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Cytochrome *bd* from *Escherichia coli* catalyzes peroxynitrite decomposition ☆

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ABSTRACT

Cytochrome *bd* is a prokaryotic respiratory quinol oxidase phylogenetically unrelated to heme-copper oxidases, that was found to promote virulence in some bacterial pathogens. Cytochrome *bd* from *Escherichia coli* was previously reported to contribute not only to proton motive force generation, but also to bacterial resistance to nitric oxide (NO•) and hydrogen peroxide (H_2O_2). Here, we investigated the interaction of the purified enzyme with peroxynitrite ($ONOO^-$), another harmful reactive species produced by the host to kill invading microorganisms. We found that addition of $ONOO^-$ to cytochrome *bd* in turnover with ascorbate and *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) causes the irreversible inhibition of a small (≤15%) protein fraction, due to the NO• generated from $ONOO^-$ and not to $ONOO^-$ itself. Consistently, addition of $ONOO^-$ to cells of the *E. coli* strain GO105/pTK1, expressing cytochrome *bd* as the only terminal oxidase, caused only a minor (≤5%) irreversible inhibition of O_2 consumption, without measurable release of NO•. Furthermore, by directly monitoring the kinetics of $ONOO^-$ decomposition by stopped-flow absorption spectroscopy, it was found that the purified *E. coli* cytochrome *bd* in turnover with O_2 is able to metabolize $ONOO^-$ with an apparent turnover rate as high as ~10 mol $ONOO^-$ (mol enzyme)$^{-1}$ s^{-1} at 25 °C. To the best of our knowledge, this is the first time that the kinetics of $ONOO^-$ decomposition by a terminal oxidase has been investigated. These results strongly suggest a protective role of cytochrome *bd* against $ONOO^-$ damage.

1. Introduction

Peroxynitrite ($ONOO^-$) is a cytotoxic effector produced in mammalian immune cells to kill invading microbes (reviewed in [1,2]). In response to microbial infections, macrophages generate simultaneously nitric oxide (NO•) and superoxide anion (O_2^- •) by activating the inducible nitric oxide synthase (iNOS) and NADPH oxidase, respectively. The two radical species then combine at a diffusion limited rate ($k \sim 10^{10}$ M^{-1} s^{-1}) to yield high levels of $ONOO^-$, particularly in the phagosomes where invading microbes are internalized to be destroyed. Under physiological conditions, $ONOO^-$ is in equilibrium with peroxynitrous acid (ONOOH, $pK_a = 6.8$), both species being highly reactive, particularly with protein metals and thiols. Peroxynitrite is a strong nucleophilic oxidant. Together with its reactive secondary intermediates, $ONOO^-$ reacts with a number of targets in the microbial cell, causing DNA damage, lipid oxidation, protein modifications and eventually cell death [3]. Therefore, it is not surprising that during evolution microorganisms have developed defense mechanisms to prevent $ONOO^-$ formation or to promote its decomposition, through the enzymatic detoxification of NO• (by NO•-dioxygenases and NO•-reductases such as flavohemoglobin and flavodiiron protein), O_2^-• (by superoxide dismutase and superoxide reductase) or $ONOO^-$ itself (by peroxiredoxins in primis).

In eukaryotes, among other pathways, $ONOO^-$ affects the mitochondrial electron transport chain through irreversible damage of NADH dehydrogenase, succinate dehydrogenase and ATPase [4–6]. Excess $ONOO^-$ was also reported to irreversibly inhibit purified cytochrome *c* oxidase [7–10], although this was not observed in functional mitochondria [4–6], while at stoichiometric $ONOO^-$ concentrations a peroxynitrite-reductase activity of the enzyme was reported with no information on the kinetics of the reaction [8]. Based on these results, and given the antimicrobial activity of $ONOO^-$, it seems of relevance to investigate the interaction of $ONOO^-$ with the bacterial counterparts of the mitochondrial respiratory complexes, an issue that has not been addressed in detail yet.

Abbreviations: NO•, nitric oxide; $ONOO^-$, peroxynitrite; DTPA, diethylenetriaminepentaacetic acid; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine

☆ The aerobic respiratory chain of *E. coli* contains two *bd*-type terminal oxidases: *bd*-I and *bd*-II. Unless otherwise stated, we refer to cytochrome *bd*-I throughout the manuscript.

