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Biochemical and Biophysical Research Communications 355 (2007) 97–102
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Redox control of fast ligand dissociation from *Escherichia coli* cytochrome *bd*

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Received 18 January 2007
Available online 30 January 2007

Abstract

Bacterial *bd*-type quinol oxidases, such as cytochrome *bd* from *Escherichia coli*, contain three hemes, but no copper. In contrast to heme–copper oxidases and similarly to globins, single electron-reduced cytochrome *bd* forms stable complexes with O_2, NO and CO at ferrous heme *d*. Kinetics of ligand dissociation from heme d^{2+} in the single electron- and fully-reduced cytochrome *bd* from *E. coli* has been investigated by rapid mixing spectrophotometry at 20 °C. Data show that (i) O_2 dissociates at 78 s^{-1}, (ii) NO and CO dissociation is fast as compared to heme–copper oxidases and (iii) dissociation in the single electron-reduced state is hindered as compared to the fully-reduced enzyme. Presumably, rapid ligand dissociation requires reduced heme b_{595}. As NO, an inhibitor of respiratory oxidases, is involved in the immune response against microbial infection, the rapid dissociation of NO from cytochrome *bd* may have important bearings on the patho-physiology of enterobacteria.

Keywords: Bacterial respiration; Nitrosative stress; Pathogenicity; Stopped-flow spectroscopy

Cytochrome *bd* (cyt *bd*) is a bacterial respiratory quinol oxidase, preferentially expressed under microaerobic conditions [1,2], proposed to enable bacteria to survive under low O_2 tension [3,4]. Cyt *bd* conserves energy in the form of transmembrane H^+ electrochemical gradient [5,6], although it is not a proton pump [7]. Cyt *bd* differs from the heme–copper respiratory oxidases in that it contains three hemes (b_{558}, b_{595}, and *d*), but no copper [8]. The low spin heme b_{558} seems to be directly involved in ubiquinol oxidation, whereas the high spin heme *d* binds O_2 and catalyzes its reduction to H_2O [6,8–12].

At variance with the other respiratory oxidases and similarly to globins, cyt *bd* forms stable complexes with gaseous ligands (O_2, NO or CO) in a single electron-reduced "mixed valence" (MV) state, in which ligand-bound heme *d* is reduced (d^{2+}) while the other two hemes (b_{558} and b_{595}) are oxidized. This peculiarity makes cyt *bd* a unique model among respiratory oxidases to explore ligand binding/dissociation to/from heme d^{2+}, as a function of the redox state of the other metal centers.

Cyt *bd* is isolated from *Escherichia coli* membranes mainly in a stable oxygenated MV state (MV-O_2). Titration of the deoxygenated MV enzyme with O_2 recently allowed to determine $K_d = 280$ nM [13]. Surprisingly, this value, although relatively low, is an order of magnitude higher than $K_d = 25$ nM, as estimated by Hill et al. [14] on the

Abbreviations: cyt, cytochrome; K_d, dissociation constant; k_{on} and k_{off}, binding and dissociation rate constants; R, fully-reduced enzyme; MV, "mixed valence" (one-electron reduced) enzyme; Mb, myoglobin; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-1,4-phenylenediamine.

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doi:10.1016/j.bbrc.2007.01.118

basis of the measured *on* and *off* rates ($k_{on} = 2 \times 10^9\ M^{-1}\ s^{-1}$ and $k_{off} = 50\ s^{-1}$), assuming a simple reversible reaction.

Discussing possible reasons of the discrepancy, Belevich et al. [13] noted that the $k_{off} = 50\ s^{-1}$ was measured in [14] by ligand-exchange experiments, using CO to displace O_2. If CO did not compete with O_2 efficiently enough, this might lead to underestimation of k_{off}. As NO combines with ferrous hemeproteins more rapidly and with higher affinity than CO, we re-addressed this issue by performing O_2 displacement experiments using NO.

