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REVIEW

Cytochrome *bd* Protects Bacteria against Oxidative and Nitrosative Stress: A Potential Target for Next-Generation Antimicrobial Agents

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Abstract—Cytochrome *bd* is a terminal quinol oxidase of the bacterial respiratory chain. This tri-heme integral membrane protein generates a proton motive force at lower efficiency than heme-copper oxidases. This notwithstanding, under unfavorable growth conditions bacteria often use cytochrome *bd* in place of heme-copper enzymes as the main terminal oxidase. This is the case for several pathogenic and opportunistic bacteria during host colonization. This review summarizes recent data on the contribution of cytochrome *bd* to bacterial resistance to hydrogen peroxide, nitric oxide, and peroxynitrite, harmful species produced by the host as part of the immune response to microbial infections. Growing evidence supports the hypothesis that *bd*-type oxidases contribute to bacterial virulence by promoting microbial survival under oxidative and nitrosative stress conditions. For these reasons, cytochrome *bd* represents a protein target for the development of next-generation antimicrobials.

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Key words: antimicrobial agents, bacteria, oxidative stress, nitrosative stress, reactive oxygen and nitrogen species, respiratory chain, terminal oxidase, virulence

GENERAL PROPERTIES

Cytochrome *bd* is a quinol:O_2 oxidoreductase of the prokaryotic respiratory chain [1-3] that has not yet been identified in eukaryotic organisms [4]. The enzyme catalyzes the four-electron reduction of molecular oxygen to water using quinols as electron donors [5, 6]. The energy released in the redox reaction is stored in the form of a transmembrane electrical potential difference [7] through a molecular mechanism that has not been fully elucidated. It is assumed that the membrane potential is mainly created by the vectorial movement of protons through a proton transfer pathway that runs from the cytoplasm to the active site located on the opposite, periplasmic side of the membrane [8-12]. It has been found that, unlike heme-copper oxidases, cytochrome *bd* does not function as a proton pump [8-15]. Thus, it works with a lower energy efficiency as compared to heme-copper respiratory enzymes. For cytochrome *bd*, the H^+/e^- ratio (the number of protons transported across the membrane upon the transfer of one electron) is equal to 1, whereas for most heme-copper oxidases is equal to 2 [12, 16-19].

Cytochrome *bd* oxidases have been identified in both harmless and pathogenic bacteria, such as *Mycobacterium tuberculosis* [20], *Klebsiella pneumoniae* [21], *Shigella flexneri* [22], *Listeria monocytogenes* [23], *Streptococcus* [24], *Brucella* [25, 26], *Salmonella* [27, 28], and members of the Bacteroides class [29]. In these pathogens, a positive correlation between virulence and the expression level of cytochrome *bd* was noted [30].

Abbreviations: k, observed rate constant; K_i, apparent inhibition constant; $ONOO^-$, peroxynitrite; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine.

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Cytochrome *bd* expression is enhanced under unfavorable growth conditions, for example at low oxygen tension, in the presence of poisons (cyanide) [31] or uncouplers (protonophore) [32] in the environment, upon alkalization of the medium [31], or at high temperature [33]. In nitrogen-fixing bacteria, cytochrome *bd* contributes to protect nitrogenase against oxygen inactivation [34-36]. Cytochrome *bd* oxidase was also found to extend the oxygen concentration range at which anoxygenic phototrophic bacteria can grow [37]. Moreover, cytochrome *bd*-I from *Escherichia coli* participates in the regulation of disulfide bond formation during protein folding [38], as well as in heme biosynthesis (namely, at the level of the protoporphyrinogen IX oxidase enzyme) [39].

The three-dimensional structure of cytochrome *bd* is still unknown. The enzyme exhibits no homology with any heme-copper or alternative cyanide-resistant terminal oxidase [1, 7, 40, 41]. The enzymes from *E. coli* and *Azotobacter vinelandii* are mainly isolated as stable oxygenated complexes [42-44]. This is probably due to the high affinity of the enzyme for oxygen [45, 46]. Cytochrome *bd*-I from *E. coli* has been studied in detail. Until recently, the enzyme had been assumed to be composed of only two different integral membrane polypeptides, subunits I (CydA, 57 kDa) and II (CydB, 43 kDa). However, more recently it was shown that cytochrome *bd* contains an additional (4 kDa) polypeptide, CydX [47, 48]. This small polypeptide was suggested to be the third subunit of the oxidase, as it is required for maintenance of the enzyme activity and stabilization of the heme prosthetic groups [47-49].

