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Examination of the Reaction of *Escherichia coli* Cytochrome *bd*-I in the Fully Reduced State with Cyanide Using Absorption and Circular Dichroism Spectroscopy

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Abstract—We have earlier begun to investigate the reaction of isolated solubilized dithionite-reduced cytochrome *bd*-I of *Escherichia coli* with cyanide [Borisov, V. B., and Arutyunyan, A. M. (2024) The fully reduced terminal oxidase *bd*-I isolated from *Escherichia coli* binds cyanide, *J. Inorg. Biochem.*, **259**, 112653]. The present work is a continuation of this study. Using absorption and CD spectroscopy, the following new results were obtained: (i) The membrane form of the fully reduced enzyme is also capable of binding cyanide. The apparent dissociation constant and second-order rate constant values are 81.1 ± 7.8 mM KCN and 0.11 ± 0.01 $M^{-1}\cdot s^{-1}$, respectively. This contradicts the data of other studies according to which the *bd*-I oxidase located in native membranes, in the fully reduced state does not bind cyanide. (ii) CO added to the cyano adduct of both membrane and isolated solubilized forms of the fully reduced cytochrome *bd*-I displaces cyanide, resulting in the formation of the CO/enzyme complex. This indicates the reversibility of cyanide binding to the protein. To saturate the oxidase binding site with CO in the presence of 100 mM KCN, much more CO is required than upon CO addition to the enzyme not pre-treated with cyanide. CO and cyanide compete for the binding to the same site in the oxidase (heme d^{2+}). Being a stronger ligand, CO "wins" the competition with cyanide. (iii) The effect of cyanide on the optical activity of dithionite-reduced cytochrome *bd*-I was studied. The CD spectra of the enzyme obtained before and after cyanide treatment indicate that the formation of the cyano adduct of heme d^{2+} leads to a significant weakening of the excitonic interactions between heme d^{2+} and heme ${b_{595}}^{2+}$. Schemes of the interaction of cyanide and CO in the presence of excess cyanide with the enzyme active site are proposed.

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INTRODUCTION

The processes taking place in aerobic respiratory electron transport chains of bacteria include a stepwise oxidation of NADH [$E^{0\prime}$ (NAD^+/NADH) ~ –320 mV] with molecular oxygen [$E^{0\prime}$ (O_2/H_2O) ~ +820 mV] that involves several enzyme complexes. Terminal oxidases are the final portion of respiratory chains. These enzyme complexes transfer electrons from ferrocytochrome *c* or quinol (usually ubiquinol, menaquinol, or demethylmenaquinol) to O_2 [1-3]. Enzymatic reduction of molecular oxygen to water is accompanied by the generation of a difference in electrochemical potentials of hydrogen ions on different sides of the coupling membrane ($\Delta\mu H^+$) [4-8], which is utilized by the bacterial cell to synthesize ATP and perform

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other useful work [9]. Bacterial plasma membrane contains terminal oxidases from two different superfamilies, heme-copper and *bd*-type (cytochromes *bd*), that are different in their primary structure [10-14]. The heme-copper oxidase superfamily includes both cytochrome *c* oxidases and quinol oxidases. So far, all biochemically characterized members of the *bd*-type superfamily are quinol oxidases [15, 16]. Unlike *bd*-type enzymes, heme-copper oxidases form $\Delta\mu H^+$ by acting as proton pumps, which increases their energy efficiency by 1.5-2 times [17-21]. A lower energy efficiency of cytochromes *bd* lacking the proton pump activity is compensated by their unique physiological properties which are absent in heme-copper oxidases [22-27]. For example, *bd*-type oxidases are more resistant to nitric oxide [28-33], peroxynitrite [34], hydrogen peroxide [35-40], carbon monoxide [41-43], sulfide [44-46], ammonia [47], cyanide [44, 48], and some antibiotics [49, 50]. In many pathogenic bacteria, cytochrome *bd* is a key terminal respiratory oxidase that increases the virulence of these microorganisms. Genes encoding *bd*-type oxidases are absent in the genomes of humans and animals. For the above reasons, cytochromes *bd* can be promising protein targets for the next-generation antibacterial drugs [51, 52].

The *Escherichia coli* cytochrome *bd*-I is often used as a model *bd*-type oxidase. It is encoded by the *cydABX* operon which is expressed under conditions of O_2 deficiency [53]. The latter correlates with a high affinity of cytochrome *bd*-I for O_2 [54-56]. According to the structural analysis by single-particle cryo-electron microscopy (cryo-EM), the *bd*-I oxidase consists of four subunits: CydA, CydB, CydX, and CydH/CydY [57, 58] (Fig. 1). CydA contains the Q-loop that provides quinol binding and oxidation, and three hemes – one low-spin hexacoordinate heme (b_{558}) and two high-spin pentacoordinate hemes (b_{595} and *d*). Heme b_{558} serves as a primary electron acceptor in the oxidation of quinol. Heme *d* is a site of oxygen reduction [59]. It was suggested that in the process of catalysis, heme *d* is converted sequentially from the oxygenated state to the peroxy, ferryl, and oxidized forms [8, 18, 60, 61]. As a result, within about 1 ms, one oxygen molecule bound to heme *d* is reduced by four electrons to two water molecules [8, 18]. The role of heme b_{595} in the enzyme functioning remains obscure. The distance between the iron atoms of hemes b_{595} and *d* (~11 Å [57, 58]) is too large for these hemes to form a *structural* binuclear center. At the same time, the distance between the edges of these hemes (less than 4 Å [57, 58]) makes van der Waals contacts between them possible. This is consistent with the experimental data obtained by spectroscopy and electrometry [8, 62-74] that indicate the interaction between hemes b_{595} and *d* and suggest the formation of a *functional* diheme center. Presumably, the function of heme b_{595} in such a center is rapid electron transfer to heme *d* in the process of oxygen reductase reaction [18, 75, 76].

