Cytochrome *bd*, a key oxidase in bacterial survival and tolerance to nitrosative stress

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Abstract. Cytochrome *bd* is a quinol respiratory oxidase widely distributed among bacteria, where its expression favours survival under low O_2 tensions, and in the presence of nitric oxide (NO) produced by the host immune system. NO reacts with and reversibly inhibits the haem-copper terminal oxidases (HCO) where it binds at the active site, containing a haem-iron and a copper (Cu_B). NO reacts also similarly with the copper-lacking active site of cytochrome *bd*, a structural peculiarity that allows one to address the question of whether Cu_B plays a role in the reaction with NO (and other ligands). In this minireview we discuss the properties of the reactions between *bd*-type oxidases and NO, and highlight consequences to cell/bacteria physiology.

Key words: Cytochromes, ROS, Nitric oxide, Microbiology.

INTRODUCTION

Cytochrome *bd* as purified from *Escherichia* coli and *Azotobacter vinelandii* is a respiratory oxidase, which couples the oxidation of ubi- or menaquinol to the reduction of O_2 to H_2O (1). The overall reaction is electrogenic (2), but not coupled to proton pumping (3). The enzyme does not display homology to haem-copper oxidases (HCO), the latter comprising a haem iron and a copper metal (Cu_B) in the active site (4). It is a heterodimer of two integral membrane polypeptides, subunit I and II, carrying three haem cofactors, but not copper. Subunit I contains the low spin haem b_{558}, the site of quinol oxidation. O_2 chemistry occurs at the high spin haem *d* in subunit II, whereas the function of the haem b_{595} is not fully understood yet. It has been proposed that this redox site may play the role of Cu_B in HCO, forming together with haem *d* a bimetallic active site (5, 6). Cytochrome *bd* shows a peculiar high affinity for O_2, and is expressed preferentially under stress conditions such as microaerobicity (1); this property allows strict anaerobic bacteria to survive even in the presence of nanomolar O_2 concentrations (7). Consistently, genetic deletion of cytochrome *bd* in pathogens (e.g., *Brucella abortus, Shigella flexneri, Mycobacterium tuberculosis* (8-10)) results into a lower capacity to infect the host oxygen-poor environments. Notably, nitric oxide (NO) is produced by the immune system to counteract bacterial infection, by targeting a variety of biomolecules and inactivat-ing critical enzymes. On the other hand, microorganisms have evolved mechanisms to degrade NO.

The respiration of *E. coli* cells expressing cytochrome *bd* has been reported to be sensitive to NO (11), as it is respiration of mammalian cells where cytochrome *c* oxidase (CcOX) in the mitochondrial respiratory chain is the primary target of NO (12-14). Mitochondrial CcOX has been shown to be reversibly inhibited by nanomolar levels of NO in both an oxygen dependent and oxygen independent manner (15-19). In the former case prevailing at high electron fluxes, NO binds to Fe^{2+} of haem a_3 yielding a nitrosyl adduct, whereas in the latter NO is metabolised to nitrite, that in turn binds to Fe^{3+} of haem a_3, thus also inhibiting the enzyme. Inhibition of CcOX by NO has been proposed to have both direct (control of cell respiration) and indirect (cell signalling) effects (20). Under reducing conditions, and in the absence of O_2, some bacterial terminal oxidases are able to catalyse a slow, but significant reduction of NO to N_2O (21-23), consistently with the hypothesis that haem-copper oxidases are phylogenetically related to the bacterial NO reductase (24, 25).

Of patho-physiological relevance, the ability of bacterial HCO to metabolise NO (in the presence/absence of O_2) might provide an additional pathway to dispose the NO produced by the host immune system. Since cytochrome *bd* is expressed in numerous human pathogenic bacteria, the understanding of the mechanism by which this peculiar enzyme reacts with NO is of interest. Moreover, the

absence of copper in cytochrome *bd* makes this enzyme a unique model to test the role of Cu_B in the reaction with both O_2 and NO.