Moreover, we have determined the dissociation rates of NO and CO from *E. coli* cyt *bd* both in the MV and in the fully-reduced (R) state to assess whether ligand dissociation from heme d^{2+} is affected by the redox state of the hemes *b*. We observed that the dissociation rates of NO and CO are unusually high and increased when the hemes *b* are reduced. Such an information is relevant from a patho-physiological viewpoint, since NO is an inhibitor of respiratory oxidases [15] and an antimicrobial agent involved in the immune response against bacterial infection [16].

Materials and methods

Stock solutions of NO or CO were prepared by equilibrating degassed water with the pure gases at 1 atm and at room temperature, yielding 2 mM NO and 1 mM CO in solution.

Recombinant cyt *bd* was expressed in the *E. coli* strain GO105 devoid of cyt bo_3 and cyt *bd* oxidases according to [17]. Cyt *bd*, isolated according to [6,18], was mainly in the O_2-bound MV state (MV-O_2) with a minor fraction of enzyme in the ferryl-oxo (F) state. Enzyme concentration was determined from the reduced-*minus*-'as isolated' spectrum using $\Delta\varepsilon_{628-607} = 10.8\ mM^{-1}\ cm^{-1}$ [11]. Deoxygenated MV cyt *bd* was prepared by N_2-equilibration, followed by addition of glucose (2–3 mM), glucose oxidase (4–8 U/mL), and catalase (130–260 U/mL) to scavenge O_2. Cyt *bd* in the R state was prepared by incubating anaerobically the deoxygenated MV enzyme with 2 mM ascorbate and 0.5 μM TMPD. The CO- and NO-derivatives of cyt *bd* (MV-CO, R-CO, MV-NO, and R-NO) were prepared by addition to the enzyme in the R or the MV state of a slight excess of the gases in solution, immediately before the experiment. Oxygenated Mb (Mb-O_2) was prepared as reported in [19] and its concentration determined using $\varepsilon_{580} = 14.4\ mM^{-1}\ cm^{-1}$ and $\varepsilon_{542} = 13.9\ mM^{-1}\ cm^{-1}$.

Stopped-flow experiments were carried out with an instrument (Applied Photophysics, Leatherhead, UK), equipped with a monochromator or with a photodiode array detector. Ligand-exchange experiments were performed using monochromatic low-intensity light to prevent photodissociation. Data analysis was carried out using the software MATLAB (The Mathworks, South Natick, MA). Assays were performed at 20 °C in 50 mM K/phosphate buffer, pH 7.0, 0.05% *N*-lauroyl-sarcosine.

The dissociation rates of NO or CO from heme d^{2+} were determined by mixing in the stopped-flow apparatus ligand-bound cyt *bd* with air-equilibrated buffer. In the case of NO, experiments were carried out in the presence of Mb-O_2. In this type of experiments, originally carried out with bovine cyt *c* oxidase [19], NO dissociating from cyt *bd* is rapidly scavenged by Mb-O_2 ($k = 3$–$4 \times 10^7\ M^{-1}\ s^{-1}$ at 20 °C [20]), yielding met-Mb. Under proper conditions, met-Mb accumulation proceeds at the rate of NO dissociation and can be followed at 581 nm, which is isosbestic for the reaction of NO-bound cyt *bd* with O_2.

Results

O_2 dissociation

As shown in Fig. 1 (top panel), after mixing cyt *bd* 'as isolated' (i.e., mainly in the MV-O_2 state) with an excess of NO, two processes can be time resolved. Starting from the initial species (spectrum 1), within less than 100 ms the α band of heme *d* weakens and shifts from 645 to 641–642 nm (spectrum 2); thereafter, a further slower blue shift to 636 nm is observed concomitant with disappearance of the 680 nm shoulder (spectrum 3). The first reaction intermediate, detected at 75 ms after mixing