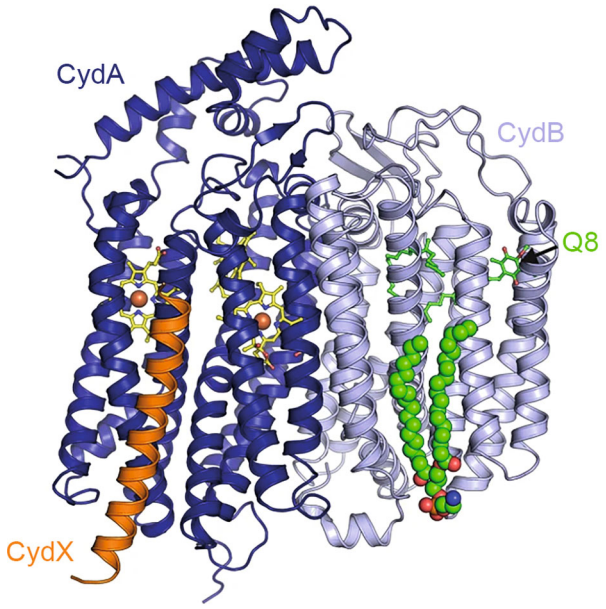

Fig. 1. Three-dimensional cryo-electron microscopy structure of *E. coli* terminal oxidase *bd*-I at a 3.3 Å resolution (PDB ID 6RX4). The CydA subunit carries hemes b_{558}, b_{595}, and *d*. The CydB subunit contains ubiquinone-8 (Q8) and glycerophospholipid (shown with spheres). Reprinted from Theßeling et al. [58] under the terms of the Creative Commons Attribution 4.0 International Public License.

Cyanide can have a major impact on the biochemistry and physiology of bacteria by inhibiting aerobic respiration, as well as acting as a signaling molecule [77]. Cyanide inhibits terminal oxidases from both superfamilies, but their sensitivity to this compound varies [1]. The molecular mechanisms of cyanide inhibition of respiratory enzymes, especially *bd*-type oxidases, have not yet been elucidated, which makes studying the reaction of cytochrome *bd* with cyanide especially relevant.

Recently, we found that the isolated solubilized *E. coli* cytochrome *bd*-I in the fully reduced form binds cyanide and determined the apparent dissociation constant (K_d = 52 mM KCN) and the second-order rate constant (k_{on} ~ 0.1 $M^{-1}\cdot s^{-1}$) [78]. These data clearly contradict the results of other studies according to which the *bd*-I oxidase in the fully reduced state does not bind cyanide [79, 80]. Moreover, Mitchell et al. [79] suggested that the inability to bind cyanide in the fully reduced state is a distinctive feature of *bd*-type terminal oxidases. It should be emphasized that the studies [79, 80] were performed on the cytochrome *bd*-I-containing membrane vesicles and not on the isolated solubilized enzyme as in our recent work [78]. Note that the two forms of cytochrome *bd*-I in these

studies had different molecular environment, namely bacterial lipids in [79, 80] and detergent micelles in [78]. There is a growing body of evidence that the membrane environment can significantly affect the structure, function, and dynamics of membrane proteins [81, 82], in particular, terminal oxidases. As reported in [83], solubilization of bovine heart submitochondrial particles containing cytochrome *c* oxidase led to a 100 to 1000-fold increase in the affinity of the oxidized enzyme for cyanide [83]. It was demonstrated that the membrane environment modulates the ligand-binding properties of the oxidized and reduced forms of the *E. coli* cytochrome *bd*-I toward cyanide and CO, respectively [84, 85]. The membrane-bound and solubilized forms of the *bd*-I oxidase differ significantly in their sensitivity to the inhibition by CO [41, 42, 86]. In this regard, we decided to study interactions of the membrane form of the reduced *bd*-I oxidase with cyanide in order to elucidate the reason for the discrepancy between the results of our recent work [78] and data reported in [79, 80]: whether it could be explained by the different environment of the protein or the authors of [79, 80] overlooked the binding of the membrane form of the reduced cytochrome *bd*-I with cyanide. The present work involved three stages. Having found that the membrane form of the reduced enzyme was capable of binding cyanide, first, we repeated on the membranes the measurements performed before on the isolated enzyme [78] in order to establish to what extent the two forms of the *bd*-I oxidase differ in the ligand-induced spectral changes and K_d and k_{on} values. At the second stage, in order to understand whether the observed reaction is reversible and to obtain confirmation that cyanide binds to the same site in the protein as CO, we compared spectral changes for the membrane and solubilized forms of the reduced enzyme titrated with the increasing concentrations of CO in the presence and absence of KCN. To our knowledge, no experiments on the ligand competition for binding to the active site of a *bd*-type terminal oxidase have been performed before. At the third stage, we used CD spectroscopy to investigate the effect of cyanide on the optical activity of the reduced cytochrome *bd*-I to find out how the binding of this ligand to heme d^{2+} affects excitonic interactions between this heme and the high-spin heme $b_{595}{}^{2+}$. No such studies have been performed before as well.