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Unlike the mitochondrial electron transport chain, the microbial respiratory chains are typically branched [11]. In particular, many bacteria in addition to or instead of a heme-copper oxidase contain a copper-less tri-heme terminal oxidase, called cytochrome *bd* (reviewed in [12–14]). The enzyme catalyzes the reduction of molecular oxygen to water, using quinols as the physiological electron donors. The reaction is associated with the generation of a proton motive force via transmembrane charge separation, without involving a proton pumping mechanism [15–22]. Cytochrome *bd* is composed of two major membrane-spanning polypeptides, subunits I (CydA, 57 kDa) and II (CydB, 43 kDa), and a small protein, named CydX (4 kDa). The latter has been recently shown to be essential for enzymatic activity and proposed to be an additional subunit of the complex, needed for either the assembly or the stability of the enzyme [23–25]. Cytochrome *bd* bears two *b*-type hemes, the low-spin b_{558} and the high-spin b_{595}, and one chlorin, heme *d*. Heme b_{558} accepts electrons from the quinol substrate, whereas heme *d* is the site where the oxygen chemistry takes place. Less clear is the function of heme b_{595}. Although the X-ray structure of the protein is not available, existing data suggest that heme b_{595} could facilitate O_2 reduction at heme *d*, forming with the latter a di-heme active site [18,20,26–37]. Nevertheless, data inconsistent with the existence of a bimetallic O_2-reducing site were also reported [13,38]. Finally, heme b_{595} was also suggested to provide a second binding site for O_2 [39,40].

Relevant to the present study, cytochrome *bd* is present in many pathogenic bacteria that are able to use O_2 as the terminal electron acceptor and, for some of these pathogens, virulence was shown to depend on the expression of cytochrome *bd* (see [14,41,42] and references therein). The rationale for the latter observation could be that, based on the available literature, beyond its role in cell bioenergetics, cytochrome *bd* seems to accomplish several important physiological functions that enable bacterial survival in different ecological niches and, importantly, resistance to the hostile conditions created by the immune system in response to microbial infections (reviewed in [14,41,42]). In agreement with this hypothesis, there is growing evidence in the literature pointing to a protective role of cytochrome *bd* against a number of stressors, including H_2O_2 [23,42–52], NO• [41,53–58], CO [59], nitrite [60,61] and excess O_2 itself [62]. Intriguingly, all these species are implicated in the host immune response against microbial infections.

Based on the information above, and given the prominent role of $ONOO^-$ in the host immune response, in this work, we have examined the interaction of the cytochrome *bd* from *E. coli* with $ONOO^-$ by amperometric and time-resolved spectroscopic techniques. The data presented here show that the purified enzyme in turnover with O_2 not only resists $ONOO^-$ damage, but is also able to rapidly metabolize $ONOO^-$.

2. Materials and methods

2.1. Reagents and enzyme purification

Na-ascorbate, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD), diethylenetriaminepentaacetic acid (DTPA) and *N*-lauroyl-sarcosine were purchased from Sigma-Aldrich. Peroxynitrite ($ONOO^-$) was from Calbiochem and its concentration was determined using $\varepsilon = 1670\ mM^{-1}\ cm^{-1}$ at 302 nm [1]. Stock solutions of NO• (Air Liquide) were prepared by equilibrating degassed water with the NO• pure gas at 1 atm at room temperature yielding 2 mM NO• in solution. Cytochrome *bd* from *E. coli* strain GO105/pTK1 was isolated as reported [63,64]. The purified enzyme displayed high oxidase activity (see below) and characteristic absorption spectra both 'as prepared' and after dithionite reduction (not shown). This confirms that in the isolated enzyme all the three hemes, including the di-heme active site components, heme b_{595} and heme *d*, were present as expected for a *bd*-type oxidase [14]. Based on recent reports [23–25], this suggests that the purified enzyme likely included the newly discovered small subunit CydX. The enzyme concentration was determined from the dithionite-reduced-*minus*-'as prepared' difference absorption spectrum using $\Delta\varepsilon_{628-607} = 10.8\ mM^{-1}\ cm^{-1}$ [30].

2.2. Amperometric assays

Oxygraphic measurements were carried out at 25 °C in 100 mM Na/phosphate pH 7.0, 20 μM DTPA, using a high-resolution respirometer (Oxygraph-2k, Oroboros Instruments) equipped with 1.5 mL-chambers. For assays with purified cytochrome *bd*, the buffer was supplemented with 0.05% *N*-lauroyl-sarcosine. In parallel to O_2 consumption, the concentration of NO• in solution was also recorded using a NO•-selective electrode (World Precision Instruments), calibrated by sequential additions of NO• from the stock solution. The O_2-reductase activity of purified cytochrome *bd* was measured in the presence of an excess of the reductants ascorbate and TMPD. In these assays, we could not use ubiquinone-1 plus an excess of dithiothreitol to sustain the cytochrome *bd* turnover, because dithiothreitol was found to react with $ONOO^-$ ($k = 500\ M^{-1}\ s^{-1}$ at 25 °C, Supplemental Fig. 1). Under the tested experimental conditions, we noted that, after the addition of cytochrome *bd* to air-equilibrated buffer containing ascorbate and TMPD, the O_2-reductase activity of the enzyme increased with time, reaching a maximal value within approximately half an hour. This phenomenon resembled the so-called 'pulsing effect' previously described for the mitochondrial cytochrome oxidase [65]. Given the observed spontaneous activation of the enzyme in turnover, to evaluate the effects of $ONOO^-$ or NO• on the O_2-reductase activity of cytochrome *bd*, prior to the addition of these effectors, the enzyme was allowed to turn over with O_2 and excess reductants until the O_2-consumption rate became constant and the oxygraphic trace took a linear profile.

