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

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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 H_2O 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 H_2O_2 showed to activate *Mtbd*, which would allow for fast activation in response 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 bo_3, cytochrome $bcc{:}aa_3$ 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 *Ecbd*[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 om *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.