MATERIALS AND METHODS

Reagents and biological preparations. Sodium dithionite and carbon monoxide from Merck (Germany), EDTA, CHES, HEPES from Serva (Germany), and sodium *N*-lauroyl sarcosinate from Fluka (Switzerland) were used in this work. Other reagents were of domestic production and of chemically pure grade. All aqueous solutions were prepared in deionized water purified with a MilliQ system (Millipore, USA). CO solution (1 mM) used in the experiments on the competition of CO and cyanide for the binding to cytochrome *bd*-I was obtained by equilibrating degassed water with pure gas at 1 atm at room temperature. To obtain the membrane and solubilized forms of cytochrome *bd*-I, the *E. coli* strain GO105/pTK1, which contains a plasmid carrying the corresponding genes for further enzyme overexpression, was used. This strain also contains a deletion of the gene coding for the terminal heme-copper quinol oxidase bo_3 that allowed us to obtain cytochrome *bd*-I preparations free of the contamination with the bo_3 oxidase. *E. coli* cells were grown aerobically in a medium containing 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% glycerol, 0.8 mM magnesium sulfate, 0.18 mM iron (II) sulfate, 0.1 mM copper (II) sulfate, 0.005% kanamycin, and 0.01% ampicillin (pH 7.2). *E. coli* membrane preparations (membrane vesicles) containing cytochrome *bd*-I were obtained from bacterial cells using a French press. To isolate the enzyme, the membranes were solubilized with sucrose monolaurate. Cytochrome *bd*-I was purified by ion-exchange chromatography on a DEAE-Sepharose Fast Flow column (Sigma, USA). A detailed protocol for cell growth, isolation of subcellular vesicles, and solubilization and purification of cytochrome *bd*-I is reported in [70]. Cytochrome *bd*-I concentration was determined from the difference absorption spectrum (sodium dithionite-reduced enzyme minus air-oxidized enzyme) using the millimolar extinction coefficient $\Delta\varepsilon_{628\text{-}607}$ = 10.8 $mM^{-1}\cdot cm^{-1}$ [65].

Absorption and CD spectroscopy and data analysis. Optical absorption was recorded with an Aminco-SLM DW-2000 spectrophotometer (SLM Instruments, USA) in the spectral (split beam) and kinetic (dual wavelength) modes. CD spectra were recorded with a Mark V dichrograph (Jobin Yvon, France) modified by A. M. Arutyunyan. Experiments were carried out at 21°C under anaerobic conditions in cuvettes with a 10-mm optical path in the main incubation medium containing 50 mM HEPES, 50 mM CHES, and 0.5 mM EDTA (pH 8.0). In the case of the solubilized enzyme, the medium also contained 0.05% sodium *N*-lauroyl sarcosinate. To reduce cytochrome *bd*-I, an excess of dry sodium dithionite (on a spatula tip) was added to the cuvette containing the enzyme and a magnetic stir bar. The resulting reaction mixture was stirred on a magnetic stirrer with the cuvette lid closed. The course of cytochrome *bd*-I reduction was monitored in real time by recording changes

in the absorption spectrum; completion of the reduction process was indicated by the cessation of spectral changes. Concentrated KCN aqueous solution (4 M) adjusted to pH 8.0 with MES was used to introduce cyanide to the cytochrome *bd*-I solution.

Data analysis was performed with Origin (OriginLab Corporation, USA) and GIM (Scientific Graphic Interactive Management System; developed by A. L. Drachev, Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University).

RESULTS AND DISCUSSION

The addition of KCN to the dithionite-reduced cytochrome *bd*-I contained in *E. coli* membrane vesicles resulted in spectral changes shown in Fig. 2. The line shapes of the difference absorption spectra (cyanide + dithionite-reduced protein minus dithionite-reduced protein) at different ligand concentrations were similar. The visible region was characterized by the hypsochromic shift of the absorption band with a maximum at 600 nm and a minimum at 634 nm (Fig. 2, curves *1* and *2*). In the Soret region, a W-shaped response with minima at 424 and 443 nm was detected (Fig. 2, curves *1* and *2*). Similar spectral changes were recorded upon the addition of cyanide to the isolated solubilized enzyme in the same redox state [78]. This observation, however, contradicts the data reported in [79, 80] according to which the addition of cyanide caused no changes in the absorption spectrum of *E. coli* membranes containing the dithionite-reduced *bd*-I. Since the cyanide-induced spectral response was relatively small even at the maximum concentration of the added ligand ($\Delta A_{600\text{-}633}$ ~ 0.015 at 100 mM KCN and 1.55 μM cytochrome *bd*-I), it is possible that it was missed by the authors of [79, 80] due to the low content of cytochrome *bd*-I in bacterial membranes and/or insufficient sensitivity of the measuring equipment.

