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Review

Cytochrome *bd* oxidase and bacterial tolerance to oxidative and nitrosative stress☆

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ABSTRACT

Cytochrome *bd* is a prokaryotic respiratory quinol:O_2 oxidoreductase, phylogenetically unrelated to the extensively studied heme–copper oxidases (HCOs). The enzyme contributes to energy conservation by generating a proton motive force, though working with a lower energetic efficiency as compared to HCOs. Relevant to patho-physiology, members of the *bd*-family were shown to promote virulence in some pathogenic bacteria, which makes these enzymes of interest also as potential drug targets. Beyond its role in cell bioenergetics, cytochrome *bd* accomplishes several additional physiological functions, being apparently implicated in the response of the bacterial cell to a number of stress conditions. Compelling experimental evidence suggests that the enzyme enhances bacterial tolerance to oxidative and nitrosative stress conditions, owing to its unusually high nitric oxide (NO) dissociation rate and a notable catalase activity; the latter has been recently documented in one of the two *bd*-type oxidases of *Escherichia coli*. Current knowledge on cytochrome *bd* and its reactivity with O_2, NO and H_2O_2 is summarized in this review in the light of the hypothesis that the preferential (over HCOs) expression of cytochrome *bd* in pathogenic bacteria may represent a strategy to evade the host immune attack based on production of NO and reactive oxygen species (ROS). This article is part of a Special Issue entitled: 18th European Bioenergetic Conference.

1. Introduction

Cytochrome *bd* oxidase [1–3] is a respiratory oxidase confined to the prokaryotic world (Eubacteria and Archaea), phylogenetically unrelated to the extensively studied protein family of heme–copper oxidases (HCOs) [4–7]. Similarly to HCOs, this integral membrane protein catalyzes the complete, four-electron reduction of O_2 to $2H_2O$, using quinols as physiological reducing substrates [2] (Fig. 1). The overall reaction is electrogenic [8], resulting in the generation of a proton motive force across the membrane that is used for ATP synthesis by the ATP synthase [8–12]. The reaction, however, is not coupled to proton pumping [9] and it is therefore characterized by a lower energetic efficiency, as compared to HCOs. At variance from HCOs, cytochrome *bd* does not contain copper, but only three hemes as cofactors: two *b*-type hemes (hemes b_{558} and b_{595}) and a heme *d*, where O_2 and other gaseous ligands (carbon monoxide (CO) and NO) bind (Fig. 1). The enzyme is reportedly characterized by high affinity for O_2 and, accordingly, it is preferentially expressed at low O_2 tension.

Relevant to patho-physiology, cytochrome *bd* oxidase is expressed in a number of bacterial pathogens. These include *Salmonella* [13,14], *Mycobacterium tuberculosis* [15], *Shigella flexneri* [16], *Streptococcus* [17], *Listeria monocytogenes* [18], *Brucella* [19,20], members of the strict anaerobe *Bacteroides* class [21] and *Klebsiella pneumoniae* [22]. Surprisingly, in some of these pathogens, virulence was shown to depend on cytochrome *bd* expression, pointing to a role of the enzyme in facilitating bacterial survival during infection. In *M. tuberculosis*, for example, cytochrome *bd* was reported to be up-regulated in vivo in the transition from acute to chronic infection of mouse lungs, promoting bacterial virulence: consistently, attenuated virulence was observed for a mutant strain unable to express cytochrome *bd* [15]. Similarly, in *Brucella abortus* mutation of cytochrome *bd* was found to affect pathogen survival in a mouse machrophage-like cell line and bacterial virulence in mouse models [19]. Very similar observations have been reported also for *S. flexneri*, where both intracellular survival and virulence were interestingly even shown to positively correlate with cytochrome *bd* expression levels [16]. All together, this evidence strongly suggests that the *bd*-type oxidases are required for adaptation to host immunity,

Abbreviations: HCO, heme–copper oxidase; NO, nitric oxide; ROS, reactive oxygen species; CO, carbon monoxide; DTT, dithiothreitol; Q_1, 2,3-dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-1,4-benzoquinone; CcO, bovine cytochrome *c* oxidase; EPR, electron paramagnetic resonance; NO^+, nitrosonium ion

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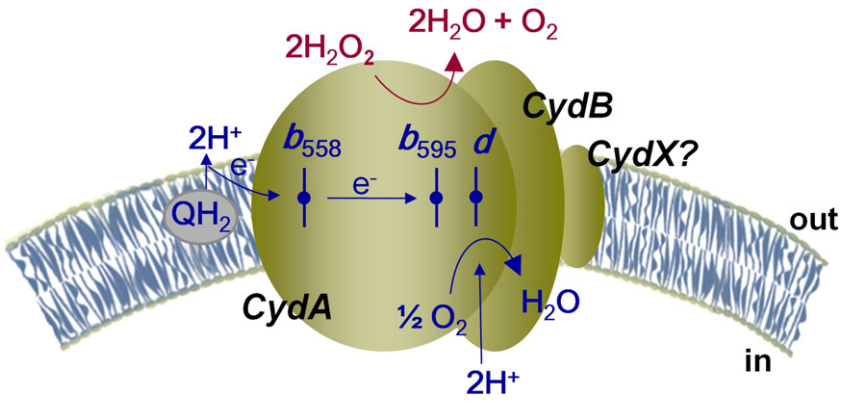

Fig. 1. Schematic representation of cytochrome *bd*. The enzyme catalyzes the reduction of O_2 to $2H_2O$, using quinol as the reducing substrate. The reaction is electrogenic, but it is not coupled to proton pumping. In addition, a catalase activity was reported for cytochrome *bd-I* from *E. coli* [61]. The small protein CydX is required for the function of the enzyme and has been proposed to be an additional subunit of the complex [28,29].

playing a role in pathogenesis. This makes these enzymes of interest also as drug targets.

Numerous observations suggest that, beyond its role in cell bioenergetics, cytochrome *bd* accomplishes several additional physiological functions in the bacterial cell. Serving as an O_2-scavenger, the enzyme favours the colonization of O_2-poor environments inside the host by both pathogenic and non-pathogenic bacteria [15,20,21,23]. It facilitates the growth of anoxygenic bacterial phototrophs in a wider range of ambient O_2 concentrations [24] and it prevents the inactivation of O_2-sensitive enzymes (such as nitrogenase in diazotrophic bacteria) (see refs. [3,25] and references therein). In *E. coli*, cytochrome *bd*-I[1] was shown to act as a source of oxidizing power, thus enabling disulfide bond formation by DsbA–DsbB during protein folding [26], and allowing the action of protoporphyrinogen IX oxidase (HemG) in heme biosynthesis [27]. Moreover, the enzyme seems to be also implicated in the response of the bacterial cell to a number of stress conditions, such as high temperature, high pH, membrane de-energization by uncouplers, low O_2 concentrations and metals or poisons in the medium (see refs. [3,25] and references therein).

