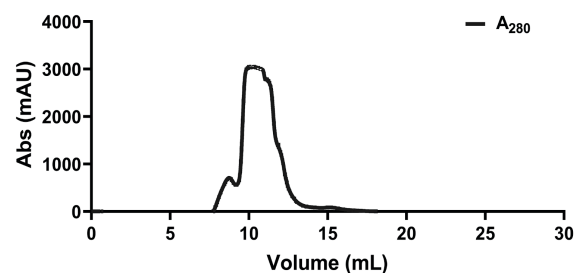


SDS-page



NDH2

Gel filtration



SDS-page

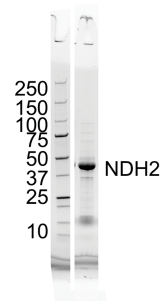


Figure S4.1. Gel filtration and SDS-Page (TGX stain) of the purified, *Mtbd*, *Ecbd* and NDH2.

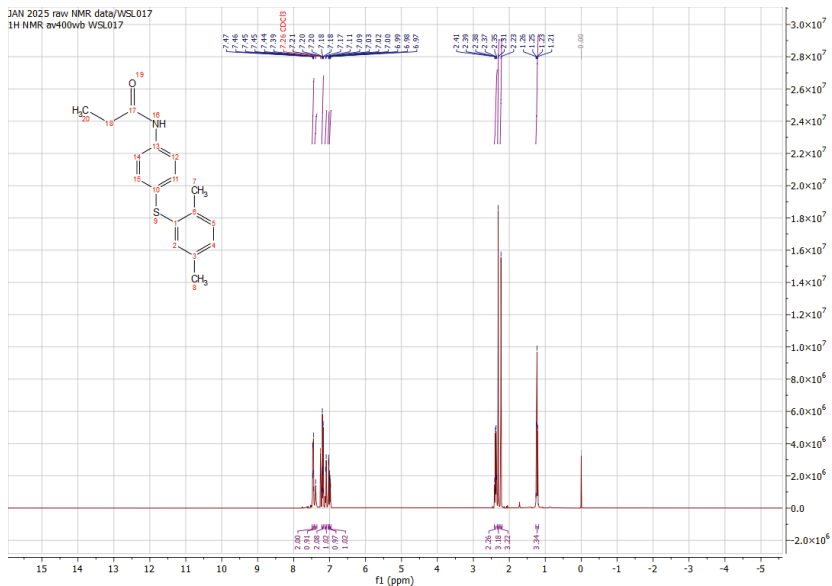


Figure S4.2. ^1H NMR of N-(4-((2,5-dimethylphenyl)thio)phenyl)propionamide (WSLo17)

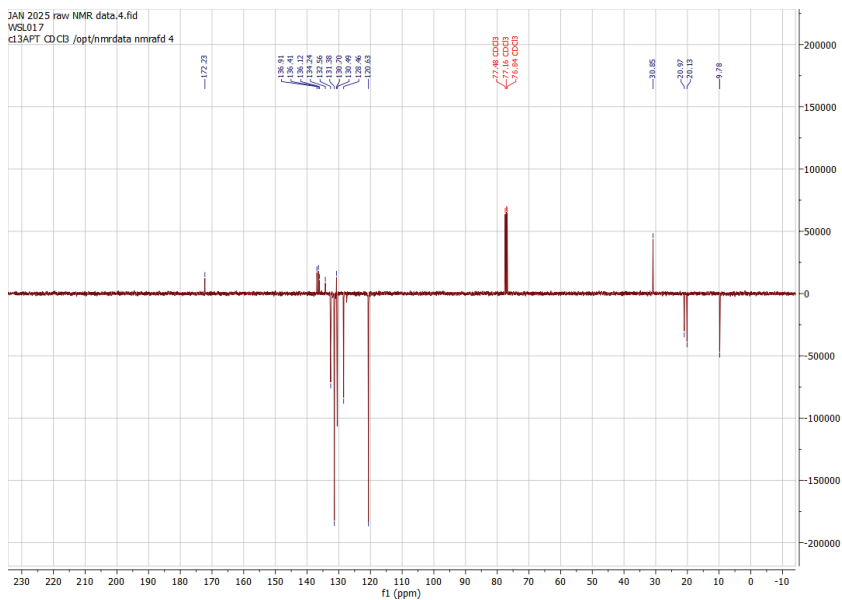


Figure S4.3. ^{13}C NMR of N-(4-((2,5-dimethylphenyl)thio)phenyl)propionamide (WSL017)

Table S4.1. Screening results and hit confirmation in detergent and proteoliposomes

Compound number	SMILES	Z-score	Hit confirmation detergent (P value)	Hit confirmation Liposomes (P value)
496	<chem>CC(NC(=O)C=C)C1(CC1)S(=O)(=O)C</chem>	4.42	0.0001	0.9991
573	<chem>C=CC(=O)N1CCC(CC1)C(=O)N2CCC(CC=3N=CON3)CC2</chem>	3.23	0.0008	0.9998
575	<chem>CCN(CC(=O)NC1CCN(C(=O)C)C=2C=CC=CC12)C(=O)C=C</chem>	4.47	0.5493	0.94
576	<chem>OC1CCCN(C1)C(=O)C2(CCN(CC2)C(=O)C=C)C=3C=CC=CC3</chem>	4.62	0.0114	0.9999
653	<chem>CCN(CC=1C=CC=C2OCOC21)C(=O)C=C</chem>	4.22	0.0009	0.9999
778	<chem>OCCNC(=O)C1CCN(CC1)C(=O)C=C</chem>	3.36	0.0001	0.2583
964	<chem>CC=1C=CC(SC=2C=CC(NC(=O)CC1)=CC2)=CC1C</chem>	3.85	0.0001	0.2583
1148	<chem>CC=1C=CC(C)=C(SC=2C=CC(NC(=O)CC1)=CC2)C1</chem>	3.69	0.0001	0.1612
1377	<chem>FC(F)(F)CC(=O)N1CCN(CC1)C(=O)CC1</chem>	3.01	0.0014	0.9991
1413	<chem>ClCC(=O)NC1CN(C1)S(=O)(=O)C=2C=CC=3OCCOC3C2</chem>	3.18	0.0006	0.9993
1513	<chem>CC(Cl)C(=O)N1CC(C)CN(CC=2C=CC=CC2)CC1C</chem>	3.56	0.015	0.999
1515	<chem>CC(Cl)C(=O)N1CC(C1)N2C=C(N=N2)C(=O)N</chem>	4.53	0.0006	0.9994
1516	<chem>CC(Cl)C(=O)N(CC1=CN=CS1)C2CCN(CC2)C(=O)C</chem>	3.59	0.0011	0.9986
1594	<chem>CC(Cl)C(=O)NC=1C=CC(C)=C(C1)S(=O)(=O)N2CCOCC2</chem>	3.11	0.0031	0.9986
1595	<chem>ClCC(=O)N(CC1=CC=CO1)CC=2C=CC=CC2</chem>	3.43	0.0003	0.9999
1596	<chem>CC(Cl)C(=O)NC=1C=CC=C(Cl)C1</chem>	4.55	0.002	0.9994
2165	<chem>CC=1C=CC=CC1C(NC(=O)CC1)C=2C=CC=CC2C</chem>	9.09	0.0001	0.9996
2570	<chem>COC=1C=CC(C=C(C#N)C(=O)NC=2C=CC(=CC2)[N+](=O)[O-])=CC1</chem>	11.72	0.0001	0.9863

Breathing in Thin Air

3053	<chem>CC=1C=CC(C=C(C#N)C(=O)NC=2C=CC=C3N=CC=CC23)=C C1</chem>	3.80	0.1058	0.9812
3075	<chem>CC1=NN(C)C(C)=C1C=C(C#N)C(=O)NC=2C=CC=CC2Cl</chem>	3.23	0.0048	0.9998
3076	<chem>CCCCNC(=O)C(=CC=1C=CC=C(Cl)C1Cl)C#N</chem>	3.01	0.0036	0.9998
3084	<chem>CCCCNC(=O)C(=CC=1C=CC(Cl)=CC1)C#N</chem>	3.03	0.0114	0.9993
3153	<chem>NC(=O)C(=CC1=CSC(COC=2C=CC=CC2)=N1)C#N</chem>	3.10	0.0014	0.998
3155	<chem>CC(=CC=1C=CC=CC1)C=C(C#N)C(=O)NC2CC2</chem>	3.16	0.0329	0.998
3156	<chem>CC1=NC=CN1CC=2C=CC(NC(=O)C(=CC3=CC=CO3)C#N)=C C2</chem>	3.45	0.0012	0.9991
3207	<chem>ClCC(=O)NCC=1C=CC(=CC1)C(=O)N2CCOCC2</chem>	3.24	0.0003	0.9863
3374	<chem>CS(=O)(=O)C=1C=CC(CNC(=O)CCl)=CC1</chem>	7.57	0.0099	0.6406
3501	<chem>ClCC(=O)N1CCN(CC1)S(=O)(=O)C=2C=CC=3OCCOC3C2</chem>	7.07	0.0031	0.9995
3726	<chem>CN1N=CC=C1CN2C[C@@H](F)[C@H]2CNC(=O)CCl</chem>	4.11	0.0048	0.9993
3929	<chem>CN1N=CC=C1C(CO)NC(=O)CCl</chem>	3.24	0.0017	0.9988
4797	<chem>CC1=NC(=NO1)C=2C=CC(CNC(=O)C=C)=CC2</chem>	3.92	0.0006	0.9812
4997	<chem>CC=1C=CC=C(C1)C2(CCC2)NC(=O)C=C</chem>	5.65	0.0011	0.9988
5038	<chem>CN1C=C(N=N1)C(C)(C)NC(=O)C=C</chem>	3.20	0.0036	0.9994
5085	<chem>C=CC(=O)NC1C2CCOC2C31CCCC3</chem>	3.55	0.0131	0.9997
5676	<chem>C=CC(=O)NC=1N=C2N=CC=CN2N1</chem>	4.40	0.0224	0.9986
6185	<chem>CC1(CCCN1C(=O)C=C)C=2C=CC=CC2</chem>	5.97	0.0086	0.9988
6717	<chem>C=CC(=O)NC1CCN2CCCC2C1</chem>	4.23	0.0196	0.6696
6820	<chem>F5(=O)(=O)N1CCN(CC1)C=2C=CC=CC2</chem>	4.34	0.0476	0.6406
7026	<chem>CC(=O)N1CCC2(CC1)CCC=3C=CC(OS(=O)(=O)F)=CC3O2</chem>	3.01	0.0255	0.0487

7182	<chem>CC=1C=C(C(=O)NC=2C=CC(OS(=O)(=O)F)=CC2)C(C)=NC1C</chem>	8.49	0.0114	0.1483
7502	<chem>OC(=O)C=1C=CC=CC1NC(=O)C(=CC=2C=CSC2)C#N</chem>	3.61	0.0002	0.4968
7527	<chem>FC=1C=CC(CNC(=O)C(=CC=2C=CC=CC2)C#N)=CC1</chem>	3.51	0.0001	0.0721
7614	<chem>FC=1C=CC(CCNC(=O)C(=CC2=CC=CO2)C#N)=C(F)C1</chem>	3.24	0.0086	0.998
7642	<chem>ClC=1C=CC(=CC1)N2C=C(C=C(C#N)C(=O)NCC=C)C=N2</chem>	3.22	0.0001	0.0358
7725	<chem>O=C(NC1CC1)C(=CC=2C=CC(=CC2)N3CCCCC3)C#N</chem>	3.72	0.0099	0.9984
8695	<chem>CC#CC(=O)N(C)CC1=NC(=CS1)C(F)(F)F</chem>	13.52	0.0114	0.9982
9524	<chem>CC(Cl)C(=O)N1CC2(CCOCC2)C1C3CC3</chem>	6.99	0.0373	0.8525
9953	<chem>OB(O)C=1C=C2C=CC=CC2=NC1O</chem>	3.24	0.0086	0.9996
10255	<chem>CCS(=O)(=O)N1CCC(CB2OC(C)(C)C(C)O2)C1</chem>	3.64	0.0027	0.9997
10257	<chem>CC1(C)OB(OC1(C)C)C2=CCN(CC2)C(=O)C=3C=CC=C4C=CN=CC34</chem>	3.87	0.0224	0.9996
10333	<chem>CC(C)NC(=O)NC=1C=CC=CC1NC(=O)C(F)Cl</chem>	3.77	0.2564	0.9995
10334	<chem>CC(NC(=O)C(F)Cl)C1=NC(=CS1)C=2C=CC=CC2</chem>	3.16	0.0604	0.9993

Table S4.2. Cryo-EM data collected, refinement and validation statistics.

	<i>Ec</i> bd dimer WSL017 (EMDB-54823) (PDB 9SFH)
Data collection and processing	
Magnification	130,000
Voltage (kV)	200
Electron exposure ($e^-/\text{\AA}^2$)	100
Defocus range (μm)	-0.8 - -2.0
Pixel size ($\text{\AA}$)	0.880
Symmetry imposed	C2
Initial particle images (no.)	5,721,267
Final particle images (no.)	193,848
Map resolution ($\text{\AA}$)	2.60
FSC threshold	0.143
Map resolution range ($\text{\AA}$)	2.5-2.7
Refinement	
Initial model used (PDB code)	9RZV
Model resolution ($\text{\AA}$)	2.6
FSC threshold	0.143
Map sharpening B factor ($\text{\AA}^2$)	-60
Model composition	
Non-hydrogen atoms	15849
Protein residues	1902
Ligands	4 HEM, 2 A1JN4, 6 LPP, 2 PGT, 4 UQ8, 2 OXY, 2 A1JT9
Water	15
B factors ($\text{\AA}^2$)	
Protein	112.53
Ligand	117.20
R.m.s. deviations	
Bond lengths ($\text{\AA}$)	0.009

Bond angles (°)	0.83
Validation	
MolProbity score	1.24
Clashscore	4.69
Poor rotamers (%)	0.39
Ramachandran plot	
Favored (%)	98.36
Allowed (%)	1.64
Disallowed (%)	0.00

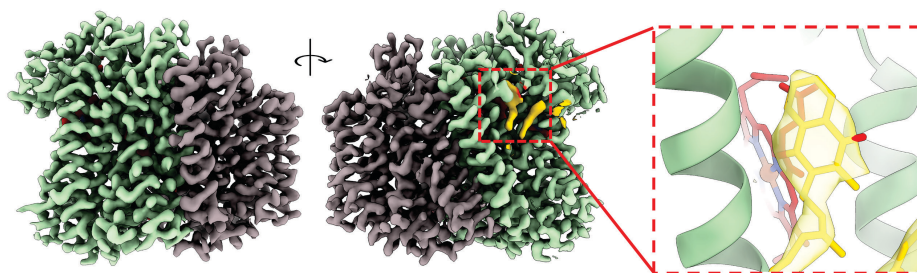


Figure S4.4. Colored Cryo-EM map of *MtbD* resolved in the presence of WSL017 (3.1 Å). CydA is shown in green with CydB in grey. No change in the density of MK-9 is observed.

