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ACCELERATED PUBLICATION

Interaction of *bd*-Type Quinol Oxidase from *Escherichia coli* and Carbon Monoxide: Heme *d* Binds CO with High Affinity

V. B. Borisov

Department of Molecular Energetics of Microorganisms, Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, 119991 Moscow, Russia; fax: (495) 939-3181; E-mail: bor@genebee.msu.su

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Abstract—Comparative studies on the interaction of the membrane-bound and detergent-solubilized forms of the enzyme in the fully reduced state with carbon monoxide at room temperature have been carried out. CO brings about a bathochromic shift of the heme *d* band with a maximum at 644 nm and a minimum at 624 nm, and a peak at 540 nm. In the Soret band, CO binding to cytochrome *bd* results in absorption decrease and minima at 430 and 445 nm. Absorption perturbations in the Soret band and at 540 nm occur in parallel with the changes at 630 nm and reach saturation at 3-5 μM CO. The peak at 540 nm is probably either β-band of the heme *d*–CO complex or part of its split α-band. In both forms of cytochrome *bd*, CO reacts predominantly with heme *d*. Addition of high CO concentrations to the solubilized cytochrome *bd* results in additional spectral changes in the γ-band attributable to the reaction of the ligand with 10-15% of low-spin heme b_{558}. High-spin heme b_{595} does not bind CO even at high concentrations of the ligand. The apparent dissociation constant values for the heme *d*–CO complex of the membrane-bound and detergent-solubilized forms of the fully reduced enzyme are about 70 and 80 nM, respectively.

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Cytochrome *bd* is a terminal quinol oxidase of the respiratory chain of aerobic and facultatively anaerobic bacteria catalyzing the four-electron reduction of molecular oxygen to water.

To date, the two classes of the respiratory oxidases have been discovered. The first class comprises a numerous family of the heme/Cu-containing enzymes, which function as proton pumps and contain a binuclear oxygen-reducing active site that includes a high-spin heme and a copper ion. A typical example of the heme–copper oxidases is an aa_3-type cytochrome *c* oxidase; its structural and functional defects cause serious human pathologies [1-3]. The second class of terminal respiratory enzymes comprises the *bd*-type oxidases. The heme–copper oxidases are already quite well studied. In particular, with the use of X-ray analysis it was possible to resolve a three-dimensional structure of the aa_3-type cytochrome *c* oxidases from beef heart and from bacteria *Paracoccus denitrificans* and *Rhodobacter sphaeroides* [4-9], a ba_3-type cytochrome oxidase from a thermophilic bacterium *Thermus thermophilus* [10], and also a bo_3-type ubiquinol oxidase from *Escherichia coli* [11]. In comparison with the typical representatives of the heme–copper class such as aa_3- and bo_3-type oxidases, the cytochrome *bd* complex remains poorly studied [12, 13].

Cytochrome *bd* has no apparent homology with other known oxidases, such as cytochrome *c* oxidase and bo_3-type ubiquinol oxidase [13-15], does not contain copper and non-heme iron [16, 17], and does not function as a transmembrane proton pump though it generates a membrane potential [18-22]. Besides, cytochrome *bd* has unusually low sensitivity to cyanide, azide, and divalent metal cations [23]. The isolated enzyme is a stable oxygenated complex [24-26], which is possibly due to a high affinity of heme *d* for oxygen [27, 28].

The *bd*-type terminal oxidase is a key energy-transducing respiratory enzyme both in microorganisms harmless for humans and animals and in bacteria causing various diseases such as dysentery [29], pneumonia [30], sal-

Abbreviations: AEBSF) 4-(2-aminoethyl)benzenesulfonyl fluoride; k_{on}) second order binding rate constant; k_{off}) dissociation rate constant; Mb) myoglobin from sperm whale skeletal muscle; MCD) magnetic circular dichroism.

monellosis [31, 32], periodontitis [33], brucellosis [34], typhoid [32], and tuberculosis [35]. A positive correlation between virulence of bacterial pathogens responsible for these diseases and level of cytochrome *bd* expression was reported.

The cytochrome *bd* contents increase under unfavorable conditions, such as lower oxygen concentration [36], the presence of poisons in the environment (for example, cyanide), uncouplers-protonophores [37], sharp change in ambient temperature, and in a number of other cases when the traditional terminal oxidases are not able to function, whereas the "alternative" cytochrome *bd* successfully works [38, 39]. In nitrogen-fixing bacteria, cytochrome *bd* takes part in respiratory protection of nitrogenase against oxygen [40]. Cytochrome *bd* from *E. coli* is involved in regulation of the disulfide (-S–S-) bond formation upon protein folding in a cell [41]. The data on unusually high NO dissociation from the cytochrome *bd* active site (in comparison with that of cytochrome *c* oxidase and other heme–copper oxidases) elucidate how a *bd*-type enzyme can help a pathogenic enterobacterium to overcome immune protection of the host cell [42-44].

The accepted scheme for conversions of the oxygen intermediates in the cytochrome *bd* active site [21, 22, 45-47] is the following: O $\rightarrow$ R + oxygen $\rightarrow$ A $\rightarrow$ P $\rightarrow$ F $\rightarrow$ O, where O, R, A, P, F are oxidized, reduced, oxygenated, peroxy, and oxo-ferryl forms of cytochrome *d*, respectively.