In addition to these functions, growing evidence supports the idea that cytochrome *bd* plays a role in protecting the bacterial cell also against oxidative and nitrosative stress conditions, i.e., against the negative effects produced by O_2, NO and related reactive species. Given that these species are produced as part of the host immune response to control microbial infections, this evidence contributes to elucidate the mechanism by which cytochrome *bd* promotes bacterial virulence.

2. The reaction with O_2

Cytochrome *bd* is composed of two integral membrane polypeptides, subunits I (CydA, 57 kDa) and II (CydB, 43 kDa), and a small protein, named CydX (4 kDa), that has been recently found to be important for the enzymatic activity and proposed to be an additional subunit of the complex [28,29] (Fig. 1). The enzyme contains three heme cofactors: the low spin hexacoordinate heme b_{558} and the high spin pentacoordinate hemes b_{595} and *d* [30] (Fig. 1). Although three-dimensional structures of cytochrome *bd* are still lacking, studies of the protein topology in the membrane suggest that all the three hemes are located near the periplasmic space. This implies that the generation of the membrane potential mainly results from proton transfer from the cytoplasm towards the active site on the opposite site of the membrane (Fig. 1), rather than from inter-heme electron transfer. Heme b_{558} in subunit I is involved in quinol oxidation, whereas heme *d* is the site where O_2 binds and is reduced to H_2O. The function of heme b_{595} is still unclear. This redox site has been proposed to form together with heme *d* a functional binuclear active site [10,31–38], somewhat similarly to the heme/copper O_2-reducing site in HCOs. This hypothesis is supported by spectroscopic studies showing that the Fe to Fe distance between hemes b_{595} and *d* is 10 Å [39].

Cytochrome *bd* and HCOs apparently share the same key intermediates of the catalytic cycle, as revealed by flow-flash [40] investigation of the reaction of fully (three-electron) reduced cytochrome *bd* ($\mathbf{R^{3\,2}}$) with O_2. In these studies, the reaction was monitored by spectroscopic and electrometric techniques [8,10–12,41], following laser photolysis of the CO-bound $\mathbf{R^3}$ enzyme in the presence of O_2. Fig. 2 illustrates the detected intermediates. The unliganded $\mathbf{R^3}$ enzyme promptly generated upon CO-photolysis binds O_2 very rapidly, forming the species $\mathbf{A^3}$ with ferrous-oxy heme *d*. Formation of $\mathbf{A^3}$ occurs at high rates ($k_{on} \sim 2 \times 10^9\ M^{-1}\ s^{-1}$ [11,41,42]) without generating electric potential [8,11]. Afterwards, a rapid non-electrogenic oxidation of heme b_{595} was observed. The resulting short-lived species, originally denoted as **P**, was discovered at 21 °C by microsecond time-resolved absorption spectroscopy and electrometry [11,12]. It was suggested [11,12] that this is either a true peroxy complex or a ferryl species with an amino acid radical or a porphyrin π-cation radical. Later, by investigating the reaction at 1 °C by ultra-fast freeze-quench trapping combined with electron paramagnetic resonance (EPR) and UV–visible spectroscopy, Paulus et al. [43] reported that **P** is a ferryl porphyrin π-cation radical intermediate. Accordingly, **P** was renamed as **F***. Therefore, in this intermediate, splitting of the O═O bond seems to have already occurred as a result of a four-electron transfer process: three of these electrons are thought to be donated by heme b_{595} (1 electron, $Fe^{2+} \rightarrow Fe^{3+}$) and heme *d* (2 electrons, $Fe^{2+} \rightarrow Fe^{4+}$), whereas the fourth electron is supposedly provided by the heme *d* porphyrin ring, thus forming a radical. It is worthy to note, however, that some concern still remains on whether the **P** intermediate observed in [11,12] is actually the same as **F*** measured at lower temperature in [43]. Further work may be required to clarify the precise chemical structure of this transient species. In the reaction of bovine cytochrome *c* oxidase (CcO) with O_2, a ferryl intermediate resembling **F*** in the *bd* oxidase [43] is populated, that anyway for historical reasons was named '**P**' (as incorrectly assigned to a peroxo-species) (see [44] and references therein): in the CcO intermediate, however, the radical does not reside on the porphyrin ring, as suggested for

[1] *E. coli* possesses the heme-copper cytochrome bo_3 quinol oxidase and two *bd*-type oxidases, called *bd*-I and *bd*-II. Unless otherwise stated, we refer to cytochrome *bd*-I throughout the manuscript.

[2] The superscript number denotes the total number of electrons in the enzyme species.