Cytochrome *bd* has no copper, but it contains three redox-active hemes: the low-spin heme b_{558} and the high-spin hemes b_{595} and *d* [50, 51]. Heme b_{558} is located on subunit I, whereas hemes b_{595} and *d* are probably located at the interface between subunits I and II [52]. According to current views, the three hemes are all located closer to the outer (periplasmic) side of the membrane [53]. The hexacoordinate heme b_{558} is likely involved in quinol oxidation, whereas heme *d* binds molecular oxygen, being directly involved in its four-electron reduction to H_2O. The role of the pentacoordinate heme b_{595} is still unclear; some authors suggested that its function is to mediate the electron transfer between heme b_{558} and heme *d* [54, 55]. According to other researchers, heme b_{595} represents a second redox center capable of reacting with oxygen [56, 57]. Finally, the data obtained with the enzymes from *E. coli* and *A. vinelandii* suggest that heme b_{595} may participate in the reduction of oxygen, forming together with heme *d* a di-heme oxygen-reducing center, similarly to the heme/Cu binuclear center in heme-copper oxidases [9, 58-68]. On the other hand, cytochrome *bd* from *Geobacillus thermodenitrificans* revealed no significant interaction between hemes b_{595} and *d*, at variance from cytochrome *bd*-I from *E. coli* [69]. This may reflect substantial differences in the arrangement of the active center between the two enzymes.

It is widely accepted that, during the catalytic cycle (Fig. 1), cytochrome *bd* undergoes the following transitions:

$$\mathbf{A^1} \rightarrow \mathbf{A^3} \rightarrow \mathbf{'P'} \rightarrow \mathbf{F} \rightarrow \mathbf{O^1} \rightarrow \mathbf{A^1},$$

where $\mathbf{A^1}$ and $\mathbf{A^3}$ are the heme *d* ferrous oxygenated forms of the enzyme with one ($b_{558}^{3+}b_{595}^{3+}d^{2+}{-}O_2$) and three ($b_{558}^{2+}b_{595}^{2+}d^{2+}{-}O_2$) electrons, respectively; **'P'** is a short-lived state originally proposed to be a peroxo intermediate of heme *d*; **F** is the intermediate with ferryl heme *d* ($b_{558}^{3+}b_{595}^{3+}d^{4+}{=}O^{2-}$), and $\mathbf{O^1}$ is the one-electron-reduced form of the enzyme with ferric hemes *d* and b_{595} ($b_{558}^{2+}b_{595}^{3+}d^{3+}{-}OH$). Under steady-state conditions, the $\mathbf{A^1}$ and **F** forms of the enzyme predominate, being the main catalytic intermediates [70]. Consistently, these intermediates are detected in preparations of the isolated and membrane-bound enzyme. Under the same conditions, a small fraction of the $\mathbf{O^1}$ intermediate is also detected [70]. $\mathbf{A^3}$ and **'P'** are short-lived species that at room temperature can only be detected by "fast kinetic" methods [10, 11]. The existence of the **'P'** intermediate was first reported by Belevich et al. [10]. The **'P'** compound is possibly a ferryl intermediate, but with a π-cation radical on the porphyrin ring of heme *d* and one electron on heme b_{558} ($b_{558}^{2+}b_{595}^{3+}d^{*4+}{=}O^{2-}$) [71]. The fully oxidized (**O**, $b_{558}^{3+}b_{595}^{3+}d^{3+}{-}OH$) and fully reduced ($\mathbf{R^3}$, $b_{558}^{2+}b_{595}^{2+}d^{2+}$) forms (Fig. 1) most likely are not intermediates of the catalytic cycle [70-72], but can be obtained artificially. It is worth noting that the ferryl complex of cytochrome *d*, very similar spectroscopically to the bona fide catalytic intermediate **F** [10, 11], can be obtained by adding an excess of hydrogen peroxide to the enzyme, either "air-oxidized" or treated with a lipophilic oxidant [8, 73-75]. In the latter case, the reaction with hydrogen peroxide is quite fast, proceeding at an observed second-order rate constant *k* of 600 $M^{-1}{\cdot}s^{-1}$ [74].

INTERACTION WITH HYDROGEN PEROXIDE (H_2O_2)

A large body of evidence suggests that cytochrome *bd* contributes to bacterial resistance against the oxidative stress induced by hydrogen peroxide. *Escherichia coli* cytochrome *bd*-defective mutants are extremely sensitive to H_2O_2 [33]. Accordingly, the expression level of cytochrome *bd* in *E. coli* increases upon exposure to the peroxide [76]. Korshunov and Imlay [77], using an *E. coli* strain devoid of some antioxidant enzymes (the KatG and KatE catalases and the NADH-peroxidase Ahp), showed that, upon a sudden switch from anaerobic to aerobic growth conditions, a *bd*-type enzyme reduces the formation of intracellular H_2O_2. They suggested that