REACTIONS OF NO WITH CYTOCHROME *bd*

In the Cu-lacking *bd*-type oxidases, NO, as CO and O_2, binds to ferrous haem *d* with high affinity, yielding a nitrosyl-adduct (26, 27). The capability of this enzyme to reduce NO to N_2O under anaerobic reducing conditions has been tested amperometrically (28). In this study we used cytochrome *bd* purified from *E. coli* or *A. vinelandii* as model. Unlike some bacterial HCO (21) cytochrome *bd* was found to lack NO-reductase activity. This finding argues against the hypothesis that this oxidase can metabolise NO to N_2O, while it suggests that a non-haem metal in the active site (copper in HCO or iron in bacterial NO-reductase) is required for reduction of NO. Moreover, since the Cu_B-containing mammalian CcOX is not endowed with NO reductase activity (29), one may also conclude that such an activity requires some yet unknown structural features in the surrounding of this metal.

The presence of Cu_B is instead dispensable for the inhibitory action of NO (28). The O_2 consumption catalysed by cytochrome *bd* from *E. coli* and *A. vinelandii* is indeed rapidly inhibited upon addition of $\leq 1 \mu M$ NO (Fig. 1), though activity promptly recovers upon NO depletion. This gas in air-equilibrated solution slowly degrades spontaneously by reactin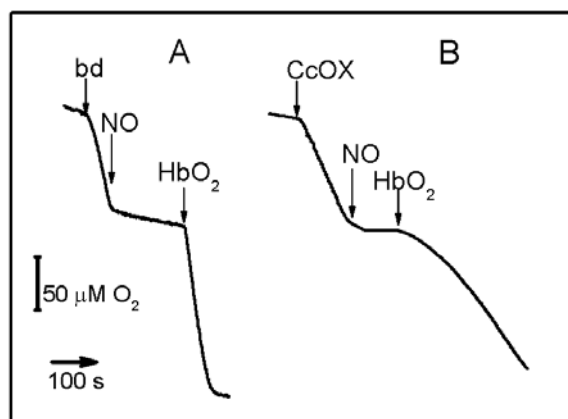g with O_2, but it is rapidly scavenged to nitrate by reacting with oxy-haemoglobin (oxy-Hb); notably, under these conditions, the reversal of NO inhibition of respiration (by oxy-Hb) is much faster in cytochrome *bd* than in mammalian CcOX (Fig. 1, see below). The estimated K_i value for cytochrome *bd* inhibition by NO (100 ± 34 nM at 70 μM O_2) is not very different from that reported for CcOX (12). Similarly to CcOX, also the NO-inhibition of cytochrome *bd* is set in competition with O_2 being more potent at lower oxygen concentrations. Therefore, *in vivo* during bacterial infection, when oxygen concentration is quite low and μM NO is produced by macrophages, inhibition of cytochrome *bd* should occur.

Fast inhibition of cytochrome *bd* by low NO concentrations has been observed even in the presence of a large excess of O_2. Since O_2 and NO are expected to have similar reactivity towards reduced haem d, we hypothesized the existence of a highly NO reactive catalytic intermediate accounting for the fast onset of the inhibition and not having haem d reduced. This hypothesis has been confirmed, as the ferryl-oxo intermediate (F) of the enzyme purified from *A. vinelandii* reacts rapidly with NO, yielding the oxidized enzyme with nitrite bound to oxidized haem *d* (30). The reaction occurs according to a 1:1 stoichiometry and proceeds at $k = 1.2 \pm 0.1 \times 10^5$ M^{-1} s^{-1} at 20°C, being even faster than the same reaction described for CcOX ($k \sim 1 - 2 \times 10^4$ M^{-1} s^{-1}). This additional reaction of NO may actually occur with cytochrome *bd* in turnover, accounting for the fast onset of inhibition observed at low NO/O_2 ratios and possibly contributing to the low K_i of inhibition (28). Since the reaction of NO with intermediate F has been observed for the mammalian enzyme and proposed to occur at Cu_B in the active site (15-19), the results obtained on the Cu-lacking cytochrome *bd* from *A. vinelandii* indicate that Cu_B is not essential or possibly not even involved in the reaction. It remains to be established whether such a reaction rather i) involves haem b_{595}, with this haem functionally mimicking Cu_B in haem-copper oxidases, or ii) proceeds by direct reaction of NO with haem d in the oxo-ferryl state ($Fe^{IV}=O$), as documented with myoglobin and haemoglobin (31, 32).