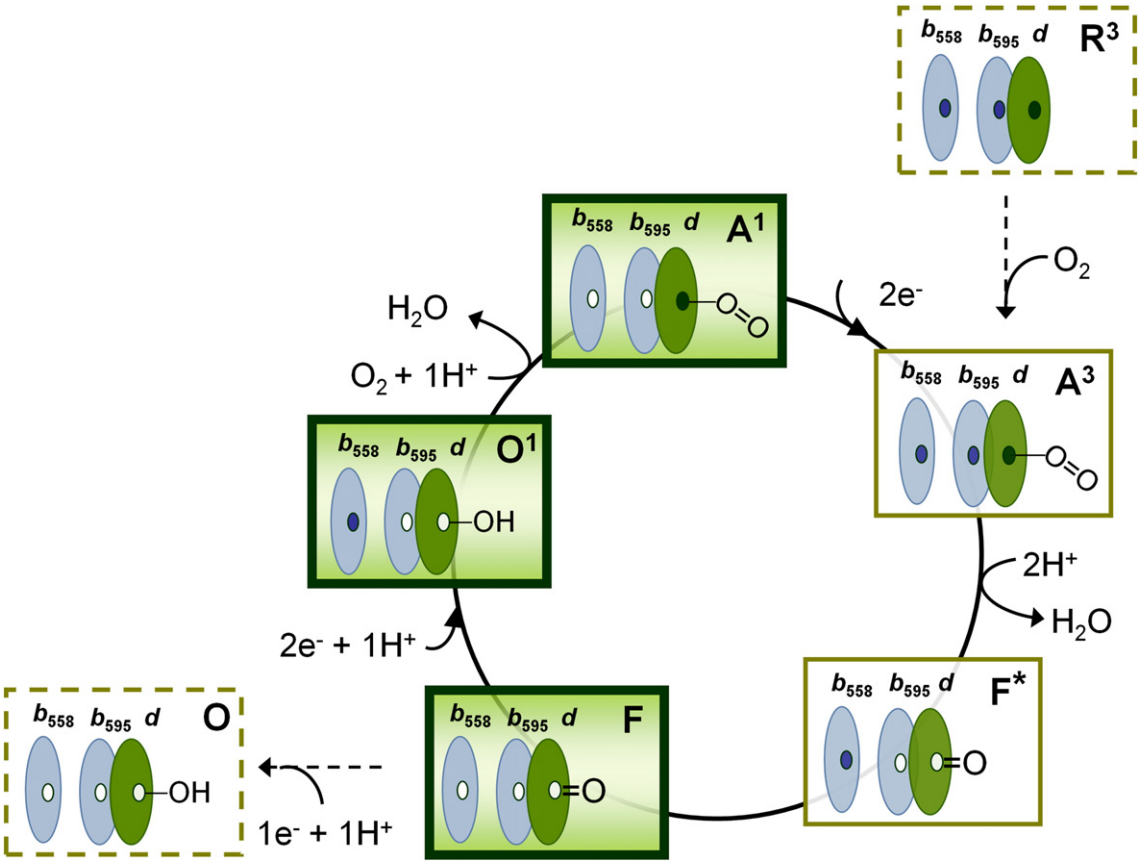

Fig. 2. Catalytic cycle. Catalytic intermediates populated in the reaction of fully reduced enzyme with O_2. In each intermediate, the superscript number denotes the total number of electrons in the enzyme and the heme redox state is indicated by open (Fe^{3+}) and closed (Fe^{2+}) circles. The **F** and **F*** intermediates contain heme *d* in the Fe^{4+} state. In the **F*** intermediate, the asterisk denotes a radical on the heme *d* porphyrin ring. The intermediates detected at steady state [50] are highlighted.

cytochrome *bd* [43], but most likely on the covalently-linked histidine–tyrosine that is close to the O_2 binding site. In cytochrome *bd*, upon electron transfer from heme b_{558}, **F*** converts into the **F** intermediate, which has the same ferryl structure but no radical. The transition to **F** is electrogenic and proceeds with $\tau = 47$ μs [8,10–12]. In the presence of bound quinol(s) copurifying with the enzyme, **F** is converted into **O** or, more likely, into the single-electron reduced, oxygenated enzyme ($\mathbf{A^1}$), since quinol can act as a two-electron donor. This last step of the reaction is electrogenic and proceeds with $\tau = 0.6$–1.1 ms [10,11].

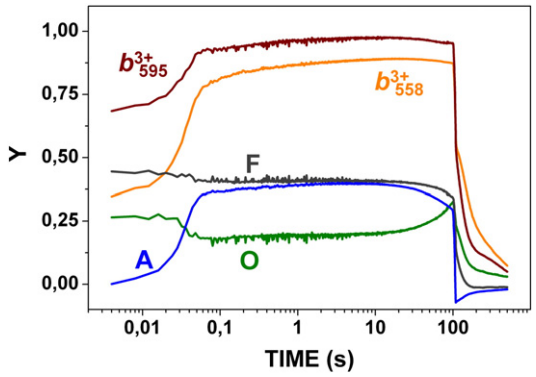

Fig. 3. Steady-state occupancy of the catalytic intermediates. Fractional occupancy of the optical species detected at steady-state after stopped-flow mixing reduced cyochrome *bd* with O_2-equilibrated buffer (see [50] for details). Concentrations after mixing: 10 μM enzyme, 5 mM DTT and 300 μM Q_1. T = 20 °C. Reprinted from [50] with permission from Elsevier.

Differently from HCOs, to which O_2 can only bind at the heme–copper binuclear site in the two-electron (fully) reduced state [45], cytochrome *bd* can bind O_2 with high affinity as a single electron reduced species ($\mathbf{R^1}$), yielding $\mathbf{A^1}$ with ferrous-oxy heme *d*. Due to the high stability of $\mathbf{A^1}$, a substantial fraction of the enzyme is isolated in such a state [42,46,47]. As measured by flow-flash technique, O_2 binds to $\mathbf{R^1}$ cytochrome *bd* with rates hyperbolically dependent on O_2 concentration [42]. In contrast, as expected for a bimolecular process assayed under pseudo-first order conditions, O_2 binds to the $\mathbf{R^3}$ enzyme with rates linearly dependent on O_2 concentration [42]. As a tentative explanation for the different behaviour, it has been proposed that the $\mathbf{R^1}$ enzyme exists in two different conformations that are in equilibrium, one O_2-accessible ('open') and the other O_2-inacessible ('closed') [42]. Consistently, also in the case of CO, the $\mathbf{R^3}$ cytochrome *bd* binds and releases the ligand more rapidly than the $\mathbf{R^1}$ enzyme [38,41,48,49]. The redox state of the heme(s) *b*, therefore, clearly modifies the ligand binding properties of heme *d*. However, both the structural determinants of this redox control and its possible mechanistic relevance remain to be assessed.

Taking advantage of the remarkable optical differences between the heme *d* and the *b*-type hemes in cytochrome *bd*, the steady-state occupancy of the catalytic O_2 intermediates in the enzyme was measured by stopped-flow multiwavelength absorption spectroscopy [50]. Global analysis of the spectral data acquired after rapidly mixing reduced cytochrome *bd* with O_2-equilibrated buffer in the presence of excess DTT (dithiothreitol) and Q_1 (2,3-dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-1,4-benzoquinone) showed that the catalytic intermediates mostly populated at steady-state are the ferryl (**F**) and oxy-ferrous (**A**) species, with a residual minor fraction of the enzyme possibly in