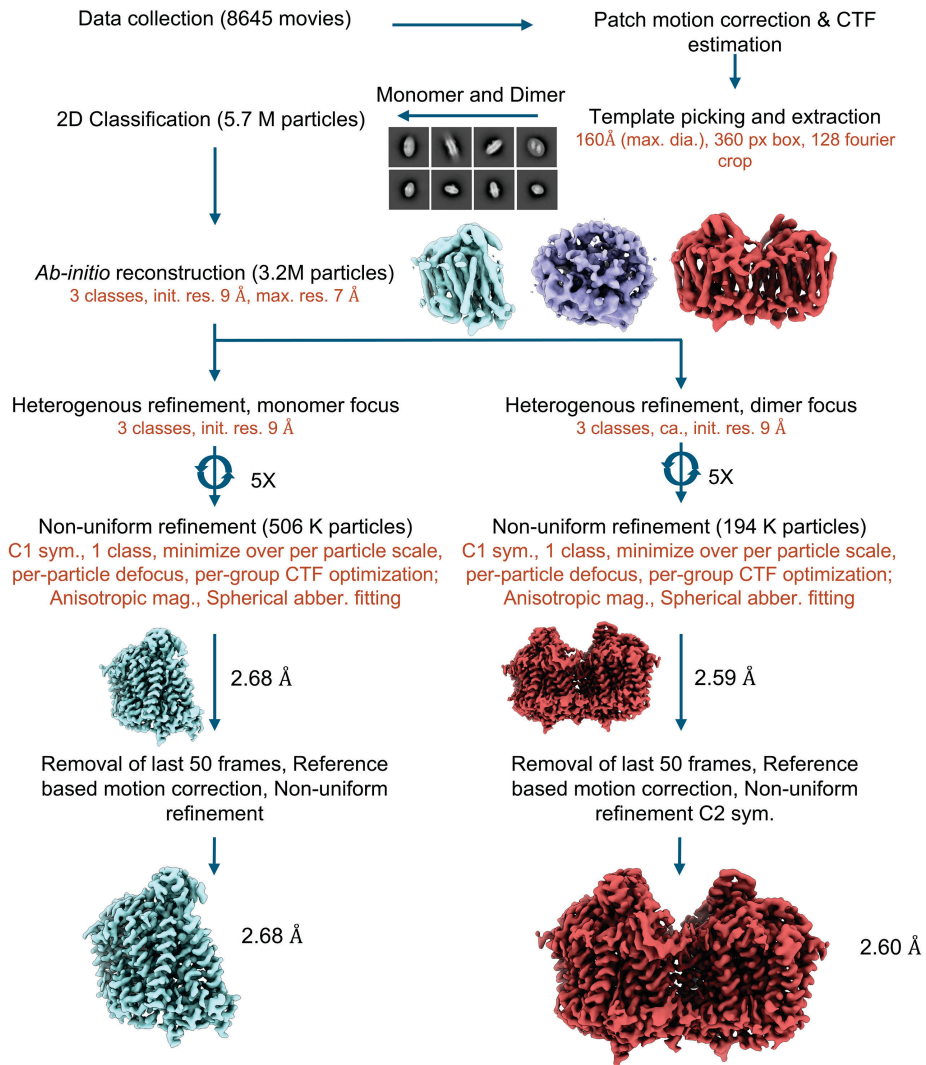


Figure S4.5. Cryo EM data processing procedure of WSL017 bound *EcbD*.

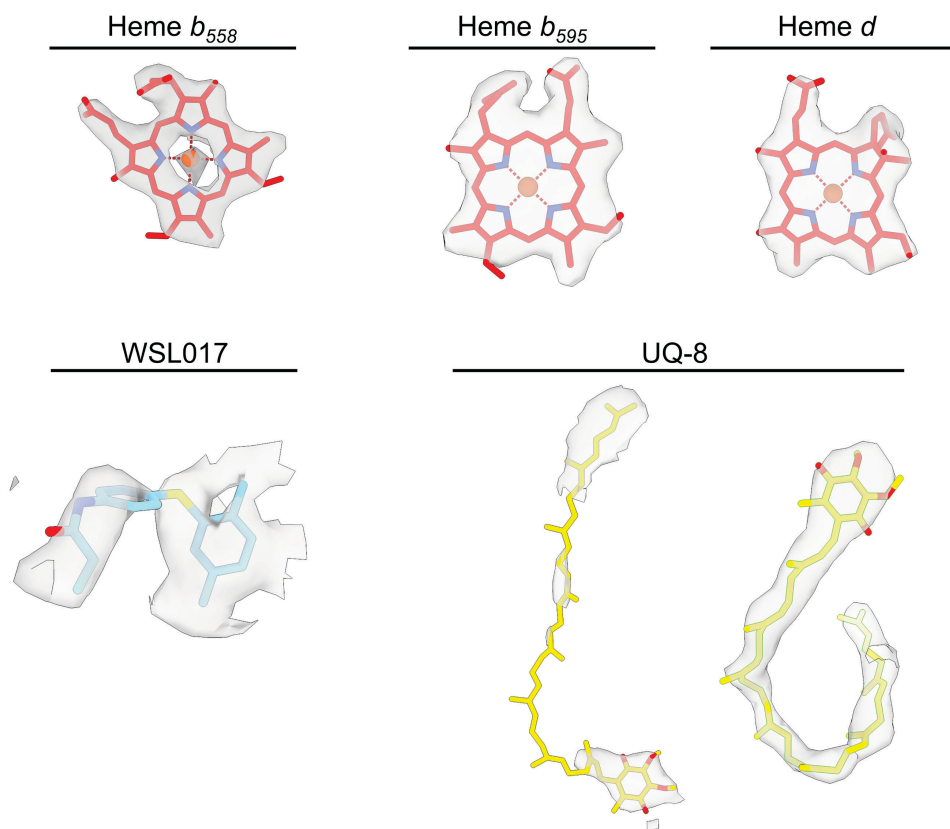


Figure S4.6. Density features of the main ligands bound to *Ecbd*

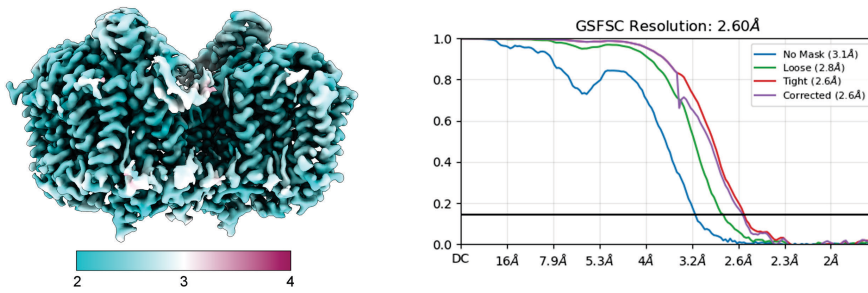


Figure S4.7. Local resolution and fsc curve of the *Ecbd* dimer structure bound to WSL017.

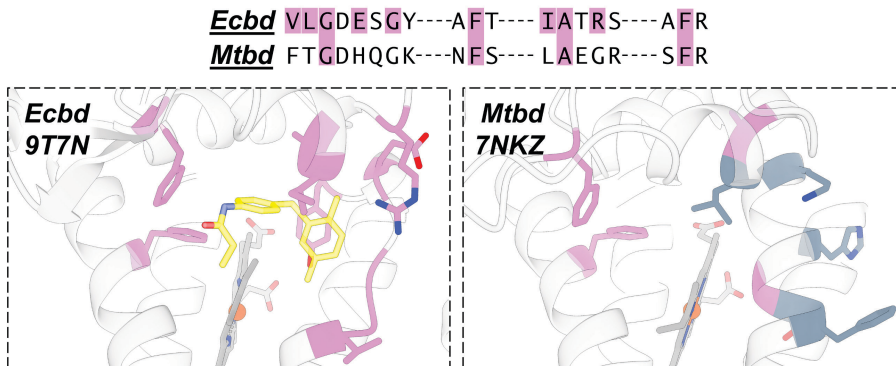


Figure S4.8. Comparison of the WSL017 binding pocket in *Ecbd*, highlighting the interacting residues in pink, with the equivalent region in *Mtbd*. The corresponding residues in *Mtbd* are shown with conserved interactions highlighted in pink.

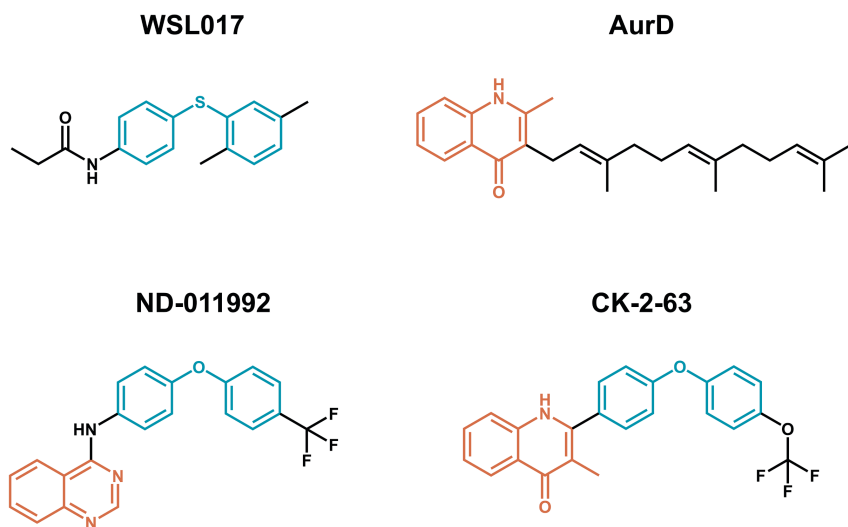


Figure S4.9. Overview if inhibitor common cytochrome *bd* inhibitor features. The Aurachin D headgroup is highlighted in orange, while the WSL017 backbone is highlighted in blue.

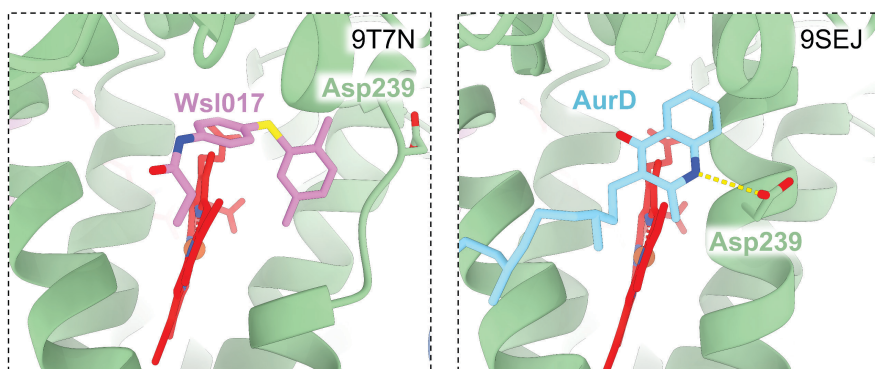


Figure S4.10. Different binding mode of Wsl017 and AurD in the *Ecbd* quinol oxidation site. AurD induced active site refolding by hydrogen bonding with Asp239^{CydA}.

Chapter

5

Towards a new cytochrome *bd* heme incorporation mechanism by direct complex formation between CydA and CydDC

Respiration is one of the principal processes that drives bioenergetics across the tree of life. In aerobic conditions, one of the central steps is the enzymatic reduction of molecular oxygen to water, catalyzed by the terminal oxidases. A subclass of these enzymes are the *bd*-type oxidases (cyt *bd*), solely found in prokaryotes and characterized by their heme-only oxygen reduction site. The incorporation of these hemes into cyt *bd* is a multistep process crucial for its oxidase activity. Recent studies have identified the type IV ATP-binding cassette (ABC) transporter CydDC as a key factor in heme delivery to cyt *bd*. However, the molecular mechanism by which heme transported across the cytoplasmic membrane is incorporated into cyt *bd* remains unresolved. Here, we combine protein purification, spectroscopy, mass spectrometry, cryo-electron microscopy and structure prediction to investigate potential interactions between CydDC and the CydA subunit of cyt *bd*. In *E. coli*, AlphaFold 3 predictions identify a high-confidence interaction interface between CydDC and CydA, suggesting direct complex formation. Fingerprinting analysis and cryo-EM are consistent with the presence of CydA in CydC pulldown samples. However, limited resolution and sample heterogeneity preclude unambiguous structural assignment. Based on these observations and structural predictions, we propose a putative model in which lateral association of CydDC with CydA enables direct heme transfer via a lateral gate formed between CydC helix 2 and CydD helix 5. This mechanism could allow direct heme incorporation during cyt *bd* maturation without a previously proposed periplasmic shuttle protein. Future experiments are proposed to further assess this model. Elucidating this pathway provides new insight into respiratory oxidase maturation and may reveal novel targets for antimicrobial intervention.