The enzyme consists of the two subunits (58 and 43 kD), each being a typical integral membrane protein. These subunits carry three redox centers: one low-spin (heme b_{558}) and two high-spin (hemes b_{595} and *d*) [48, 49]. Heme b_{558} is located on subunit I, whereas hemes b_{595} and *d* are likely to be in the area of the subunit contact [50]. According to current thinking, all the three heme redox groups are located closer to the outer (periplasmic) side of the membrane [51].

The low-spin heme b_{558} apparently takes part in the oxidation of ubiquinol. The high-spin heme *d* binds oxygen and likely takes part in the oxygen-reducing reaction. The role of the high-spin heme b_{595} remains obscure. Some authors propose that heme b_{595} participates in the reduction of oxygen forming together with heme *d* a binuclear oxygen-reducing site, similar to the heme/Cu oxygen-reducing site in the aa_3-and bo_3-type oxidases [22, 52-57]. In the opinion of other researchers, the function of heme b_{595} is limited to transferring an electron from heme b_{558} to heme *d* [58, 59]. Some authors believe that heme b_{595} can form a second site capable of reacting with oxygen [60, 61].

It is important to understand whether the presence of a binuclear redox site in terminal oxidases is a prerequisite for the reduction of oxygen to water and, if so, for which of the steps of the catalytic cycle. An alternative could be the necessity for the presence of the second redox site to pump protons through the membrane. It is worth noting that terminal oxidases lacking the binuclear oxygen-reducing site have still not been found. Furthermore, the cytochrome bo_3 mutants lacking Cu_B (the second component of the oxygen-reducing site) are not able to bind and reduce oxygen [62]. However, the data available in the literature on the presence of such a site in cytochrome *bd* can hardly be considered as sufficiently valid. Formally the reaction of reduction of oxygen to water can be divided into two steps: the actually oxidase step where O_2 is reduced to H_2O_2, and the peroxidase step where the bound peroxide undergoes further reduction to water. The enzymes carrying out either the first part of the reaction (a quinol oxidase from the sea bacterium *Pseudomonas nautica* 617 [63-65]) or the second one (many peroxidases) [66-68] but not containing a binuclear site have been found. Furthermore, cytochrome cd_1-nitrite reductase found in many denitrifying bacteria can also catalyze the four-electron reduction of oxygen to water [69]. However, cytochrome cd_1-nitrite reductase does not contain a binuclear bimetallic site; only one redox center (heme d_1) is involved in the oxygen-reducing reaction [69]. Thus, deciphering the role of high-spin heme b_{595} is of particular interest.

Studying the interaction of heme-containing enzymes with ligands is one of the efficient and frequently used approaches for establishing the arrangement and functioning of the catalytic sites of these proteins. In the present work, a comparative study on the reaction of carbon monoxide with cytochrome *bd* incorporated into the *E. coli* membrane and the purified enzyme solubilized in the detergent micelles has been carried out.

MATERIALS AND METHODS

Reagents. Yeast extract and tryptone was purchased from Difco (USA); ampicillin, DNase I, kanamycin, and sodium N-lauroyl-sarcosinate were obtained from Fluka (Switzerland); carbon monoxide, dimethylsulfoxide, sodium dithionite, KCl, and $MgSO_4$ from Merck (Germany); myoglobin from sperm whale skeletal muscle (Mb), EDTA, Ches, and Hepes from Serva (Germany); DEAE-Sepharose Fast Flow, 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), leupeptin, and benzamidine from Sigma (USA); sucrose monolaurate from Anatrace (USA). Other reagents were of chemically pure grade produced in Russia. All aqueous solutions were prepared with distilled water that was additionally purified by means of a Milli-Q System (Millipore, USA).

Bacterial strain. The work was performed using the *bd*-type oxidase from *E. coli* (strain GO105/pTK1, kindly provided by Prof. R. Gennis from the University of Illinois at Urbana-Champaign, USA). This strain is convenient due to overexpression of the enzyme achieved by introduction of a plasmid containing the cytochrome *bd* gene. Also, cytochrome bo_3 (the second terminal oxidase

in *E. coli*) has been deleted in this strain, which allows obtaining the cytochrome *bd* preparation without impurity of another oxidase.

Growth of cells. The cells were grown aerobically in a fermenter (with controlled feed of O_2) or in flasks on a shaker (at 200 rpm) at 37°C. The medium contained 80 mM sodium phosphate, 2.5 mM sodium citrate, 19 mM ammonium sulfate, 1% tryptone, 0.5% yeast extract, 0.5% casamino acids, 0.01% L-tryptophan, 2% (v/v) glycerol, 0.8 mM $MgSO_4$, 0.18 mM $FeSO_4 \cdot 7H_2O$, 0.1 mM $CuSO_4 \cdot 5H_2O$, 0.005% kanamycin, and 0.01% ampicillin, pH 7.2. The cell growth was tracked by monitoring increase in turbidity at 600 nm. The cultivation typically took 22-24 h. On the late logarithmic growth phase, the cells were sedimented (5500*g*, 10 min, 4°C) and washed twice with medium containing 172 mM NaCl, 5 mM sodium phosphate, and AEBSF (on a spatula tip), pH 7.5.