LIGAND (NO) DISSOCIATION FROM THE ACTIVE SITE

After NO depletion by oxy-Hb, NO-inhibited cytochrome *bd* recovers activity remarkably faster than CcOX under similar experimental conditions (Fig. 1). Under high electron flux conditions, NO binds to CcOX yielding a nitrosyl adduct and the activity recovery is rate-limited by the dissociation of NO from ferrous haem a_3 (33). Since cytochrome *bd*

Figure 1
Recovery from NO inhibition of cytochrome bd oxidase. O_2 consumption catalysed by (A) 17 nM cytochrome bd and sustained by 0.25 mM coenzyme Q1, 4mM DTT, in K/ phosphate buffer pH 7.0 + 0.05% N-lauroyl-sarcosine, to be compared with (B) 50nM mammalian cytochrome c oxidase, in the presence of 18 μM horse heart cytochrome c,10mM ascorbate, 0.2 mM TMPD, in K/HEPES buffer pH 7.3 + 0.1% dodecyl- β-D-maltoside. Notice that oxygen consumption inhibited by NO is fully restored after NO scavenging (μM Hb-O_2), the reactivation kinetics being faster in cytochrome bd than in mammalian oxidase.

yields a similar Fe^{2+}-NO adduct, the faster recovery of activity should correlate with a faster NO dissociation from ferrous haem d. This hypothesis was validated by time-resolved spectrophotometry. Unlike the other respiratory oxidases, cytochrome *bd* forms stable complexes with gaseous ligands (O_2, NO, CO) also in the so-called single-electron reduced "mixed valence" (MV) state, whereby haem d is reduced and ligand-bound, while the *b*-type haems are oxidized. The rate of ligand dissociation from haem d was thus measured both for the fully reduced (R) and MV enzyme (30), and compared with available information on CcOX (see Table I). The measurements were performed by mixing in a stopped-flow apparatus NO-bound cytochrome *bd* with air-equilibrated buffer in the presence of an excess of oxy-myoglobin (oxy-Mb). Under these experimental conditions, oxy-Mb rapidly scavenges NO ($k = 3\text{-}4 \times 10^7$ M^{-1} s^{-1} (34)) yielding met-Mb, which then accumulates at the rate of NO dissociation from haem d. As shown in Fig. 2, at 20°C NO dissociates mono-exponentially from R and MV cytochrome *bd* at $k = 0.133 \pm 0.005$ and 0.036 ± 0.003 s^{-1}, respectively. The results indicate that the redox state of the *b*-type haems, particularly haem b_{595}, controls the pathway and/or the kinetic barrier for ligand dissociation from the active site of cytochrome *bd*. Even more interesting, the rate of NO dissociation from reduced haem d in cytochrome *bd* is about 30 fold higher than the off-rate from fer-

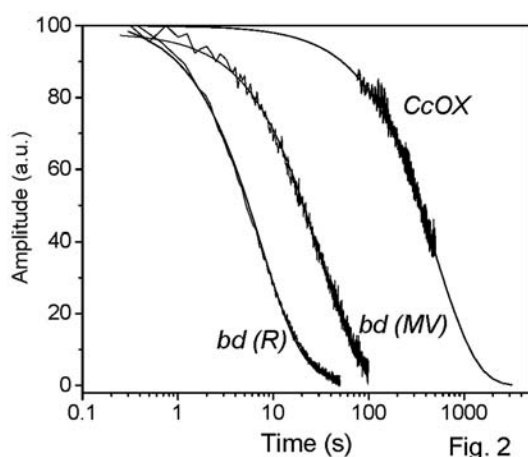rous haem a_3 of CcOX under similar experimental conditions ($k = 0.004$ s^{-1}, Table I). The higher k_{off} value for NO measured for cytochrome *bd* is consistent with the observation that NO inhibition reverts more rapidly in the case of cytochrome *bd* than in CcOX. CO dissociation from fully reduced cytochrome *bd* is also much faster than from reduced CcOX ($k = 6.0 \pm 0.2$ s^{-1} vs $k = 0.023$ s^{-1}), thus suggesting that in HCO Cu_B may control ligand escape from the nearby haem to the bulk phase (35).