Introduction

Aerobic respiration is one of the main biochemical processes that drives ATP generation across the tree of life. During this process, the enzyme class of respiratory oxidases reduce molecular oxygen to water, often displacing protons across the inner membrane to create a proton motive force required for ATP synthesis. Evolution has resulted in three distinct types of these respiratory oxidases based on their active site architecture. These include the heme-copper oxidases, characterized by a heme-copper active site, the alternative oxidases, characterized by an iron-iron active site, and the *bd* oxidases, which utilize a heme *d* co-factor to catalyze the four-electron reduction of oxygen to water.

The *bd*-type oxidases are particularly notable for their role in energy conservation under microaerobic and stress conditions, highlighted by their remarkable affinity for oxygen[46,47]. The *bd* oxidases are selectively found in prokaryotes, and in pathogens contribute to virulence, antibiotic resistance, and oxidative stress tolerance[48,58,59,112]. Structurally, *bd* type oxidases consist of two core subunits, CydA and CydB, with additional small subunits such as CydX and CydH found in some species including *E. coli* cytochrome *bd*-I (*Ecbd*)[83]. The CydA subunits harbors three heme groups required for catalysis, while the CydB unit is hypothesized to have a structural role providing stability to the complex[91]. In *E. coli*, a secondary *bd* oxidase, cyt *bd*-II, resembles the cyt *bd*-I architecture including its heme cofactors, although being expressed at near-anaerobic conditions to maintain respiration when the *bo₃* and *bd*-I oxidase lack oxygen affinity[87,144]. The assembly and function of *bd* oxidases depends on a multistep process that requires coordinated integration of the characteristic heme cofactors into the CydA subunit, a process that remains incompletely understood.

Central to this process is the type IV ATP-binding cassette (ABC) transporter CydDC, a heterodimer composed of CydD and CydC subunits. Genetic studies have shown that CydD and CydC often are adjacent on the same operon as cyt *bd*[95], and that deletion of CydD or CydC leads to loss of the characteristic cyt *bd* heme signal and oxidase activity[196]. CydDC has been proposed to export

low-molecular-weight thiols such as glutathione and cysteine, thereby modulating the redox environment of the periplasm[95]. However, emerging evidence indicates a direct role in heme transport[197,198], raising fundamental questions how the heme transported by CydDC gets incorporation into cyt *bd*.

Despite its importance, the molecular mechanism by which CydDC contributes to cyt *bd* assembly has remained elusive. Biochemical and structural studies have shown heme binding and displacement into the core of the CydDC transmembrane domain[198]. The heme release mechanism of CydDC, however, remains speculative. Molecular dynamics simulations indicate a rapid opening of the CydDC gate towards the periplasm, allowing the release of heme[198]. Current hypotheses point to an unknown periplasmic partner protein that can shuttle the exported heme from CydDC to CydA to form mature cyt *bd*[197,198], although direct evidence for this hypothesis is lacking. Heme transport and enzyme maturation are key to respiration and given its potential as a target for antimicrobial intervention, resolving the heme incorporation mechanism by CydDC is of great interest.

Here, we explore the alternative hypothesis that CydDC directly associates with the CydA subunit during cyt *bd* biogenesis. Using structural predictions and experimental approaches, we investigate whether transient CydDC–CydA (CydADC) complex formation could enable direct heme transfer into the CydA core.

Results

Co-evolution of CydA and CydDC indicates a common binding interface

The genes for cyt *bd* and CydDC are adjacent on the *E. coli* genome, although they are regulated under distinct promoters (Fig. 5.1A)[199,200]. Expression analysis indicates coordinated expression between CydDC and both *E. coli* cyt *bd* oxidases (*bd*-I: CydA, CydB ; *bd*-II: AppA, AppC), lacking coordination with the *ba*₃ oxidase (CyoA, CyoB)[201], hinting at a correlated functional role

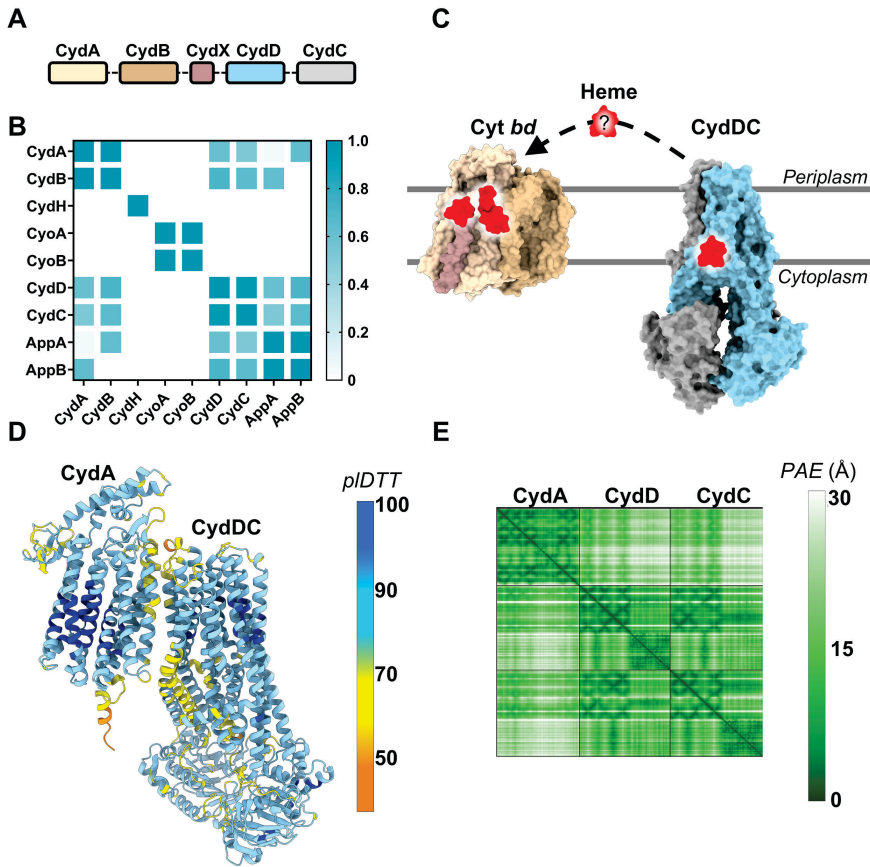


Figure 5.1 (A) Genomic organization of *E. coli* *cyt bd*, *CydA*, *CydB*, *CydX* and *CydDC*. (B) Co-expression of the *cyt bd* and *CydDC* subunits according to the STRING database[201] (C). Proposed heme incorporation mechanism, highlighting an unknown periplasmic chaperone to transfer the heme from *CydDC* to *cyt bd*[197,198]. (D) AlphaFold 3 prediction of the *CydA* and *CydDC* subunits, colored by pIDTT with its associated (E) PAE plot.

in *bd* oxidase maturation (Fig. 5.1B). Recent advances in the *CydDC* heme transport mechanism hypothesize that a periplasmic partner protein shuttles the exported heme through the periplasm into the *CydA* subunit of *cyt bd* [197,198] (Fig. 5.1C). To study this heme incorporation mechanism of *CydDC* into *CydA*, an AlphaFold 3 interactome study was performed. Surprisingly, AlphaFold 3 prediction indicates a stable complex between *CydA* subunit and *CydDC* in the absence of *CydB* in *E. coli* (Fig. 5.1D,E), and across several other species (Fig.

S5.1). These predictions suggest that CydDC may transiently associate with CydA, potentially enabling direct heme transfer prior to full cyt *bd* assembly.

Purification of a putative CydADC complex

To probe the existence of a CydA-CydDC (CydADC) complex, we have cloned the CydDC genes into the pET17bCydABX-linkerstrep plasmid. During cloning, CydDC with a C-terminal His tag on CydC was used to replace the CydB to enable affinity purification of the transporter. The removal of the CydB subunit was performed to minimize binding competition for CydA after heme incorporation by CydDC (Fig. 5.2A, Fig. S5.1). This plasmid was transformed into BL21 Rosetta cells and expressed overnight in M9 minimal media. The protein was purified using IMAC affinity purification of the CydC subunit followed by gel filtration to study the pulldown of CydA. SEC MALS analysis of the purified complex indicates the existence of multiple oligomeric species, as indicated by a shoulder of the main peak with increasing molecular weight between 200 and 400 kDa (Fig. 5.2B). The purified protein showed clear bands for both the CydC and CydD subunits, as confirmed by western blot (Fig. 5.2C). Additionally, a faint band was observed at the molecular weight of CydA. To provide indirect evidence of CydA binding, a western blot against the strep tagged CydX was performed, which specifically associates with CydA in the mature cyt *bd* complex[83]. This indicates co-purification of the CydX subunit, suggesting the presence of CydA after CydC pulldown (Fig. 5.2C).

To detect the presence of specific heme species in the purified protein preparation, a reduced minus oxidized UV-vis spectrum was taken. This difference spectrum shows signals corresponding to the presence of *b*-type hemes, without a clear heme *d* signature as is observed in mature *Ec**bd* (Fig. 5.2D). This indicates that if CydA is bound to CydDC, it has not yet fully matured its heme *d* oxygen reduction site. Additionally, it should be noted that purified CydDC has previously been shown to contain a heme signal corresponding to heme *b* [202], which means that no heme signal can unambiguously be assigned to CydA. Mass spectrometry-based fingerprinting following trypsin digestion detected significant enrichment of CydA in comparison

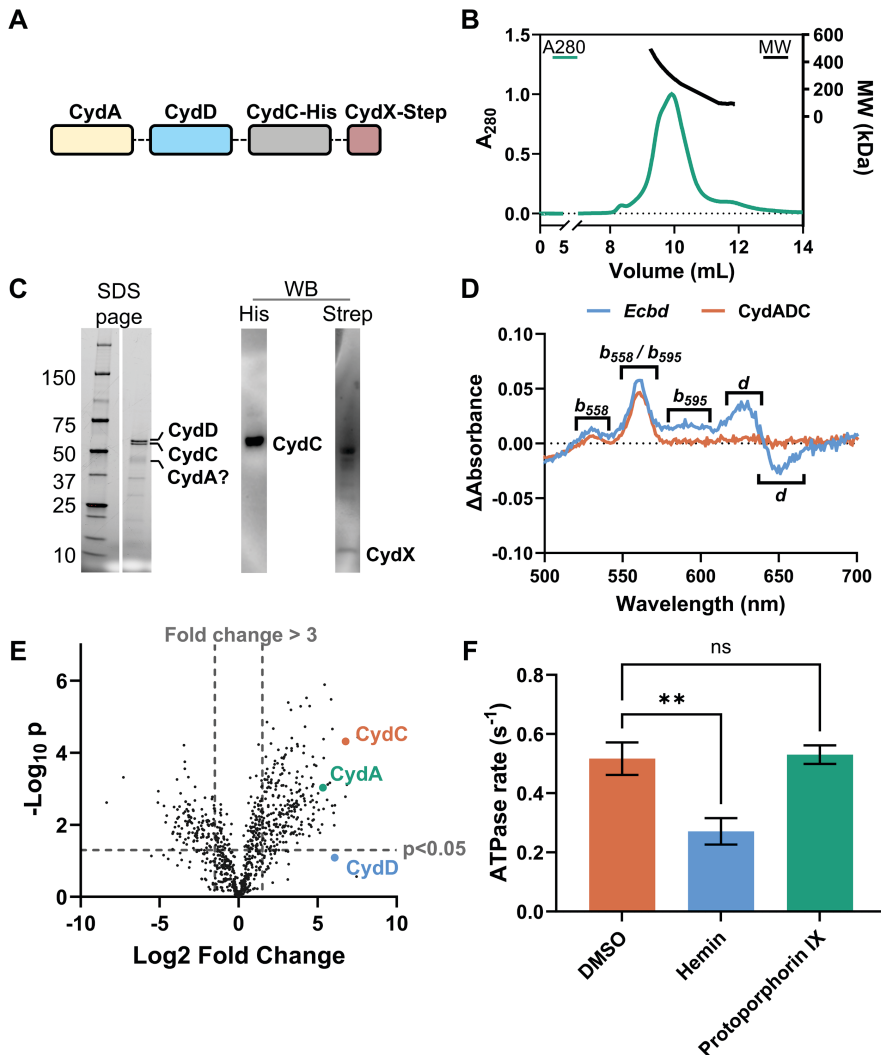


Figure 5.2 Purification and characterization of the putative CydADC complex. (A) Organization of genes and purification tags in the CydADC expression construct. (B) SEC-MALS analysis of the purified complex. (C) SDS page and western blot against CydC (His) and CydX (Strep). (D) Reduced minus oxidized UV vis spectrum of the purified complex in comparison to purified *Ecdbd*. Data is normalized to the Soret band (417 nm) in the oxidized spectrum (20 mM MES pH 6.0, 50 mM KCl, 0.001% LMNG). (E) Fingerprinting results of the purified complex (N=3), indicating the presence of CydA. (F) ATPase activity of the purified complex (N=2), inhibited upon exposure to hemin (20 mM Tris-HCl (pH 7.0), 50 mM KCl, 4 mM MgCl₂, 350 μ M PEP, 350 μ M ATP, 350 μ M NADH, 2.5 v/v% pyruvate kinase and lactate dehydrogenase solution (ThermoFisher), 0.002% LMNG, 500 nM substrate in DMSO, 80 μ g/mL purified transporter (final 1 v/v% DMSO).

to a negative control (his-tagged purified *C. thermarum* NDH₂), providing additional, albeit indirect, evidence for complex formation (Fig. 5.2E). The purified preparation showed ATPase activity indicating functional integrity of the transporter (Fig. 5.2F). Interestingly, exposure to hemin decreased the ATPase activity, an effect not seen with protoporphyrin IX. This indicates a kinetic profile that differs to previously characterized CydDC[197,198], and might indicate a specific effect the iron containing hemin has on the putative CydADC complex.