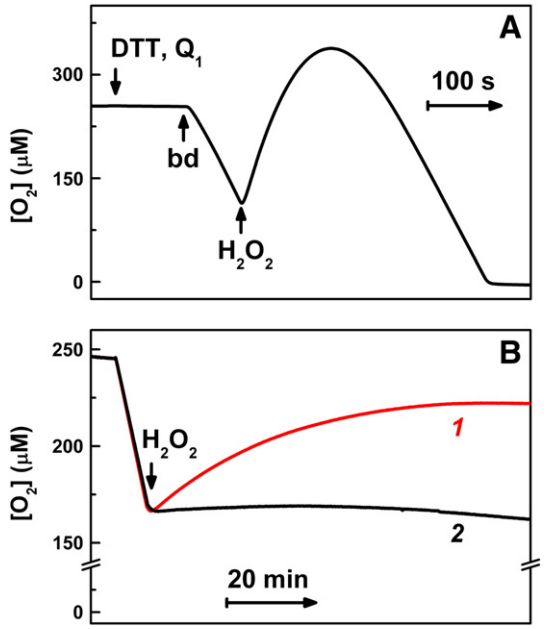

Fig. 4. Cytochrome *bd* catalase activity. A) O_2 evolution measured after addition of 1 mM H_2O_2 to the purified *E. coli* cytochrome *bd-I* in turnover with 10 mM DTT and 250 μM Q_1. B) Catalase activity observed after addition of 235 μM H_2O_2 to respiring *katE*/*katG*-deficient *E. coli* cells over-expressing cytochrome *bd* (trace 1). O_2 evolution is much less pronounced after addition of H_2O_2 to the same catalase deficient strain not over-expressing cytochrome *bd* (trace 2). Reprinted from [61] with permission from Elsevier.

the $\mathbf{O^1}$ state (i.e., with ferric heme *d* and one electron on hemes b_{558}) (Fig. 3). These results are consistent with the proposal that the fully ferric **O** species is not a catalytic intermediate [51]. Notably, different results were obtained with CcO, using cytochrome *c* as the reducing substrate: for this enzyme the steady-state occupancy of the oxygen catalytic intermediates was reported to be very low (<10%, [52]).

3. Cytochrome *bd* and oxidative stress

Several lines of evidence point to a role of cytochrome *bd* in bacterial protection against oxidative stress. In *E. coli*, for example, mutants defective in cytochrome *bd* display increased levels of intracellular H_2O_2 [53], as inferred from the enhanced expression of the gene coding for KatG catalase, also known as HPI. Consistently, these mutants show an increased sensitivity to external H_2O_2 [53,54], although conflicting results were also reported more recently [29]. Along the same line, defects in *E. coli* cytochrome *bd* are associated to characteristic phenotypes, including higher temperature-sensitivity and defects in stationary phase growth, that were interestingly found to be corrected upon treatment with reducing agents or exogenous antioxidant enzymes, such as catalase and superoxide dismutase [55]. Moreover, expression of *E. coli* cytochrome *bd* increases when bacterial cells are exposed to external H_2O_2 [53], again pointing to a defence role of the enzyme against oxidative stress. It is also interesting that such a role does not seem to be unique to cytochrome *bd* from *E. coli*. In the Gram-negative diazotrophic bacterium *A. vinelandii*, for example, mutation of cytochrome *bd* also results in a hypersensitivity to H_2O_2, metals (Cu^{2+} and Zn^{2+}) and other compounds exerting oxidative stress, such as paraquat and plumbagin [56]. In line with these observations, it was recently reported that disruption of cytochrome *c* maturation in *M. tuberculosis* results in the overexpression of cytochrome *bd* oxidase along with a hyper-resistance of the bacterium to H_2O_2 [57,58]. Particularly interesting is also the case of *Brucella abortus*, in which the lack of cytochrome *bd* expression is associated not only with an increased sensitivity to H_2O_2 [19,28], but also with a reduced bacterial virulence in a murine infection model, and both effects were shown to completely revert upon bacterial transformation with a vector harbouring genes encoding catalase or superoxide dismutase [19]. All together, these observations suggest that, in addition to their bioenergetic role, *bd*-type oxidases from several bacterial sources are also able to afford protection against oxidative stress, and this may represent an advantage particularly for pathogenic bacteria that have to cope with the hostile oxidative conditions created by the immune system in response to microbial infection.

Despite this large body of evidence, the molecular mechanisms by which cytochrome *bd* enhances bacterial resistance to oxidative stress are not completely understood. Two non-mutually exclusive possibilities can be envisaged: cytochrome *bd* may act by reducing the production of intracellular H_2O_2 and other ROS, and/or by metabolizing these species. The former possibility has been repeatedly suggested based on the notion that the enzyme, due to its high affinity for O_2, is expected to reduce the intracellular O_2 level, and thus ROS formation. Along the same line, more recently it was suggested that, in *E. coli*, cytochrome *bd* can act as an electron sink and decrease electrons availability for fumarate reductase, an important source of H_2O_2, thereby diminishing the rate of H_2O_2 formation by this enzyme when anaerobic cultures are exposed to O_2 [59]. As outlined above, in addition to these possibilities, cytochrome *bd* may act by directly removing H_2O_2 and other ROS. In support of this hypothesis, a low peroxidase activity was documented for *E. coli* cytochrome *bd-I* [60] and, more recently, Borisov et al. [61] reported the unprecedented observation that the same enzyme displays high catalase activity (Fig. 1).

Experimental evidence for the catalase activity of *E. coli* cytochrome *bd-I* was obtained by monitoring the increase in the O_2 concentration, following the addition of H_2O_2 to the enzyme, either purified or overexpressed in a catalase-deficient *E. coli* strain (Fig. 4) [61]. A remarkable activity was observed with the purified enzyme both in the 'as isolated' state and in turnover with O_2 sustained by an excess of reductants (DTT and Q_1). O_2 concentration was not found to have an effect on this catalase activity; however, intriguingly, no activity was measured following full reduction of the enzyme by DTT and Q_1 under anaerobic conditions [61]. As expected, the activity proportionally increased with the enzyme concentration and disappeared after thermal denaturation of the enzyme. Surprisingly, the catalase activity was found not to compete with the O_2-reducing activity of the enzyme [61], suggesting that the H_2O_2 chemistry takes place at a site other than heme *d*, where O_2 binds and reacts. Consistently, heme *d*-targeting inhibitors, such as CO or NO, proved to be unable to inhibit the catalase activity of *E. coli* cytochrome *bd-I*, though preventing the reaction with O_2. The observed insensitivity to NO strongly argues against the idea that the catalase activity of cytochrome *bd* arises from a contaminant bona fide catalase, as the latter enzyme is strongly inhibited by NO. In agreement with this conclusion, the activity was observed also in an *E. coli* strain devoid of both *katE* and *katG* genes, following overexpression of cytochrome *bd-I* (Fig. 4) [61].