The decrease in the absorption in the region of the α-band of heme d^{2+} (634 nm) most likely indicated cyanide binding to the heme. Spectral changes around 600 nm, in the region of the α-band of heme $b_{595}{}^{2+}$, indicated that the ligand binding to the enzyme led to changes in the heme spectrum. This might suggest complex formation between heme $b_{595}{}^{2+}$ and cyanide. It is known that when cytochromes *a*, *b*, or *c* bind a ligand, absorption changes in the Soret region are at least 5-10 times greater than those in the visible range of the spectrum [54, 87]. However, in the case of cytochrome *bd*-I, the cyanide-induced responses in the γ-band and the visible region were comparable in magnitude (Fig. 2, curves *1* and *2*). It could also be assumed that the resulting cyano adduct had the bridge structure of

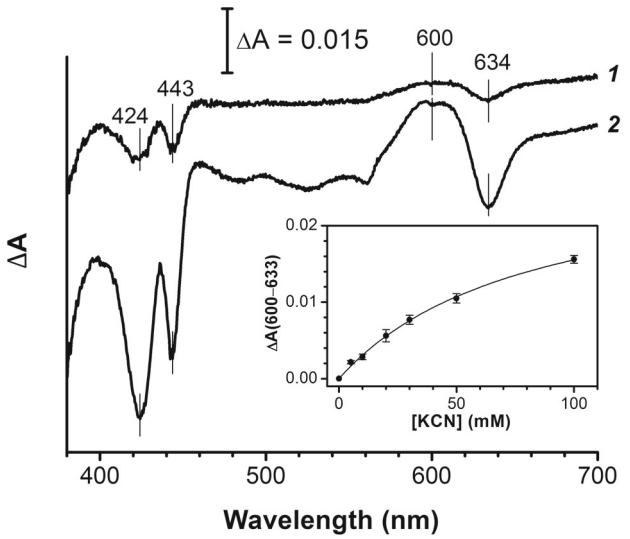

Fig. 2. Cyanide-induced spectral changes in the membrane form of fully reduced *E. coli* cytochrome *bd*-I. Difference absorption spectra of *bd*-I-containing bacterial membranes obtained by subtracting the spectra recorded after reduction with dithionite but before addition of cyanide from the spectra recorded after dithionite reduction and cyanide treatment. KCN concentration: 10 mM (curve *1*) and 100 mM (curve *2*). Inset: K_d evaluation by analysis of the dependence of changes in absorbance at 600-633 nm of the membrane form of fully reduced *E. coli* cytochrome *bd*-I on the cyanide concentration; $\Delta A_{600\text{-}633}$ values were determined from the corresponding difference absorption spectra (KCN + dithionite-reduced enzyme minus dithionite-reduced enzyme). To determine K_d, experimental data were approximated by a hyperbola equation using the built-in "Hyperbola function" in the drop-down menu tab "Analysis → Non-linear Curve Fit → Advanced Fitting Tool" of the Origin program. The apparent K_d value was 81.1 ± 7.8 mM KCN (n = 3). The concentration of cytochrome *bd*-I in the experiments represented in the main panel and inset was 2.17 and 1.55 μM, respectively.

the heme $d(Fe^{2+})$–C=N–heme $b_{595}(Fe^{2+})$ type. However, taking into account that the Fe atoms of hemes b_{595} and *d* are located at a distance of ~11 Å from each other [57, 58], this is quite unlikely. Therefore, the cyanide-induced spectral changes in the membrane form of the fully reduced *bd*-I oxidase indicate that the ligand binds to heme *d* and not to the *b*-type hemes (b_{595} or b_{558}). Notably, the cyanide-induced W-shaped response in the Soret region (Fig. 2, curves *1* and *2*) resembled that observed upon the CO addition [65, 67, 68, 70, 74, 86, 88-90]). In the case of CO, such a response in the Soret band was explained by the complex formation between heme d^{2+} and CO accompanied by a slight change in the spectrum of heme $b_{595}{}^{2+}$ [68]. Therefore, we assume that the W-shaped response in the Soret region observed upon cyanide addition to the membrane-bound cytochrome *bd*-I (Fig. 2, curves *1* and *2*) also indicates changes in the absorption spectrum of heme $b_{595}{}^{2+}$ caused by the complex formation between cyanide and heme d^{2+}.