Cryo-EM and structure prediction of the CydADC complex

To further explore the existence of the putative CydADC complex, we performed cryogenic electron microscopy (cryo-EM) on the purified transporter sample. The sample was concentrated to 1.5 mg/mL and blotted on a 300 mesh copper grid. A total of 7408 movies were collected on a titan Glacios microscope at 50 $e^-/\text{\AA}^2$ and a 0.88 $\text{\AA}$ calibrated pixel size (Fig. 5.3A). The movies were imported into CryoSPARC live and subjected to patch-based motion correction followed by blob picking, multiple rounds of 2D classification, and finally unsupervised *ab-initio* model generation (Fig. 5.3B). Data collection and image processing revealed a dominant particle population corresponding to CydDC in a conformation consistent with previously solved structures. These particles displayed well-defined side and top views characteristic of the CydDC heterodimer[197,198] (Fig. 5.3C). A smaller subset of particles exhibited an asymmetric detergent micelle and additional lateral density. *Ab-initio* reconstruction of this subset revealed density compatible with a three-subunit CydADC assembly. However, the resolution and structural definition were insufficient to confidently assign the additional density to CydA (Fig. 5.3D). These results suggest that if a CydADC complex exists, it is likely transient, low-abundance, or structurally heterogeneous, precluding unambiguous visualization under the conditions tested.

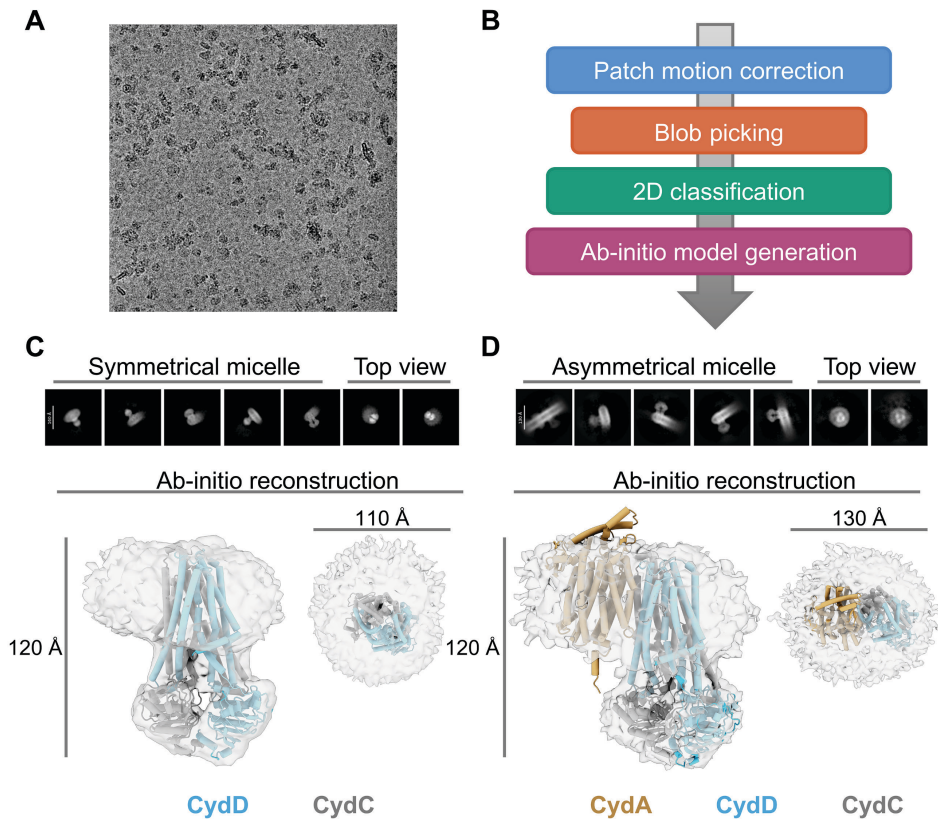


Figure 5.3. (A) Representative micrograph of the collected dataset (B) Schematic data processing strategy in CryoSPARC (C) Selected 2D classes and corresponding ab initio volume of the CydDC complex. (D) Selected 2D classes and ab initio volume of the putative CydADC complex, highlighted by an asymmetrical micelle and threefold density seen from the top.

Discussion

Recent advances in the structure and function of CydDC have highlighted a clear relationship with the heme maturation mechanism *cyt bd*. Despite extensive research into the heme transport mechanism of CydDC, no experimental evidence has shown how heme is released from CydDC for downstream incorporation into *cyt bd*. Existing models invoke periplasmic heme release and capture by an unidentified shuttle protein, despite the absence of direct experimental evidence[197,198]. Using structural predictions of AlphaFold 3, we hypothesize that CydDC can bind laterally to the CydA subunit during *cyt bd* maturation, allowing for direct heme incorporation without the need for an additional binding partner. After heme incorporation, the CydA subunit would be released from CydDC, and subsequently stabilized by binding to the CydB, CydX and CydH subunits.

Co-expression and purification of CydA and CydDC shows to contain species of varying molecular weight, with an additional faint band at the molecular weight of CydA indicating the presence of multiple purified protein complexes. Western blot and protein fingerprinting underline co-purification of CydA and CydX, suggesting complex formation. Heme analysis, however, show the absence of the characteristic α -type heme signal, indicative of incomplete maturation of the co-purified CydA subunit. The complex purified here shows significant inhibition rather than stimulation of ATPase activity upon exposure to substrate hemin. Similar decreases in ATPase activity have been observed for the *E. coli* methionine and *Methanosarcina acetivorans* molybdate transporter upon exposure to its substrate and was attributed to the stabilization of a state which decreases futile ATP hydrolysis[203,204]. The decrease in ATPase activity observed here is in stark contrast with previously characterized CydDC, suggesting a change in heme handling[197,198]. Regretfully, our purified protein complex was shown to be heterogenous, prohibiting direct comparison of transporter kinetics with a single protein state. Potentially, the subpopulation existing as a CydADC complex exhibits an altered structural landscape upon binding to heme, explaining the distinct kinetic response upon heme exposure. However, due to the sample heterogeneity, unambiguous conclusions cannot be made. Furthermore, it should be noted that CydDC has

not yet been separately purified as a control to our experiments, warranting caution in interpretation of the characteristics presented here.

Interestingly, CydDC has not yet been probed for the transport of *d*-type hemes, the characteristic active site co-factor in cyt *bd*. Direct transport of this heme subtype by CydDC would be interesting as current discussions propose the formation of *d*-type hemes *in situ* after incorporation of a *b*-type heme into its host protein[205,206]. Heme *d* is formed by lactone formation of the propionate group of heme *b*, introducing a chiral carbon on the porphyrin ring of heme *d*, presenting both *cis* and *trans* variants. If heme *d* formation occurs *in situ*, this could indicate why different heme *d* chirality's are reported in cyt *bd* oxidases[153,192], potentially imposed by the local environment during lactone formation. Whether heme *d* chirality affects cyt *bd* activity remains to be determined.

Regretfully, despite biochemical indications of CydA co-purification, cryo-EM analysis was unable to produce explicit structural features in line with the CydADC complex. Potentially, the CydA subunit rapidly releases from CydDC after heme incorporation, hindering stable complex formation for purification and structural integration. Regardless, the structure of CydADC predicted by AlphaFold 3 provides a putative framework for proposing a mechanistic model of heme incorporation. The model suggests that lateral association of CydDC with the CydA subunit forms a transient assembly that enables direct heme transfer without the requirement for an additional periplasmic carrier protein. In this configuration, heme bound to CydDC in the previously described heme-confined state[196,197] must first be repositioned toward the midplane of the membrane through the transmembrane helices of CydDC. Upon reaching this membrane-embedded position, the cofactor can be inserted laterally into CydA, allowing sequential incorporation of all three heme groups (Fig. 5.4A). Notably, this model diverges from earlier proposals suggesting periplasmic release. Instead, it requires formation of a lateral gate in the outward-open conformation of CydDC. We propose that this gate is formed at the interface between helix 2 of CydC and helix 5 of CydD and opens upon ATP binding to the CydC NBD. A laterally open conformation has not been observed in molecular dynamics simulations of *E. coli* CydDC[197,198], however, such a state may be

stabilized only in the presence of CydA, which was absent in previous simulations. Interestingly, CydDC from *M. smegmatis* has been solved in an outward open state using cryo-EM[197]. This state shows the formation of a lateral gate between helix 1 and 6 of CydC, as well as helix 5 of CydC and helix 2 of CydD[197]. This lateral opening in CydDC is directly opposite to the CydADC interface in our predicted structures (Fig. S5.2). While this argues against the model proposed here, the structural landscape of CydDC potentially changes upon binding to CydA, allowing for the opening of the lateral gate between CydC helix 2 and CydD helix 5 for direct heme incorporation. Notably, lateral gate opening has precedent in other type IV ABC transporters, including the lipid flippase MsbA[207], supporting the mechanistic plausibility of intramembrane heme transfer.

Assuming this lateral docking model is correct, a stepwise mechanism of heme incorporation can be derived (Fig. 5.4B). During assembly of cyt *bd*, CydA initially associates laterally with CydDC. In the inward-open state of CydDC, heme binds within the transmembrane region. Release of ADP from the CydC NBD promotes translocation of heme into the central cavity, corresponding to the heme-confined state[198]. Subsequent ATP binding induces conformational rearrangements that drive heme toward the transmembrane mid membrane plane and trigger opening of the proposed lateral gate between CydC and CydD, thereby establishing direct access to CydA. Following ATP hydrolysis, CydDC returns to the inward-open state, resetting the transporter for another cycle. This process is repeated three times to achieve complete incorporation of the three heme cofactors into CydA. Upon completion, CydA disengages from CydDC and stabilizes its structure through association with CydB, CydH and CydX. Formation of the mature cyt *bd* complex then enables initiation of respiratory activity.

Future studies are required to unambiguously prove the formation of the CydADC complex. Most likely, this requires optimization of the expression and purification protocol to isolate the intact complex. Stabilization strategies, such as ATPase-inactive CydDC mutants (H85A^{CydC} or E500Q^{CydC}), chemical crosslinking, dual-affinity purification of CydDC and CydA, or cell-free co-expression, may facilitate stable complex purification for future

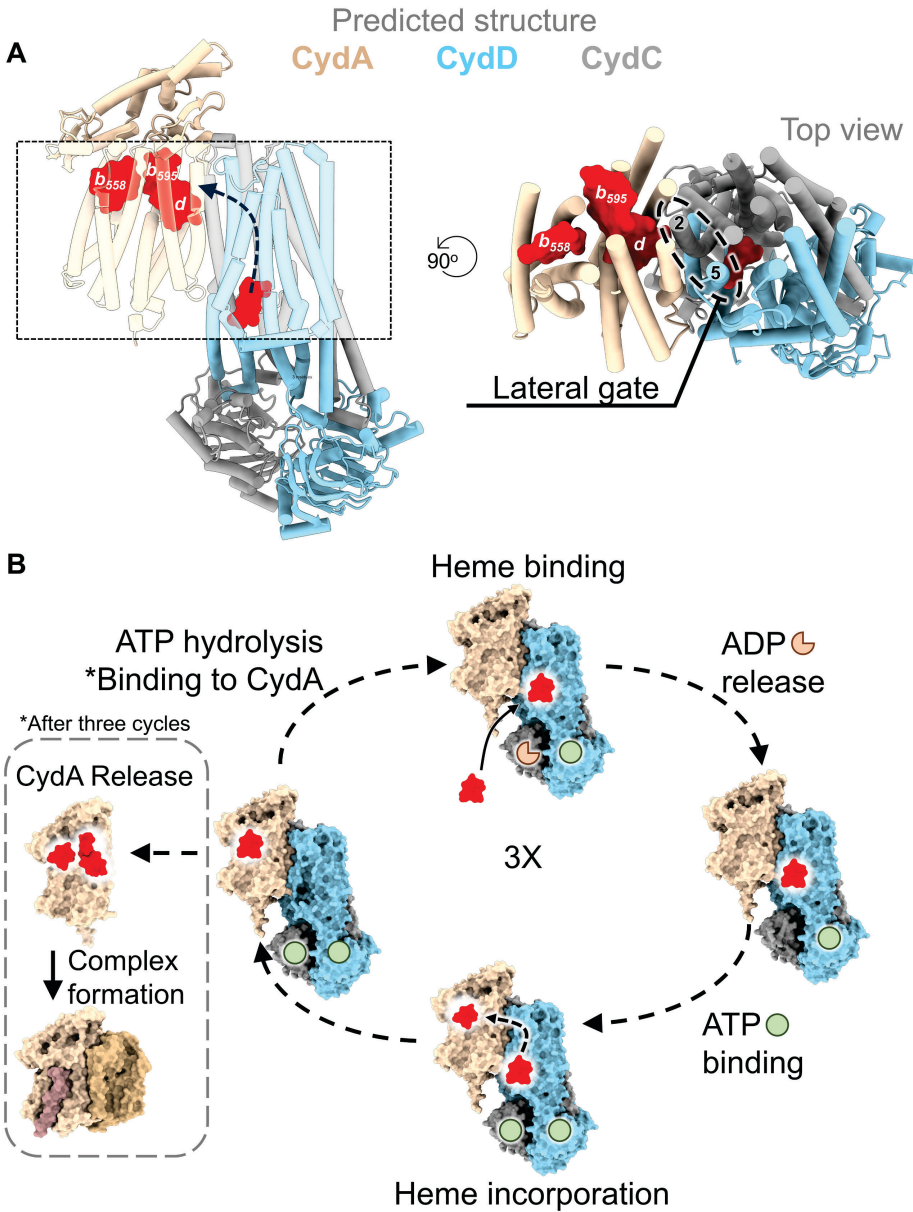


Figure 5.4 (A) Representation of the putative heme transport pathway in the CydADC complex, moving the heme from the confined state in CydDC into the CydA subunit. This mechanism would require the opening of a lateral gate between helix 2 of CydC and helix 5 of CydD. (B) Putative heme incorporation mechanism based on the predicted CydADC complex.

structural characterization. Alternatively, genome wide analysis of co-expression or an interactome studies using proximity-based MS labeling might point to CydDC binding partners, whether this is CydA or an unknown binding partner. Additionally, reliable control samples are needed, both CydDC for kinetic evaluation, and non-CydA interacting alternatives for pulldown, to validate the overall biochemical evaluation. Solving the heme transport of CydDC and heme incorporation mechanism into cyt *bd* provides fundamental information into the maturation of this important class of enzymes and provides further foundation for antibiotic development targeting microbial respiration.