Membrane preparation. The washed cells were suspended in medium containing 20 mM Tris, 0.5 mM EDTA, 5 mM $MgSO_4$, 20 mM benzamidine, 1 μM leupeptin, pH 8.3. Just before the cell disruption, AEBSF (on a spatula tip), 1 mM 1,4-dithiothreitol, and DNase I (0.01 mg/ml) were added to the suspension. The suspension was passed twice through a French press with 35 ml portions. Intact and partially destroyed cells were removed by centrifugation (14,500*g*, 15 min, 4°C). Subcellular vesicles were sedimented from the supernatant by ultracentrifugation (160,000*g*, 2 h, 4°C) and then homogenized with a desired medium. The membranes were stored at −70 to −80°C or in liquid nitrogen.

Isolation and purification of the enzyme. Cytochrome *bd* was solubilized and purified as described earlier by Miller and Gennis [70] with some modifications.

The thawed *E. coli* membranes were homogenized with a medium for equilibration of a chromatography column without detergent (50 mM potassium phosphate, 25 mM KCl, 5 mM EDTA, AEBSF, pH 6.5). The homogenate was supplemented with sucrose monolaurate (1.8% final concentration) and incubated in an ice bath upon stirring for 2 h. The suspension was centrifuged (160,000*g*, 60 min, 4°C), the pellet was discarded and the supernatant loaded on a DEAE-Sepharose Fast Flow column equilibrated with 50 mM potassium phosphate buffer also containing 25 mM KCl, 5 mM EDTA, and 0.1% sucrose monolaurate, pH 6.5. The column was washed with 100 ml of the above buffer at 100-120 ml/h flow rate and 4°C (the flow rate was set with a peristaltic pump (Pharmacia, Sweden)), then the elution was performed with a KCl gradient (25-350 mM) at 90-100 ml/h flow rate. Fractions of 7 ml were collected (LKB 2070 Ultrorac II Fraction Collector), monitoring absorption at 405 nm (spectrophotometer UA-5 with a flow-through cell, ISCO). The fractions with an absorbance ratio of $A_{412}/A_{280} \geq 0.7$ were collected for further work. These fractions were pooled and concentrated by ultrafiltration with the aid of an Amicon cell and a YM-30 membrane filter. The resulting solution was dialyzed at 4°C against 50 mM sodium phosphate buffer containing 5 mM EDTA and 0.05% sodium N-lauroyl-sarcosinate, pH 7.0, with buffer change every 5 h for two times. Sometimes gel filtration on a PD10 column prepacked with Sephadex G-25 (Pharmacia) was used instead of dialysis. The enzyme was stored with small portions (100-200 μl) in liquid nitrogen.

Spectroscopy. Absorption changes were recorded in an Aminco-SLM DW 2000 spectrophotometer in split beam mode in standard cuvettes at room temperature. The medium composition used in the experiments is described in the figure legends.

Protein concentration was measured by the methods of Lowry et al. [71] and Bradford [72] using bovine serum albumin as a standard. Cytochrome *bd* concentration was determined from the difference absorption spectra (dithionite-reduced *minus* "air-oxidized") using a millimolar extinction coefficient value of $\Delta\varepsilon_{628\text{-}607} = 10.8\ mM^{-1} \cdot cm^{-1}$ [54].

CO concentration was estimated assuming a solubility of this gas in water at 20°C and 1 atm to be 1 mM [73].

Treatment of the experimental data. Computer analysis of the experimental data was carried out with the aid of the software package GIM (Scientific Graphic Interactive Management System) developed by A. L. Drachev in the A. N. Belozersky Institute of Physico-Chemical Biology, M. V. Lomonosov Moscow State University.

Analysis of carbon monoxide titration curves of cytochrome *bd* spectral changes. CO titration curves of spectral changes of the dithionite-reduced cytochrome *bd* were processed using the GIM package according to Eq. (1):

$$A = 0.5\ \varepsilon(K_d + [d]_t + [CO]_t - \sqrt{(K_d + [d]_t + [CO]_t)^2 - 4[d]_t[CO]_t}), \quad (1)$$

where A is $\Delta A_{644\text{-}624}$ estimated from the absorption difference of the dithionite-reduced cytochrome *bd* in the presence and absence of CO, ε is extinction coefficient for the heme *d*–CO complex ($\Delta\varepsilon_{644\text{-}624}$), K_d is an apparent dissociation constant for the heme *d*–CO complex, $[d]_t$ is total concentration of cytochrome *d*, $[CO]_t$ is concentration of added CO.

Upon processing of the titration curves, ε and K_d were given as sought values.

Equation (1) was derived from the following equations:

$$K_d = \frac{[d]_f[CO]_f}{[d\text{–CO}]},$$

$$[CO]_f = [CO]_t - [d\text{–CO}],$$

$$[d]_f = [d]_t - [d{-}CO],\ A = \varepsilon\,[d{-}CO]\,L,$$

where L is light path length.