2.3. Spectrophotometric measurements

Static UV-visible absorption spectra were recorded in an Agilent Cary-60 spectrophotometer. Stopped-flow experiments were carried out at 25 °C in a thermostated instrument (DX.17MV, Applied Photophysics, Leatherhead, UK), equipped with a 1 cm-light path observation chamber that allows the rapid (1–2 ms) mixing of two solutions in a 1:1 ratio. After mixing, time-resolved absorption changes were collected according to a logarithmic time scale. Peroxynitrite decomposition in the presence or absence of cytochrome *bd* was monitored at 310 nm, using $\varepsilon = 1600\ mM^{-1}\ cm^{-1}$ [66]. The non-enzymatic degradation of $ONOO^-$ was investigated by mixing a freshly prepared, air-equilibrated solution of $ONOO^-$ (80–90 μM) in 10 mM NaOH with degassed buffer (100 mM Na/phosphate, pH 7.0, 100 μM DTPA, 0.05% *N*-lauroyl-sarcosine) alone or containing ascorbate and TMPD. The ability of cytochrome *bd* to catalyze the degradation of $ONOO^-$ was tested by mixing the aforementioned aerobic alkaline solution of $ONOO^-$ with degassed buffer containing the enzyme 'as isolated' or pre-reduced with ascorbate and TMPD. At higher cytochrome *bd* concentration, the optical contribution of the enzyme to the absorption changes measured at 310 nm was independently evaluated in control experiments carried out in the absence of $ONOO^-$, and subtracted from the traces acquired in the presence of $ONOO^-$ at the same wavelength. The measured time courses of $ONOO^-$ decomposition were fitted to single exponentials. The apparent turnover rate (*TN*) at which cytochrome *bd* catalyzes the degradation of $ONOO^-$ was estimated using the following formula:

$$TN = \frac{([ONOO^-]_i \times k'_{bd})}{[bd]}$$

where $[ONOO^-]_i$ is the initial concentration of $ONOO^-$, $[bd]$ is the concentration of cytochrome *bd* in the assay and k_{bd}' is the difference between the observed rate constant measured in the presence of both the enzyme

and the reductants (enzymatic plus non-enzymatic degradation of $ONOO^-$) and the rate constant measured in the presence of the reductants alone (non-enzymatic degradation of $ONOO^-$).

2.4. Data analysis

Data analysis was carried out using MATLAB (The Mathworks, South Natick, MA) and Origin (OriginLab Corporation).

3. Results

The effect of $ONOO^-$ on the O_2-reductase activity of cytochrome *bd* from *E. coli* was investigated by adding up to 100 μM $ONOO^-$ to the purified enzyme in turnover with O_2 and excess reductants (ascorbate and TMPD), and measuring the O_2 consumption rate. In these assays, the concentration of NO• in solution was also measured in parallel, using a NO•-selective electrode. As a representative dataset, we report in Fig. 1A the O_2 and NO• traces collected on addition of 50 μM $ONOO^-$. Comparison of the two traces clearly shows that, following the addition of $ONOO^-$, a transient arrest of O_2 consumption by cytochrome *bd* occurs concomitantly with the production of approximately 4 μM NO•. Afterward, the O_2 consumption resumes as NO• disappears from solution by reacting with O_2 and with the ferryl intermediate of cytochrome *bd*, highly populated at steady state [67], yielding in both cases nitrite as the main product according to previous reports [54,68].