Experimental procedures

Cloning and mutagenesis

The CydDC gene with a hexahistidine tag on the CydC C-terminus was ordered from Genscript and cloned into the pET17bCydABXlinkerstrep plasmid, exchanging the CydB gene for CydDC using Gibson assembly (Fig. S5.1). The newly created pET17BCydADCHis construct was confirmed using nanopore whole plasmid sequencing.

Expression and purification of the CydA-CydDC complex

The pET17BCydADCHis was transformed into *E. coli*/BL21 Rosetta and incubated overnight at 37°C to select positive transformants. Expression and purification were based on prior CydDC purifications[198]. A streak of colonies was inoculated in LB supplemented with 100 µg/mL ampicillin and grown overnight at 37°C, 200 rpm. The culture was diluted 1:100 in M9 media (100 µg/mL ampicillin) and grown to OD 0.8. Prior to induction, the culture was cooled to 16°C followed by addition of 1 mM IPTG. The CydADC complex was expressed for 16 hours before harvesting (7540 rcf, 20 min, 4°C). Cells were resuspended in 20 mM Tris-HCl, 300 mM NaCl, 100 mM KCl, 4 mM MgCl₂, pH 8.0, cOmplete protease

inhibitor before lysis using and Stansted cell homogenizer operating at 270 MPa. Cell debris were pelleted (10,000 rcf, 20 min 4°C) before membrane harvesting (200,000 rcf, 1h, 4°C). Membranes were resuspended in 20 mM Tris-HCl, 300 mM NaCl, 100 mM KCl, 4 mM MgCl₂, pH 8.0 and adjusted to a total protein concentration of 10 mg/mL as determined by BCA assay (ThermoFisher). Solubilization of the membrane protein was achieved by addition of 0.5% LMNG and gentle mixing for 1 hour at 4°C. Remaining insoluble materials were pelleted (200,000 rcf, 1h, 4°C) before application of the soluble fraction on a 5mL Hitrap Talon column (Cytiva). Sample application was performed at 0.5 mg/mL, following a wash of 10 column volumes using 20 mM Tris-HCl, 300 mM NaCl, 100 mM KCl, 4 mM MgCl₂, 10% glycerol, 0.002% LMNG, pH 8.0 at 1 mL/min. Sample elution was achieved using a linear gradient from 0 to 240 mM imidazole over 40 column volumes. Peak fractions were pooled and further purified using gel filtration using a Superdex 10/300 200 column at 0.5 mL/min (20 mM MES pH 6.0, 50 mM KCl, 0.001% LMNG). Peak fractions were pooled, concentrated and stored at -80°C until further use.

Reduced minus oxidized UV vis spectroscopy

Heme content of the purified transporter was analyzed using an oxidized minus reduced UV vis spectrum. CydADC was oxidized with 0.25 mM hexacyanoferrate in 20 mM MES pH 6.0, 50 mM KCl, 0.001% LMNG, and reduced by adding a few grains of sodium dithionite. Specific heme reduction was found by subtracting the oxidized from the reduced spectrum. The spectra were normalized to the Soret band at 417 nm of the respective oxidized spectra.

SEC-MALS analysis

The CydADC sample (1 mg/mL) in LMNG detergent was injected and characterized on a SEC-MALS system running 50 mM Tris HCl, 200 mM NaCl, 0.005% LMNG at 0.5 mL/min. The system was comprised of a miniDAWN® TREOS®, Optilab differential refractometer (Wyatt technology) and 1260 Infinity II multiple wavelength absorbance detector (Agilent).

ATPase activity

ATPase activity of the purified transporter was performed as described previously[198]. Briefly, heme substrates were dissolved in DMSO and added (500 nM final concentration) to the ATPase activity buffer when required (20 mM Tris-HCl (pH 7.0), 50 mM KCl, 4 mM MgCl₂, 350 μM phosphoenolpyruvate (PEP), 350 μM ATP, 350 μM NADH, 2.5 v/v % of pyruvate kinase and lactate dehydrogenase enzyme solution (Sigma Aldrich), 0.002% LMNG). This mix was preheated for 5 minutes in a FLUOstar platereader set to 37°C prior to the addition of 80 μg/mL purified transporter. ATPase activity was measured for 60 minutes by determining the coupled NADH oxidation rate (340 nm).

Protein fingerprinting

To establish the presence of CydA in the CydADC pulldown, purified protein was subjected to fingerprinting. To control for aspecific co-purification of CydA, His purified *C. thermarum* NDH₂ (as previously described[159]) was used as a control. Each protein sample was precipitated in 80 vol/vol% ice cold acetone at 5 ug total protein and stored at -20°C for one hour. Subsequently, the precipitated protein was pelleted by centrifugation at 4000 g for 10 min, followed by removal of the supernatant. The protein pellet was resuspended in 8M urea and incubated at 37°C for 30 minutes while shaking to achieve protein denaturation. Subsequently, 3.1 mg/mL DTT was added for disulfide bond reduction and incubated at 37°C for 15 minutes. The reduced cysteines were capped by addition of iodoacetamide (7.4 mg/mL) and incubation at 37°C for 30 min. Proteins were digested using 200 ng trypsin (ThermoFisher) and incubated overnight at 37°C. The following day, C18 silica stage tips were prepared by washing with methanol, followed by 0.5% (vol/vol) formic acid (FA) in 80% (vol/vol) acetonitrile (ACN)/MilliQ and 0.5% (vol/vol) FA in MilliQ. Digested protein samples were loaded onto the stage tips by centrifugation (400 rcf). After loading, the stagetips were washed with 0.5% (vol/vol) FA in MilliQ, followed by elution into fresh low binding tubes using 0.5% (vol/vol) FA in 80%

(vol/vol) ACN/MilliQ. The samples were evaporated to dryness using a speedvac (45°C), followed by freshly dissolving into 0.1% TFA in MilliQ prior to Mass spec injection.

Peptides were separated via nanoflow reversed-phase liquid chromatography using a Vanquish Neo UHPLC system setup in a trap-elute configuration. For trapping peptides, a PepMap Neo 5 μm C18, 300 μm x 5 mm trap (Thermo Scientific, 174500) was used which was eluted into an Easy-Spray PepMap Neo 2 μm C18, 75 μm x 500 mm analytical column (Thermo Scientific, ES75500PN) kept at 50°C with a constant flowrate of 0.3 $\mu\text{L}/\text{min}$. The mobile phase consisted of a mixture of mobile phase A (0.1% FA in ULC-MS grade water (Biosolve) and mobile phase B (0.1% FA in 80% ULC-MS grade acetonitrile (Biosolve)), and the separation was performed with a gradient from 4 – 44% B over 40 min, followed by an increase to 90% B over 5 min and a column wash with 90% B for 14 min before column re-equilibration. Peptides were introduced to an Orbitrap Eclipse Tribrid Mass Spectrometer (Thermo Scientific) with a spray voltage of 1.5 kV. Data was acquired in data-dependent acquisition (DDA) mode with the following scan sequence: MS1 master scan (Orbitrap analysis, 240K resolution, 375-1500 m/z, RF lens 30%, 80 ms maximum injection time) with dynamic exclusion enabled (repeat count 1, exclusion duration 60 s, mass tolerance 10 ppm, dependent scan on single charge state per precursor only). The top precursors were then selected for MS2 analysis with an intensity threshold of $1.0 \text{ E}4$ within a 1.7 second duty cycle through quadrupole isolation (1.2 m/z isolation window) followed by higher-energy collisional dissociation in the ion routing multipole (normalized collision energy 30%) and analysis of the resulting fragments in the orbitrap (15K resolution, 100 ms maximum injection time, centroid mode). The data was searched using FragPipe v23.1 used using the pre-defined workflow LFQ-MBR[197]. A database was created by adding reverse entries to reviewed human proteome (Uniprot, February 2025, 20402 entries) supplemented with a list of contaminant proteins[200]. MSFragger[208,209] (version 4.3) was used to search DDA-PASEF data using the default settings. In the validation step, MSBooster (version 1.1.11) was implemented on both spectra and RT levels, and subsequently Percolator[210] and ProteinProphet[211] integrated in Philosopher (version 5.0.0) were used for

PSM validation and protein inference. Quantification was performed by IonQuant[212] (version 1.11.11) using proteotypic peptides only. Normalization was turned off.

Cryo-EM Sample Preparation and Data Collection

C-flat R1.2/1.3 grids (mesh 300) were glow discharged with a PELCO easiGlow device at 15 mA for 90s. CydADC in LMNG (4 μ L, 1.5 mg/ml) was applied to the grid, followed by blotting at 4°C, 100% humidity, 10 blot force for 4 seconds using a Vitrobot IV device (Thermo Fisher) immediately before plunge freezing in liquid ethane.

Grids were imaged on a Titan Glacios (ThermoFisher Scientific) operating at 200 kV with a Falcon 4i detector and selectris energy filter with a slit width of 20 eV. Movies were gathered in electron counting mode using aberration-free image-shift (AFIS) in EPU (ThermoFisher Scientific). A total dose of 50 $e^-/\text{\AA}^2$ at 130,000 $\times$ magnification with a calibrated pixel size of 0.880 $\text{\AA}$, at and a defocus range of -1.0 to -2.0 μ m.

Cryo-EM Data analysis

Data processing was performed in CryoSPARC. Image preprocessing was performed in CryoSPARC live[150] using Patch Motion Correction and Patch CTF estimation. The processed micrographs and particles were exported to CryoSPARC for further processing. Initial particle picking was performed with blob picking, followed by template generation for ABC transporter additional density in the micelle. Template particle picking was performed followed by multiple rounds of 2D classification to remove junk particles. Three volume unsupervised ab initio model generation was performed to generate initial and bait volumes for further particle cleanup. Further rounds of heterogeneous refinement were performed but did not yield improvements in the density of CydDC.

AlphaFold prediction and model analysis

The CydADC complex was predicted using the AlphaFold 3 servers using different combinations of the CydC, CydD, CydA and CydB subunits from various species. To visualize heme binding pockets, the predicted CydADC complex was aligned to known structures of cytochrome *bd*(6RKO) and CydDC (7ZDG). Models and ab initio Cryo-EM maps were assessed and analyzed in ChimeraX^[176] (version 1.7.1).

Author contributions

All experiments were performed by T.T. van der Velden. Mass spec data collected was performed by B. Gagestein from the mass spec facility. The chapter was written by T.T. van der Velden under the supervision of L.J.C. Jeuken.

Supplementary information

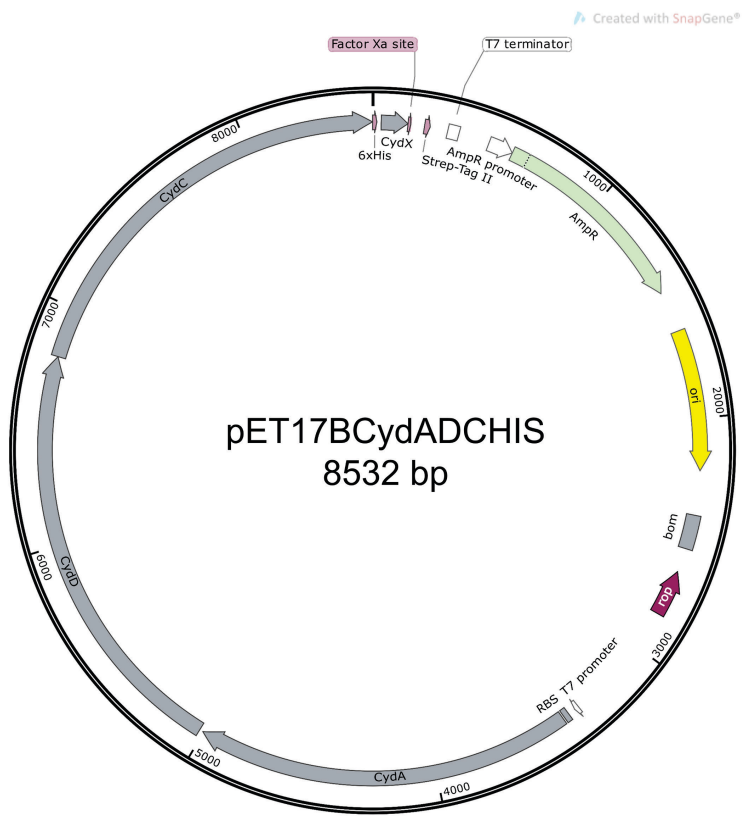


Figure S5.1. Plasmid map of the pET17BCydADCHis expression construct.

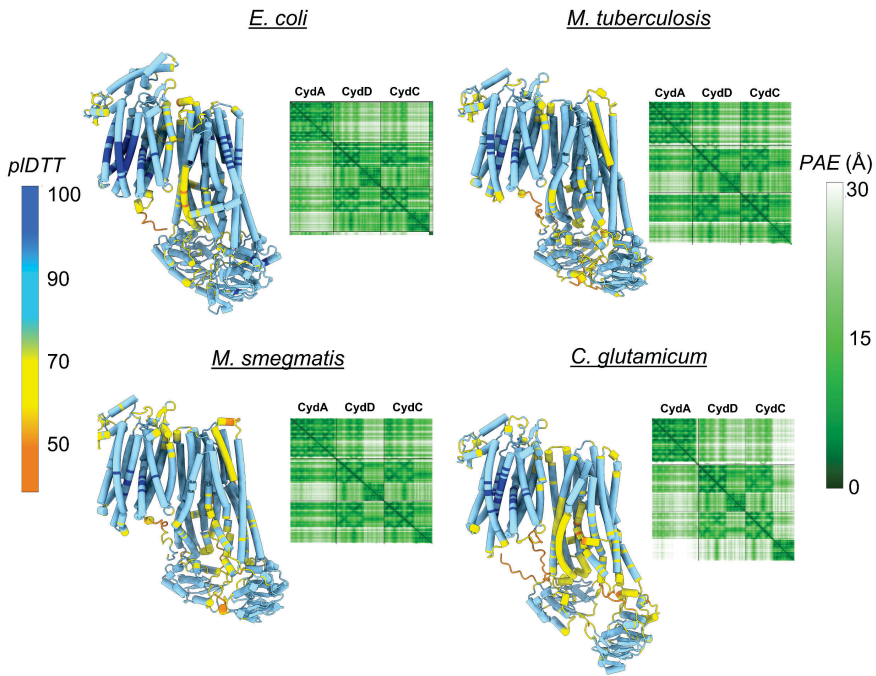


Figure S5.2. AlphaFold prediction of the putative CydADC complex from *E.coli*, *M. tuberculosis*, *M. smegmatis*, and *C. glutamicum*, colored by pIDTT.

