ISSN 0006-2979, Biochemistry (Moscow), 2010, Vol. 75, No. 4, pp. 428-436. © Pleiades Publishing, Ltd., 2010.
Published in Russian in Biokhimiya, 2010, Vol. 75, No. 4, pp. 520-530.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM09-271, February 14, 2010.

Peroxidase Activity of Cytochrome *bd* from *Escherichia coli*

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Received September 12, 2009
Revision received October 19, 2009

Abstract—Cytochrome *bd* from *Escherichia coli* is able to oxidize such substrates as guaiacol, ferrocene, benzohydroquinone, and potassium ferrocyanide through the peroxidase mechanism, while none of these donors is oxidized in the oxidase reaction (i.e. in the reaction that involves molecular oxygen as the electron acceptor). Peroxidation of guaiacol has been studied in detail. The dependence of the rate of the reaction on the concentration of the enzyme and substrates as well as the effect of various inhibitors of the oxidase reaction on the peroxidase activity have been tested. The dependence of the guaiacol-peroxidase activity on the H_2O_2 concentration is linear up to the concentration of 8 mM. At higher concentrations of H_2O_2, inactivation of the enzyme is observed. Guaiacol markedly protects the enzyme from inactivation induced by peroxide. The peroxidase activity of cytochrome *bd* increases with increasing guaiacol concentration, reaching saturation in the range from 0.5 to 2.5 mM, but then starts falling. Such inhibitors of the ubiquinol-oxidase activity of cytochrome *bd* as cyanide, pentachlorophenol, and 2-*n*-heptyl 4-hydroxyquinoline-N-oxide also suppress its guaiacol-peroxidase activity; in contrast, zinc ions have no influence on the enzyme-catalyzed peroxidation of guaiacol. These data suggest that guaiacol interacts with the enzyme in the center of ubiquinol binding and donates electrons into the di-heme center of oxygen reduction via heme b_{558}, and H_2O_2 is reduced by heme *d*. Although the peroxidase activity of cytochrome *bd* from *E. coli* is low compared to peroxidases, it might be of physiological significance for the bacterium itself and plays a pathophysiological role for humans and animals.

DOI: 10.1134/S000629791004005X

Key words: aromatic substrates, cytochrome *bd*, *Escherichia coli*, oxidation of phenols, peroxidase, respiratory chain, terminal oxidase

Terminal oxidases are the final enzyme complexes of respiratory chains of organisms catalyzing the four-electron reduction of molecular oxygen into two molecules of water without release of intermediate products of partial reduction of oxygen into the medium. In the aerobic respiratory chain of *Escherichia coli*, the two types of such enzymes, cytochromes bo_3 and *bd*, can be found [1-5]. Both enzymes use ubiquinol-8 as a donor of reducing equivalents (although cytochrome *bd* can also oxidize menaquinol-8), coupling the transfer of electrons from the respiratory substrate to O_2 with the generation of the difference of electrochemical potentials of H^+ ions on the cytoplasmic membrane [6-10]. Efficiency of the *bd*-oxidase as a generator of membrane potential is twice lower than that of the bo_3-type enzyme due to the lack of a proton pump mechanism [6]. Nevertheless, in their life bacteria often express cytochrome *bd* as the predominant, or even the sole, terminal oxidase. For instance, recently a systematic mutational analysis was performed to elucidate the role of the respiratory chain in the ability of both harmless symbiotic and pathogenic enterohemorrhagic *E. coli* to colonize the mouse intestine [11]. It was found that the mutants lacking cytochrome *bd* but possessing the bo_3-oxidase are incapable of colonization, whereas the mutants without cytochrome bo_3 but with the *bd*-oxidase can colonize as well as the wild type where both terminal oxidases are present [11]. This is probably due to the fact that cytochrome *bd* is endowed with a number of important physiological functions. The cytochrome *bd* contents increase under unfavorable conditions such as low oxygen concentrations [12-14], alkalization of the medium [15],

Abbreviations: E_m, midpoint potential; HOQNO, 2-*n*-heptyl 4-hydroxyquinoline-N-oxide; Tween-20, polyoxyethylene sorbitan monolaurate (polysorbate 20).