Based on these data, we propose a mechanism by which microaerobic bacteria can increase their pathogenicity. During infection, these bacteria, by preferentially expressing *bd*-type, rather than haem copper-type oxidases enhance both their reactivity towards O_2 and tolerance to nitrosative stress, thus evading the host immune attack based on NO production.

CONCLUDING REMARKS

Depending on the intracellular concentration, NO plays a key role in a wide range of physiological and pathological processes. Due to its cytotoxic effects, in higher eukaryotes, NO is produced by the immune system to fight microbial infection, thus an enhanced tolerance to nitrosative stress may promote pathogenicity of some microorganisms. NO detoxification in microbes is mainly accomplished by enzymes endowed with NO-reductase or NO-dioxygenase activity, such as the NO-reductase flavorubredoxin (36, 37) and the flavohemoglobin (38) in *E. coli*. Cytochrome *bd* oxidase does not reduce NO to N_2O under anaerobic conditions, however likely contributes to enhance bacterial tolerance to nitrosative stress since, compared to CcOX: i) the F catalytic intermediate can metabolise NO to the less toxic nitrite at a rate one order magnitude higher (39), ii) the inhibited nitrosylated enzyme is more unstable as testified by the higher rate of NO dissociation from the reduced adduct. The structural features accounting for such differences are yet unknown, as the X-ray structure of cytochrome *bd* is not available.

We propose that the expression of cytochrome *bd* might be a tool for microaerophylic microorganisms not only to survive low O_2 tension but also to counteract the NO produced by the host immune system, thus increasing virulence. This conclusion is supported by the recent finding that in some pathogenic bacteria expression of cytochrome *bd* enhances during specific phases of the infection (10) or when bacteria are exposed to sublethal levels of NO (40).

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Figure 2
Kinetics of NO dissociation. NO dissociation from cytochrome bd in the mixed valence (MV) or in the reduced (R) state; absorbance changes followed at 581nm, after mixing the NO-bound adduct with air equilibrated buffer in the presence of Mb-O_2 in excess (2 fold) with respect to the oxidase concentration (T = 20°C). Buffer: K/ phosphate buffer, pH 7 + 0.05 % N-lauroyl-sarcosine. The experimental traces were fitted to a single exponential decay, yielding k = 0.133 $\pm$ 0.005 and k= 0.036 $\pm$ 0.003 s^{-1} for R and MW cytochrome bd, respectively. The kinetic profile of the NO dissociation from reduced mammalian cytochrome c oxidase is shown for comparison, k = 0.002 $\pm$ 0.001 s^{-1}.

Table 1
Ligand dissociation rate from cytochrome bd and mitochondrial aa₃ oxidase (T = 20°C).

	Cytochrome bd	Cytochrome c oxidase
R-NO	$0.133 \pm 0.005 \ s^{-1(a)}$	$0.004 \ s^{-1(b)}$
MW-NO	$0.036 \pm 0.003 \ s^{-1(a)}$	
R-CO	$6.0 \pm 0.2 \ s^{-1(a)}$	$0.023 \ s^{-1(c)}$
MW-CO	$4.2 \pm 0.34 \ s^{-1(a)}$	

[a]*ref 30*
[b]*ref 15*
[c]*Gibson & Greenwood Biochem. J. 86,541-54 1963*

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