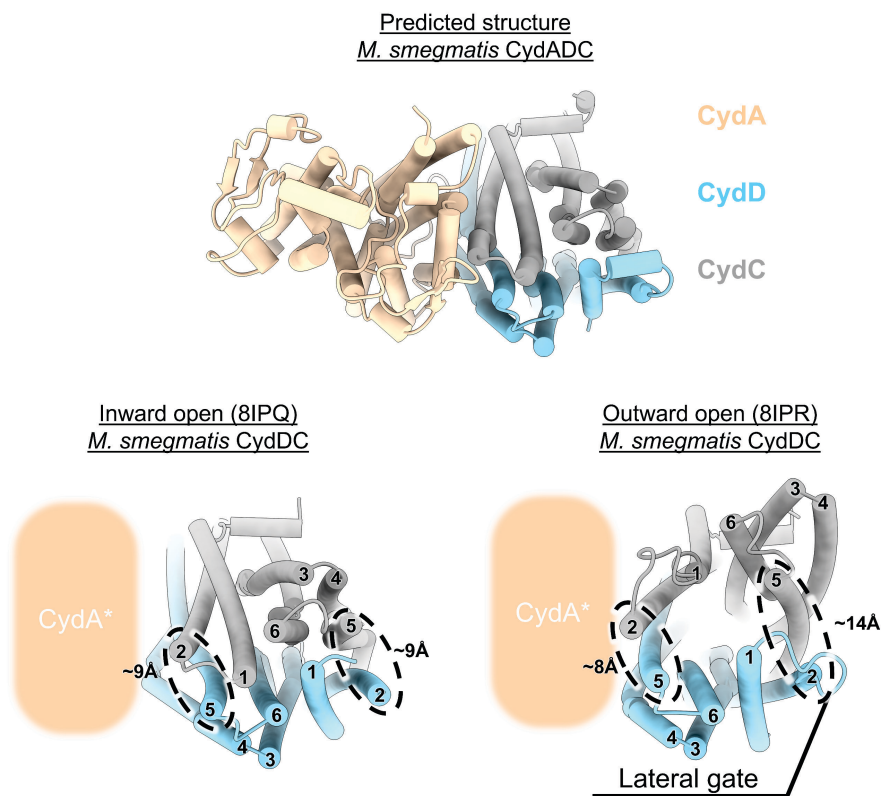


Figure S5.3. Top view of the predicted *M. smegmatis* CydADC complex in comparison with the solved cryo-EM structures of *M. smegmatis* CydDC in the inward (8IPQ) and outward (8IPR) open states.

Chapter

6

Discussion

Ever since the great oxygenation event, increasing the oxygen levels in our atmosphere[213], the reduction of molecular oxygen to water has become central to cellular energetics for many life forms. For obligate and facultative aerobes, this oxygen reduction reaction serves as the terminal electron sink for their respiratory chains, a series of enzymes that generate a proton or sodium motive force across the cell or organelle membrane. This system has evolved to be an efficient machinery in which the proton motive force is used to generate ATP, the central energetic molecule that drives biochemical processes.

Evolution has produced three types of respiratory oxidases to catalyse the terminal oxygen reduction reaction: the heme-copper oxidases[214], the alternative oxidases[215], and the *bd* oxidases[79]. While all three oxidases evolved to perform the same catalytic function, they utilize different molecular structures and catalytic mechanisms. The heme-copper and the alternative oxidases have well described molecular mechanisms, whereas the *bd* oxidases lack behind. The *bd* oxidases are not only interesting from an enzymatic point of view, having an exceptional high affinity for oxygen, but they are also exclusively found in prokaryotes. Their sole conservation in prokaryotes and critical role in ATP generation has made the *bd* oxidases an interesting antibiotic target. This thesis has described several advances in this field, shedding light on the evolution, catalytic mechanism, regulation, therapeutic intervention, and finally the heme-maturation mechanism of *bd* oxidases. A broader view of literature reveals many underexplored features of this enzyme class, which seems to keep broadening its importance in microbial adaptation and survival. In this chapter, the main findings of this thesis are discussed in a broader literature context and complemented with future directions of study.

The physiological role bd oxidases

The *bd* oxidases are classically characterized as oxidases with an exceptional high affinity for oxygen[46,47], allowing for respiration in oxygen poor niches such as found during infection. How the *bd* oxidases achieve such high affinity

for oxygen while remaining insensitive to stressors remains poorly understood. Better understanding of this oxygen affinity requires direct structural and sequence-based comparison of different oxidases, mutagenesis of the oxygen channels, and direct physicochemical study of the signature trans-heme *d* oxygen reduction site to delineate conserved features unique to the *bd* oxidases. Unfortunately, it remains technically challenging to directly measure the oxygen affinity of the enzymes by Michaelis Menten kinetics, requiring specialist setups which can reliably measure kinetics rates at extremely low oxygen concentrations[115]. Especially in the age of protein design, it would be highly interesting to fundamentally understand how this high oxygen affinity is achieved to design oxygen scavenging proteins.

In addition to its central role in bacterial energetics, scientist keep finding new attributes that *bd* oxidases impose upon its host bacteria[112]. To date, *bd* oxidases have been shown to play key roles in respiration, defence against various toxic compounds, and tolerance against antibiotics[48,56,59,154]. With an ever-growing list of attributes, it is becoming increasingly clear that the integrated role of the *bd*oxidases is centered in a complex system of bacterial homeostasis, rather than the limited view of an oxidase with a high affinity for oxygen. While this thesis has contributed to the understanding of the complex biochemical and structural features of this enzyme, many questions remain. Given that *bd* oxidases are unlikely to detoxify stressors such as nitrite or antibiotics directly, their protective effect likely stems from alleviating a more generalized form of cellular toxicity. Disentangling this mechanism will require a deeper understanding of bacterial stress responses that moves beyond correlating the presence of a stressor with its presumed harmful effect. For instance, it has been proposed that many antibiotics, regardless of their primary molecular targets, exert bactericidal activity through the generation of reactive oxygen species (ROS)[216]. Exploring this hypothesis requires close collaboration between biochemists and cell biologists, particularly to establish whether ROS production is a causal or secondary phenomenon. For example, it is well established that cell wall inhibitors are potent antibiotics, and how they work on enzymes that are responsible for cell wall synthesis. However, if the

direct breakdown of the cell wall is causative of the bactericidal effects, or if this includes the formation of ROS remains unclear[216].

The protective feature of *bd* oxidases against many different stressors may be explained by their peroxidase activity[49], which enables them to reduce ROS to H₂O and thus confer broad tolerance to ROS-generating conditions. This feature can unify the activities observed *in vitro* to the effects found *in vivo*. Alternatively, the insensitivity of the *bd* oxidases to noxious chemicals can provide defence by maintaining respiration in hostile environments, supplying the proton motive force required for survival when other components shut down. Insights into how cells reroute electrons to *bd* oxidases under stress can now be obtained using remission spectroscopy, which directly monitors the redox state of respiratory hemes in cell suspensions[217]. This approach offers an important bridge between *in vitro* enzymology and *in vivo* physiology and may serve as a powerful tool to delineate the role of cytochrome *bd* during cellular stress.

Other studies have implied cytochrome *bd* as a relieve valve on the respiratory chain[185]. A defining feature of cytochrome *bd* is that it is a non-proton pumping oxidase, translocating only the chemical protons associated with quinol oxidation and oxygen reduction across the periplasmic membrane. While this may appear energetically disadvantageous compared to the heme-copper, proton-pumping oxidases, it introduces another layer of respiratory flexibility. For example, inhibition of ATP synthase can lead to proton motive force accumulation, generating backpressure that disrupts cellular redox balance, damages membranes, and increases ROS formation through incomplete respiratory reactions[31]. By translocating less protons per quinol oxidized, the selective use of *bd* oxidases over their pumping counterparts may allow for tuning of the proton flux, deliberately slowing proton motive force generation upon back pressure to alleviate the toxic effects. Consistent with this hypothesis, it was shown that inhibition of the *bd* oxidase is synergistic with inhibition of the ATP synthase[190], pointing towards a hampered ability of bacteria to deal with the resulting backpressure.

Supporting this concept, Harrison *et al.* showed that upon inhibition of the ATP synthase, electrons are instantly rerouted to the *bd* oxidase, showing both flexibility and selective activation[185]. In chapter 2 of this thesis, we have shown that the *bd* oxidase from *M. tuberculosis* (*Mtbd*) has a characteristic disulfide bond, uniquely found in actinobacteria[82], that can be readily oxidized and reduced *in vitro* based on environmental redox pressures. Oxidation of the disulfide bond by H₂O₂ showed to activate *Mtbd*, which would allow for fast activation in response to the ROS generated by ATP synthase inhibition. Whether the protective role of *Mtbd* to this backpressure comes from peroxidase activity, directly removing ROS, or lowering the rate of proton motive force generation, remains to be studied. As such it would be interesting to be able to probe the response of the *Mtbd* disulfide bond *in vivo* upon exposure to various environmental stressors. This would require trapping the disulfide reduction state *in vivo* before analysis. Once achieved, it can be studied by mobility shifts on a non-reducing SDS page as performed in chapter 2. To adapt this system to whole cell lysates, a reporter-conjugated protein binder or antibody against *Mtbd* CydA is required to determine its position on gel. However, as the shift is relatively minor, it will be complex to analyse without direct comparison to *Mtbd* with a known disulfide state. Alternative to non-reducing SDS page, the cysteine reduction state can be probed by rapid alkylation of free cysteine *in vivo*. Downstream proteomics with a different alkylation reagent allows for specific probing which cysteines were free for alkylation *in vivo*, indicative of a reduced disulfide bond[218]. This approach requires proteomic coverage of the Q-loop disulfide bond and depends on *in vivo* alkylation outpacing a cellular response which can adapt the disulfide bond state.

The bd oxidase as an antibiotic target

The central contribution of *bd* oxidases to bacterial defence has positioned them as highly attractive targets for antibiotic development. Their exclusive conservation in prokaryotes provides an additional advantage, as inhibitors can be designed without risk of cross-reactivity with human respiratory enzymes. Although *bd* oxidases are essential in some pathogens such as *S. aureus* [156],

others require simultaneous inhibition of multiple respiratory components to achieve bactericidal effects, underscoring the diverse respiratory plasticity across species[183]. Consequently, no single inhibition strategy will be universally effective, and pathogen-specific studies are required to reveal vulnerabilities. Developing inhibitors against a range of respiratory chain enzymes will generate a versatile therapeutic toolkit that can be deployed in tailored combinations. Moreover, targeting the quinol pool, either directly or by inhibiting quinone biosynthesis, offers a complementary strategy to disrupt respiration globally through depletion of the central substrate, thereby bypassing species-specific respiratory weaknesses[219].

Historically, *bd* oxidase inhibition has focused on competitive inhibitors that occupy the quinol oxidation site. This approach has led to compounds such as Aurachin D[100], ND-011992[101], CK-2-63[135], and WSL017 developed in chapter 4. Interestingly, WSL017 shows potent inhibition for a small fragment, especially as it is not a quinolone derivative. This indicates that the Aurachin D-type moiety is not strictly necessary for inhibition and broadens the pool of available chemotypes that inhibit *bd* oxidases. In chapter 3, we show that inhibition of *E. coli* cytochrome *bd*-I by Aurachin D triggers refolding of the active site, turning CydA helix 6 from a loop to an α -helical structure by hydrogen bonding to Asp239^{CydA}. Contrarily, WSL017 does not trigger similar refolding and works via textbook competitive binding. The WSL017 fragment provides many opportunities for medicinal chemical optimization, such as introducing π - π stacking and hydrogen bonding interactions throughout the pocket, as described in chapter 4. We hypothesize that refolding of the active site can be a potent inhibition strategy, as the energetic barrier to bind substrates will increase due to the intermolecular contacts within CydA helix 6. It would be interesting to develop a small molecule which triggers this refolding without utilizing the Aurachin D headgroup moiety to alleviate potential cytotoxic effects. We anticipate that both the Aurachin D bound and WSL017 bound structures presented in this thesis will form a solid basis for structure guided drug design, with or without triggering active site refolding. The sequence conservation presented in chapter 3 highlights several conserved residues such Glu263^{CydA} (*Mtbd* numbering) that can be

therapeutically targeted, highlighting interactions with limited chance of mutation to create resistant enzyme variants.

While these inhibitors show effective and open the possibility for development of the scaffold, many other strategies can be utilized to generate clinical candidates. In chapter 2 we have shown that *Mtbd* can be inhibited by ubiquinone, probably because of a mismatch in reduction potential between ubiquinol and the *Mtbd*/heme chain[159]. Expanding on these results allows for the therapeutic administration of a quinone that affects *Mtbd* electron transfer but maintains functional within the human respiratory chain. The therapeutic potential of ubiquinone has been shown beneficial before for several indications[220,221], and has recently been shown to inhibit mycobacterial growth[222], although this strategy has yet to be assessed in an infectious model.

In addition to targeting the quinol oxidation site of the *bd*-oxidase, selective compounds against the proton or oxygen channel leading to heme *d* can be developed. As both channels are extensively described in *bd* oxidases from several species, there is a solid basis for structure guided drug design. It would be interesting to perform docking strategies across the *bd* oxidase structures to gain inhibitors of different chemotypes and mechanisms of action compared to the compounds targeting the quinol reduction site. Other options include the generation of molecular glues which stabilize the putative interactions between CydA and the CydDC heme transporter as shown in chapter 5. Stabilizing these interactions renders the complex stuck during maturation and may result in an inactive state. This approach would require a deep understanding of the interfacial interactions between CydA and CydDC, along with extensive chemical efforts to bind this transient state, which have not been unambiguously shown to date.

