Interaction of the bacterial terminal oxidase cytochrome *bd* with nitric oxide

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Abstract Cytochrome *bd* is a prokaryotic terminal oxidase catalyzing O_2 reduction to H_2O. The oxygen-reducing site has been proposed to contain two hemes, *d* and b_{595}, the latter presumably replacing functionally Cu_B of heme-copper oxidases. We show that NO, in competition with O_2, rapidly and potently ($K_i = 100 \pm 34$ nM at ~70 μM O_2) inhibits cytochrome *bd* isolated from *Escherichia coli* and *Azotobacter vinelandii* in turnover, inhibition being quickly and fully reverted upon NO depletion. Under anaerobic reducing conditions, neither of the two enzymes reveals NO reductase activity, which is proposed in heme-copper oxidases to be associated with Cu_B.

Keywords: Bacterial respiration; NO metabolism; Nitrosative stress; Inhibition mechanism; Respiratory chain

1. Introduction

Cytochrome *bd* is a terminal oxidase of the respiratory chain, which is expressed by numerous bacteria preferentially under low O_2 tension or other "stress" conditions [1]. The enzyme catalyses the reduction of O_2 to H_2O by ubi- or menaquinol [2–5]. The reaction is electrogenic [6–8], but is not coupled to transmembrane proton pumping [9].

Although the X-ray structure of cytochrome *bd* is not available yet, this oxidase does not reveal apparent sequence homology to oxidases of the heme-copper superfamily [10–12]. Cytochrome *bd* does not contain copper [13] and it is composed of two different membrane-integrated subunits, carrying three redox cofactors: two protoheme IX groups (hemes b_{558} and b_{595}) and a chlorin (heme *d*) [14]. The low spin heme b_{558} is presumed to be involved directly in quinol oxidation [2]. The high spin heme *d* is the place where O_2 chemistry occurs [2], whereas the high spin heme b_{595} has been proposed to play a role analogous to that of Cu_B in heme-copper oxidases, forming together with heme *d* a bimetallic O_2-reducing site [15–21].

Currently there is growing interest in interactions of terminal oxidases with nitric oxide (NO) [22–24]. Particular emphasis is on the mechanism by which NO rapidly and potently inhibits these enzymes in competition with O_2, as well as on the chemistry occurring at the heme-Cu binuclear site following NO binding. Following the hypothesis that heme-copper oxidases are phylogenetically related to bacterial NO-reductase [25,26], it has been reported that some bacterial heme-copper oxidases under anaerobic reducing conditions are able to reduce NO to N_2O [27–29]; such an activity has never been tested in *bd*-type oxidases. Recently, cytochrome *bd* has been shown to enable strictly anaerobic, pathogenic bacteria to survive even in the presence of nanomolar O_2 concentrations [30]. In bacterial infections, NO is produced by the host as part of the immune response, so it seems important to investigate how NO interacts with *bd*-type oxidases.

In this work, we characterized the interaction of NO with cytochrome *bd*-type oxidases, specifically those isolated from *Escherichia coli* and *Azotobacter vinelandii*. In particular, we investigated the inhibition pattern of these enzymes by NO, as well as their ability to consume NO under anaerobic reducing conditions.

2. Materials and methods

Cytochrome *bd* from *E. coli* strain GO105/pTK1 was isolated as described [31], omitting the final hydroxyapatite chromatographic step. Cytochrome *bd* from *A. vinelandii* strain MK8 overproducing cytochrome *bd* was isolated essentially as previously reported [32].

Static absorption spectra were recorded with a double beam UV/Vis spectrophotometer (Jasco V-550) with a light path of 1 cm.

Cytochrome *bd* concentration was determined from the reduced *minus* 'air-oxidized' difference absorption spectra using $\Delta\varepsilon_{628-607}$ of 10.8 $mM^{-1}\,cm^{-1}$ for the *E. coli* enzyme [18], and $\Delta\varepsilon_{628-605}$ of 12 $mM^{-1}\,cm^{-1}$ for the *A. vinelandii* enzyme [32]. The concentration of human hemoglobin (Hb), purified according to [33], is expressed on the heme basis, using ε_{555} of 12.5 $mM^{-1}\,cm^{-1}$ for the deoxygenated state.