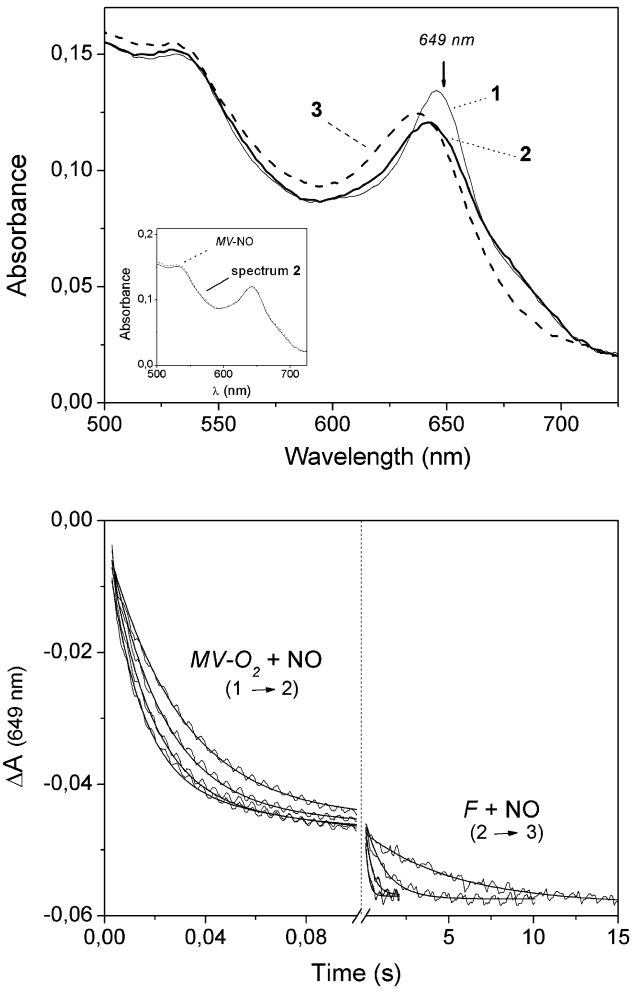

Fig. 1. Reaction of cytochrome *bd* 'as isolated' with NO. (Top) Absorption spectra collected at 2.5 ms (spectrum 1, thin solid), 75 ms (spectrum 2, thick solid), and 10 s (spectrum 3, dashed) after mixing cyt *bd* 'as isolated' with NO. O_2 concentration in the enzyme solution was reduced (<30 μM) prior to mixing by flushing N_2. (Inset) spectrum 2 from the main panel (solid) as compared to the spectrum of MV-NO cyt *bd* (dotted), independently obtained at 50 ms after mixing deoxygenated MV cyt *bd* with NO. Concentrations after mixing: enzyme = 6.5 μM; NO = 500 μM (main panel) or 50 μM (inset). (Bottom) Time courses collected at 649 nm (arrow in the top panel) after mixing 25.4 μM cyt *bd* 'as isolated' with NO at increasing concentration (0.1, 0.3, 1, and 2 mM, from right to left). Traces are shown with their best fits to the sum of two exponentials.

(spectrum 2), is the MV enzyme with NO bound at reduced heme *d* (MV-NO), as assessed independently by exposing deoxygenated MV cyt *bd* to NO (inset to Fig. 1). Thus, after mixing with NO, O_2 bound to heme d^{2+} is rapidly displaced and replaced by NO without redox changes at the level of the hemes (transition from spectrum 1 to spectrum 2 in Fig. 1). Thereafter, the loss of the 680 nm band suggests that this ligand-exchange process is followed by the slow (several seconds) reaction of NO with the minor fraction of ferryl-oxo (F) enzyme initially present in cyt *bd* 'as isolated' (transition from spectrum 2 to spectrum 3 in Fig. 1).