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high temperature [16, 17], the presence of poisons in the environment (for instance, cyanide at low concentrations [18]) and uncouplers-protonophores [15, 19, 20]. Cytochrome *bd* confers resistance to nitrosative stress in a bacterial cell [21-26]. The *bd*-type oxidase often predominates not only in the respiratory chain of *E. coli* but also in bacteria that cause diseases such as dysentery [27], pneumonia [28], salmonellosis [29, 30], periodontitis [31], brucellosis [32], typhoid [30], and tuberculosis [33]. A positive correlation between virulence of bacterial pathogens responsible for these diseases and level of cytochrome *bd* expression has been reported.

Cytochrome *bd* consists of the two different subunits, I (CydA, 58 kDa) and II (CydB, 43 kDa), each being a typical integral membrane protein. These subunits carry three heme redox centers: one low-spin (heme b_{558}) and two high-spin (hemes b_{595} and *d*), whereas no copper ions are found in their composition [34, 35]. 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 [36]. According to current thinking, all the three hemes are located closer to the outer (periplasmic) side of the membrane [37]. Heme b_{558} is probably involved in the oxidation of ubiquinol [38, 39]. Heme *d* probably plays a key role in the catalytic act – it binds molecular oxygen with high affinity [40, 41] and is directly involved in the oxygen-reductase reaction. Heme b_{595} most likely participates in the reduction of oxygen as well. The data accumulated to date suggest that this heme can form together with heme *d* a common di-heme site for successive reduction of O_2 [8, 9, 42-50].

Several relatively stable forms of the enzyme corresponding to intermediates of the catalytic cycle have been identified. Under aerobic conditions, cytochrome *bd* is predominantly in the one-electron-reduced oxygenated state (**S**$^{\mathbf{1}}$, $b_{558}^{3+}b_{595}^{3+}d^{2+}$-$O_2$), with a noticeable admixture of the oxoferryl (**F**, $b_{558}^{3+}b_{595}^{3+}d^{4+}{=}O^{2-}$) and fully oxidized forms (**O**, $b_{558}^{3+}b_{595}^{3+}d^{3+}$-OH). Under anaerobic conditions, the reduced forms of the enzyme lacking an oxygen ligand with one (**R**$^{\mathbf{1}}$, $b_{558}^{3+}b_{595}^{3+}d^{2+}$) and three (**R**$^{\mathbf{3}}$, $b_{558}^{2+}b_{595}^{2+}d^{2+}$) electrons can be generated. Besides, studies on the oxidation of the fully reduced enzyme being originally in a CO complex, with oxygen, using methods of fast kinetics, showed that following photodissociation of CO from heme *d*, a short-lived complex of the three-electron reduced cytochrome *bd* with oxygen (**S**$^{\mathbf{3}}$, $b_{558}^{2+}b_{595}^{2+}d^{2+}$-$O_2$) [7, 9, 41, 51] and oxygen intermediate **P** (from "peroxy") that is likely to be a true peroxy complex ($b_{558}^{2+}b_{595}^{3+}d^{3+}$-OOH) [9] and compound **F** (from "ferryl", $b_{558}^{3+}b_{595}^{3+}d^{4+}{=}O^{2-}$) [7, 9, 51] are formed sequentially. Compound **F** of cytochrome *bd* can also be generated by addition of excess hydrogen peroxide to the enzyme being in the **O** or **S**$^{\mathbf{1}}$ state [7, 52, 53]. Based on the fact that the **F** species observed in the "as-prepared air-oxidized" enzyme preparation, as well as in the reaction of **R**$^{\mathbf{3}}$ with oxygen and in the reaction of **O** or **S**$^{\mathbf{1}}$ with H_2O_2 have similar absorption bands in the visible spectral region with a maximum at 680 nm, one can suggest that these species also have similar or even identical heme *d* configurations. At present, a scheme of the catalytic cycle of cytochrome *bd* is accepted (Fig. 1) according to which the enzyme *in vivo* undergoes the following transformations: $\mathbf{S^1 \rightarrow S^3 \rightarrow P \rightarrow F \rightarrow R^1 \rightarrow S^1}$.

Intermediates of this cycle are close to those that occur in the catalytic cycle of heme–copper-containing terminal oxidases, of which the most studied to date is the mitochondrial cytochrome *c* oxidase. Earlier it was found that cytochrome *c* oxidase under certain conditions reveals a small peroxidase activity towards potassium ferrocyanide [54] as well as some aromatic compounds [55]. In this work, we sought to find whether peroxidase activity is inherent to cytochrome *bd* from *E. coli*. To detect the peroxidase reaction, it was necessary to select such substrates that under aerobic conditions are not oxidized at a noticeable rate in the oxidase reaction but start to undergo oxidation upon addition of hydrogen peroxide. The electron donors tested that meet these requirements appeared to be guaiacol (*o*-methoxyphenol), ferrocene, benzohydroquinone, and potassium ferrocyanide.