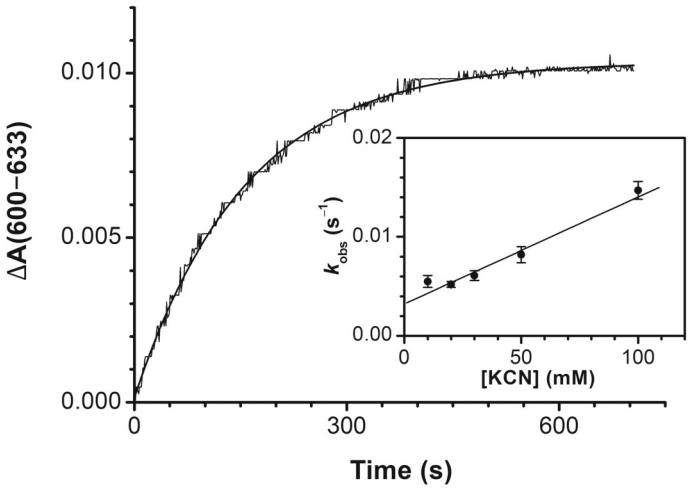

Fig. 3. Kinetics of the cyanide reaction with the membrane form of fully reduced *E. coli* cytochrome *bd*-I. To initiate the reaction, KCN (final concentration, 30 mM) was added to the cuvette containing the membrane-bound terminal oxidase *bd*-I reduced with dithionite. The reaction was recorded in the dual-wavelength mode at 600 nm relative to 633 nm. The experimental points (curve with noise) were approximated by the single exponential function (smoothed curve) with k_{obs} = 0.0064 s^{-1}. Inset: k_{on} assessment by analysis of the dependence on the ligand concentration of the pseudo-first-order rate constant (k_{obs}) of the cyanide reaction with the membrane form of fully reduced *E. coli* cytochrome *bd*-I. For this purpose, the experimental points were approximated by the linear function $k_{obs} = k_{on}$·[KCN] + k_{off} [equation for the pseudo-first-order reaction ([KCN] >> [*bd*-I]), where k_{on} is the second-order rate constant and k_{off} is the dissociation rate constant] by executing the "Fit Linear" procedure in the drop-down tab of the "Analysis" menu of the Origin program. The k_{on} value was 0.11 ± 0.01 M^{-1}·s^{-1} (n = 3). Cytochrome *bd*-I concentration, 2.17 μM.

Figure 2 (inset) shows the dependence of the absorption changes at 600-633 nm of the membrane form of the fully reduced *E. coli* cytochrome *bd*-I on the cyanide concentration. Analysis of the obtained data allowed us to determine the K_d value of the ferroheme *d* complex with the ligand (81.1 ± 7.8 mM KCN), which was approximately 1.5 times higher than the corresponding K_d value for the isolated solubilized enzyme (52 mM) [78]. Presumably, this difference is due to different lipid and protein environments of the enzyme. The obtained value was significantly higher than the K_d values for cyanide complexes with the reduced forms of the *E. coli* cytochrome bo_3 (7 mM) [79], cytochrome *c* oxidase from bovine heart mitochondria (0.1-1 mM) [91-94], and horseradish peroxidase (~1 mM) [95, 96]. In contrast, the affinity of reduced globins (horse heart myoglobin and human hemoglobin) for cyanide is much lower, with the K_d values of ~0.4 M [97] and 1 M [98], respectively. The lower affinity of the dithionite-reduced cytochrome *bd*-I for cyanide compared to that of the heme-copper superfamily oxidases may contribute to its relative insensitivity to this toxic compound [44, 48].

We also studied the kinetics of cyanide reaction with the membrane form of the dithionite-reduced *bd*-I oxidase. Fig. 3 shows a representative kinetic curve at [KCN] = 30 mM. Within the studied range of ligand concentration (10-100 mM KCN), the reaction rate was well described by the monoexponential function. The monophasic nature of the reaction indicates the presence of a single ligand binding site in cytochrome *bd*-I. This confirms our conclusion based on the analysis of static absorption spectra (Fig. 2) according to which only one of the three hemes forms a complex with cyanide. The binding rate increased proportionally to the increase in the ligand concentration (Fig. 3, inset). The second-order rate constant (k_{on}) was 0.11 ± 0.01 M^{-1}·s^{-1}, which was very close to the k_{on} value obtained for the isolated solubilized enzyme (~0.1 M^{-1}·s^{-1}) [78]). Hence, the lipid and protein environment most likely does not affect the binding rate of the reduced *bd*-I oxidase to cyanide. Interestingly, the k_{on} values for the cyanide reaction with the *E. coli* cytochrome bo_3 (572 M^{-1}·s^{-1}) [79], cytochrome *c* oxidase from bovine heart mitochondria (35-235 M^{-1}·s^{-1}) [91-94], and horseradish peroxidase (29-115 M^{-1}·s^{-1} [95, 96] were much higher. A relatively low rate of binding of the fully reduced cytochrome *bd*-I to cyanide (k_{on}) might explain its low affinity for the ligand (K_d).

In contrast to cyanide, the addition of CO to the fully reduced cytochrome *bd*-I resulted in a bathochromic shift by 5-6 nm of the α-band of heme d^{2+} at 629 nm in the visible region of the absolute absorption spectrum (Fig. 4c). Accordingly, the CO-induced difference spectrum contained a band with a maximum at 643-645 nm and a minimum at 622-625 nm [65, 67, 68, 70, 74, 86, 88-90]. As a result, the spectral changes induced in the visible region by CO and cyanide differed significantly (Fig. 4c), allowing

us to monitor the competition between these ligands for the enzyme binding site. The addition of 1 mM CO to cytochrome *bd*-I reduced with excess dithionite and then complexed with cyanide by treatment with 100 mM KCN resulted in the absolute absorption spectrum almost identical to that obtained by the addition of 1 mM CO to the fully reduced enzyme not treated with cyanide (Fig. 4c). The observed spectral changes can be explained as follows: CO added to the cyano adduct of the fully reduced cytochrome *bd*-I displaced cyanide, resulting in the formation of the enzyme complex with CO with a 80-90% yield. However, as can be seen from the CO titration curves (Fig. 4, a and b), much more CO was required to saturate the protein binding site with CO in the presence of 100 mM KCN than in the case of untreated cytochrome *bd*-I. The $K_{0.5}$ values in the presence and absence of 100 mM KCN were 5.7 ± 0.2 and 0.8 ± 0.1 μM CO for the membrane form of the oxidase (Fig. 4a) and 4.4 ± 0.3 and 1.1 ± 0.1 μM CO for the isolated solubilized enzyme, respectively (Fig. 4b). Therefore, the data obtained indicate that the binding of cyanide to the enzyme is reversible. CO and cyanide compete for binding to the same site in cytochrome *bd*-I (heme d^{2+}). Being a stronger ligand, CO "wins" this competition with cyanide.