The time courses observed by monitoring the reaction at 649 nm at increasing NO concentration can be fitted by the sum of two exponentials (Fig. 1, bottom panel). The first phase accounts for the O_2/NO exchange at heme d^{2+}, with a rate constant approaching a plateau value of $\sim 78\ s^{-1}$ at 1 mM NO (Fig. 2). This rate-limiting value, observed also starting from partially oxygenated heme d^{2+} (not shown), is assigned to the k_{off} of O_2 bound to heme d^{2+} in the MV enzyme. The slower kinetic phase, assigned to the reaction of NO with the fraction of cyt *bd* initially in the F state, displays a rate constant linearly dependent on [NO], with an apparent second order rate constant $k = 5.0 \pm 0.5 \times 10^3\ M^{-1}\ s^{-1}$ (not shown). A very similar optical transition, although faster ($k = 1.2 \pm 0.1 \times 10^5\ M^{-1}\ s^{-1}$) has been recently reported [21] for the reaction of NO with the F intermediate of *Azotobacter vinelandii* cyt *bd*.

Over the explored NO concentration range, the amplitudes of both the fast and the slow kinetic phases do not vary (Fig. 1, bottom panel), indicating that ligand exchange is complete even at the lowest NO concentration tested (50 μM after mixing). NO is thus very efficient in displacing O_2 from heme d^{2+}. In contrast, when carbon monoxide (CO) was employed in similar ligand-exchange experiments, significantly lower O_2 dissociation rates were measured (Fig. 2); this shows that CO, used to determine the k_{off} of O_2 from cyt *bd* in previous studies [14], is not as efficient as NO in displacing O_2.

NO dissociation

NO-bound cyt *bd*, both in the MV and the R state, was mixed in the stopped-flow apparatus with air-equilibrated buffer containing Mb-O_2 and the release of NO from the enzyme followed monitoring the accumulation of met-Mb at 581 nm (see Materials and methods). In these experiments, free NO is scavenged by Mb-O_2 within the dead-time of the instrument (<2 ms); thereafter, the NO bound to heme d^{2+}, displaced by O_2, dissociates and oxidizes Mb-O_2.

As shown in Fig. 3, NO dissociates monoexponentially at $k = 0.133 \pm 0.005$ and $0.036 \pm 0.003\ s^{-1}$ from R and MV cyt *bd*, respectively. We noticed that with the MV enzyme, no further processes occur after NO displacement, whereas with the R enzyme, the NO/O_2 exchange is followed by complete oxidation of cyt *bd*. As expected, upon mixing Mb-O_2 with NO in solution, the oxidation of Mb-O_2 occurs within the dead-time of the instrument (not shown).

CO dissociation

In a similar experiment, the kinetics of CO dissociation from cyt *bd* in the MV and R state was investigated using O_2 as the displacing ligand. CO displacement from the MV enzyme was detected as a monoexponential absorption

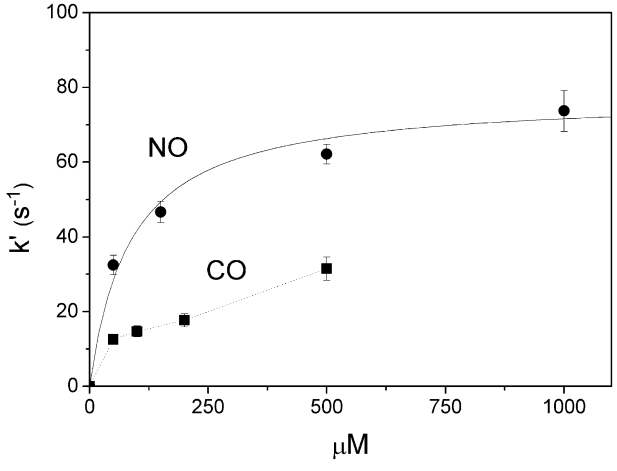

Fig. 2. O_2 dissociation. Rate constants observed by displacing O_2 bound to MV cyt *bd* with either NO (circles) or CO (squares) at different concentrations. In the case of NO, the rate constants of the fast phase (traces in Fig. 1, bottom panel) are shown together with their best fit to a hyperbola, yielding a plateau value (k_{off} for O_2 dissociation) of $78 \pm 0.5\ s^{-1}$. Experimental conditions as in Fig. 1.