Of particular interest are recent studies which indicate that antibiotic susceptibility is linked to bioenergetic stress[223,224]. By inducing a stress-generated imbalance in ATP production and consumption, bacterial cells were shown to induce ROS mediated mutagenesis, accelerating the generation of resistant cells. Rather than inhibiting the respiratory chain, it was proposed to

stimulate respiration to decrease bioenergetic stress and increase antibiotic susceptibility[223,224]. Unfortunately, this approach has not seen any subsequent study to assess its therapeutic potential. Regardless, this counterintuitive strategy remains an interesting concept for evaluation. It has been shown that the interaction of cardiolipin with respiratory chain proteins stimulate activity[123,225]. While the underlying mechanism of the activation by cardiolipin remains unresolved, it does prove the concept of stimulation through interaction with ligands. This finding supports the feasibility of enhancing respiration via small-molecule ligands, which could yield both mechanistic insights and therapeutic benefit. Additionally, the screening method described in chapter 4 could be adapted to search for stimulatory compounds, allowing for subsequent study of its underlying mechanism and binding mode to different respiratory chain components.

More importantly, *bd* oxidase inhibitors need to advance from simple biochemical evaluation through the pre-clinical stage. This requires moving their development into *in vivo* model systems such as mice or zebrafish to assess treatment potency and potential cytotoxic side effects. This critical stage will give a more complete view of the therapeutic potential of the found inhibitors and inform us of the weaknesses that require further development. Only if successful at this stage can *bd* oxidase inhibitors advance to clinical assessment and result in new therapeutic modalities against pathogens such as *M. tuberculosis*.

Structural features and challenges of the *bd* oxidases

Solving the structure of proteins has proven to be instrumental in understanding their function. In chapter 3 we describe the first quinone bound structures of a *bd* oxidase to describe its catalytic cycle. Interestingly, we show that the characteristically flexible Q-loop undergoes a disorder-to-order transition during quinol turnover. This is particularly notable because it represents a divergence from the canonical paradigm in which catalytic competence arises from pre-organized, rigid active sites. Taking a broader view at quinol oxidases such as cytochrome *bo₃*, cytochrome *bcc:aa₃* and the

alternative oxidases, none of these have evolved such active site flexibility while mastering the same catalytic reaction of oxidizing quinols. This raises the question what evolutionary pressure pushed the *bd* oxidases to evolve and maintain such flexible active site. As discussed in chapter 3, we hypothesize that the flexibility of the quinol oxidation site allows the *bd* oxidase to maintain a low K_m while increasing k_{cat} , as the energetic barrier to form the active site allows for specificity to the transition state while maintaining low product affinity[167]. This might help the *bd* oxidase to compensate for its lack of proton pumping by increasing the turnover rate in comparison to its proton pumping counterparts.

A major question remaining is the location of the quinol oxidation site in the short Q-loop *bd* oxidases, among which is *Mtbd*. Regardless of the addition of high affinity inhibitors, none seem to form stable interactions or alter the structure of these *bd* oxidases in cryo-EM. As we have shown in chapter 3 for *Ecbd*, we only find ligand binding in the dimeric state, where part of the Q-loop is stabilized to capture quinols. This raises the question whether other *bd* oxidases require dimerization or complex formation with another binding partner to trigger structural changes and stabilize the quinone bound active site. However, *Ecbd* dimerization depends on CydX, a subunit present only in a subset of long Q-loop oxidases, implying that other members of the family must stabilize their active sites through alternative mechanisms. A further complication is that detergent solubilization may disrupt native oligomeric states or partner interactions, potentially yielding structures that do not fully reflect *in vivo* biology. As such, interactions with partner proteins or multimer states could be disrupted, resulting in misrepresentation in structural studies. While it remains challenging to ensure isolation of the protein in a native state, especially after overexpression, the solubilization step of the protein from the membrane can be optimized using milder detergent or SMALPS, although this does not guarantee a native protein state. It should be noted, however, that the isolated monomeric *Mtbd* remains active, showing that substrate binding and turnover must occur, even if this state is highly transient and therefore not captured by cryo-EM. This quinol bound state could be stabilized by sample freezing in anaerobic conditions, blocking the oxygen reduction reaction and

thereby trapping cytochrome *bd* during catalysis. This might prove challenging due to the high oxygen affinity of the *bd* oxidase, retaining turnover at near-anaerobic conditions[46,47]. Blocking the oxygen reduction reaction can be assisted chemically by the addition of high concentration carbon monoxide or cyanide, directly binding to the heme *d* active site.

Time-resolved cryo-EM offers an alternative route to visualize transient intermediates[226]. By initiating quinol reduction immediately before vitrification, one may capture short-lived conformations inaccessible to static imaging. Implementing this approach will require rapid photoreduction strategies with carefully optimized photosensitizers, electron donors, and reaction conditions capable of driving quinone reduction on microsecond timescales. A more complex variant involves photoreducing NAD⁺ to NADH and relying on an NADH dehydrogenase to transfer electrons to the quinone pool, though the kinetic control required would be complex.

While direct structure determination of *Mtbd* in a bound state remains challenging, indirect probing of the active site can be realized through mass spectrometry (MS) studies. Hydrogen deuterium exchange (HDX) MS has previously been used to probe the Q-loop as the active site in *Ec bd*[83]. Utilizing a similar approach in *Mtbd* might show a global hotspot on the enzyme where quinol oxidation or inhibitor binding takes place. This method unfortunately suffers from low resolution to the level of digested peptides and requires good coverage to confidently identify the global location of the active site. In a similar approach, a covalent quinol/quinone analogue can be used to probe binding spots using MS analysis[227,228]. Unfortunately, this still requires the presence of an amino acid in the active site that can bind the covalent probe. Additionally, many *bd* oxidases and other respiratory enzymes have been shown to bind to quinones in a structural rather than a catalytic manner, further complicating the assessment of the active site location.

The menaquinone binding site positioned at CydA Trp9 in *Mtbd* has been proposed as an active site for quinol oxidation. We have challenged the capability for quinol oxidation at this binding site due to a lack of semiquinone stabilizing amino acids, as well as the observation of stable menaquinone

binding in the presence of large molar excess of Aurachin D. Recent molecular dynamics studies on *M. smegmatis* cytochrome *bd*-II has shown that menaquinol can bind at Trp9^{CydA} and near the Q-loop with similar affinity and one electron potentials[229]. Additionally, phylogenetic analysis shows that both CydA Arg8 and Trp9 are conserved in the qOR1-short Q-loop *bd* oxidases, suggesting them as important residues. It should therefore not be disregarded that the menaquinone bound at Trp9 in *Mtbd* represents the active site, and the current resolution limitations might misrepresent the potential exchange of MK for Aurachin D.

An interesting but overlooked detail is that *M. smegmatis* cytochrome *bd* is isolated with a reduced Q-loop disulfide bond and no quinone bound at Trp9^{CydA}, while *Mtbd* expressed and isolated from the same bacterial system does contain both the disulfide bond and a bound menaquinone. Potentially, *Mtbd* responds differently to the periplasmic redox poise than *M. smegmatis* cytochrome *bd*, and is therefore isolated in a different state. This offers the possibility that formation of the disulfide bond increases the affinity for the menaquinone, maintaining a bound state during isolation. Regardless, we have shown in chapter 2 that *in vitro* reduction of the disulfide bond not only reduced oxidase activity but also maintained MK bound at Trp9^{CydA}.

Advancing our mechanistic understanding of *bd* oxidases will illuminate fundamental principles underlying enzyme flexibility, clarify bacterial respiratory physiology, and accelerate the development of new therapeutic strategies. In an era increasingly focused on fundamental protein characteristics and design, the *bd* oxidases can serve as a valuable model for function that emerges not from static architecture but from controlled structural disorder, a feature still underrepresented in current computational and experimental frameworks.

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Summary

Breathing in Thin Air: Structure, Function and Therapeutic Opportunities of *bd* type Oxidases

Respiration is a key biochemical process responsible for the majority of ATP generation in organisms under non-fermenting conditions. During aerobic respiration, the class of respiratory oxidases catalyze the terminal reaction, reducing molecular oxygen to water. This thesis investigates the structure, mechanism, maturation, regulation, and inhibition of cytochrome *bd* oxidases, a class of prokaryotic terminal oxidases that play a critical role in bacterial respiration and represent promising target for antibiotic development. In particular, the work focuses on understanding how these enzymes function at the molecular level and how this knowledge can be leveraged for drug discovery against pathogenic bacteria such as *Mycobacterium tuberculosis*. A general introduction and thesis outline is provided in **chapter 1**.

Chapter 2 examines cytochrome *bd* from *Mycobacterium tuberculosis* (*Mtbd*), a menaquinol oxidase essential for bacterial survival under infectious conditions. Using a minimal respiratory chain consisting of *Caldalkalibacillus thermarum* NADH dehydrogenase type 2 (NDH₂) and *Mtbd*, we show that *Mtbd* specifically oxidizes menaquinol. Both ubiquinol and demethylmenaquinol can bind but are not turned over, resulting in inhibition of *Mtbd*. We hypothesize on a thermodynamic or mechanistic barrier prohibiting the turnover of ubiquinol or demethylmenaquinol. *Mtbd* activity is further shown to be regulated by the redox state of its Q-loop disulfide bond. Reduction of this bond decreases catalytic activity by up to 90%, effectively deactivating *Mtbd*. Cryo-EM analysis of the disulfide reduced enzyme reveals local disorder within the Q-loop residues 279^{CydA} to 288^{CydA}. However, a stabilizing salt bridge between Arg290^{CydA} and Glu263^{CydA} preserves partial structural integrity, enabling rapid re-oxidation and reactivation upon exposure to oxidants. These findings identify *Mtbd* as the first terminal oxidase regulated by a redox-sensitive disulfide switch, suggesting a potential mechanistic explanation for its rapid activation during infection and respiratory chain inhibition.

Chapter 3 focuses on *Escherichia coli* cytochrome *bd-I* (*Ecbd*) and establishes a structural framework for quinol oxidation and inhibition across *bd*-type

oxidases from the qOR-1 long Q-loop family. High-resolution cryo-EM structures demonstrate that *Ecbd* exists not only as a monomer, long considered its canonical form, but also as a physiologically relevant dimer. Dimerization stabilizes the Q-loop and enables the first complete visualization of the quinol oxidation site. Three quinone-binding pockets are identified per protomer: the primary catalytic site adjacent to heme *b*₅₅₈, a structural ubiquinone site at the CydA-CydX interface, and a non-catalytic pocket within CydB. Mutagenesis confirms that the quinol binding residues Tyr243^{CydA} and Arg298^{CydA} are essential for catalysis. Whereas the Q-loop is disordered in monomers, dimerization allows the Q-loop lid to undergo a disorder-to-order transition upon quinol binding, completing the active site and resulting in a doubling of catalytic efficiency. The chapter further reveals the inhibition mechanism of Aurachin D, a potent quinone analogue specific to cyt bd. Rather than merely occupying the active site, Aurachin D induces a striking structural refolding of *Ecbd* helix 6 into an α -helix, collapsing the quinol-binding pocket. Asp239^{CydA} plays a central role in this inhibitor-triggered rearrangement, providing hydrogen bonding to the Aurachin D amine group. We highlight a dual role of Asp239^{CydA}, maintaining viability of the quinol oxidation site by hydrogen bonding with Trp2^{CydX}, while providing the main interaction for inhibition with Aurachin D. Together, these findings provide the first comprehensive structural and mechanistic description of quinol turnover and inhibition in a *bd*-type oxidase.

Chapter 4 turns to drug discovery and introduces a rapid *in vitro* screening platform for *Mtbd* inhibitors. By coupling *Mtbd* with NDH2, NADH oxidation serves as a rapid spectroscopic proxy for *Mtbd* turnover, enabling the screening of more than 10,000 covalent fragments. 52 primary hits were identified, 5 retained activity in liposomal assays, and the most promising candidate, compound 1148, proved to act through an unexpected non-covalent mechanism. A derivative lacking the covalent warhead, Wslo17, maintained low-micromolar potency and competitively inhibited both *Mtbd* and *Ecbd* without affecting NDH2. Although cryo-EM analysis of *Mtbd* did not resolve bound Wslo17, consistent with the persistent difficulty of trapping ligands bound to *Mtbd*, the inhibitor's binding pose was visualized in the *Ecbd* dimer. In

Ecbd, Wslo17 was shown to occupy the same shallow Q-loop cavity as menaquinone and forming key interactions with Tyr243^{CydA}. Wslo17 inhibited growth of *M. tuberculosis* H37ra at 25 μ M and showed modest improvement when combined with the cytochrome *bcc:aa₃* inhibitor Q203, supporting the therapeutic rationale for dual oxidase inhibition. As a non-quinone chemotype, Wslo17 expands the inhibitor landscape beyond toxic quinone analogues such as Aurachin D derivatives.

Chapter 5 addresses the maturation of cytochrome *bd* and proposes initial indications of a revised mechanism for heme incorporation. Traditionally, the ABC transporter CydDC has been thought to export heme into the periplasm, where an unidentified chaperone inserts heme into CydA. Evidence presented here, including weak but reproducible co-purification of CydA with CydDC, heme-responsive ATPase modulation, cryo-EM observations, and AlphaFold 3 structural predictions, supports an alternative model in which CydDC interacts directly with CydA to mediate direct heme transfer. Unfortunately, structural analysis did not yield unambiguous identification of the CydADC complex. However, structure prediction identifies a plausible lateral docking interface linking CydA to a lateral gate between CydC helix 2 and CydD helix 5. This lateral gate could open cyclically during ATP hydrolysis to deliver heme directly into the CydA core. In this model, CydDC loads the three hemes into CydA through sequential cycles, after which CydA dissociates and associates with CydB, CydX and CydH to form the mature oxidase. This direct-transfer mechanism aligns with paradigms in other ABC transporters and identifies cytochrome *bd* maturation as a potential new antimicrobial target. Further investigations are required to strengthen this putative new model of cytochrome *bd* maturation.