Determination of apparent dissociation constant (K_d) for the heme *d*–CO complex. An apparent K_d for the heme *d*–CO complex was determined according to Eq. (2):

$$K_2 = \frac{K_1[\text{Mb}{-}\text{CO}]([d]_t - [d{-}\text{CO}])}{[d{-}\text{CO}]([\text{Mb}]_t - [\text{Mb}{-}\text{CO}])}, \qquad (2)$$

where K_1 is an apparent dissociation constant for the Mb–CO complex; K_2 is an apparent dissociation constant for the heme *d*–CO complex; $[d]_t$ is total concentration of cytochrome *d*; $[\text{Mb}]_t$ is total concentration of Mb determined from $\Delta\varepsilon_{579\text{-}594} = 6.4\ \text{mM}^{-1}\cdot\text{cm}^{-1}$ for difference spectra (carboxymyoglobin (Fe^{2+}-CO) *minus* deoxymyoglobin (Fe^{2+})), from $\Delta\varepsilon_{435\text{-}480} = 121\ \text{mM}^{-1}\cdot\text{cm}^{-1}$ for absolute spectra of reduced myoglobin (deoxymyoglobin), and from $\Delta\varepsilon_{502\text{-}600} = 10.2\ \text{mM}^{-1}\cdot\text{cm}^{-1}$ for absolute spectra of oxidized myoglobin (Fe^{3+}) ($\Delta\varepsilon$ values for Mb were taken from the report of Wood [74]); [Mb–CO] is concentration of the Mb–CO complex determined as $[\text{Mb}]_t\cdot(\Delta A^{x\ \mu\text{M CO}}_{579\text{-}594})/(\Delta A^{1\ \text{mM CO}}_{579\text{-}594})$ (ΔA was taken from the difference spectra (CO + reduced *minus* reduced)); [*d*–CO] is concentration of the heme *d*–CO complex determined as $[d]_t\cdot(\Delta A^{x\ \mu\text{M CO}}_{643\text{-}623})/(\Delta A^{1\ \text{mM CO}}_{643\text{-}623})$ (ΔA was taken from the difference spectra (CO + reduced *minus* reduced)); $[\text{CO}]_t$ is concentration of added CO.

Equation (2) was derived from the following equations:

$$K_1 = \frac{[\text{Mb}]_f[\text{CO}]_f}{[\text{Mb}{-}\text{CO}]},$$

$$K_2 = \frac{[d]_f[\text{CO}]_f}{[d{-}\text{CO}]},$$

$$[\text{CO}]_f = [\text{CO}]_t - [\text{Mb}{-}\text{CO}] - [d{-}\text{CO}],$$

$$[\text{Mb}]_f = [\text{Mb}]_t - [\text{Mb}{-}\text{CO}],$$

$$[d]_f = [d]_t - [d{-}\text{CO}],$$

where $[\text{CO}]_f$ is concentration of free carbon monoxide, $[\text{Mb}]_f$ is concentration of free Mb, $[\text{d}]_f$ is concentration of free cytochrome *d*.

RESULTS

CO-induced changes of reduced cytochrome *bd* absorption spectra. *Membrane form of cytochrome bd.* Addition of CO to the membrane-bound reduced cytochrome *bd* results in spectral changes shown in Fig. 1a. Difference (CO + dithionite-reduced *minus* dithionite-reduced) spectra at low (17 μM) and high (1 mM) concentrations of the ligand are almost identical except for slight differences in the Soret region (see Fig. 1c, spectrum *2*) and are in agreement with the published data [48, 75]. In the visible region, a band with $\lambda_{max} = 644$ nm and $\lambda_{min} = 624$ nm ($\Delta\varepsilon_{644\text{-}624} \sim 13\text{-}14\ \text{mM}^{-1}\cdot\text{cm}^{-1}$), a peak at 540 nm ($\Delta\varepsilon_{540\text{-}500} \sim 4.4\ \text{mM}^{-1}\cdot\text{cm}^{-1}$), and a "W"-shaped trough in the Soret region with two minima, at 430 and 445 nm, and a local maximum near 437 nm ($\Delta\varepsilon_{470\text{-}445} \sim 23\text{-}25\ \text{mM}^{-1}\cdot\text{cm}^{-1}$) are observed.

Solubilized enzyme. Addition of CO to the solubilized *bd* complex gives rise to a band with $\lambda_{max} = 644$ nm and $\lambda_{min} = 624$ nm, a peak at 540 nm, and a characteristic "W"-shaped curve in the Soret region with the two minima, at 430 and 444 nm, and a local maximum at ~437 nm in the difference absorption spectrum of the enzyme (Fig.