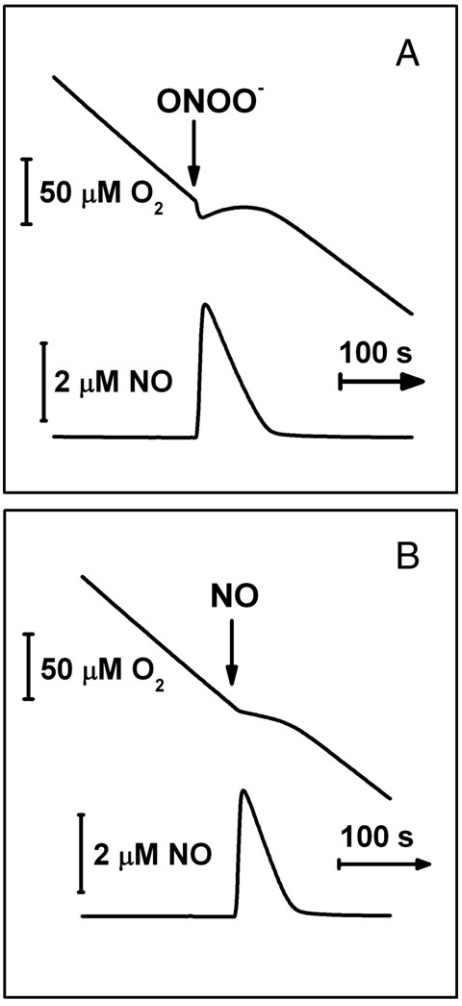

Fig. 1. Effect of $ONOO^-$ and NO• on cytochrome *bd* oxidase activity. Effect of 50 μM $ONOO^-$ (A) or 4 μM NO• (B) on the O_2 consumption of cytochrome *bd* (upper traces). In these assays the NO• concentration was measured in parallel (lower traces). Conditions: 100 mM Na/phosphate pH 7.0, 20 μM DTPA, 0.05% *N*-lauroyl-sarcosine, 10 mM ascorbate, 0.5 mM TMPD and 0.1 μM cytochrome *bd*. T = 25 °C.

Fig. 1B shows a control oxygraphic trace in which ~4 μM authentic NO• has been added to the reaction chamber instead of 50 μM $ONOO^-$. Addition of NO• reveals almost the same inhibitory pattern observed with $ONOO^-$, with the enzyme activity being temporarily stopped and eventually resuming after the NO• decay. The observed rapid and reversible inhibition of cytochrome *bd* by NO• is in agreement with previous reports [53,69]. The two oxygraphic traces shown in Fig. 1 differ from each other in that, in contrast to NO•, addition of $ONOO^-$ not only stops oxygen consumption, but also leads to a slight short-term evolution of O_2 (Fig. 1A, upper trace). Since H_2O_2 is a contaminant of commercially available $ONOO^-$ preparations and can be produced as a secondary product of $ONOO^-$ decomposition, the observed evolution of O_2 likely originates from the reaction of H_2O_2 with *E. coli* cytochrome *bd*, that has been recently reported to display a NO•-insensitive catalase activity [51].

Careful analysis of the O_2 consumption rates measured before and after the addition of $ONOO^-$ shows that, following the NO• decay, the enzyme activity does not return to the initial level. Interestingly, identical results were obtained in the control experiments performed with authentic NO•. By comparing the effects of $ONOO^-$ and authentic NO• on the maximal O_2-reductase activity recovered after reversal of enzyme inhibition, it can be seen that if such activity (expressed as a percentage of the initial control value) is plotted as a function of the concentration of NO•, added (*triangles*) or generated from $ONOO^-$ (*circles*), the two datasets perfectly match (Fig. 2). Therefore, we conclude that the small irreversible inhibition of cytochrome *bd* observed