Yet far from being elucidated, the molecular mechanism underlying the cytochrome *bd* catalase activity remains elusive. Assays with inhibitors have ruled out that the catalytic decomposition of H_2O_2 involves thiol groups or the quinol-binding site of the enzyme [61]. On the other hand, the catalase activity proved to be ~1000-times more sensitive to cyanide than the O_2-reducing activity of the enzyme: cyanide inhibition of the catalase activity indeed occurs with K_i ~ 2.5 μM, whereas the O_2-reducing activity of the enzyme is known to be inhibited only at millimolar cyanide concentrations [62]. The high cyanide sensitivity points to the involvement of a heme in the catalase reaction of *E. coli* cytochrome *bd-I*, that Borisov et al. [61] tentatively proposed to be heme b_{595}. However, based on the finding that cyanide added at a concentration sufficient to completely inhibit the catalase activity induces minimal spectral changes in the isolated protein, it was suggested that only a minor fraction (4% or less) of the purified enzyme is catalytically competent in metabolizing H_2O_2 [61].

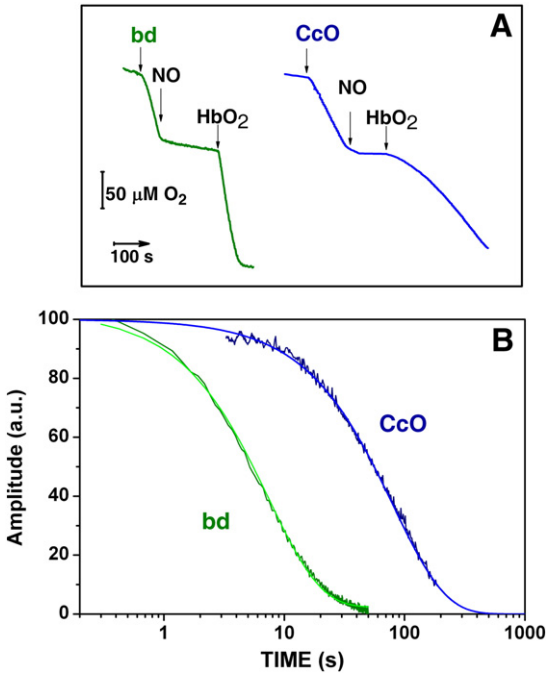

Fig. 5. Reversible inhibition of cytochrome *bd* and CcO by NO. A) Oxygraphic traces showing NO inhibition of *E. coli* cytochrome *bd-I* and CcO, and activity recovery after addition of oxy-haemoglobin (HbO_2) to scavenge free NO. The recovery is remarkably faster in cytochrome *bd* as compared to CcO. B) Kinetics of NO dissociation from fully reduced NO-bound *E. coli* cytochrome *bd-I* and CcO, measured after mixing the NO-bound proteins with air-equilibrated buffer in the presence of oxy-myoglobin in excess over the oxidases (see [116] and [48] for details). NO dissociation is much faster in cytochrome *bd* than in CcO.

These observations collectively suggest that *E. coli* cytochrome *bd-I* is mostly purified in a state devoid of catalase activity, along with a sub-population of the enzyme exhibiting a high catalase activity. Both enzyme populations seem to co-exist in *E. coli* cells, although it is yet unknown i) in which respect one enzyme population differs from the other one from a structural point of view, ii) whether conversion between the two populations is allowed and, iii) if this is the case, how this process is controlled in living cells. At this stage, it is unknown whether *bd*-type cytochromes from bacterial sources other than *E. coli* are also endowed with a catalase activity, which represents an important task for future studies. In this regard, it may be worth mentioning that a reduced catalase activity was measured in cell extracts of *A. vinelandii* by comparing a cytochrome *bd*-mutant with the wild-type strain [56]. This finding could be indicative of a catalase activity associated with the cytochrome *bd* from *A. vinelandii*, though this remains to be demonstrated.

The unprecedented observation that *E. coli* cytochrome *bd-I* displays high catalase activity may acquire patho-physiological relevance, particularly if such activity is common to *bd*-type oxidases from other pathogenic bacteria. Notably, it provides a reasonable mechanistic basis for the finding that these enzymes enhance bacterial resistance to oxidative stress, as a possible strategy to evade the host immune response and thus increase bacterial virulence.

4. Cytochrome *bd* and nitrosative stress

During evolution, microorganisms have developed a number of strategies to detoxify NO and survive nitrosative stress. NO and related reactive species, produced from either abiotic or biotic sources, are indeed commonly found in the natural habitats of prokaryotes, and are also generated as part of the immune response to control microbial infections. Infectious bacteria have, therefore, to cope with nitrosative stress to enhance their pathogenicity. NO-reductases and NO-dioxygenases (O_2-denitrosylases), such as the NO-reductase flavorubredoxin [63,64] and the flavohemoglobin [65,66] in *E. coli*, are mainly responsible for NO detoxification in microbes. The former enzymes anaerobically reduce NO to dinitrogen oxide (N_2O), while NO-dioxygenases degrade NO to nitrate (NO_3^-), using O_2 as co-substrate. Flavohemoglobin is typically characterized by a relatively low apparent affinity for O_2 (K_M ~ 20–100 μM O_2, [67–69]): though promptly scavenging NO under aerobic conditions, the enzyme is therefore much less efficient under microaerobic conditions.

NO is a pleiotropic signalling molecule. Over and above controlling numerous physiological and pathological processes, such as vasodilation, platelet aggregation, neuromodulation, cell death and host immunity, NO is also an effective inhibitor of HCOs (reviewed in [70–76]). As shown by extensive studies on CcO, the enzyme is potently and rapidly inhibited by sub-micromolar NO concentrations, with important patho-physiological consequences (reviewed in [72–79]). Inhibition occurs even in the presence of O_2 in excess (over NO), but it reverts if NO in solution is degraded. Two different reaction pathways have been demonstrated for CcO inhibition by NO (reviewed in [70–76]): the 'nitrosyl pathway' in which NO binds to ferrous heme a_3 in the active site, yielding a stable nitrosyl adduct, and the 'nitrite pathway', where a nitrite-adduct of ferric heme a_3 is generated by the reaction of NO with enzyme species containing fully oxidized heme a_3/Cu_B or ferryl heme a_3. While the nitrosyl pathway occurs simply through the binding/release of NO to/from ferrous heme a_3, the nitrite pathway was proposed to follow a multi-step mechanism, involving the transient formation of nitrosonium ion (NO^+) upon reaction of NO with oxidized Cu_B, and its subsequent hydroxylation to nitrous acid/nitrite [80–83]. The O_2-competitive nitrosyl pathway prevails at higher electron flux and lower O_2 concentration, whereas the nitrite pathway at lower electron flux and higher O_2 concentration [84]. Both inhibition pathways are reversible; however, in the absence of free NO in solution activity recovery occurs with notably different kinetics in the two cases. The nitrosyl enzyme recovers activity slowly, at the rate of NO dissociation form ferrous heme a_3 ($k = 0.0035\ s^{-1}$ at 20 °C, [84]) (Fig. 5) whereas, in the case of nitrite-bound CcO, activity is promptly recovered following the fast ejection of nitrite from heme a_3 upon reduction of this site [85].