MATERIALS AND METHODS

Reagents. Hydrogen peroxide (30% Suprapur) and dithionite from Merck (Germany), Mops, 2-*n*-heptyl 4-hydroxyquinoline-N-oxide (HOQNO), KCN, potassium ferrocyanide, and potassium ferricyanide from Sigma (USA), Tween-20 and EDTA from Serva (Germany), and benzohydroquinone from Fluka (Switzerland) were used in this work. 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. Cytochrome *bd* from the *E. coli* strain GO105/pTK1, kindly provided by Prof. R. Gennis from the University of Illinois at Urbana-Champaign (USA), was used. This strain is a cytochrome *bd* overproducer due to introduction of a plasmid containing the genes coding for this enzyme. Also, this strain lacks cytochrome bo_3, which allows obtaining cytochrome *bd* preparation without impurity of another quinol-oxidase.

Growth of cells, membrane preparation, and isolation of the purified enzyme. The *E. coli* cells were grown aerobically in a fermenter with controlled feed of oxygen in the growth medium as described by Borisov [56]. To obtain the membranes, the grown cells were disrupted by passing through a French press; the method was described in detail by Borisov [56]. Cytochrome *bd* was solubilized and purified as described earlier by Miller and Gennis [57] with some modifications. A zwitterionic detergent *N*-dodecyl-*N*,*N*-dimethylammonio-3-propane-sulfonate (SB-12) was used as a solubilizing agent. The chromatography was performed using DEAE-Sepharose CL-6B.