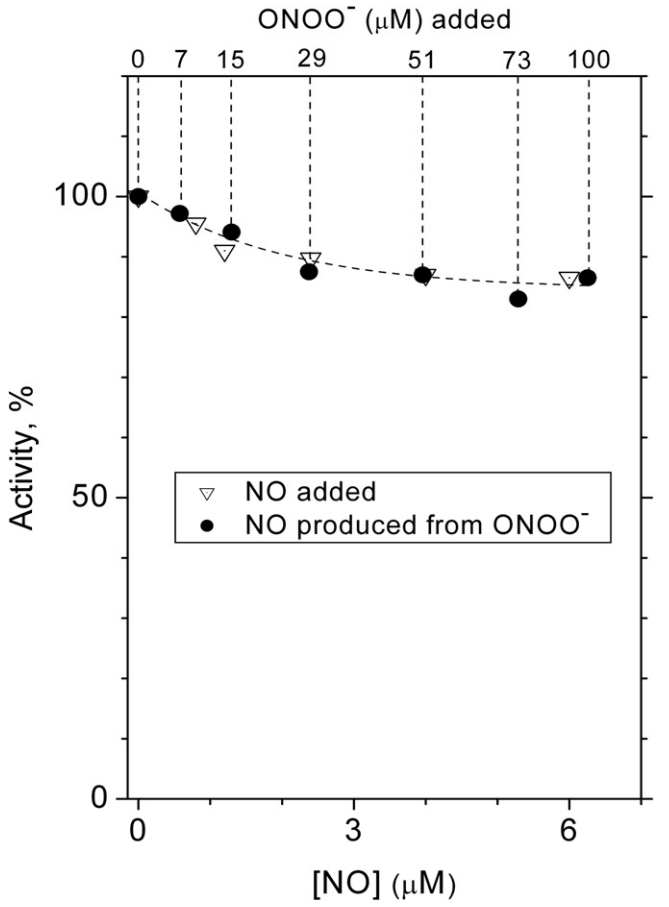

Fig. 2. Cytochrome *bd* activity recovered after reversal of NO• inhibition. O_2-reductase activity of cytochrome *bd* observed after disappearance of NO•, initially added or produced from $ONOO^-$. Values are expressed with reference to the activity measured before addition of $ONOO^-$ or NO• taken as 100%. Conditions as in Fig. 1.

after exposure to $ONOO^-$ is due to NO• and not to $ONOO^-$ itself. Such irreversible inhibition can only be observed at high (micromolar) concentrations of NO•, with a maximum of ~15% inhibition detected at ~6 μM NO•, either externally added or produced from 100 μM $ONOO^-$ (Fig. 2).

Fig. 3 reports the amount of NO• detected in solution in the experiments described above after the addition of $ONOO^-$ to cytochrome *bd* in turnover in the presence of an excess of ascorbate and TMPD. The data clearly show that the NO• released increases proportionally with the concentration of the $ONOO^-$ added. Moreover, it is evident that the formation of NO• is also observed in the absence of the enzyme, but to a much lesser extent than in its presence. This finding indicates that, in the presence of ascorbate and TMPD, cytochrome *bd* by reacting with $ONOO^-$ facilitates its (partial) conversion to NO•. In control experiments we tested that varying the enzyme concentration in the 15–300 nM range, while proportionally increasing the O_2 consumption rate, did not change the amount of NO• released from $ONOO^-$ (Supplemental Fig. 2).

To provide direct evidence for the ability of cytochrome *bd* to metabolize $ONOO^-$ and gain insight into the kinetics of the reaction, we carried out stopped-flow experiments, in which the kinetics of $ONOO^-$ decomposition was investigated at 25 °C in the presence and in the absence of the enzyme, by monitoring the absorption changes at 310 nm. Fig. 4 shows that under the tested experimental conditions, 42 μM $ONOO^-$ decays spontaneously within 10 s with a rate constant k' ~0.35 s^{-1} (dotted line). However, in the presence of cytochrome *bd* (1.1 μM) in turnover with O_2 and excess reductants (0.5 mM ascorbate and 50 μM TMPD), the same amount of $ONOO^-$ disappears from the solution at a higher rate (k' ~0.52 s^{-1}, solid line). Importantly, neither the enzyme nor the reductants, when assayed independently and at the same concentrations indicated above, were found to elicit $ONOO^-$ decomposition to a significant extent (inset to Fig. 4). Therefore, after subtracting the contribution of the spontaneous, non-enzymatic decay of $ONOO^-$, one can conclude that under the tested experimental conditions 1.1 μM cytochrome *bd* in turnover with O_2 is able to metabolize 42 μM $ONOO^-$ with k' ~0.15 s^{-1}, which allows the estimation of an apparent initial turnover rate of ~5.7 mol $ONOO^-$ (mol enzyme)$^{-1}$ s^{-1} for the enzyme-mediated degradation of $ONOO^-$. By investigating the kinetics of $ONOO^-$ decomposition at different cytochrome *bd* concentrations (from 0 to 5 μM) in the presence of 1.5 mM ascorbate and 150 μM TMPD, we found $ONOO^-$ to be metabolized with an observed rate