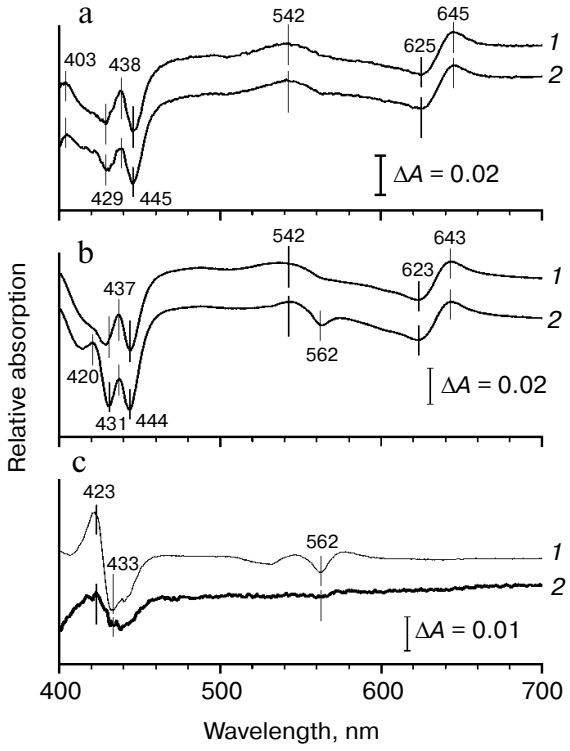

Fig. 1. CO-induced spectral changes of cytochrome *bd* from *E. coli*. Panels (a) and (b) show difference absorption spectra of the cytochrome *d*-containing membranes (a) and the solubilized enzyme (b): treated with 17 μM CO (curves *1*) and 1 mM CO (curves *2*) after reduction with dithionite *versus* dithionite-reduced. Panel (c) shows difference between the spectra at high and low CO concentrations for the solubilized enzyme (curve *1*) and the membrane form of cytochrome *bd* (curve *2*). Spectra were recorded in the split-beam mode. The optical cuvette contains cytochrome *bd* (1.6 μM) in medium containing 50 mM Hepes, 50 mM Ches, 0.1 mM EDTA, pH 8.0. In case of the solubilized enzyme, the detergent 0.025% sodium N-lauroyl-sarcosinate was also present.

1b). The spectrum of cytochrome *bd* at low CO concentration (Fig. 1b, curve *1*) is analogous to that of the CO complex with the membrane enzyme (Fig. 1a). However, in case of the isolated enzyme, the differences between the spectra at high (1 mM) and low (17 μM) CO concentrations are much more pronounced (compare spectra *1* and *2* in Fig. 1c): the difference spectrum shows a band with a maximum at 422 nm, a minimum at 434 nm, and a small shoulder at ~440 nm in the Soret band ($\Delta\varepsilon_{422\text{-}434}$ ~ 19 $mM^{-1}\cdot cm^{-1}$), and the two minima, at 562 and 531 nm, in the visible region, $\Delta\varepsilon_{578\text{-}562}$ ~ 4 $mM^{-1}\cdot cm^{-1}$ (Fig. 1c, curve *1*).

The CO-induced shift of the absorption band near 630 nm is usually assigned to heme *d*. Absorption changes at about 436 nm could be due to contribution from heme b_{595} [76]. We have tried to test homogeneity of the CO-induced absorption changes in different parts of the spectrum of the membrane and solubilized forms of the dithionite-reduced cytochrome *bd*.

CO concentration dependence of the reduced cytochrome *bd* absorption changes. *Membrane form of cytochrome bd*. Figure 2a shows CO concentration dependence of cytochrome *bd* absorption changes at different wavelengths. It can be seen that the changes in the Soret band (curve *1*), as well as the development of a peak at 540 nm (curve *3*) titrate with CO in parallel with the absorption increase in the α-band of the heme *d*–CO complex (curve *2*) reaching saturation at 3-5 μM CO, which is not much higher than the concentration of the enzyme (1.6 μM). A hardly appreciable second phase of the spectral changes is observed in the Soret region, which continues to grow even at 1 mM CO and does not contribute substantially to the dependence shown in Fig. 2a. The difference spectrum of the second phase (curve *2* in Fig. 1c) shows a band with λ_{max} = 423 nm, λ_{min} = 436 nm ($\Delta\varepsilon_{423\text{-}436}$ ~ 5-6 $mM^{-1}\cdot cm^{-1}$) and a very small minimum at 561 nm in the visible ($\Delta\varepsilon_{561}$ ~ 0.3-0.5 $mM^{-1}\cdot cm^{-1}$). This spectrum possibly reflects the interaction of the ligand with a very small part of heme b_{558} (1-3%). The saturation curves observed at different wavelengths fit well the Eq. (1) (see "Materials and Methods" section). This allows making only an upper estimate of the K_d value (0.1-0.2 μM) since the latter is much lower than the concentration of the enzyme (1.6 μM): the theoretical curves for this K_d range are virtually indistinguishable from each other.