The final discussion in **chapter 6** integrates these findings to portray cytochrome *bd* as a flexible, stress-responsive respiratory enzyme whose distinctive biochemical properties enable *M. tuberculosis* to survive the hostile conditions of infection. The redox-regulated Q-loop of *Mtbd* together with the *Ecbd* dynamic Q-loop lid architecture, dimerization-dependent catalysis, newly characterized inhibitor binding modes, and revised heme-delivery pathway together reveal a respiratory system finely tuned for resilience. By combining mechanistic and structural insight with new experimental tools for

inhibitor discovery, this thesis establishes a strong foundation for the rational development of next-generation therapeutics targeting *cyt bd*, with significant implications for combating drug-resistant tuberculosis.

Samenvatting

Ademen in ijle lucht: structuur, functie en therapeutische mogelijkheden van *bd*-oxidases

Ademhaling is een essentieel biochemisch proces dat verantwoordelijk is voor het grootste deel van de ATP-productie onder niet-fermenterende condities. Tijdens aerobe ademhaling katalyseert de klasse van respiratoire oxidases de eindreactie, waarbij moleculaire zuurstof wordt gereduceerd tot water. Dit proefschrift onderzoekt de structuur, het mechanisme, heme incorporatie, de regulatie en de remming van cytochroom *bd*, een klasse van prokaryotische terminale oxidases die een cruciale rol spelen in de bacteriële ademhaling en veelbelovende doelwitten vormen voor de ontwikkeling van antibiotica. Het onderzoek richt zich met name op het begrijpen van de werking van deze enzymen op moleculair niveau en hoe deze kennis kan worden benut voor de ontwikkeling van geneesmiddelen tegen pathogene bacteriën zoals *Mycobacterium tuberculosis*. Een algemene inleiding en een overzicht van het proefschrift zijn te vinden in **hoofdstuk 1**.

Hoofdstuk 2 onderzoekt cytochroom *bd* van *Mycobacterium tuberculosis* (*Mtb*), een menaquinol-oxidase die essentieel is voor de overleving van bacteriën onder infectieuze omstandigheden. Met behulp van een minimale ademhalingsketen bestaande uit *Caldalkalibacillus thermarum* NADH-dehydrogenase type 2 (NDH2) en *Mtb*, tonen we aan dat *Mtb* specifiek menaquinol oxideert. Zowel ubiquinol als demethylmenaquinol kunnen binden, maar worden niet geoxideerd, wat resulteert in remming van *Mtb*. We veronderstellen een thermodynamische of mechanistische barrière die de oxidatie van ubiquinol of demethylmenaquinol verhindert. Het belangrijkste resultaat is dat de activiteit van *Mtb* wordt gereguleerd door de redoxstatus van de disulfidebinding in de Q-loop. Reductie van deze binding vermindert de katalytische activiteit met 90%, waardoor *Mtb* effectief wordt gedeactiveerd. Cryo-EM-analyse van het gereduceerde enzym onthult lokale flexibiliteit binnen de Q-loop residuen 279^{CydA} tot 288^{CydA}. Een stabiliserende zoutbrug tussen Arg290^{CydA} en Glu263^{CydA} behoudt gedeeltelijk de structurele integriteit, waardoor snelle re-oxidatie en reactivering mogelijk is bij blootstelling aan oxidanten. Deze bevindingen identificeren *Mtb* als de eerste terminale oxidase die wordt gereguleerd door een redoxgevoelige disulfidebinding, wat

een mechanistische verklaring kan bieden voor de snelle activering van *Mtbd* tijdens infectie en remming van de ademhalingsketen.

Hoofdstuk 3 richt zich op *Escherichia coli* cytochroom *bd-I* (*Ecbd*) en schetst een structureel kader voor quinoloxidatie en remming bij *bd*-type oxidases uit de qOR1-lange Q-loop familie. Hoge resolutie cryo-EM-structuren tonen aan dat *Ecbd* niet alleen bestaat als monomeer, wat lange tijd als de standaard vorm werd beschouwd, maar ook als een fysiologisch relevant dimeer. Dimerisatie stabiliseert de Q-loop en maakt de eerste volledige visualisatie van quinoloxidatie mogelijk. Per protomeer worden drie quinone bindingsplekken geïdentificeerd: de primaire katalytische plek naast hema b_{558} , een structurele ubiquinonplek op het CydA-Cydx-grensvlak en een niet-katalytische plek in CydB. Mutagenese bevestigt dat Tyr243^{CydA} en Arg298^{CydA} essentieel zijn voor de katalyse. Terwijl de Q-loop in monomeren ongeordend is, maakt dimerisatie het mogelijk dat de deksel van de Q-loop een overgang van een ongeordende naar geordende structuur ondergaat bij binding van quinol, waardoor de actieve site wordt voltooid en de katalytische efficiëntie verdubbelt. Het hoofdstuk onthult verder het remmingsmechanisme van Aurachin D, een krachtig quinon-analoog specifiek voor cyt *bd*. In plaats van directe binding aan de actieve site, induceert Aurachin D een opvallende structurele heropvouwing van helix 6 van *Ecbd* tot een α -helix, waardoor de quinol bindingsplek omvormd. Asp239^{CydA} speelt een centrale rol in deze door de remmer geïnitieerde herschikking door waterstofbruggen te vormen met de aminegroep van Aurachin D. We benadrukken de dubbele rol van Asp239^{CydA}: het in stand houden van de structurele integriteit van de quinoloxidatieplek door waterstofbinding met Trp2^{Cydx}, terwijl het tegelijkertijd de belangrijkste interactie voor remming met Aurachin D verzorgt. Samen bieden deze bevindingen de eerste uitgebreide structurele en mechanistische beschrijving van quinolomzetting en remming in een *bd*-type oxidase.

Hoofdstuk 4 behandelt de ontdekking van nieuwe geneesmiddelen en introduceert een snel *in vitro* screeningsplatform voor *Mtbd* remmers. Door *Mtbd* te koppelen aan NDH₂, dient NADH-oxidatie als een snelle spectroscopische indicator voor de katalytische activiteit van *Mtbd*, waardoor

de screening van meer dan 10.000 covalente fragmenten mogelijk wordt. Er werden 52 primaire hits geïdentificeerd, waarvan er 5 hun activiteit behielden in liposomale assays. De meest veelbelovende kandidaat, verbinding 1148, bleek te werken via een onverwacht niet-covalent mechanisme. Een derivaat zonder de covalente reactieve groep, Wslo17, behield een lage micromolaire potentie en remde zowel *Mtbd* als *Ecbd* competitief zonder NDH₂ te beïnvloeden. Hoewel cryo-EM-analyse van *Mtbd* het gebonden Wslo17 niet kon detecteren, wat consistent is met de aanhoudende moeilijkheid om liganden gebonden aan *Mtbd* te vangen, werd de bindingspositie van de remmer wel gevisualiseerd in het *Ecbd*-dimeer. In *Ecbd* werd aangetoond dat Wslo17 dezelfde ondiepe Q-loopplek bezet als menaquinone en belangrijke interacties aangaat met Tyr243^{CyDA}. Wslo17 remde de groei van *M. tuberculosis* H37ra bij 25 µM en vertoonde een bescheiden verbetering in combinatie met de cytochroom *bcc:aa₃*-remmer Q203, wat de therapeutische rationale voor dubbele oxidase-remming ondersteunt. Als een niet-quinon-chemotype breidt Wslo17 het spectrum aan remmers uit voorbij toxische quinon analogen zoals Aurachin D derivaten.

Hoofdstuk 5 behandelt de maturatie van cytochroom *bd* en geeft eerste aanwijzingen voor een herzien mechanisme waarbij CydA direct bindt aan de CydDC transporter. Traditioneel werd aangenomen dat de ABC-transporter CydDC heam naar het periplasma exporteert, waar een niet-geïdentificeerd chaperone-eiwit heam in CydA inbouwt. Het hier gepresenteerde bewijs, waaronder milde maar reproduceerbare co-zuivering van CydA met CydDC, modulatie van heam-responsieve ATPase, cryo-EM-waarnemingen en structurele voorspellingen met AlphaFold 3, ondersteunt een alternatief model waarin CydDC rechtstreeks met CydA interacteert om directe heamoverdracht mogelijk te maken. Helaas leverde de structurele analyse geen eenduidige identificatie van het CydADC complex op. De structuurvoorspelling identificeert echter een plausibele laterale interactievlak die CydA verbindt met een laterale poort tussen helix 2 van CydC en helix 5 van CydD. Deze laterale poort zou cyclisch kunnen openen tijdens ATP-hydrolyse om heam rechtstreeks in de kern van CydA te bouwen. In dit model laadt CydDC de drie heamgroepen in CydA via opeenvolgende cycli, waarna CydA dissocieert en

zich bindt aan CydB, CydX en CydH om de volwassen oxidase te vormen. Dit directe overdrachtsmechanisme sluit aan bij paradigma's in andere ABC-transporters en identificeert de heam incorporatie van cytochroom *bd* als een potentieel nieuw antibacterieel doelwit. Verder onderzoek is nodig om dit vermeende nieuwe model van cytochroom *bd*-maturatie te onderbouwen.

De afsluitende discussie in **hoofdstuk 6** integreert deze bevindingen om cytochroom *bd* af te schilderen als een flexibel, stressgevoelig ademhalingsenzym waarvan de kenmerkende biochemische eigenschappen *M. tuberculosis* in staat stellen de vijandige omstandigheden van een infectie te overleven. De redox-gereguleerde Q-loop van *Mtbd*, samen met de dynamische Q-loop-architectuur van *Ec bd*, dimerisatie-afhankelijke katalyse, nieuw gekarakteriseerde bindingswijzen van remmers en een herziene heam-afgifte route, onthullen een ademhalingssysteem dat perfect is afgestemd op veerkracht. Door mechanistisch en structureel inzicht te combineren met nieuwe experimentele methoden voor de ontdekking van remmers, legt dit proefschrift een sterke basis voor de rationele ontwikkeling van de volgende generatie therapieën gericht op cytochroom *bd*, met belangrijke implicaties voor de bestrijding van geneesmiddelresistente tuberculose.

Curriculum vitae

Tijn van der Velden was born in Zoetermeer, The Netherlands, on January the 31st 1997. In 2014 he finished his higher secondary education at the Erasmus College in Zoetermeer, raising his interest in chemistry and biology. That same year he started the bachelor's program Chemistry at the Leiden University of Applied Sciences, where he gained interest in how the simple molecules found in pharmaceuticals are able to affect the complex human physiology. To better understand the development of pharmaceuticals and their mechanism of action he moved to Leiden university to start the bachelor program Biopharmaceutical sciences in 2015. In 2018 he completed the program, where he wrote his bachelor thesis at the Leiden Academic Centre of Drug Research (LACDR) in the group of Prof. Dr. Joke Bouwstra on the effect of the ceramide lipid chain length on phase separation in the skin lipid matrix. This same year he started as an analyst next generation sequencing at the company Baseclear, gaining industry experience and broadening his research skills. In 2019, Tijn moved back to Leiden university to start the master's program Biopharmaceutical sciences, deepening his knowledge in pharmaceutical research and science commercialization. He completed the program (cum laude) with a double specialization in Biotherapeutics, and Business studies. Tijn performed internships in the group of Prof. Dr. Joke Bouwstra (LACDR), Dr. Thomas Sharp (LUMC), and the organ-on-a-chip company MIMETAS. During these research internships he studied adjuvant incorporation into hybrid nanoparticle vaccines, and pentraxin-mediated complement activation on lipid cell mimetics. At MIMETAS, he learned how to convey scientific insights from organ-on-a-chip technology to a broader audience by the development of application notes, webinars, e-books and an online conference. After his Masters, Tijn moved to the group of Prof. Dr. Lars Jeuken for his PhD, the results of which are presented in this thesis. During his PhD, Tijn presented his work at several national and international conferences including Metals in biology 2022, France (Travel grant and poster prize), Surface modification for chemical and biochemical sensing, Poland (Poster prize), European Bioenergetics conference, Austria (Travel grant), circuits of life, UK, and the Kroese Duijster Symposium (Poster award). In addition to his research, he completed courses in project management, scientific writing, presentation skills and pharmaceutical CMC.

List of publications

T.T. van der Velden, A. Halimi, J. pols, W.S. Lam, S. M Hacker, L.J.C. Jeuken, (2026) *Discovery of a novel chemotype targeting Mycobacterium tuberculosis cytochrome bd through rapid screening and structural elucidation.* (Submitted)

T.T. van der Velden, K. Kayastha, F. pelser, S. Brünle, L.J.C. Jeuken, (2026) *Visualizing the mechanism of quinol oxidation and inhibition of a bd-type oxidase using cryo-EM.* Science Advances, DOI:10.1126/sciadv.aec9946

T.T. van der Velden, K. Kayastha, C.Y.J. Waterham, S. Brünle, L.J.C. Jeuken, (2025) *Menaquinone-specific turnover by Mycobacterium tuberculosis cytochrome bd is redox regulated by the Q-loop disulfide bond.* Journal of Biological Chemistry. DOI: 10.1016/j.jbc.2024.108094

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D.P. Noone, M.M.E. Isendoorn, S.M.W.R. Hamers, M.E. Keizer, J. Wulffelé, **T.T. van der Velden**, D.J. Dijkstra, L.A. Trouw, D.V. Filippov, & T.H. Sharp, (2024) *Structural basis for surface activation of the classical complement cascade by the short pentraxin C-reactive protein.* PNAS DOI: 10.1073/pnas.2404542121.

D.P. Noone, **T.T. van der Velden**, T. Sharp, (2021) *Cryo-Electron Microscopy and Biochemical Analysis Offer Insights Into the Effects of Acidic pH, Such as Occur During Acidosis, on the Complement Binding Properties of C-Reactive Protein.* Frontiers in Immunology. DOI: 10.3389/fimmu.2021.757633

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