The rate constant of the enzyme-cyanide complex decay (k_{off}) obtained from the kinetic data presented in the inset in Fig. 3, was approximately 0.0032 s^{-1} (at [KCN] = 0; according to the equation $k_{obs} = k_{on}\cdot[KCN] + k_{off}$). This value was different from the k_{off} value (0.0089 s^{-1}) calculated according to the equation $k_{off} = K_d\cdot k_{on}$, where K_d = 81.1 mM as determined from the equilibrium titration data (Fig. 2, inset) and k_{on} = ~0.11 $M^{-1}\cdot s^{-1}$ as determined from the kinetic data (Fig. 3, inset). There are two possible explanations for this discrepancy. The first one is that the reaction of cyanide with the protein may be irreversible, as for example, the reaction of cytochrome *bd*-I with hydrogen peroxide [99, 100]. However, this explanation contradicts the results of the equilibrium titration with CO (Fig. 4) which clearly indicated the reversibility of the cyanide binding to the enzyme. Another possible explanation is that the reaction is reversible but occurs in two stages rather than one. The binding of cyanide to heme d^{2+} is followed by a slower, reversible conversion of the resulting complex into a more stable, spectrally similar adduct, the decay of which is the rate-limiting step of the reverse reaction. Further research is needed to fully elucidate this issue.

Finally, we investigated the effect of cyanide on the optical activity of the fully reduced cytochrome *bd*-I. The CD spectrum of the dithionite-reduced enzyme (Fig. 5a) was almost identical to that published by Arutyunyan et al. [69]. The spectrum showed a small positive signal with a maximum at 630 nm ($\Delta\varepsilon_{630\text{-}653}$ ~ 21 $mM^{-1}\cdot cm^{-1}$) that coincided with the absorption maximum of heme d^{2+} in the same wavelength range. The Soret region contained a large asymmetric signal consisting of an intense negative band at 440 nm and a small positive extremum at 417 nm, with a zero-crossing point at 426 nm.

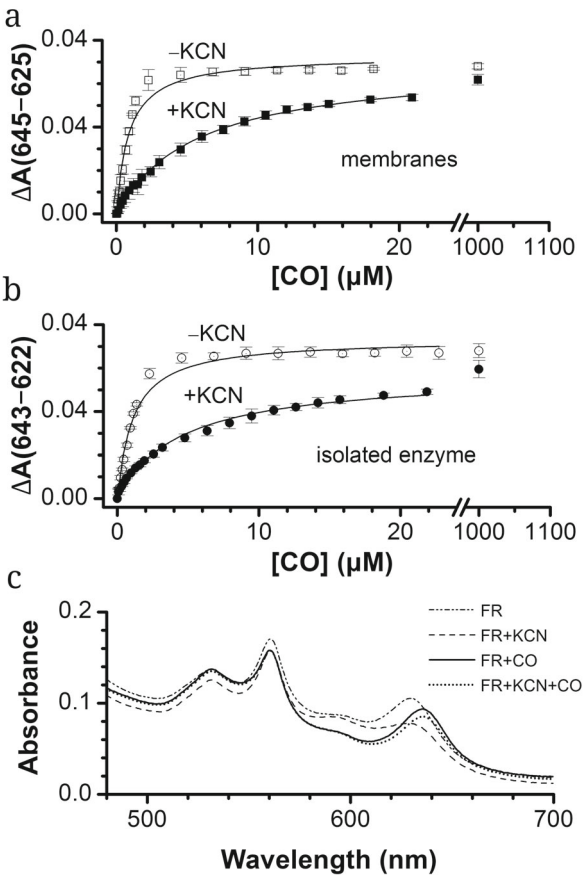

Fig. 4. The dependence of spectral changes of the membrane form (a) and isolated solubilized form (b) of fully reduced *E. coli* cytochrome *bd*-I on the concentration of CO in the presence and absence of KCN (100 mM). To exclude the influence of spectral changes caused by the decay of the cyanide complex with cytochrome *bd*-I on the CO titration curves, we used a pair of wavelengths at which such influence was insignificant: 645 minus 625 nm (a) and 643 minus 622 nm (b). To estimate $K_{0.5}$ (the concentration of added CO at which heme d^{2+} was half-saturated with CO), the experimental data were approximated by a hyperbola equation using the built-in "Hyperbola function" in the drop-down menu tab "Analysis → Non-linear Curve Fit → Advanced Fitting Tool" of the Origin program. The $K_{0.5}$ values in the presence and absence of 100 mM KCN were 5.7 ± 0.2 μM CO (n = 3) and 0.8 ± 0.1 μM CO (n = 3), respectively, for the membrane form of cytochrome *bd*-I, and 4.4 ± 0.3 μM CO (n = 3) and 1.1 ± 0.1 μM CO (n = 3), respectively, for the isolated solubilized enzyme. c) Absolute absorption spectra of cytochrome *bd*-I in the fully reduced state (FR) and its complexes with cyanide (100 mM) and CO (1 mM). The concentration of cytochrome *bd*-I was 2.32 μM (a), 2.53 μM (b), and 2.67 μM (c).