Similarly to CO [86] and O_2 [42,47], in *bd*-type oxidases, NO binds to ferrous heme *d* with high affinity, leading to formation of a tight nitrosyl adduct with characteristic UV–visible absorption and EPR spectra [87,88]. Hemes b_{595} [87,88] and b_{558} [35] in the reduced state were also proposed to bind NO, but only at high, non-physiological ligand concentrations. The kinetics of NO binding to the ferrous uncomplexed heme *d* has not been investigated yet; the reaction is anyway expected to be very fast, possibly proceeding at a rate comparable to that measured for O_2 binding to the enzyme in the $\mathbf{R^3}$ state (k_{on} ~ $2 \times 10^9\ M^{-1}\ s^{-1}$ [11,41,42]). Ferrous heme *d* in the $\mathbf{A^1}$ oxy-intermediate (Fe^{2+}–O_2) also reacts with NO, yielding the nitrosyl heme *d* adduct, as a result of O_2 displacement and subsequent NO binding to the heme; in this case, however, the reaction was shown to be rate-limited by the off-rate of O_2 from the reduced heme ($k = 78\ s^{-1}$ at 20 °C, [48]) (Fig. 6). Working on the enzyme purified from *A. vinelandii*, fast ($k = 1.2 \pm 0.1 \times 10^5\ M^{-1}\ s^{-1}$ at 20 °C) reaction of NO with the **F** intermediate was also documented [89], yielding nitrite bound at ferric heme *d* (Fig. 6). It is yet unclear whether the reaction occurs through the direct reaction of NO with ferryl heme *d* iron, as reported for myoglobin and haemoglobin [90,91], or involving heme b_{595} too. The reaction of NO with the **F** intermediate was previously shown to occur at a lower rate (k ~ 1–2 × $10^4\ M^{-1}\ s^{-1}$) in CcO, where Cu_B in the active site was suggested to be the primary site of the reaction [82,83]. Based on the results obtained on the Cu-lacking cytochrome *bd*, one may conclude that Cu_B is dispensable or possibly not involved at all in the reaction. On the contrary, Cu_B seems to play an important role in the reaction of NO with ferric heme in the fully oxidized (**O**) active site of terminal oxidases, when comparing the results

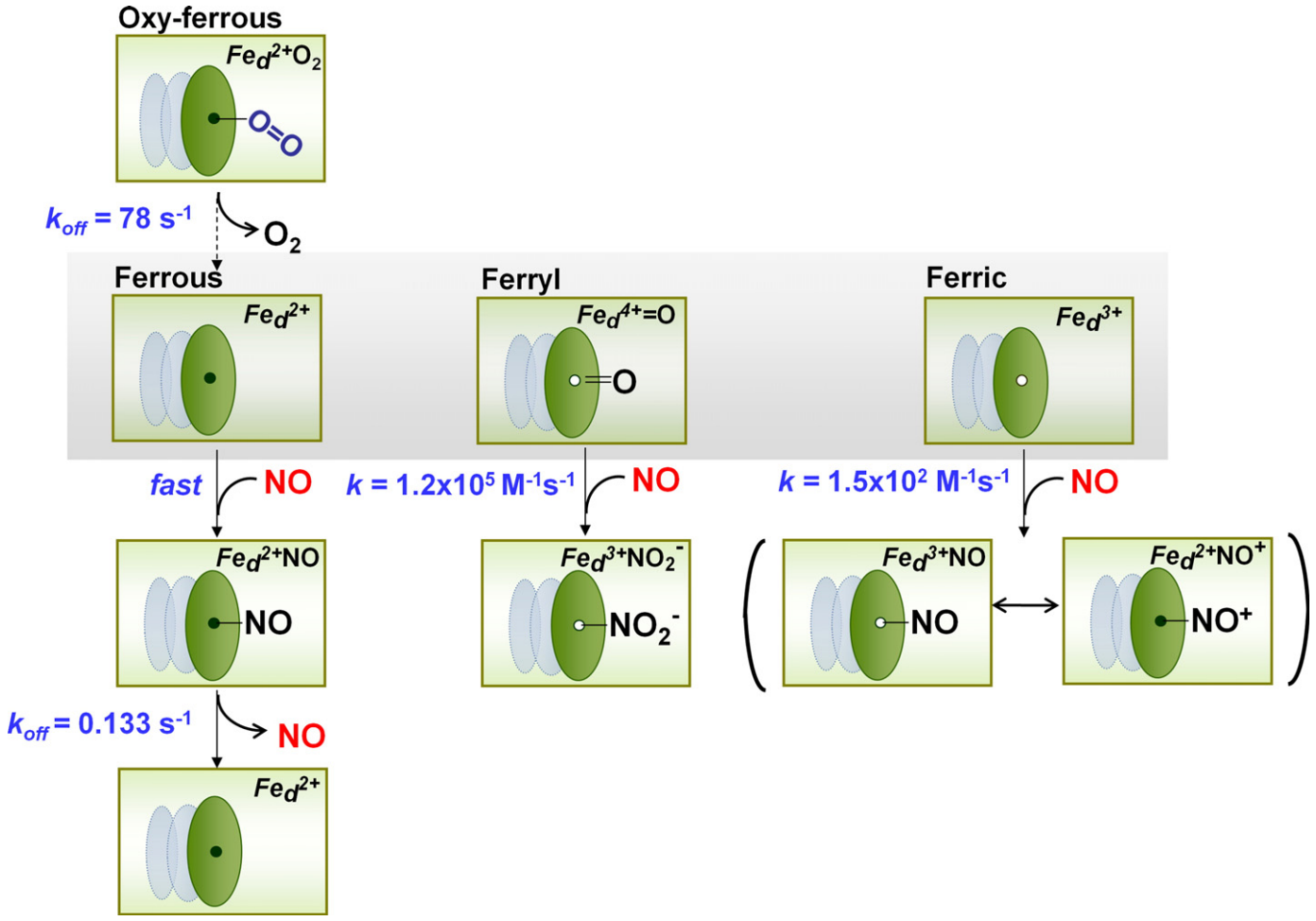

Fig. 6. Reactions of heme *d* with NO. Ferrous and ferryl heme *d* reacts with NO quickly, yielding a nitrosyl ferrous (Fe^{2+}–NO) or a nitrite–ferric (Fe^{3+}–NO_2^-) derivative, respectively. Reaction of NO with oxy-ferrous heme *d* is rate-limited by the dissociation of O_2. Ferric heme *d* reacts with NO, yielding a nitrosyl ferric (Fe^{3+}–NO ↔ Fe^{2+}–NO^+) adduct.