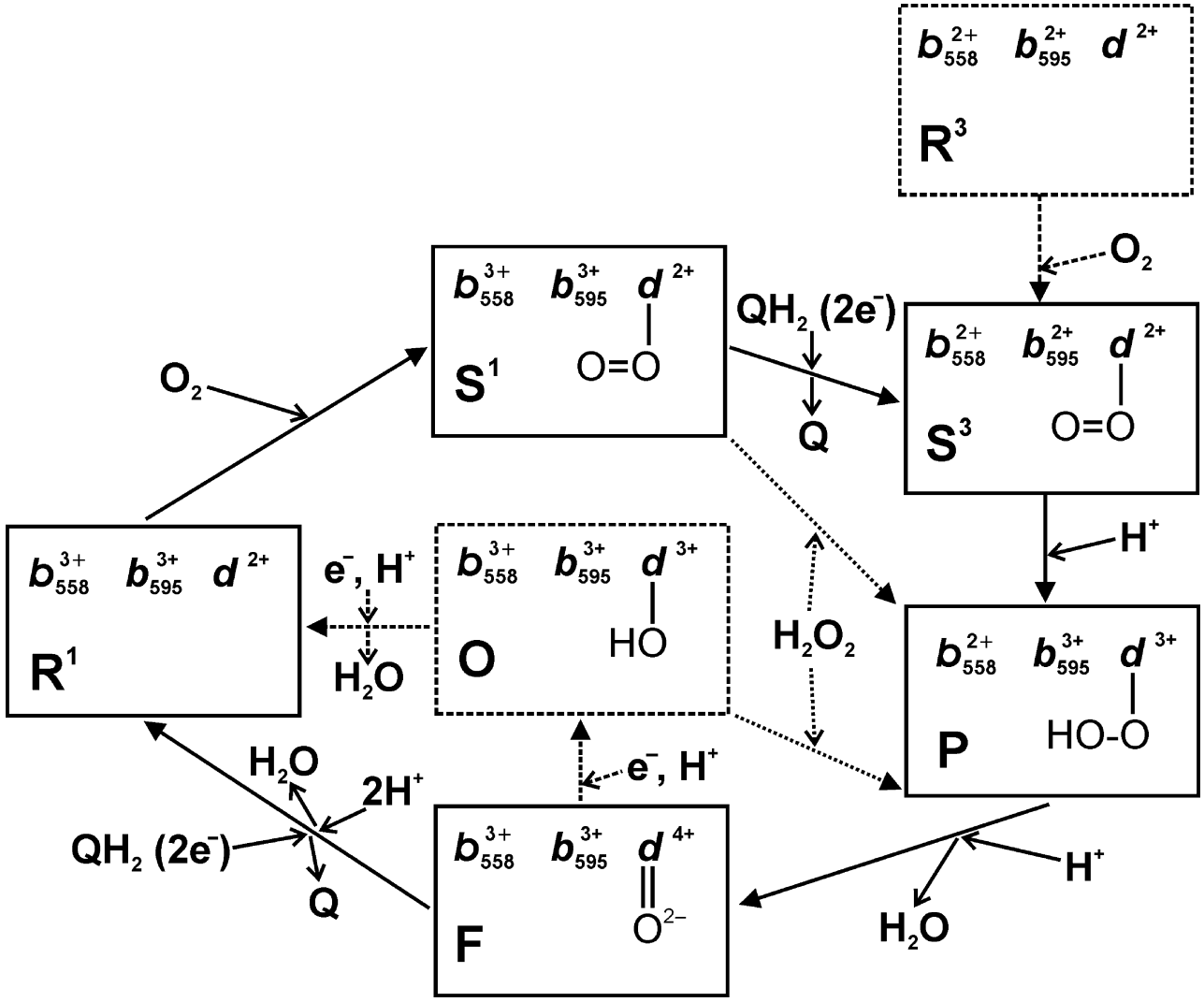

Fig. 1. Catalytic cycle of cytochrome *bd*. This scheme is based on the works of Junemann et al. [76], Matsumoto et al. [66], Belevich et al. [9], and Yang et al. [74]. **S**^{1} and **S**^{3} – one-electron-reduced and fully reduced forms of the oxygen-bound enzyme (complex of reduced heme *d* with oxygen, d^{2+}-O_2, designated as "**S**" (from "superoxo") because formally it is a complex of the oxidized heme *d* with superoxide, d^{3+}-O_2^{-1}); **R**^{3}, **O**, and **F** – fully reduced, fully oxidized, and oxo-ferryl forms of cytochrome *bd*; **R**^{1} – one-electron-reduced form of the enzyme; **P** – peroxide-bound form of cytochrome *bd*. QH_2 and Q – reduced and oxidized forms of quinone. Solid arrows show the natural catalytic reaction pathway. Dotted arrows indicate transitions that are not part of the catalytic cycle but can be observed experimentally. The **O** form of the enzyme is most likely not to be an intermediate of the catalytic cycle [74]. The **F** form of the enzyme can also be obtained *in situ* by adding excess hydrogen peroxide to cytochrome *bd* in **O** or **S**^{1} state [7, 52, 53]. The reaction of the enzyme with H_2O_2 most likely proceeds via transient formation of a short-lived intermediate **P** ($b_{558}^{3+}b_{595}^{3+}d^{3+}$-(H)OOH or $b_{558}^{2+}b_{595}^{3+}d^{3+}$-(H)OOH). Possible mechanisms of this reaction are discussed by Borisov et al. [52].

Spectroscopy. The work was performed in spectral (split beam) and kinetic (dual wavelength, 470 minus 580 nm) mode using an Aminco-SLM DW-2000 spectrophotometer (SLM Instruments, USA). Kinetic data were also obtained with Cary-219 spectrophotometer (Varian, USA) at 470 nm. A standard 10 mm light path cuvette was employed. The experiments were performed under aerobic conditions at 22°C in a basic medium containing 0.1 M Mops, 0.5 mM EDTA, and 0.4% Tween-20, pH 7.0. According to the data of Lorence et al. [58], quinol oxidase activity of the enzyme in a medium with such a detergent is maximal.

Determination of the concentration of enzyme, hydrogen peroxide, and guaiacol oxidation product. 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 $mM^{-1}\cdot cm^{-1}$ [45]. Hydrogen peroxide concentration was determined by optical absorption using a molar extinction coefficient of ε_{240} =

43.6 $mM^{-1}\cdot cm^{-1}$ [59]. Guaiacol oxidation can be seen as an absorption change at 470 nm. In peroxidation of guaiacol, two molecules of the latter are converted into one molecule of 3,3′-dimethoxy-4,4′-biphenylquinone [60]. According to the work of Capeillere-Blandin [61], the extinction coefficient of this guaiacol oxidation product of ε_{470} is 5.58 $mM^{-1}\cdot cm^{-1}$ and two electrons are consumed to produce one molecule of the product.

Treatment of the experimental data. The experimental data were analyzed with the aid of the software package GIM (Scientific Graphic Interactive Management System, developed by A. L. Drachev in Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University) and Origin 7 (OriginLab Corporation, USA).