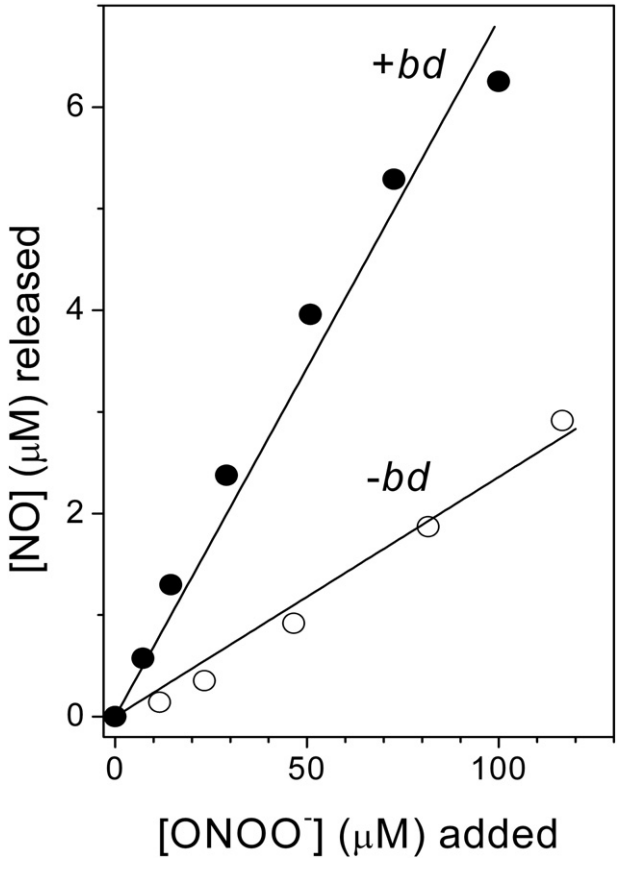

Fig. 3. NO• released from $ONOO^-$ in the presence or absence of cytochrome *bd*. NO• detected in solution following the addition of increasing amounts of $ONOO^-$ to 100 mM Na/phosphate pH 7.0, 20 μM DTPA, 0.05% *N*-lauroyl-sarcosine, 10 mM ascorbate and 0.5 mM TMPD alone (open circles) or plus 0.1 μM cytochrome *bd* (closed circles). Conditions as in Fig. 1.

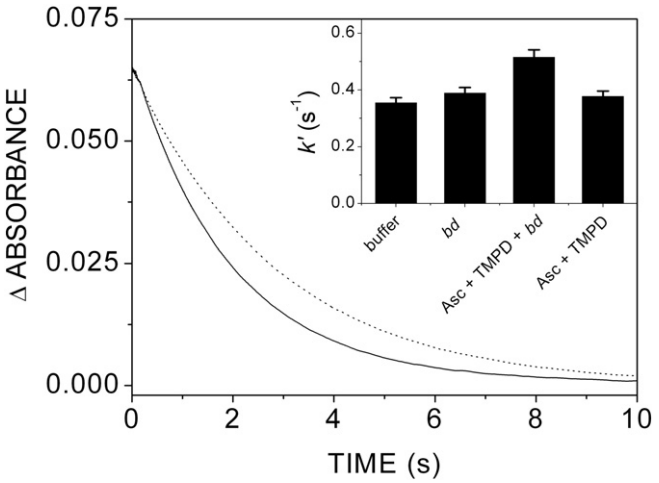

Fig. 4. $ONOO^-$ decomposition by cytochrome *bd* in turnover. Time course of $ONOO^-$ decomposition acquired at 310 nm after stopped-flow mixing at 25 °C an air-equilibrated solution of 84 μM $ONOO^-$ in 10 mM NaOH with anaerobic buffer alone (100 mM Na/phosphate, pH 7.0, 100 μM DTPA, 0.05% *N*-lauroyl-sarcosine, dotted line) or containing 2.2 μM cytochrome *bd* pre-reduced by 1 mM ascorbate and 100 μM TMPD (solid line). The reported concentrations are before mixing. Inset: rate constants observed for $ONOO^-$ decomposition measured in the presence of: buffer alone ('buffer') or plus 1.1 μM cytochrome *bd* as isolated ('bd'), 1.1 μM cytochrome *bd* pre-reduced by 0.5 mM ascorbate and 50 μM TMPD ('Asc + TMPD + bd') or just 0.5 mM ascorbate and 50 μM TMPD ('Asc + TMPD'). The reported concentrations are all after mixing.