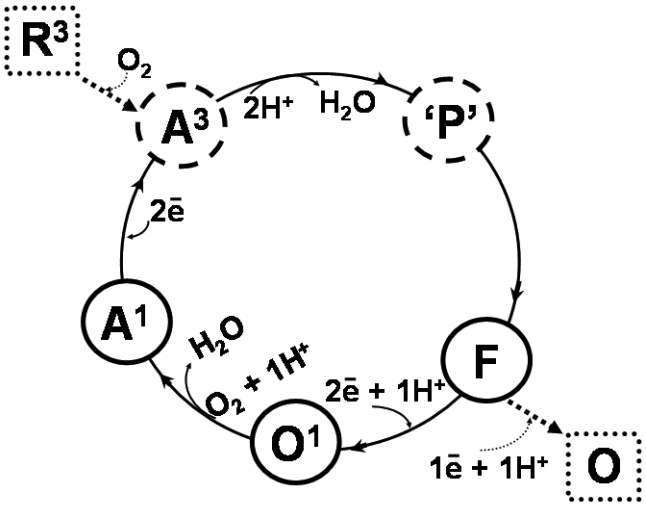

Fig. 1. Catalytic cycle of cytochrome *bd*. Solid arrows show the catalytic reaction pathway. Dotted arrows indicate transitions that are not part of the catalytic cycle.

cytochrome *bd* accomplishes this function indirectly, by diverging reducing equivalents from fumarate reductase, a key H_2O_2-generator [77].

Recently, we found that cytochrome *bd*-I from *E. coli* is able also to directly decompose H_2O_2 [78, 79]. In all likelihood, for this purpose the enzyme can use two different mechanisms, exhibiting both catalase [78] and peroxidase [79] activity. A high catalase activity of cytochrome *bd*-I was described by Borisov et al. [78]. This activity was assessed polarographically by measuring the rate of O_2 formation upon addition of H_2O_2 to the enzyme [78]. The activity was observed with both the isolated solubilized enzyme (Fig. 2a) and in cells of the catalase-deficient *E. coli* UM2 strain overexpressing cytochrome *bd*-I (solid line in Fig. 2b).

The reaction of H_2O_2 with the isolated enzyme was studied in more detail [78]. It turned out that the oxidase exhibits notable catalase activity not only in the "air-oxidized" state (Fig. 2a), but also in turnover with O_2 (in the presence of ubiquinol-1 and an excess of dithiothreitol, serving as the reducing system). In turnover, the catalase and oxygen reductase activities were found not to compete with each other, suggesting that different active sites are responsible for these activities in the enzyme. Upon O_2 depletion and consequent full reduction of the enzyme, the catalase activity disappears. The activity thus depends on the redox state of cytochrome *bd*-I.

As expected, the reaction rate was found to be proportional to the concentration of the oxidase, as well as to the concentration of H_2O_2 up to 0.5 mM. At higher H_2O_2 concentrations, the reaction rate tends to saturate, possibly due to a partial inactivation of the enzyme. It was shown that the reaction product (O_2) does not inhibit the catalase activity of the enzyme, as almost identical rates have been measured under aerobic (~255 μM O_2) and microaerobic (3-15 μM O_2) conditions. The reaction proceeds with the formation of about half a mole of O_2 per mole of H_2O_2, whereas no O_2 generation was observed in control experiments (i.e. with the thermoinactivated enzyme, in the absence of the enzyme, or without the substrate).

A number of experiments have been carried out with different inhibitors to gain insight into the nature of the site responsible for the observed catalase activity. The lack of effects by N-ethylmaleimide rules out that thiol groups of the protein are responsible for the catalase reaction. The involvement of a quinol binding site was also excluded as the reaction proved to be insensitive to antimycin A. Inhibitors targeting the reduced heme *d*, such as NO and CO, did not affect the catalase activity, thus arguing against a participation of this heme in the reaction. The lack of effects by NO, an effective inhibitor of *bona fide* catalases [78, 80], allowed us to exclude that the observed catalase activity was due to contaminant catalases in the cytochrome *bd* preparations.