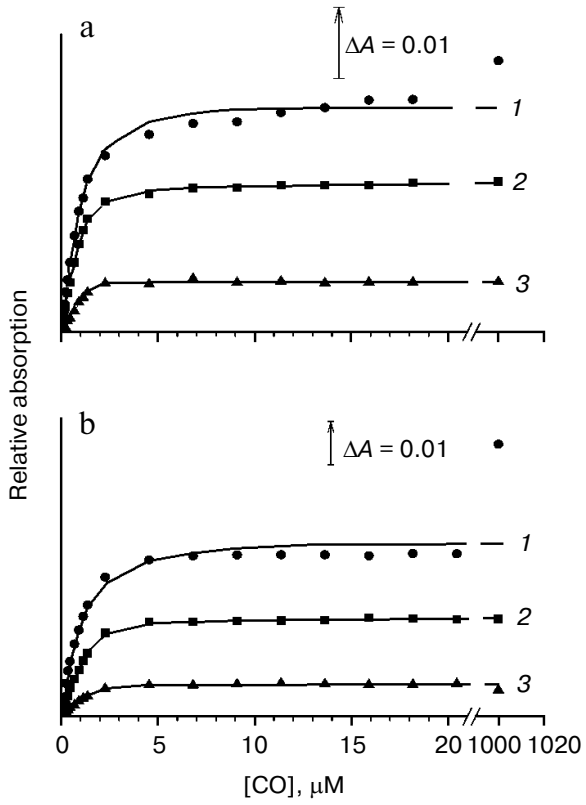

Fig. 2. Dependence of the spectral changes of cytochrome *bd* from *E. coli* at different wavelength ranges on CO concentration: a) membrane form of the enzyme; b) solubilized enzyme. Shown are the curves obtained by processing of the corresponding difference absorption spectra (CO + dithionite-reduced *minus* dithionite-reduced) with the use of the software package GIM: *1*) 470-444 nm; *2*) 644-624 nm; *3*) 540 nm *versus* an arbitrary baseline connecting the 500 and 590 nm points. Other conditions as in Fig. 1.

Solubilized enzyme. As for the membrane form of cytochrome *bd*, the spectral changes of the solubilized enzyme at 540 nm and 644-624 nm titrate with CO in parallel, look like saturation curves, and apparently reflect the same process—CO binding to heme *d* (Fig. 2b, curves *2* and *3*). The peak at 540 nm may be either β-band of the heme *d*–CO complex or part of its split α-band. These curves can also be described by the equation used for the K_d determination. However, the K_d values obtained (0.1-0.25 μM CO) are only an upper estimate, as for the membrane form of cytochrome *bd*. CO concentration dependence of the spectral changes in the Soret band is clearly biphasic: the first phase corresponds to the changes in the visible region; the second phase does not saturate even at 1 mM CO and makes the greater contribution to the total amplitude of the changes in the γ-band than in case of the membrane form of the enzyme (compare spectra *1* and *2* in Fig. 1c). It is worth noting that the amplitude of the second phase apparently corresponding to the interaction of the ligand with part of heme b_{558} (about 10-15%) is not a constant but preparation-dependent. Usually larger amplitude of the second phase is observed with the enzyme kept for a long time.

Determination of an apparent dissociation constant (K_d) for the CO complexes of the membrane and solubilized forms of the reduced cytochrome *d*. *Membrane form of cytochrome bd*. Since we failed to obtain the exact K_d value from the data on the concentration dependence of the CO-induced absorption changes of the reduced cytochrome *bd*, another approach has been used. To determine K_d for the CO complex with heme *d* of the bac-

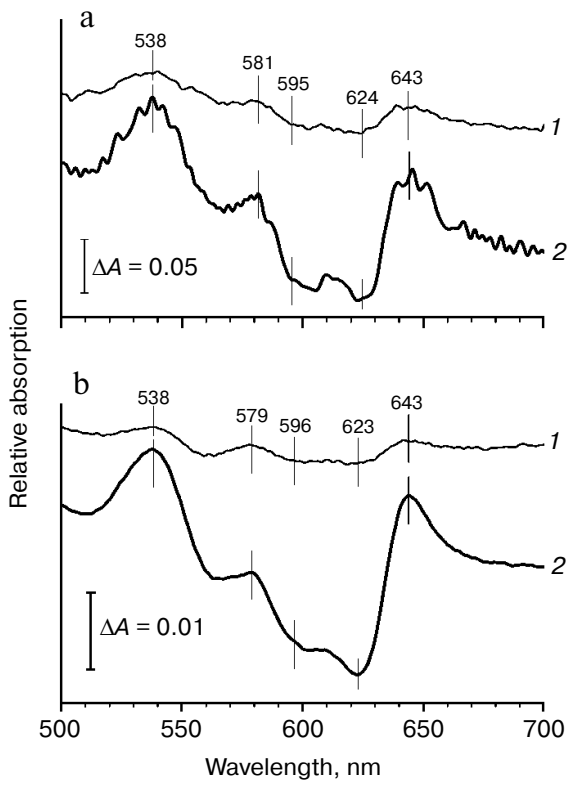

Fig. 3. Spectral changes of the mixture of cytochrome *bd* from *E. coli* and sperm whale skeletal muscle myoglobin induced by CO. a) Difference spectra of the cytochrome *d*-containing membranes (1.21 μM) with myoglobin (1.31 μM): treated with 0.4 and 4 μM CO (curves *1* and *2*, respectively) after reduction with dithionite *versus* dithionite-reduced. b) Difference spectra of the solubilized enzyme (2.33 μM) with myoglobin (2.1 μM): treated with 0.7 and 5.5 μM CO (curves *1* and *2*, respectively) after reduction with dithionite *versus* dithionite-reduced. Spectra were recorded in the split-beam mode in 10 mM potassium phosphate buffer, pH 7.0. In case of the solubilized enzyme, the detergent 0.025% sodium N-lauroyl-sarcosinate was also present.