Stock solutions of NO (Air Liquide, Paris, France) were prepared by equilibrating degassed water at room temperature with pure NO at 1 atm. NO concentration in stock solutions was measured by spectrophotometric titration of bovine cytochrome *c* oxidase.

Simultaneous amperometric NO and O_2 measurements were performed using Clark-type selective electrodes (ISO_2 and ISO-NO MarkII, World Precision Instruments, Sarasota, FL, USA) interfaced with a computer. The instrument is equipped with 2 mm probes inserted into a 1 mL gas-tight chamber. The assays were performed in 50 mM potassium phosphate buffer (pH 7.0) containing 40 μM EDTA,

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Abbreviations: TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; DTT, dithiothreitol; Hb, human hemoglobin

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and either 0.05% *N*-lauroyl-sarcosine (the *E. coli* enzyme) or 0.02% dodecyl-β-D-maltoside (the *A. vinelandii* enzyme).

The apparent inhibition constant (K_i) for NO inhibition was estimated by processing the amperometric traces using the software MATLAB (The Mathworks, South Natick, MA). The extent of the enzyme inhibition was assessed by first derivative analysis of O_2 traces and plotted as a function of the NO concentration measured in parallel.

3. Results

By absorption spectroscopy, we show that cytochrome *bd* both from *E. coli* and *A. vinelandii* in the reduced state binds NO at the level of heme *d* yielding a Fe^{2+}–NO nitrosyl-adduct, in agreement with previous reports [18,32,34]. Addition of micromolar concentrations of NO to the reduced enzyme indeed shifts the absorption peak of ferrous heme *d* from 629 to 641 nm, giving rise to a symmetric first derivative-shaped difference spectrum (not shown). In the present study, ascorbate and *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) were used as reductants to avoid the use of dithionite which promptly reacts with NO [35].

Fig. 1 shows that NO is a potent inhibitor of the O_2-reductase activity of cytochrome *bd* from *E. coli* with a fast complete inhibition at [NO] $\geqslant$ 0.5 μM. Analysis of the NO and O_2 experimental traces recorded in parallel yields an apparent inhibition constant (K_i) for NO of 100 ± 34 nM ($n = 10$) at [O_2] $\sim$ 70 μM (Fig. 1, inset). This value is not significantly different for the *A. vinelandii* enzyme and is in agreement with the observation that under similar experimental conditions direct addition of 100 nM NO to the cytochrome *bd* in turnover results in 50–60% inhibition of the O_2-reductase activity (not shown). At higher O_2 concentration ($\sim$1 mM O_2), a significantly greater apparent K_i value ($\sim$230 nM NO) was measured, proving competition between NO and O_2 (not shown).

NO in aerobic solution is spontaneously degraded by reacting with O_2 and, when NO concentration decays below 200 nM, cytochrome *bd* inhibition reverts and the enzyme gradually recovers the initial (prior to the NO addition) activity (Fig. 1). Kinetics of activity recovery was measured by using oxy-Hb as NO scavenger. As shown in Fig. 2, upon NO depletion after Hb addition, a rapid and full recovery of the O_2-reductase activity of cytochrome *bd* is observed. Similar patterns were observed using the enzyme isolated from *A. vinelandii* (not shown).

The ability of cytochrome *bd* oxidases from both *E. coli* and *A. vinelandii* to degrade NO under anaerobic reducing conditions was tested by amperometry in the 3–30 μM NO concentration range according to [36], by using either dithiothreitol (DTT)/Q_1 or ascorbate/TMPD as reductants. As shown in Fig. 3, such an activity is not detected with the *E. coli* enzyme, and similar results are obtained using the *A. vinelandii* oxidase (not shown). In the presence of excess reductants, NO indeed slowly decomposes at a rate that is essentially unchanged upon addition of cytochrome *bd*, either pre-reduced or as isolated.

4. Discussion

Cytochrome *bd* has been shown to enable strictly anaerobic, pathogenic bacteria to survive even in the presence of nanomolar O_2 concentrations [30]. As NO is produced by the host as part of the immune response to bacterial infections, we investigated how NO interacts with two different *bd*-type oxidases (from *E. coli* and *A. vinelandii*) under both aerobic and anaerobic conditions.

