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

Velden, T.T. van der

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 a 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, Wsl017, maintained low-micromolar potency and competitively inhibited both *Mtbd* and *Ecbd* without affecting NDH2. Although cryo-EM analysis of *Mtbd* did not resolve bound Wsl017, consistent with the persistent difficulty of trapping ligands bound to *Mtbd*, the inhibitor's binding pose was visualized in the *Ecbd* dimer. In

Ecbd, Wsl017 was shown to occupy the same shallow Q-loop cavity as menaquinone and forming key interactions with $Tyr243^{CydA}$. Wsl017 inhibited growth of *M. tuberculosis* H37ra at 25 μM and showed modest improvement when combined with the cytochrome *bcc:aa*$_3$ inhibitor Q203, supporting the therapeutic rationale for dual oxidase inhibition. As a non-quinone chemotype, Wsl017 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.