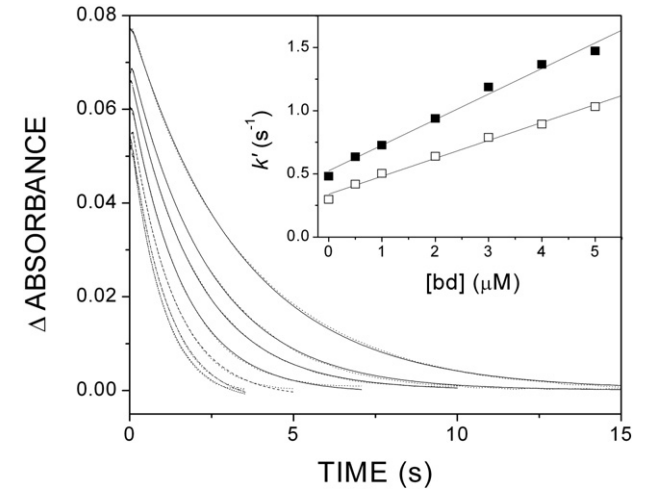

Fig. 5. Effect of cytochrome *bd* and TMPD concentration on $ONOO^-$ decomposition. Decomposition of 37–45 μM $ONOO^-$ measured in the presence of 1.5 mM ascorbate, 150 μM TMPD and cytochrome *bd* at the following concentrations: 0, 0.5, 1, 2, 3, 4 and 5 μM (traces from right to left). Other conditions as in Fig. 4. The reported concentrations are all after mixing. The depicted traces, shown with their best fit to single exponentials, were obtained after subtracting the optical contribution of the enzyme independently measured in the absence of $ONOO^-$. Inset: rate constants observed for $ONOO^-$ decomposition at increasing concentrations of cytochrome *bd* in the presence of 1.5 mM ascorbate and 150 μM (open symbols) or 300 μM TMPD (closed symbols).

constant linearly dependent on the enzyme concentration (Fig. 5). Moreover, upon increasing the TMPD concentration from 150 to 300 μM, at each enzyme concentration a faster decomposition of $ONOO^-$ was measured (solid vs open symbols, inset to Fig. 5), consistent with an increase of the apparent turnover rate for cytochrome *bd*-mediated $ONOO^-$ decomposition from ~7 to ~10 mol $ONOO^-$ (mol enzyme)$^{-1}$ s^{-1}.

All together, these data show that i) cytochrome *bd* is not inactivated by up to 100 μM $ONOO^-$ and ii) the enzyme in turnover with O_2 is able to rapidly metabolize $ONOO^-$ with an apparent turnover rate increasing at higher concentrations of the reducing substrates. These conclusions are fully consistent with the results obtained in control experiments carried out with cells of the *E. coli* GO105/pTK1 strain, expressing plasmid-encoded cytochrome *bd* as the only respiratory terminal oxidase [70]. As shown in Fig. 6, similar to the isolated enzyme (Fig. 1A), addition of $ONOO^-$ to the cells respiring on endogenous substrates led to a short-term evolution of O_2, due to contaminant H_2O_2 in the $ONOO^-$ solution. However, at variance from the isolated enzyme (Fig. 1A), addition of a relatively large amount of $ONOO^-$ (80 μM) to the cells did not lead to a measurable NO• release (lower trace in Fig. 6), thus causing only a minor (≤5%) irreversible inhibition of O_2 consumption (upper trace in Fig. 6). This finding is consistent with the conclusion that cytochrome *bd* is highly resistant to $ONOO^-$ damage.

4. Discussion

Previously, it was suggested that *E. coli* cytochrome *bd* contributes to bacterial resistance to NO• and H_2O_2 (see [14,41,42] and references therein), which is likely to have patho-physiological consequences in vivo, since both these species are generated as part of the host immune response to microbial infections. This prompted us to investigate in the present study the interaction of *E. coli* cytochrome *bd* with $ONOO^-$, another highly reactive species produced by the host to combat microbial pathogens, that is known to give rise to oxidative and nitrosative stresses in *E. coli* [71] and other microorganisms (reviewed in [2]). The aim of this work was twofold: on the one hand to determine whether the enzyme is irreversibly inhibited by $ONOO^-$, thus representing a potential target for $ONOO^-$ in the *E. coli* cell, and on the other hand to test if cytochrome *bd* can metabolize $ONOO^-$.

We found that up to 100 μM $ONOO^-$ does not damage the *bd*-type terminal oxidase. Following the addition of $ONOO^-$ to the purified enzyme in the presence of ascorbate and TMPD, we have observed a release of NO• and a transient inhibition of the O_2 reductase activity of the *bd* enzyme (Fig. 1A). Control experiments carried out with authentic NO• gas showed the same pattern of inhibition. On this basis, we concluded that the observed inhibition was due to the NO• generated from $ONOO^-$ (Figs. 1 and 2). The amount of NO• produced from the $ONOO^-$ added to cytochrome *bd* in turnover was indeed in the μM range, i.e., well above the apparent K_i value (100 nM ± 34 nM NO• at ~70 μM O_2, [53]) determined for NO• inhibition of cytochrome *bd*. In addition, it was found that NO• at high (μM) levels causes a small (~15%) irreversible inhibition of purified cytochrome *bd* (Fig. 2). In this regard, it should be noted that NO• and $ONOO^-$ were both reported to cause an irreversible inhibition of the mitochondrial cytochrome *c* oxidase purified from beef heart [7,9], used as a model of respiratory heme-copper oxidases. At low concentrations (0–3 μM NO• and 0–20 μM $ONOO^-$), NO• and $ONOO^-$ were found to irreversibly increase the K_m for O_2, presumably via nitration of specific tyrosine residues, whereas at high concentrations (>20 μM) $ONOO^-$ was shown to irreversibly affect also V_{max} [7,9]. The latter effect was attributed to irreversible damage at the redox-active metal centers of the enzyme, in particular at the binuclear Cu_A center [7]. This copper center is located in a hydrophilic domain protruding outside the inner mitochondrial membrane, being thus accessible to $ONOO^-$. As mentioned above, in the present study it is shown that, at variance from purified mitochondrial cytochrome *c* oxidase, exposure to $ONOO^-$ does not lead to a permanent impairment of the cytochrome *bd* O_2-reductase activity, as if the latter enzyme was more resistant to $ONOO^-$ damages. At this stage, we ignore what accounts for the different susceptibility between the two enzymes; however, it is tempting to speculate that the higher resistance of cytochrome *bd* might be related to the lack of copper centers in the bacterial enzyme.