In agreement with previous reports [18,32,34], by absorption spectroscopy we showed that the ferrous heme *d* of cytochrome *bd* oxidases binds NO, like CO and O_2, giving rise to a red shift of its absorption band at $\sim$630 nm. Turnover of both the *bd*-type oxidases tested in this study was promptly inhibited by NO (Figs. 1 and 2), a finding consistent with the observation that respiration of *E. coli* cells expressing cytochrome *bd* is sensitive to NO [37]. The estimated apparent K_i value (100 ± 34 nM at 70 μM O_2) is comparable to that of mammalian cytochrome *c* oxidase ($\sim$60 nM at 30 μM O_2, cf. [38]), and is dependent on O_2 concentration, as previously demonstrated for the mitochondrial enzyme [38]. The latter observation leads to the

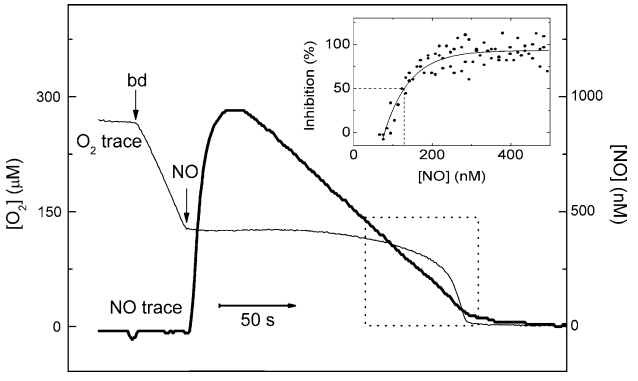

Fig. 1. Inhibition of cytochrome *bd* oxidase activity by NO. O_2 and NO traces recorded in parallel. O_2-reductase activity of cytochrome *bd* (17 nM) sustained by 4 mM DTT and 0.25 mM Q_1 is rapidly inhibited by addition of 1 μM NO, to be fully recovered at low NO concentrations (dotted box). Inset: extent of cytochrome *bd* inhibition at [NO] $\leqslant$ 500 nM. Data do not intercept the origin as invariably observed and tentatively explained by limitations in the NO electrode response at very low NO concentrations.

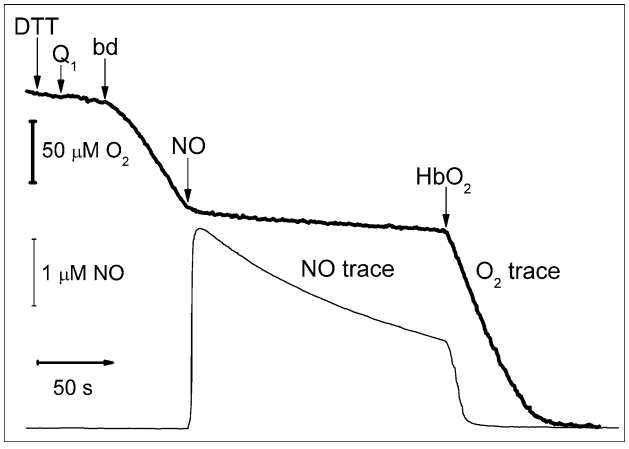

Fig. 2. Recovery of cytochrome *bd* oxidase activity upon NO scavenging. Conditions as in Fig. 1, except that, about 3 min after addition of 3 μM NO, 15 μM Hb-O_2 was added to promptly scavenge bulk NO, resulting in a fast and complete recovery of the enzyme activity.