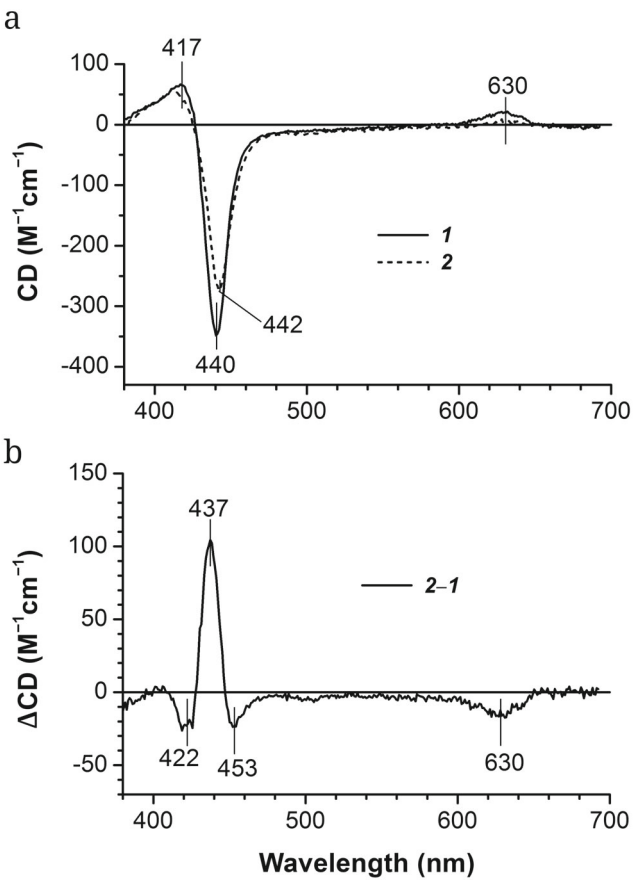

Fig. 5. CD spectra of fully reduced *E. coli* cytochrome *bd*-I and its complex with cyanide. a) Absolute CD spectra of the enzyme before and after treatment with 200 mM KCN: curves *1* and *2*, respectively. b) Difference CD spectrum obtained by subtracting the spectrum of the enzyme after dithionite reduction before KCN addition from the spectrum after dithionite reduction and KCN treatment. Cytochrome *bd*-I concentration, 3.84 μM.

As was previously found [69], the CD spectrum of cytochrome *bd*-I is dominated by the optical activity of ferroheme *d*, while individual contributions of the CD signals of the *b*-type ferrohemes, b_{558} and b_{595}, are insignificant. The magnitude of the CD signal in the Soret region ($\Delta\varepsilon_{417\text{-}440}$ ~ 415 $mM^{-1}\cdot cm^{-1}$) was much higher than that usually observed for the heme-containing proteins with only one heme or several hemes weakly interacting with each other (~10-50 $mM^{-1}\cdot cm^{-1}$) [101, 102]. Such a large magnitude of the CD signal of the fully reduced cytochrome *bd*-I (Fig. 5a) can be explained by strong electronic (primarily excitonic and dipole-dipole) interactions between the electronic transitions of heme d^{2+} and heme $b_{595}{}^{2+}$ in the Soret band [69].

The addition of cyanide to the dithionite-reduced cytochrome *bd*-I resulted in the disappearance of the positive CD signal at 630 nm belonging to heme d^{2+} (Fig. 5a). This was accompanied by a decrease in the negative CD signal in the Soret region, with the shifts of the minimum of the negative band from 440 to 442 nm, positive extremum – from 417 to 410 nm, and zero-crossing point – from 426 to 425 nm ($\Delta\varepsilon_{410\text{-}442}$ ~ 331 $mM^{-1}\cdot cm^{-1}$ (Fig. 5a). Accordingly, the difference between the CD spectra (dithionite-reduced enzyme + KCN minus dithionite-reduced enzyme, Fig. 5b) revealed a minimum at 630 nm, as well as an intense positive signal with a maximum at 437 nm and local minima at 422 and 453 nm ($\Delta\varepsilon_{437\text{-}453}$ ~ 128 $mM^{-1}\cdot cm^{-1}$). The disappearance of the positive CD signal at 630 nm caused by the addition of cyanide indicated that in the fully reduced enzyme, the ligand binds specifically to the heme d^{2+} iron and not to the protein moiety near this redox-active group. The observed pronounced change in the CD signal magnitude in the Soret region indicates that the formation of the cyanide–heme d^{2+}complex led to a significant weakening of the excitonic and, possibly, dipole-dipole interactions between hemes d^{2+} and $b_{595}{}^{2+}$. This is consistent with the observed changes in the absorption spectrum of heme $b_{595}{}^{2+}$ caused by the cyanide binding to heme d^{2+} (Fig. 2).