RESULTS

Compounds such as guaiacol, ferrocene, benzohydroquinone, and potassium ferrocyanide (at potassium ferrocyanide/potassium ferricyanide = 1 : 10) were found not to be oxidized when added to the isolated cytochrome *bd* from *E. coli* under aerobic conditions (i.e. they cannot serve as substrates for the oxidase reaction – the reduction of molecular oxygen into two molecules of water). However when hydrogen peroxide is added to the medium, the oxidation of these substrates can be observed. Such cytochrome *bd* activity with hydrogen peroxide as an electron acceptor can be called peroxidase activity.

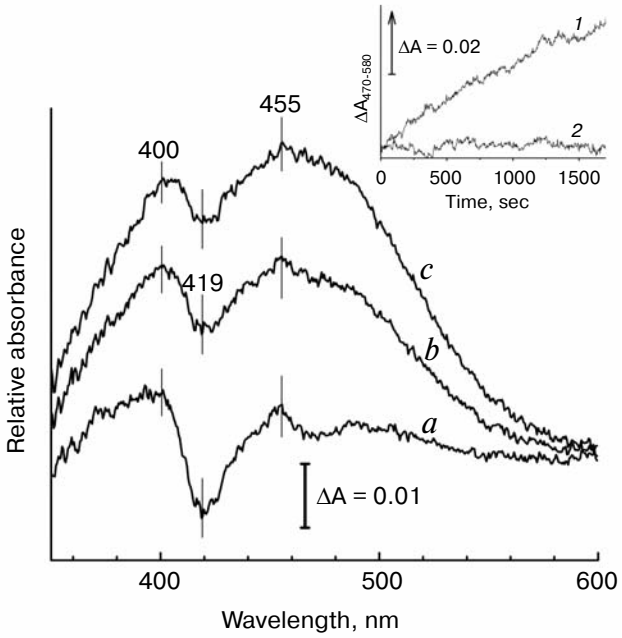

Fig. 2. Peroxidation of guaiacol by cytochrome *bd* from *E. coli*. The reaction was initiated by addition of 0.08 μM cytochrome *bd* to a mixture of 1 mM guaiacol and 0.5 mM hydrogen peroxide in incubation medium containing 0.1 M Mops, 0.5 mM EDTA, and 0.4% Tween-20, pH 7.0. Shown are the difference absorption spectra (versus the spectrum recorded 15 sec after adding the enzyme), 5 min (*a*), 20 min (*b*), and 40 min (*c*) after the start of the reaction. The inset shows the kinetics of the reaction (curve *1*) and control without the enzyme (curve *2*). The kinetic traces were recorded in dual wavelength mode, 470 minus 580 nm.

Peroxidation of guaiacol. Guaiacol is a classic substrate in studies of peroxidases, and its peroxidation by cytochrome *bd* was studied in more detail. Addition of cytochrome *bd* to a mixture of guaiacol and hydrogen peroxide causes a fast appearance of a narrow minimum near 419 nm (Fig. 2, main panel, spectrum *a*) and slower development of a broad absorption band in the region of 350-550 nm (Fig. 2, main panel, spectra *b* and *c*). The development of the minimum at 419 nm slows down over time (in the range of ten minutes). The overall increase in extinction corresponds to the formation of guaiacol oxidation product [60, 61]; its absorption spectrum has characteristic maxima at 400-410 and 450-475 nm. The narrow minimum at 419 nm might be due to both a change in the spectrum of one or more hemes in response to addition of hydrogen peroxide and their destruction in a fraction of the cytochrome *bd* molecules [53]. A typical kinetic trace of guaiacol peroxidation is shown in the inset of Fig. 2 (curve *1*). Despite a low rate, the peroxidase reaction was well reproduced and was not dependent on what it was initiated with – the addition of enzyme or hydrogen peroxide. Absorption changes at the same wavelength and the same incubation time were not observed when various controls, in the absence of the enzyme (curve *2*, inset in Fig. 2) or one of the substrates, guaiacol or hydrogen peroxide (not shown), were performed. Thus, we can conclude that there is an enzymatic oxidation of guaiacol via the peroxidase mechanism.