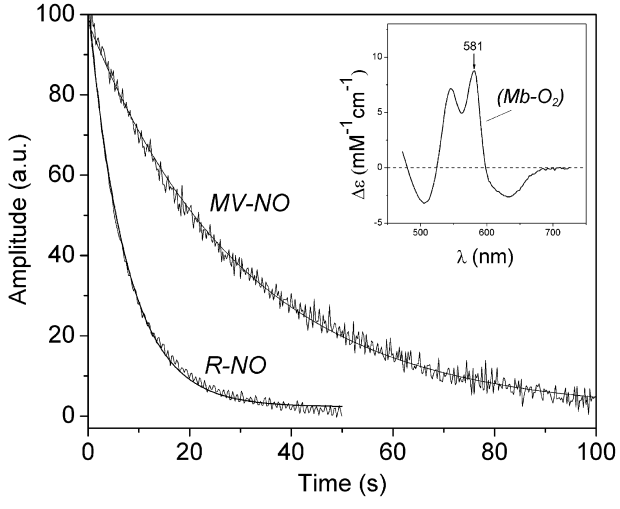

Fig. 3. NO dissociation. Time course of NO dissociation from cyt *bd*, collected at 581 nm after mixing NO-bound enzyme either in the MV or in the R state with air-equilibrated buffer containing Mb-O_2. Concentrations after mixing: enzyme = 6 μM; Mb-O_2 = 15 μM; NO = 7.5–10 μM. Traces were fitted to single exponentials, yielding $k = 0.036 \pm 0.003$ and $0.133 \pm 0.005\ s^{-1}$ for MV-NO and R-NO, respectively. (Inset) difference absorption spectrum of Mb-O_2 *minus* met-Mb.

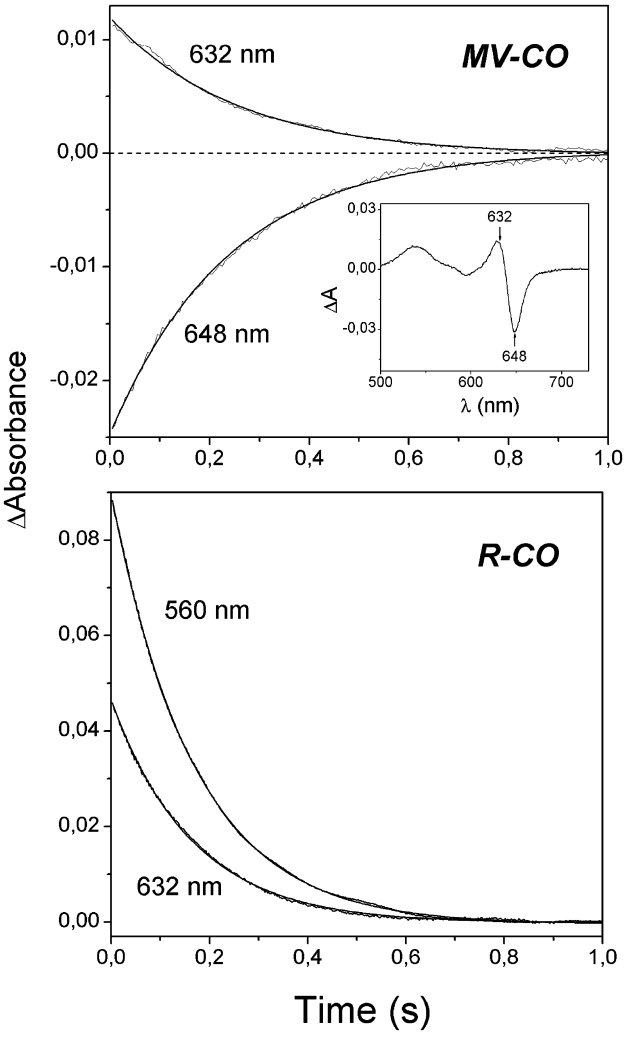

Fig. 4. CO dissociation. Time course of CO dissociation from cyt *bd*, collected after mixing CO-bound enzyme either in the MV (Top) or in the R state (Bottom) with air-equilibrated buffer. Concentrations after mixing: enzyme = 6 μM; CO = 10 μM. Traces are shown with their best fits to single exponentials, yielding $k = 4.2 \pm 0.34$ and $6.0 \pm 0.2\ s^{-1}$ for MV-CO and R-CO, respectively. (Inset) difference absorption spectrum of MV-CO *minus* MV-O_2.