terial membranes, CO titration of spectral changes of the mixture of cytochrome *bd* and Mb has been performed observing distribution of the ligand between these two proteins (Fig. 3a). K_d for heme *d* has been estimated according to Eq. (2) as described in "Materials and Methods" section taking the K_d value for the CO–Mb complex to be 37 nM [77]. This was possible to do because the spectral characteristics of the CO complexes with heme *d* and Mb in the visible region of the difference (CO + reduced *minus* reduced) absorption spectrum are different: the α-band of the heme *d*–CO complex shows a maximum at 644 nm and a minimum at 624 nm, whereas the Mb–CO complex displays a band with λ_{max} = 579 nm and λ_{min} = 594 nm. The apparent K_d value for the CO complex with heme *d* of the membrane form of the enzyme determined in such a way appeared to be ~70 nM.

Solubilized enzyme. The apparent K_d for the CO complex with the solubilized cytochrome *d* has been determined as for the membranes (Fig. 3b). Its value appeared to be ~80 nM. For comparison, the corresponding apparent K_d value for the R-state α-subunits of human hemoglobin is 2 nM [78], horseradish peroxidase – 30 nM [79], cytochrome aa_3 – 0.29 μM [80], and cytochrome bo_3 – 1.7 μM [81].

Thus, there is no evidence for the existence of a second (apart from heme *d*) CO-binding heme with K_d lower than 1 μM both in the membrane-bound cytochrome *bd* and in the solubilized enzyme.

DISCUSSION

We have compared the interaction of the native (membrane) form of the *E. coli* cytochrome *bd* and the solubilized enzyme with carbon monoxide. We aimed to determine which of the three heme centers of the *bd*-oxidase can bind CO. We showed that CO reacts predominantly with heme *d*. This is evident from the two observations:

(i) the amplitude of the CO-induced absorption changes in the visible region and in the Soret band is similar. Such a ratio is unusual for most hemoproteins but typical for cytochrome *d*, the Soret band of which is much less intense as compared to the corresponding γ-bands of the iron-porphyrin complexes of the *a*-, *b*-, *c*-, or *o*-type [75];

(ii) CO concentration dependencies of the absorption changes of cytochrome *bd* at all wavelengths tested are very similar. Since the CO-induced spectral changes of cytochrome *bd* at 540 and 644-624 nm titrate identically, the peak at 540 nm may be β-band of the heme *d*–CO complex or part of its split α-band.

It is important to emphasize that the CO-induced spectral changes of the membrane form of cytochrome *bd* are virtually identical to those observed with the solubilized enzyme at a low concentration of the ligand. In both cases, CO added at micromolar concentrations reacts predominantly with heme *d*. At the same time, the addition of high concentrations of carbon monoxide (1 mM) to the solubilized cytochrome *bd* results in additional spectral changes in the γ absorption band of the enzyme that points to binding of the ligand to a *b*-type heme. The data are fully consistent with an earlier published work [54] where, upon studying the interaction of cyanide and CO with the solubilized form of the enzyme by the magnetic circular dichroism (MCD), it was established that these ligands, added at high concentration, react with 10-20% of low-spin heme b_{558}.

At first glance, it seems surprising that CO and other ligands are capable of binding to part of the low-spin heme b_{558} of the solubilized enzyme. However, it is well-known that many soluble heme *c*-containing proteins can bind CO at atmospheric pressure [82]. Cyanide also reacts with cytochrome *c* under alkaline conditions. This

is because the sixth axial ligand of cytochrome *c* is Met [74] bonding a polypeptide with heme iron rather poorly and therefore can be replaced with strong exogenous ligands. The studies carried out by the groups of Gennis and Thomson showed that amino acid residues Met393 and His186 of subunit I can serve as the axial ligands of heme b_{558} [83, 84]. It is interesting that a mutant, in which Met393 was replaced with Leu, retains heme b_{558} but in a high-spin form. Probably one His186 is enough for maintenance of the bond between heme b_{558} and the protein [83].

It cannot be excluded that binding of CO with part of heme b_{558} in the solubilized enzyme occurs due to partial denaturation of the protein upon solubilization. However, reconstitution of the enzyme into artificial phospholipid membranes (azolectin liposomes) reverts its CO-binding properties to those typical for cytochrome *bd* of the bacterial membranes, i.e. heme b_{558} no longer binds CO upon reconstitution of the enzyme into the liposomes (V. B. Borisov, unpublished data). Therefore, if weakening the bond of Met with the heme b_{558} iron is caused by protein denaturation, this denaturation is apparently reversible.