In line with the results collected on the isolated enzyme, in experiments carried out with intact *E. coli* GO105/pTK1 cells expressing cytochrome *bd* as the only respiratory terminal oxidase, we found that addition of up to 80 μM $ONOO^-$ causes only a minor (≤5%) irreversible inhibition of O_2 consumption and no measurable release of NO• (Fig. 6). These observations are consistent with the finding that cytochrome *bd* is highly resistant to $ONOO^-$ damage, although in these assays with bacterial cells it is hard to predict the stability of $ONOO^-$ and its ability to react with cytochrome *bd* among the different cellular targets.

As an additional important result, we found that *E. coli* cytochrome *bd* in turnover with O_2 and excess reductants not only tolerates $ONOO^-$, but can also catalyze the decomposition of $ONOO^-$. The reaction is accompanied by formation of NO• (Fig. 3), as reported previously for the mitochondrial cytochrome oxidase [7], and is clearly associated with enzyme turnover (Figs. 4 and 5). By directly monitoring the decomposition of $ONOO^-$ by time-resolved absorption spectroscopy, we found that $ONOO^-$ is degraded with an observed rate constant that is proportional to the enzyme concentration and increases with TMPD concentration (i.e., with the electron flux) (Fig. 5). The acquired data revealed that *E. coli* cytochrome *bd*, in turnover with O_2 and excess ascorbate and TMPD, is able to metabolize $ONOO^-$ at 25 °C with an apparent turnover rate as high as ~10 mol $ONOO^-$ (mol enzyme)$^{-1}$ s^{-1}. We cannot rule out that the $ONOO^-$-degrading activity of cytochrome *bd* could be even higher in vivo with the enzyme membrane-integrated and readily reduced by its physiological quinol substrates (ubiquinol, menaquinol and demethylquinol). Although the molecular mechanism of $ONOO^-$ decomposition by cytochrome *bd* is yet to be established, we hypothesize that it may involve the reaction of $ONOO^-$ with one or more enzyme's turnover intermediates. This is the first time that a terminal oxidase has been shown to metabolize $ONOO^-$ by directly measuring the kinetics of $ONOO^-$ decay by time-resolved spectroscopy. Moreover, to the best of our knowledge, cytochrome *bd* is the first enzyme from *E. coli* that has been shown to metabolize $ONOO^-$.

In conclusion, in this study we found that *E. coli* cytochrome *bd* in turnover with O_2 not only resists $ONOO^-$ damage, but is also able to catalyze the rapid degradation of $ONOO^-$. The newly discovered $ONOO^-$-degrading activity of cytochrome *bd* may be relevant

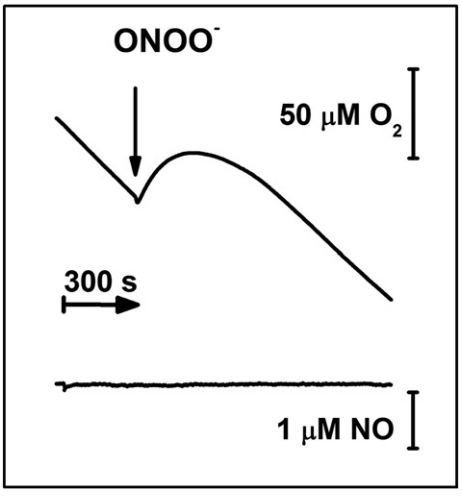

Fig. 6. Effect of $ONOO^-$ on O_2 consumption of *E. coli* cells. Effect of 80 μM $ONOO^-$ on the O_2 consumption of *E. coli* GO105/pTK1 cells (OD_{600} ~ 0.25, upper traces). Trace measured in parallel using a NO•-selective electrode (lower traces). Buffer: 100 mM Na/phosphate pH 7.0, 20 μM DTPA. T = 25 °C.

physiologically, contributing to the enhancement of bacterial resistance to nitroxidative stress and, thus, pathogenicity.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2014.10.006.

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