decrease at 632 nm and a synchronous absorption increase at 648 nm, proceeding at $k = 4.20 \pm 0.34\ s^{-1}$ (Fig. 4, top panel). The reaction with O_2 of the CO-bound enzyme in the R state, monitored at 632 and 560 nm, was faster and monoexponential ($k = 6.0 \pm 0.2\ s^{-1}$), rate-limiting the complete oxidation of the enzyme by O_2 (Fig. 4, bottom panel).

Discussion

In this work, we measured the rates of ligand dissociation from cyt *bd* purified from *E. coli*, specifically focusing on the dissociation of O_2 and NO, because the latter reactions are directly relevant to bacterial physiology. Two aspects of the results obtained are to be emphasized.

Oxygen affinity of cytochrome bd

The K_d value for O_2 binding to cyt *bd* recently determined by equilibrium titrations (280 nM, [13]) proved to be much higher than $K_d = 25$ nM previously estimated by Hill et al. [14] from the measured rate constants ($k_{on} = 2 \times 10^9\ M^{-1}\ s^{-1}$ and $k_{off} = 50\ s^{-1}$), assuming a simple reversible reaction. Belevich et al. [13] suggested that the experimental procedure used in [14], based on O_2 displacement by CO, might have led to underestimation of k_{off} for O_2, if CO was not able to efficiently compete with O_2. Therefore, we have re-addressed this issue by using NO, instead of CO, to displace O_2 bound at heme d^{2+}, since NO binds to ferrous hemes with significantly higher rates and affinities as compared to CO.

Our data confirm that NO displaces O_2 more efficiently than CO (Fig. 2). Nevertheless, the k_{off} for O_2 determined with NO is only slightly higher than reported in [14] ($78 \pm 0.5\ s^{-1}$ vs $50\ s^{-1}$). Accordingly, the K_d of ~40 nM, calculated from $k_{off} = 78\ s^{-1}$ herein reported and $k_{on} = 2 \times 10^9\ M^{-1}\ s^{-1}$ [14], is still lower than the K_d value of 280 nM reported in [13]. Hence, the discrepancy between the data in Refs. [13] and [14] does not simply originate from the use of CO in ligand-exchange experiments, as proposed in [13].

Two possible reasons for this discrepancy may be as follows. First, O_2 binding might be a multi-step process, possibly including spectroscopically silent steps. Second, the k_{on} for O_2 binding was determined for cyt *bd* in the R state [14], whereas K_d was measured with the MV enzyme [13]; therefore, the data would be reconciled, if k_{on} for O_2 binding to the MV enzyme was ~7-fold slower than in the R state. Previous studies on cyt *bd* from *A. vinelandii* [22] showed that CO recombination to the MV enzyme is only ~1.5 times slower than that determined in the R state. In agreement with previous reports [23], we found that ligand dissociation from heme d^{2+} is slower in the MV state than in the R state (see below). It would be thus interesting to directly compare the k_{on} for O_2 binding to cyt *bd* in the MV and R states.

Fast ligand dissociation from cytochrome bd as compared to heme–copper oxidases.