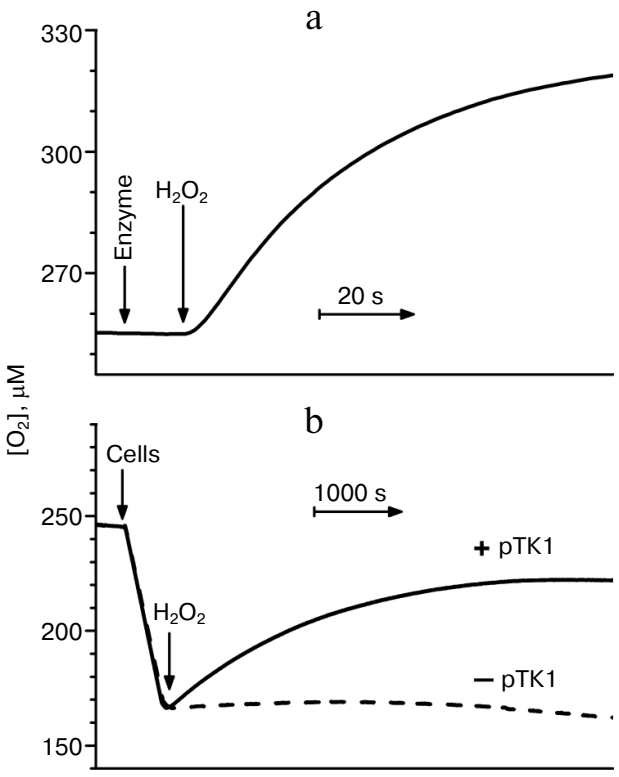

Fig. 2. Catalase activity of cytochrome *bd*-I from *E. coli*. a) Effect of addition of 100 μM H_2O_2 to the isolated solubilized enzyme. b) Effect of addition of 235 μM H_2O_2 to catalase-deficient *E. coli* cells of the UM2 strain (devoid of the KatG and KatE catalases). The cells, when overexpressing cytochrome *bd* (due to the presence of the pTK1 plasmid carrying the operon encoding cytochrome *bd*-I), show catalase activity (solid line, +*pTK1*). In the absence of cytochrome *bd*-I overexpression, the cells do not exhibit a notable catalase activity (dashed line, –*pTK1*). The experimental details are given in Borisov et al. [78].

The molecular mechanism at the basis of the catalase activity of cytochrome *bd* is unclear. The catalase activity of the enzyme is three orders of magnitude more sensitive to cyanide than the oxygen reductase one [78], pointing to the participation of a heme group in the catalase activity. As the oxygen reductase activity is directly linked to heme *d* and this activity does not compete with the catalase reaction, heme *d* should not be involved in the latter reaction. On this basis, it was tentatively suggested that the site responsible for the catalase activity was the high-spin heme b_{595} [78]. It was found that cyanide at a concentration completely inhibiting the catalase activity causes only small changes in the absorption spectrum of the "air-oxidized" enzyme, accounting for a binding of this ligand to maximally 4% of the hemes *b*. Therefore, in the preparations of the solubilized cytochrome *bd*-I, only a small fraction of the enzyme is endowed with the catalase activity (with a high apparent turnover number of at least 3250 s^{-1}) [78]. It is important to emphasize that the observed catalase activity is not an artifact of the isolation/purification procedure and is not due to the contamination by a bacterial *bona fide* catalase, as cytochrome *bd*-I, when overexpressed, shows a notable catalase activity in intact cells devoid of the KatG and KatE catalases (solid line in Fig. 2b). Thus, one can conclude that in *E. coli* cytochrome *bd*-I can have a protective role against oxidative stress, in addition to the bacterial catalases.

Cytochrome *bd*-I from *E. coli* was shown to be endowed also with a peroxidase activity in the presence of different substrates, such as guaiacol, ferrocene, benzohydroquinone, and potassium ferrocyanide [79]. The guaiacol peroxidase activity was investigated in more detail. In particular, inhibitors of the oxygen reductase activity of the enzyme, such as cyanide, pentachlorophenol, and 2-*n*-heptyl 4-hydroxyquinoline-N-oxide proved to inhibit the peroxidase activity of *E. coli* cytochrome *bd*-I to a similar extent [79]. It is assumed that guaiacol donates electrons to cytochrome *bd*-I *via* a quinol binding site, and the reduction of H_2O_2 occurs in the oxygen-reducing center. Although the peroxidase activity of the solubilized enzyme towards guaiacol appeared to be rather low (the apparent turnover number was ~4 s^{-1}), such an activity *in vivo*, i.e. with the physiological electron donors (such as quinols), may be much higher, thereby contributing to H_2O_2 detoxification in the bacterial cell.

The molecular mechanism through which the peroxide-utilizing activities of cytochrome *bd*-I are regulated in the *E. coli* cell is still unknown, as well as whether *bd*-type oxidases from other pathogenic bacteria are endowed with such activities. In this regard, it is interesting to note that disruption of the cytochrome *c* maturation system in *Mycobacterium tuberculosis*, the causative agent of tuberculosis, was reported to lead to a significant increase in both the expression of cytochrome *bd* and bacterial resistance to H_2O_2 [81]. This observation suggests that in *M. tuberculosis* the *bd*-type oxidase plays a role in protecting the pathogen against oxidative stress, by metabolizing H_2O_2 through a catalase and/or a peroxidase activity [82].