obtained with the Cu-lacking cytochrome *bd* [92] with those achieved with CcO [80,81]. In CcO, the reaction of NO with the fully oxidized enzyme is fast ($k = 2.2 \times 10^5$ M^{-1} s^{-1} at 20 °C, [81]), unless chloride is bound at the oxidized heme a_3–Cu_B site [81], and was proposed to proceed through the oxidation of NO to nitrosonium ion (NO^+) at Cu_B, followed by hydroxylation of NO^+ to nitrous acid/nitrite, eventually binding to ferric heme a_3 [80]. In contrast, regardless of chloride being present, NO reacts with cytochrome *bd* in the **O** state at a much lower rate ($k = 1.5 \pm 0.2 \times 10^2$ M^{-1} s^{-1} at 20 °C), yielding a nitrosyl adduct (heme d^{2+}-NO^+ or heme d^{3+}-NO), rather than the nitrite-bound enzyme [92] (Fig. 6).

Summing up, several catalytic intermediates of cytochrome *bd* are reactive towards NO: some of them just bind NO, while others seem to be able to oxidatively degrade it into nitrite. This raises the question as to whether *bd*-type oxidases are also able to *reductively* metabolize nitrite (to NO) or NO (to N_2O). Nitrite was reported to react with reduced cytochrome *bd* in membrane particles of *E. coli* only very slowly [93]. As for NO reduction, it is of interest that some bacterial HCOs [94–96], but not mitochondrial CcO [97], can function as NO-reductases at low rates, which may be consistent with the notion that HCOs are believed to share a common phylogenesis with heme b_3-containing bacterial NO-reductases [98,99]. Differently from these enzymes, however, neither cytochrome *bd-I* purified from *E. coli* nor the homologous enzyme isolated from *Azotobacter vinelandii* proved to be endowed with a measurable NO reductase activity in amperometric experiments carried out under anaerobic conditions making use of a selective NO electrode [100].

Further experimental work aimed at testing whether NO is an inhibitor of *bd*-type oxidases, as shown for CcO. The first evidence that this is actually the case was provided working on *E. coli* mutant cells lacking the heme-copper cytochrome bo_3 quinol oxidase, and measuring the sensitivity of the residual cytochrome *bd*-mediated respiration to NO [101]. This finding was later confirmed and expanded by measuring the effect of NO on the O_2 consumption catalyzed by cytochrome *bd* isolated from *E. coli* or *A. vinelandii* in the presence of DTT and Q_1 [100]. In this study, relatively low amounts (≤1 μM) of NO were reported to completely inhibit each one of the two *bd*-type oxidases [100] (Fig. 5A), further demonstrating that the presence of Cu_B is not essential for terminal oxidases inhibition by NO. For *E. coli* cytochrome *bd-I*, the IC_{50} value measured for NO inhibition (100 ± 34 nM at 70 μM O_2 [100]) is comparable to that reported for CcO [102,103]. Possibly relevant to microbial physiology, the inhibition was found to be stronger at lower O_2 concentrations. In experiments carried out on cytochrome bo_3-lacking *E. coli* cells, an IC_{50} linearly dependent on [O_2] was measured (at least up to ~150 μM O_2), showing that NO inhibition of cytochrome *bd* is set in competition with O_2 [104]. One may therefore expect that, in host infecting bacteria, inhibition of cytochrome *bd* is likely to occur when these microorganisms colonize O_2-poor environments and are challenged by the relatively high concentrations (μM) of NO produced by the host immune system as a defence against microbial infection.

The molecular mechanism by which NO inhibits cytochrome *bd* in turnover with O_2 is still a matter of debate. The O_2-competitive nature of NO inhibition [100,104] suggests that NO targets one or more enzyme species with uncomplexed heme d^{2+}, that are clearly reactive with O_2

too. NO binding to ferrous heme *d* is likely to occur, owing to the high affinity of heme d^{2+} for NO. On the other hand, if NO was reacting only with heme d^{2+}, at low [NO]:[O_2] ratios the onset of inhibition should be relatively slow; both ligands are indeed expected to bind heme d^{2+} with comparable rate constants, and O_2 present in large molar excess would therefore outcompete NO binding to heme d^{2+}. This is however in conflict with the observation that, even in the presence of a large excess of O_2 (100–200 μM), the enzyme is inhibited quickly by relatively low NO concentrations (≤1 μM), as observed also for CcO. To account for this discrepancy, one may hypothesize that, similarly to CcO, when the enzyme is in turnover, one or more catalytic intermediates without ferrous uncomplexed heme d^{2+} are populated that, in spite of their low or even absent reactivity towards O_2, are promptly targeted by NO, resulting in enzyme inhibition. The hypothesis is supported by the recent observation that the O_2-unreactive oxy-ferrous (**A**) and ferryl (**F**) intermediates, both able to react with NO, are the main catalytic intermediates populated at steady-state [50] (Fig. 3).