According to the cryo-EM structures of the *E. coli* cytochrome *bd*-I reported in [57, 58], all the three hemes and their axial ligands are located in the CydA subunit. There is a consensus regarding the nature of the axial ligands of hemes *b*: b_{558} is coordinated by His186 and Met393, and b_{595} is coordinated by Glu445. However, it is still debated whether the axial ligand of heme *d* is His19 [57] or Glu99 [58]. His19 and Glu99 are located on opposite sides of the heme *d* porphyrin ring plane. In the course of oxygen reductase reaction, O_2 presumably binds to Fe^{2+} of heme *d* on the side where Glu99 is located. According to the resonance Raman spectroscopy, ferroheme *d* in a complex with cyanide remains in the pentacoordinate high-spin form [103]. If CN^- binds to the heme iron on the same side of the macrocycle plane as the oxygen molecule, two variants of cyanide binding to heme d^{2+} in the fully reduced cytochrome *bd*-I can be proposed (Fig. 6). If the axial ligand of heme *d* is His19 (variant 1), then the binding of CN^- to the heme iron on one side of the porphyrin macrocycle plane leads to the dissociation of His19 from the other side of the ring plane. If the axial ligand is Glu99 (variant 2), then it is replaced by CN^-. In both variants, heme d^{2+} retains the pentacoordinate high-spin state after binding with cyanide.

It would also be interesting to discuss a possible mechanism of CO interaction with the cyano adduct of heme d^{2+}. It is assumed that in the fully reduced oxidase, heme d^{2+} in the complex with CO is in the hexacoordinate low-spin form [65]. Since the absolute absorption spectra recorded for the CO addition to cytochrome *bd*-I pre-treated with 100 mM KCN and untreated with cyanide were virtually identical (Fig. 4c),

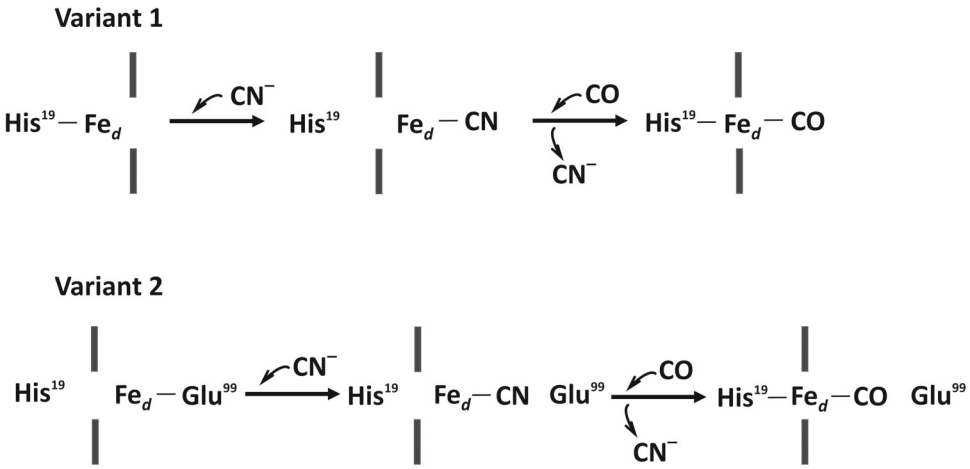

Fig. 6. Schemes of cyanide binding to unliganded heme d^{2+} and CO binding to the cyano adduct of heme d^{2+} in fully reduced *E. coli* cytochrome *bd*-I. The central iron atom of heme d^{2+} (Fe_d), porphyrin ring plane, putative axial ligand of the heme, and changes in its liganded state are shown (see the text for detailed description of variants 1 and 2). Variant 1 does not show Glu99 since in none of the putative liganded states of the heme, Glu99 is bound to Fe_d.

it can be assumed that in both cases, the state of heme d^{2+} in the complex with CO was the same (hexacoordinate low-spin). It can also be assumed that, like O_2 and cyanide, CO binds to Fe^{2+} of heme *d* on the side of the porphyrin macrocycle plane where Glu99 is located. If so, then regardless of the nature of heme *d* protein ligand, the addition of CO leads to the cyanide dissociation and CO binding to the heme iron on the same side of the macrocycle plane and this is accompanied by the binding of His19 on the other side of the plane (Fig. 6). In this regard, it should be noted that the difference in the nature of the heme *d* axial ligand (His19 or Glu99) revealed in the two cryo-EM structures of the *E. coli* cytochrome *bd*-I [57, 58] may be explained by the flexibility of the coordination bond between the heme iron and the ligand, as well as different functional state of the enzyme in frozen static structures.

Abbreviations

K_d	apparent dissociation constant
k_{obs}	observed pseudo-first-order binding rate constant
k_{off}	dissociation rate constant
k_{on}	second-order binding rate constant

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Contributions
V.B.B. developed the concept and managed the study; V.B.B. and A.M.A. conducted the experiments, analyzed and discussed the data, and wrote and edited the text of the article.

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Ethics approval and consent to participate
This work does not contain any studies involving human or animal subjects.

Conflict of interest
The authors of this work declare that they have no conflicts of interest.

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