INTERACTION WITH NITRIC OXIDE (NO)

NO is produced by the host cell as part of the immune response to microbial infections. Interestingly, in some bacteria (*E. coli* [83], *Staphylococcus aureus* [84], *M. tuberculosis* [20], *Desulfovibrio gigas* [85], *Bacillus subtilis* [86]) NO induces expression of cytochrome *bd*-encoding genes. In this regard, investigating the interaction of cytochrome *bd* with NO is of particular interest. It was found that NO effectively inhibits the oxygen reductase activity of the *bd*-oxidases from *E. coli* and *A. vinelandii* [87]. At [NO] > 0.5 μM, a fast and complete inhibition of the activity of these enzymes is achieved. Cytochrome *bd*-I from *E. coli* was investigated in more detail. It was shown that following the removal of the added (<1 μM) NO from the solution, the oxygen reductase activity of the enzyme is recovered quickly and completely [87]. In contrast, at higher (micromolar) concentrations of NO, a small (<15%) irreversible inhibition of the enzyme was observed (Fig. 3, see also Borisov et al. [88]). For cytochrome *bd*-I from *E. coli*, at $[O_2]$ = 70 μM the value of the apparent inhibition constant (K_i) for NO is equal to 100 nM [87], close to the K_i value determined for the mitochondrial cytochrome *c* oxidase under similar experimental conditions [89]. After increasing the concentration of O_2 in solution (up to 1 mM), the observed K_i value for NO appeared to be significantly higher (230 nM) [87]. This finding suggests a competition between NO and O_2 binding to reduced unliganded heme *d*:

$$Fe_d^{2+} + NO \rightarrow Fe_d^{2+}{-}NO.$$

Since the rate constants for the binding of NO and O_2 to ferrous heme *d* are likely similar [2], at low $[NO]/[O_2]$ ratio the onset of the inhibition is expected to be slow. However, this is not the case, as the enzyme is inhibited by NO rather quickly even at $[NO]/[O_2]$ ~ 0.005 [87]. This is probably due to the fact that NO can "trap" not only the unliganded ferrous heme *d*, but also some catalytic intermediates of the enzyme that are unreactive towards molecular oxygen. Accordingly, we have recently shown that the O_2-unreactive $\mathbf{A^1}$ and **F** intermediates, prevailing under steady-state conditions [70], are able to react with NO [75, 90]. Upon interacting with the $\mathbf{A^1}$ intermediate, NO displaces O_2 from heme *d*, eventually yielding the heme iron nitrosyl complex. In this reaction, the rate-limiting step is the dissociation of O_2 from heme *d* (k = 78 s^{-1}) [90]:

$$Fe_d^{2+}{-}O_2 \rightarrow Fe_d^{2+} + O_2,$$

$$Fe_d^{2+} + NO \rightarrow Fe_d^{2+}{-}NO.$$

Cytochrome *bd* apparently is not endowed with a NO reductase activity (i.e. it is unable to reduce NO to N_2O) [87]. The reaction of nitric oxide with the **F** intermediate leads to the formation of a complex of the fully oxidized enzyme with nitrite bound at the ferric heme *d*. The reaction is quite fast ($k \sim 10^5$ $M^{-1}{\cdot}s^{-1}$, for cytochrome *bd* from *A. vinelandii* [75]) and likely proceeds according to the following mechanism:

$$Fe_d^{4+}{=}O^{2-} + NO \rightarrow Fe_d^{3+}{-}NO_2^-.$$

In this reaction, NO is oxidatively degraded into the much less toxic nitrite. The reaction might therefore be physiologically relevant as a defense mechanism against NO.

Considering the interaction of NO with the different forms of cytochrome *bd*, it is worth mentioning that this ligand also reacts with heme *d* in the fully oxidized enzyme with a rate constant $k \sim 10^2$ $M^{-1}{\cdot}s^{-1}$, forming a heme *d* nitrosyl adduct [91]:

$$Fe_d^{3+} + NO \rightarrow Fe_d^{3+}{-}NO \leftrightarrow Fe_d^{2+}{-}NO^+.$$

Importantly, after exhaustion of NO in the medium, the recovery of cytochrome *bd*-I activity occurs much faster than in the case of the mitochondrial cytochrome *c* oxidase [2, 87]. This happens because in the fully reduced isolated bacterial enzyme NO dissociates from heme *d* at a much higher rate than from heme a_3 in the mitochondrial enzyme ($k = 0.133$ s^{-1} [90] versus $k = 0.0035$ s^{-1} [92]). The high rate of NO dissociation from cytochrome *bd*-I [90] has been also confirmed in intact *E. coli* cells [93]. Another important observation made on the isolated cytochrome *bd*-I is that the rate constant of the dissociation of NO from the completely reduced enzyme ($k = 0.133$ s^{-1}) is significantly higher than k_{off} from the one-electron-reduced enzyme ($k = 0.036$ s^{-1}) [90]. This suggests that the redox state of the hemes *b* (most likely heme b_{595}) affects the stability of the ferrous nitrosyl heme *d* complex, the rate of NO dissociation being maximal when heme b_{595} is in the reduced state. This unique ability of cytochrome *bd* to rapidly dissociate NO from the active site may explain why under specific conditions this particular oxidase is preferentially expressed in place of a heme-copper enzyme. The faster NO dissociation is indeed expected to speed the recovery of bacterial respiration from NO inhibition, thus conferring to the microorganism a higher resistance to nitrosative stress. Interestingly, it has been recently found that the *bd*-type terminal oxidase from *Shewanella oneidensis* also makes this Gram-negative facultative anaerobe more resistant to nitrite and NO under aerobic growth conditions [94, 95].

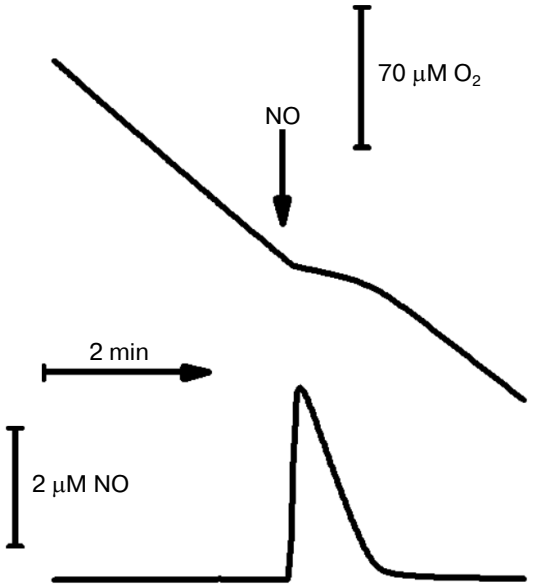

Fig. 3. Inhibition by NO of the isolated solubilized cytochrome *bd*-I from *E. coli*. The O_2 and NO traces were recorded in parallel. The O_2-reductase activity of the enzyme was sustained with the reducing substrates 10 mM ascorbate and 0.5 mM TMPD. NO added, 4 μM; cytochrome *bd*-I, 100 nM. The experimental details are given in Borisov et al. [88].

INTERACTION WITH PEROXYNITRITE ($ONOO^-$)

In response to bacterial infection, cells of the mammalian immune system produce both nitric oxide (NO) and superoxide anion at high concentrations by activating the NO synthases and NADPH oxidase, respectively. As a result of the diffusion-controlled reaction of NO with superoxide anion, peroxynitrite ($ONOO^-$) is formed. Peroxynitrite is a highly reactive toxic compound, which gives rise to both oxidative and nitrosative stress in bacteria [96]. Upon penetrating inside the bacterial cell, $ONOO^-$ can cause protein modifications [97, 98], lipid oxidation [99], and DNA damage [100].

As mentioned above, some pathogenic bacteria express cytochrome *bd* as the terminal oxidase of the respiratory chain during host infection [6]. It is therefore important to know (i) the extent of *bd*-type terminal oxidase sensitivity to $ONOO^-$, and (ii) the benefits, in terms of resistance to oxidative and nitrosative stress, granted to a pathogen expressing a *bd*-type rather than a heme-copper oxidase, such as cytochrome *c* oxidase. Previously, Cooper et al. studied in detail the interaction of cytochrome *c* oxidase isolated from mitochondria with $ONOO^-$, and they reported that $ONOO^-$ causes the irreversible inhibition of the purified cytochrome *c* oxidase [101-103].

Recently, we investigated the effect of $ONOO^-$ on the oxygen reductase activity of cytochrome *bd*-I from *E. coli* [88] and found that upon adding $ONOO^-$ to the iso-