Regardless of the exact mechanism by which cytochrome *bd* is inhibited by NO, it is interesting to notice that, following the removal of NO from solution, in cytochrome *bd* activity is notably restored much more rapidly than in CcO under similar experimental conditions [84,100] (Fig. 5A). In the case of CcO, under high electron flux conditions, reversal of inhibition proceeds at the low rate of NO dissociation from ferrous heme a_3 ($k = 0.0035\ s^{-1}$ at 20 °C, [84]). Since NO-inhibited cytochrome *bd* is likely to contain a similar nitrosyl Fe^{2+}-NO heme *d* adduct, the faster release of NO inhibition of the enzyme should result from a faster release of NO from ferrous heme *d*, compared to CcO. The off-rate of the ligand from heme *d* was thus measured for both the fully reduced ($\mathbf{R}^3$-NO) and single-electron reduced enzyme ($\mathbf{R}^1$-NO) by stopped-flow absorption spectroscopy [48]. Experiments were carried out mixing NO-bound cytochrome *bd* with air-equilibrated buffer in the presence of an excess of oxymyoglobin, acting as both a trap and an optical indicator of the NO released from heme d^{2+} after displacement by O_2. Two interesting results were obtained in this study. Firstly, the off-rate of NO from heme *d* in fully reduced cytochrome *bd* was found to be remarkably higher than in CcO ($k = 0.133\ s^{-1}$ vs $k = 0.0035\ s^{-1}$, [48,84]) (Fig. 5B), possibly accounting for the faster release of NO inhibition observed in the case of cytochrome *bd* [100]. CO dissociation from the bacterial enzyme also proved to be remarkably faster than that from the mitochondrial enzyme ($k = 6.0 \pm 0.2\ s^{-1}$ vs $k = 0.023\ s^{-1}$ [40,48]), supporting the view that Cu_B in HCOs controls the on/off ligand pathway to/from the nearby heme in the active site [105]. Secondly, a clear effect of the redox state of the *b*-type hemes on the measured rates was observed, with NO dissociating from $\mathbf{R}^3$ cytochrome *bd* more quickly than from the $\mathbf{R}^1$ enzyme ($k = 0.133 \pm 0.005$ and $0.036 \pm 0.003\ s^{-1}$) [48].

The rate of NO dissociation from *E. coli* cytochrome *bd* was measured also in cell suspensions more recently [104] and found to be similar ($0.163\ s^{-1}$ at 35 °C) to that one ($0.133\ s^{-1}$ at 20 °C) reported previously for the purified fully reduced enzyme [48]. To be noted that the NO off-rate measured for cytochrome *bd* is unusually high for a hemeprotein: it is comparable to the NO dissociation rate reported for soluble guanylate cyclase in the presence of GTP ($k = 0.18\ s^{-1}$, [106]), but much lower than the rate more recently measured for cd_1-type nitrite reductases (up to $200\ s^{-1}$, [107]). The fast release of NO from reduced cytochrome *bd* should result from structural peculiarities of heme *d* and/or its pocket that hopefully will be unveiled when the three-dimensional structure of the enzyme will be solved.

Based on the information reported above, our groups proposed [48,108] that, owing to their high NO off-rates, *bd*-type oxidases could be preferentially expressed in place of HCOs to enhance bacterial tolerance to nitrosative stress, and possibly promote bacterial pathogenicity (reviewed in [109]). Under NO stress conditions, the expression of a fast NO-dissociating cytochrome *bd* instead of HCOs can indeed allow a faster recovery of bacterial respiration after NO inhibition, in response to a decline in the NO flux, and a reduced sensitivity of respiration to NO, if the higher NO off-rate of the *bd* enzyme results into higher IC_{50} values for NO. This hypothesis was substantiated more recently by Mason et al. [104] who reported that, in *E. coli*, compared to the heme–copper bo_3-type oxidase, cytochrome *bd* is less sensitive to NO-inhibition, i.e., it is characterized by higher IC_{50} values for NO. Consistently, compared to cytochrome bo_3-deficient *E. coli* cells, in cytochrome *bd*-lacking cells NO induced a more severe growth inhibition [104]. In agreement with these results, recently cytochrome *bd* has been shown to confer the facultative Gram-negative anaerobe *Shewanella oneidensis* resistance to nitrite and NO during aerobic growth [110,111].

All together, this information suggests that the preferential expression of cytochrome *bd* (over HCOs) may represent a general strategy to enhance bacterial tolerance to nitrosative stress, a strategy that bacterial pathogens may utilize to evade the host immune attack based on NO production. In this light, it is of interest that, according to a transcriptomic analysis performed on chemostat-cultured *E. coli* [112], upon exposure to NO, expression of the cytochrome *bd* genes proved to be preferentially stimulated, whereas unaltered expression of the cytochrome bo_3 oxidase was observed. Notably, *E. coli* is not the only system in which stimulation of cytochrome *bd* expression was documented, as NO-induced expression of cytochrome *bd*-related genes was also reported for *M. tuberculosis* [15], *Staphylococcus aureus* [113], *Bacillus subtilis* [114] and *Desulfovibrio gigas* [115].

5. Conclusions and future perspectives

Growing evidence suggests that cytochrome *bd* enhances bacterial tolerance to oxidative and nitrosative stress conditions. This ability seems relevant particularly for pathogenic bacteria, as hostile conditions of this type are typically created as part of the immune response to fight microbial infection. This leads to the hypothesis that, by enabling survival under oxidative and nitrosative stress, cytochrome *bd* expression represents a strategy for pathogenic bacteria to evade the immune attack and thus enhance bacterial virulence, as already documented for specific pathogens. In this regard, cytochrome *bd* oxidases are to be considered as potential drug targets.

Studies on cytochrome *bd* unveiled functional peculiarities, apparently not shared by the better known HCOs, that suitably account for the protective role played by this enzyme against oxidative and nitrosative stress. Extensive studies on the reactivity of NO with the catalytic intermediates of cytochrome *bd* led to the discovery that the enzyme, though being inhibited by NO, exhibits an unusually fast NO dissociation rate from the active site, unlike HCOs and most hemeproteins. This high dissociation rate results in a fast activity recovery of the NO-inhibited enzyme upon NO removal from solution and, possibly, in a reduced sensitivity of the enzyme to NO inhibition. Moreover, a recent study revealed that a cytochrome *bd* (the *bd-I* oxidase from *E. coli*), both isolated and overexpressed in bacterial cells, exhibits a high catalase activity, enabling prompt degradation of H_2O_2. We think that this peculiar reactivity of cytochrome *bd* toward NO and H_2O_2 likely emerged during evolution as an adaptive strategy to enhance bacterial tolerance to oxidative and nitrosative stress conditions.

Despite these achievements, several questions remain to be addressed. First of all, studies on *bd*-type oxidases need to be extended to a wider range of bacteria, including a bigger set of pathogens, to establish whether the unusual properties recently unveiled are characteristic of these respiratory oxidases and correlate with bacterial pathogenicity. More work is then needed in the future to elucidate the yet obscure mechanism underlying the catalase activity shown by cytochrome *bd*, and its control in the bacterial cell. It is unclear if such activity results from a conformational switch, from post-translational modifications and/or assembly with additional polypeptides/proteins, such as, for instance, the small protein CydX [28,29]. Finally, solving

the three-dimensional structure of the enzyme represents an important goal also in the perspective of designing selective inhibitors of pharmacological interest.

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