We determined the rates of NO and CO dissociation from heme d^{2+} of cyt *bd* in both the R and MV states. By using Mb-O_2 as an optical probe and a trap for NO in solution, we found that in the R state, NO dissociates from heme d^{2+} at an unusually high rate, $k_{off} = 0.133 \pm 0.005\ s^{-1}$ (Fig. 3): this value is ~30-fold higher than the off-rate measured for ferrous heme a_3 of the mitochondrial cyt *c* oxidase under similar experimental conditions ($k_{off} = 0.004\ s^{-1}$ [24]). Likewise, CO dissociation from fully-reduced cyt *bd* is found to be very much faster than that in the fully-reduced bovine cyt *c* oxidase ($k_{off} = 6.0 \pm 0.2\ s^{-1}$ vs $k_{off} = 0.023\ s^{-1}$, [25]). These data are consistent with the proposal that in heme–copper oxidases Cu_B acts as a

Table 1
Kinetic and thermodynamic parameters of the reaction of *E. coli* cytochrome *bd* with O_2, CO, and NO at room temperature

Parameter	Ligand					
	O_2		CO		NO	
	MV-O_2	R-O_2	MV-CO	R-CO	MV-NO	R-NO
k_{on} (M^{-1} s^{-1})		2×10^9[b]		8×10^7[b]		
k_{off} (s^{-1})	78 ± 0.5[a] (50[b])		4.2 ± 0.34[a]	6.0 ± 0.2[a] (1.6[b])	0.036 ± 0.003[a]	0.133 ± 0.005[a]
K_d (nM)	280[c] (25[b])		75[a] (20[b])			

[a] This work.
[b] Ref. [14].
[c] Ref. [13].

gate controlling ligand binding to the heme in the active site [26].

Another remarkable feature of ligand dissociation from cyt *bd* is that the k_{off} values obtained for NO (0.036 ± 0.003 s^{-1}) and CO (4.20 ± 0.34 s^{-1}) in the MV state, although still quite high, are significantly lower than those measured with the R enzyme (Table 1). In agreement with previous studies [23], this finding shows that the redox state of the hemes *b*, presumably that of heme b_{595}, controls the kinetic barrier for ligand dissociation from the active site of cyt *bd*.

The unusually high NO dissociation rates from *E. coli* cyt *bd* found in this work may explain the observation [27] that the NO-poisoned cyt *bd* recovers respiratory function much more rapidly than the mitochondrial heme–copper cyt *c* oxidase (upon removal of free NO). NO is known to mediate part of the immune response against microbial infection [16]; therefore, the unusually fast dissociation of NO from *bd*-type oxidases leading to prompt restoration of respiration may be related to patho-physiology of bacterial infection. One can expect that expression of *bd*-type, instead of heme–copper-type, oxidases would enhance bacterial tolerance to nitrosative stress, thus promoting pathogenicity of the microorganisms. This hypothesis looks plausible in the light of the recent evidence that: (i) mutants of pathogenic bacteria deficient in cyt *bd* (e.g., *Brucella abortus*, *Shigella flexneri*, *Mycobacterium tuberculosis*) are characterized by a markedly reduced virulence [4,28,29]; (ii) expression of cyt *bd* in *M. tuberculosis* is transient and specifically up-regulated during just one specific phase of infection [4]; (iii) exposure of *M. tuberculosis* [4] and *Staphylococcus aureus* [30] to sub-lethal NO concentrations enhances expression of cyt *bd* in these pathogenic bacteria.

Acknowledgments

Work partially supported by the Russian Foundation for Basic Research (Grant 05-04-48096 to V.B.B.), by MiUR of Italy (PRIN "Meccanismi molecolari e aspetti fisiopatologici dei sistemi bioenergetici di membrana" to P.S.), by Howard Hughes Medical Institute (International Scholar Award 55005615) and Civilian Fund for Research and Development (RUB-1-2836-MO-06) to A.A.K. V.B.B. was recipient of an EMBO short-term Fellowship ASTF 118.00-05 and a short-term mobility Fellowship by CNR of Italy. We thank R.B. Gennis (Urbana, USA) for providing the *E. coli* strain GO105/pTK1, and I.F. Bukhova (Moscow, Russia) for growing *E. coli* cells.

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