lated solubilized enzyme in turnover with O_2 (with an excess of the reductants ascorbate and TMPD), two events can be observed: a transient stop of the oxygen consumption and the formation of NO (Fig. 4a). For example, upon addition of 50 μM $ONOO^-$, a release of 4 μM NO was observed. Once the NO disappears from the solution by reacting with O_2 and the ferryl complex of cytochrome *bd*-I, the oxygen consumption activity of the enzyme resumes. Detailed analysis of the oxygen consumption rates measured before and after addition of $ONOO^-$ showed that, if the concentration of NO formed following the addition of $ONOO^-$ is greater than 1 μM, the enzymatic activity does not return to its initial level. Virtually identical results have been obtained in control experiments upon adding authentic NO instead of $ONOO^-$ (Fig. 3). The latter result suggests that the small irreversible inhibition observed after addition of high $ONOO^-$ concentrations has to be attributed to NO rather than to $ONOO^-$. The maximum irreversible inhibition (~15%) has been observed at 6 μM NO, either exogenously added or produced following the addition of 100 μM $ONOO^-$ [88]. It is important to emphasize that, upon addition of $ONOO^-$ to cytochrome *bd*-I overexpressing *E. coli* cells, a rapid and reversible stop of the oxygen consumption is also observed (Fig. 4b). In this case, however, following the addition of high concentrations of $ONOO^-$ to the cells, the extent of the irreversible inhibition was very small (<5%), in agreement with the finding that no noticeable amounts of NO are produced (Fig. 4b) under these experimental conditions. This led us to conclude that the oxygen reductase activity of cytochrome *bd*-I, as isolated or in cells, is not inhibited by $ONOO^-$ per se [88]. It is interesting to note that in both cases (isolated enzyme or cells) the addition $ONOO^-$ not only leads to a temporary stop of the oxygen consumption, but also to a short-term formation of a small amount of O_2 (see the increase in the oxygen trace in Fig. 4). Since H_2O_2 may be a contaminant of the commercial preparations of $ONOO^-$ and/or a secondary product of $ONOO^-$ decay, we assume that the observed evolution of O_2 is a consequence of the NO-insensitive catalase activity of cytochrome *bd*-I [78, 88].

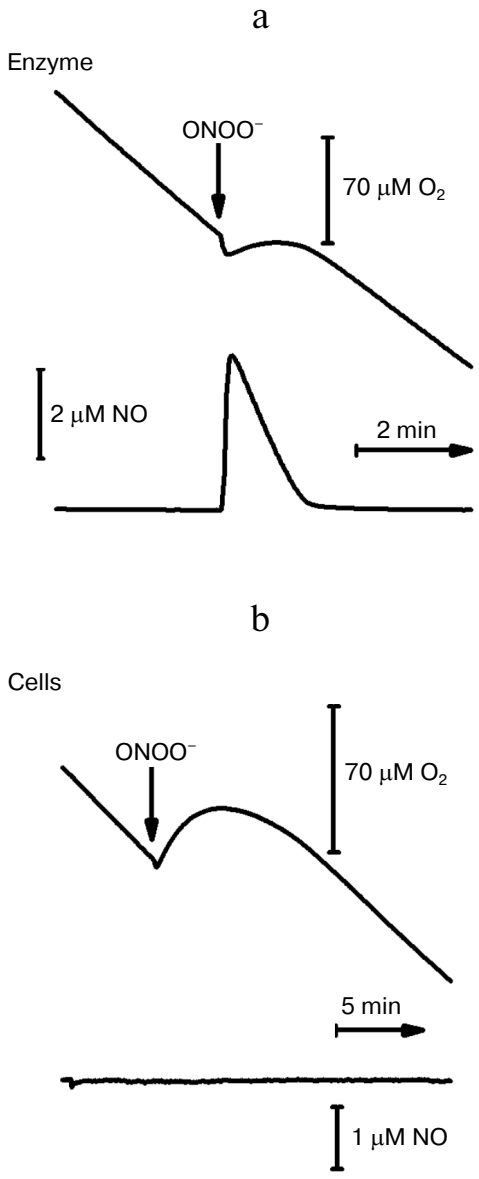

Fig. 4. Effect of peroxynitrite on the oxygen consumption rate by cytochrome *bd*-I from *E. coli*. a) $ONOO^-$ (50 μM) was added to the isolated solubilized enzyme (100 nM) in turnover with O_2 and excess reductants (10 mM ascorbate and 0.5 mM TMPD). b) $ONOO^-$ (80 μM) was added to the respiring (due to endogenous substrates) *E. coli* cells (of the GO105 strain devoid of cytochrome bo_3) overexpressing the *bd*-I oxidase (due to the presence of the pTK1 plasmid carrying the operon encoding this enzyme). The experimental details are given in Borisov et al. [88].