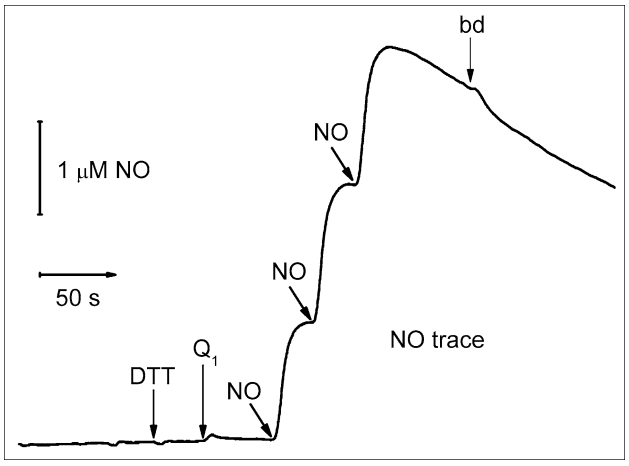

Fig. 3. NO reductase activity test. Three aliquots of 1.5 μM NO were successively added to degassed buffer containing 4 mM DTT, 0.25 mM Q_1, as well as 5 mM ascorbate and 5 units/mL ascorbate oxidase to scavenge residual O_2. Addition of 70 nM *E. coli* cytochrome *bd*, though associated with a small decrease in NO concentration (partly due to NO binding to the enzyme), did not significantly change the rate of NO consumption.

conclusion that, also in the *bd*-type oxidases, NO inhibition occurs in competition with O_2 and that in vivo at very low O_2 concentration NO inhibition should be even more effective. Assuming that NO and O_2 combine with ferrous heme *d* with similar rates, as originally proposed for mitochondrial cytochrome *c* oxidase [39], the fast onset of inhibition by NO even in the presence of a large excess O_2 suggests the existence of catalytic intermediates displaying preferential reactivity for NO over O_2 (see [23] for a review on this issue). Such a hypothesis demands further investigation.

The NO inhibition of the isolated cytochrome *bd* oxidases is fully reversible (Figs. 1 and 2), as previously shown for cytochrome *c* oxidase [38–40]. Following NO depletion upon addition of excess oxy-Hb, the recovery of activity appears to be faster in the *bd* oxidases than in mitochondrial cytochrome *c* oxidase assayed under similar conditions, i.e. in the presence of excess reductants [41]. Under such conditions, the recovery of activity of the mitochondrial enzyme is rate-limited by NO dissociation from the enzyme into the bulk phase [41]. Therefore, if a similar Fe^{2+}–NO adduct forms in the *bd* oxidases, the faster recovery of activity should correlate with a higher k_{off} of NO dissociation from reduced cytochrome *bd*.

Differently from some bacterial heme-copper oxidases [27–29], the oxidases of the *bd*-type tested in this study do not reveal NO reductase activity (<0.1 mol NO/mol *bd* × min to be compared for instance with ~100 mol NO/mol enzyme × min, as measured for the cytochrome cbb_3 oxidase from *Pseudomonas stutzeri* [28]). The inability of cytochrome *bd* to catalyze the reduction of NO suggests that Cu_B plays a key role in this reaction, as originally proposed [27]. Thus, the presence of a non-heme metal ion in the active site (either Fe_B in bacterial NO reductase [42,43], or Cu_B in heme-copper oxidases) seems to be a prerequisite for the NO reductase activity in terminal oxidases. Moreover, the lack of such an activity in the mitochondrial beef heart cytochrome *c* oxidase [36] and in the "nitric oxide reductase homolog" from *Roseobacter denitrificans* [44], both containing a copper ion in the binuclear site, suggests that the reduction of NO by terminal oxidases requires specific structural features of Cu_B surroundings [27], probably modulating the affinity of Cu_B^+ for NO. In this context it is of interest that, upon CO photolysis, Cu_B^+ acts as a better trap for the photodissociated ligand in those oxidases endowed with NO-reductase activity [45,46]. Accordingly, no transient binding of photodissociated CO to reduced heme b_{595} has been measured in *bd*-oxidases [21]. On this basis, the low affinity for CO and NO reported for ferrous heme b_{595} [15,17,18,20,32], possibly replacing functionally Cu_B in *bd* oxidases, may provide a rationale for the lack of NO-reductase activity in this enzyme. An important physiological corollary is that cytochrome *bd* does not contribute significantly to bacterial NO detoxification mechanisms, which are dominated aerobically by the flavohemoglobin Hmp [37].

In conclusion, we provide evidence that the *bd*-type bacterial terminal oxidases tested in this study are: (i) quickly, potently and reversibly inhibited by NO (as mitochondrial cytochrome *c* oxidase) and (ii) unable to degrade NO under reductive anaerobic conditions.

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