These results suggest that, upon addition of CO to both the cytochrome *d*-containing membranes and the purified solubilized enzyme, a majority of heme b_{595} (>95%) does not bind this ligand. This is in agreement with data according to which the spectral changes induced by dissociation of CO from its complex with the isolated reduced cytochrome *bd* from *Azotobacter vinelandii* at low temperatures (from −70 to −100°C) correspond to binding of the ligand to less than 5% of heme b_{595} [56]. It is likely that heme b_{595}, although in the high-spin state, is resistant to the interaction with the classical ligands of the high-spin iron-porphyrin complexes. It cannot be excluded that despite the high-spin state of the iron-porphyrin group, the specific features of the protein environment are such that this redox center is protected from interaction with the ligands. In such case, the participation of b_{595} in the oxygen reduction in cooperation with heme *d* is unlikely and its role is limited to a transfer of an electron to heme *d*. Another possible explanation that we favor is the following: (i) both heme b_{595} and heme *d* potentially can bind ligands; (ii) the hemes are located very close to each other forming a binuclear active site; (iii) the spatial proximity of hemes b_{595} and *d* results in steric restrictions allowing such a di-heme site to bind only one ligand molecule; (iv) heme *d* has a higher affinity for ligands than heme b_{595}. In that case, the final result observed upon addition of a ligand will be always the ligand binding to heme *d*, whereas heme b_{595} will remain mainly in the free state.

We determined an apparent K_d value for the CO complex with cytochrome *d* from *E. coli*. It appeared that heme *d* binds this ligand with a high affinity. The K_d values for the membrane and solubilized forms of the enzyme appeared to be about 70 and 80 nM, respectively. It has to be noted that the K_d values measured in this work are higher than K_d = 20 nM published by Hill et al. [85] for the CO complex with the isolated cytochrome *bd* from *E. coli*. However, the value of K_d = 20 nM was obtained indirectly, namely, calculated from the equation for $K_d = k_{off}/k_{on}$, taking into account the values of $k_{off} = 1.6\ sec^{-1}$ and $k_{on} = 8 \cdot 10^7\ M^{-1} \cdot sec^{-1}$ measured experimentally [85]. Very recently, using the rapid mixing (stopped-flow) method, it was established that the k_{off} value for the CO complex with the isolated cytochrome *bd* from *E. coli* is actually 6 sec^{-1} [42], i.e. several times higher than k_{off} measured by Hill et al. If the specified value of $k_{off} = 6\ sec^{-1}$ is introduced into the equation for $K_d = k_{off}/k_{on}$, the estimated K_d value appears to be 75 nM that coincides the apparent K_d values for the heme *d*–CO complex measured in this work.

It is of interest to consider a plausible structure of the heme d^{2+}–CO complex. In reactions of the fully reduced oxidases of the heme–copper family comprising cytochrome *c* oxidase and cytochrome bo_3 with carbon monoxide, the conversion of a high-spin pentacoordinate heme (*a*-, *o*-, or *b*-type) to its low-spin hexacoordinate CO complex occurs:

$$\begin{array}{ccc} | & & | \\ L{-}Fe^{2+} + CO & \rightarrow & L{-}Fe^{2+}{-}CO, \\ | & & | \end{array}$$

where L denotes the proximal ligand that is a histidine residue for most known heme–copper oxidases [86].

It is proposed that heme *d* in cytochrome *bd* is in the high-spin pentacoordinate state [87, 88]. Moreover, according to the data of Sun et el., heme *d* retains the high-spin pentacoordinate state in the oxygenated ($Fe^{2+}{-}O_2$) and oxo-ferryl ($Fe^{4+}{=}O^{2-}$) intermediates, as well as in the cyanide complex [87, 88]. Hence, one may propose that, when in the CO complex, heme *d* is also in the high-spin state. Recently, it has been reported that anaerobic reduction of cytochrome *bd* is followed by secondary absorption changes on a time scale of tens of minutes pointing to bonding of heme *d* iron to an endogenous protein ligand [89]. In the present study, CO was added to the sample immediately after anaerobic reduction of cytochrome *bd*, i.e. prior to a major part of the spontaneous spectral changes develops. According to a work of Azarkina et al. [89], anaerobic reduction of heme *d* converting a distal oxygen ligand to water can produce transiently either tetracoordinate state (without axial ligands) or pentacoordinate state (with a weak axial ligand of the heme iron on the proximal side of the heme plane). Thus, several possible ways of CO bonding to heme *d* may be considered.

Initially generated tetracoordinate heme *d* can be converted to the high-spin pentacoordinate complex with CO:

$$\begin{array}{c} | \\ Fe^{2+} \\ | \end{array} + CO \rightarrow \begin{array}{c} | \\ Fe^{2+} \\ | \end{array} - CO.$$

When bound to heme *d* upon reduction of the enzyme, a protein ligand can be replaced with carbon monoxide at the distal side of the heme plane:

$$\begin{array}{c} | \\ L-Fe^{2+} \\ | \end{array} + CO \rightarrow \begin{array}{c} | \\ L\,Fe^{2+} \\ | \end{array} - CO.$$

A protein ligand of heme *d* can be replaced with carbon monoxide at the same (proximal) side of the heme plane:

$$\begin{array}{c} | \\ L-Fe^{2+} \\ | \end{array} + CO \rightarrow \begin{array}{c} | \\ L\,OC-Fe^{2+} \\ | \end{array}.$$

Further studies are required to establish precisely the spin state and the structure of the coordination sphere of the CO-bound heme *d* iron.

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