We have also directly measured the peroxynitrite-detoxifying activity of cytochrome *bd*-I isolated from *E. coli* by stopped-flow absorption spectroscopy. In these experiments, the $ONOO^-$ concentration was monitored over time measuring the absorption at 310 nm. A number of control experiments (without the enzyme or the reducing substrates or $ONOO^-$) was also carried out, whose results were taken into account to calculate the rate constant of the enzymatic $ONOO^-$ decomposition by cytochrome *bd*-I in turnover with O_2 and excess ascorbate and TMPD reductants. As expected, this observed rate constant was found to increase linearly with the concentration of the enzyme [88]. Moreover, upon increasing the concentration of TMPD from 150 to 300 μM, at each cytochrome *bd*-I concentration tested, a faster decay of $ONOO^-$ was observed, consistent with an increase in the apparent turnover number of the $ONOO^-$-detoxifying activity of the enzyme, from 7 to 10 moles $ONOO^-$/mole enzyme per second [88].

All in all, we have found that (i) differently from mitochondrial cytochrome *c* oxidase, cytochrome *bd*-I from *E. coli* is not inactivated by $ONOO^-$ up to a concen-

tration of 100 μM; (ii) the *bd*-I enzyme, in turnover with oxygen and the reducing substrates, is capable of metabolizing $ONOO^-$ quite rapidly, thus acting as a detoxifying agent for this highly reactive toxic compound. To our knowledge, this has been the first time that the kinetics of $ONOO^-$ decomposition by a terminal oxidase was directly measured [88].

PHARMACOLOGICAL PREPARATIONS – SPECIFIC INHIBITORS OF ENERGY METABOLISM IN PATHOGENIC BACTERIA

To combat pathogenic microorganisms, a number of antibiotics with different modes of action have been used for many decades. However, in recent years there has been a significant increase in the resistance (including multidrug resistance) of pathogenic microorganisms to antimicrobial drugs, particularly to antibiotics that in the past were highly effective. As a result, the infectious diseases caused by such resistant pathogens are not amenable to standard treatment, thus leading to prolonged illness and increased risk of death. Hence, there is an urgent need for the development of new antimicrobial drugs acting through novel mechanisms. In this regard, the enzymes playing a central role in the energy metabolism represent suitable targets for novel antibacterial compounds.

Cytochrome *bd* oxidase is emerging as one of such targets. As indicated above, the enzyme is able to significantly increase the resistance of a microbial cell to oxidative and nitrosative stress, thereby presumably helping a bacterial pathogen to evade the host immune defense. Since cytochrome *bd* is found only in bacteria, selective inhibitors of *bd*-type oxidases should not have negative effects on the host energy metabolism.

The development of next-generation drugs by the world pharmaceutical industry is only in its infancy. The first next-generation drug targeting the energy metabolism of a bacterial pathogen is bedaquiline (diarylquinoline). The bactericidal effect of this anti-tuberculosis drug approved by the U.S. Food and Drug Administration (U.S. FDA) is due its ability to selectively inhibit the F_1F_o-ATP synthase of *Mycobacterium tuberculosis* [104]. The inhibition of ATP synthesis leads to disruption of the energy production system and, eventually, to the death of the microbial cell. However, compared to other frontline anti-tuberculosis drugs, such as isoniazid, bedaquiline kills the *M. tuberculosis* cells rather slowly [105]. Recently, we raised the hypothesis that cytochrome *bd* could confer to *M. tuberculosis* cells higher resistance to oxidative stress, thanks to its ability to degrade hydrogen peroxide [82]. Following our work, Berney et al. reported that the rate at which bedaquiline kills the *M. tuberculosis* cells increases remarkably, if the *bd*-type terminal oxidase is knocked-out (by replacing the *cyd*A gene with a hygromycin cassette by using specialized transduction) [106]. This is consistent with the observation that in *M. tuberculosis* cells treated with bedaquiline the expression levels of cytochrome *bd* increase substantially [105].

Another drug targeting the respiratory chain of *M. tuberculosis* is compound Q203, based on imidazo[1,2-α]pyridine [107]. Its action is based on the inhibition of the bc_1 respiratory complex [107]. Recently, the effect of a series of five different scaffolds, imidazo[1,2-α]pyridine derivatives, on clinical strains of *M. tuberculosis* was reported [108]. Although imidazo[1,2-α]pyridines completely inhibit the growth of most of the *M. tuberculosis* strains tested by inhibiting the bc_1 complex, the laboratory-adapted strains H37Rv, CDC1551, and Erdman appeared to overcome this growth inhibition [108]. This was suggested to be due to an increase in the cytochrome *bd* expression levels. Indeed, deletion of this terminal oxidase in the H37Rv strain makes the mutant more sensitive to imidazo[1,2-α]pyridines [108].

In summary, it is hoped that the use of a specific inhibitor of cytochrome *bd* in combination with inhibitors of other enzymes of the energy metabolism (e.g. bedaquiline and/or imidazo[1,2-α]pyridines) will have a synergistic effect, thereby representing an innovative pharmacological strategy to fight bacterial pathogens.

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