Dependence of reaction rate on concentration of cytochrome *bd* and hydrogen peroxide. In the range of the enzyme concentrations tested (0-0.2 μM), the rate of peroxidation of guaiacol increases linearly with increasing cytochrome *bd* concentration (Fig. 3). At relatively low concentrations of hydrogen peroxide (up to 8 mM), the reaction is accelerated linearly with increasing the H_2O_2 concentration (Fig. 4). However, at higher concentrations of hydrogen peroxide, inactivation of cytochrome *bd* can be observed. For example, at 25 mM hydrogen peroxide, the cytochrome *bd* activity is reduced towards zero within a few minutes after mixing (Fig. 5, curve *2*). Because of the observed effect of inhibition, the experiments were limited to relatively low concentrations of hydrogen peroxide.

It should be noted that the inhibition induced by hydrogen peroxide at high concentrations is characteristic of all known peroxidases [62, 63]. The mechanism of inhibition is apparently the gradual degradation of heme due to its attack by oxygen radicals. At the same time, according to Kita et al. [64], the quinol oxidase activity of cytochrome *bd* can be inhibited in the presence of 120 mM hydrogen peroxide by only 50%, i.e. the enzyme

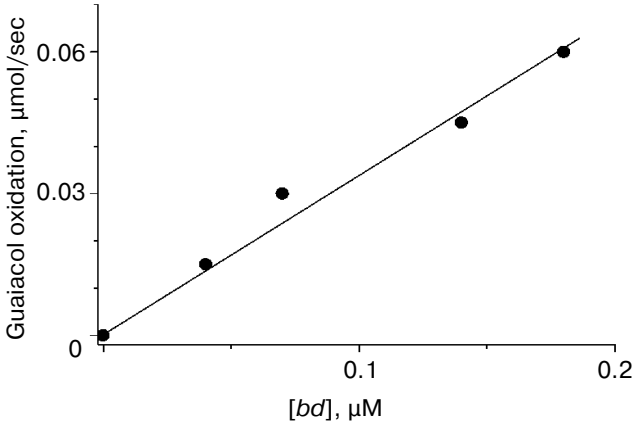

Fig. 3. Dependence of rate of guaiacol peroxidation on cytochrome *bd* concentration. The reaction was initiated by addition of hydrogen peroxide. Basic conditions as in Fig. 2 but the concentration of guaiacol is 1.3 mM.

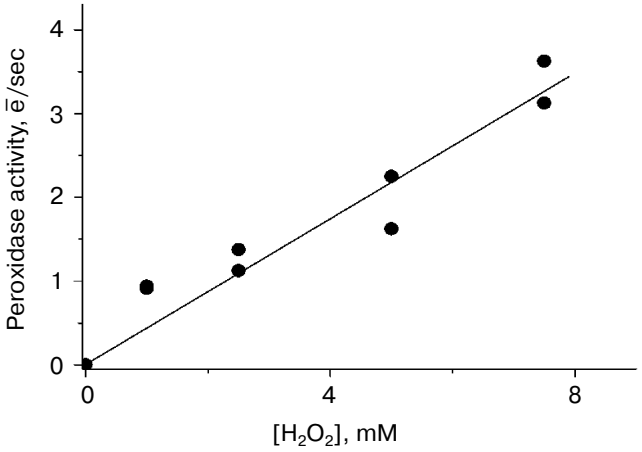

Fig. 4. Dependence of rate of peroxidase reaction on concentration of hydrogen peroxide. The reaction was initiated by addition of cytochrome *bd*. Basic conditions as in Fig. 2 but the concentrations of cytochrome *bd* and guaiacol are 0.06 μM and 1.3 mM, respectively.

is much more resistant to H_2O_2 than in our experiments. This is likely to be due to the substrate protection effect on cytochrome *bd*.

Dependence of reaction rate on guaiacol concentration. The observed dependence of the rate of the peroxidase reaction on the guaiacol concentration at a constant concentration of H_2O_2 of 1.5 mM (Fig. 6) can be roughly divided into three parts: increase in activity – up to 0.5 mM (part *1*), saturation – from 0.5 to 2.5 mM (part *2*), and decrease in activity with increasing guaiacol concentrations – more than 2.5 mM, that can be called substrate inhibition (part *3*). The initial part is characterized by a linear dependence of the reaction rate on guaiacol with no sign of saturation. The concentration of guaiacol at which the reaction rate reaches half the maximum possible, $[C]_{0.5}$, is ~0.28 mM. The concentration of guaiacol which causes half the maximum inhibition of the reaction (I_{50}) on part *3* is ~5 mM. It should be noted that inhibition by the natural substrate (quinol) is also typical of ubiquinol oxidase activity of cytochrome *bd* [65, 66].

At low guaiacol concentrations (less than 200 μM), the rate of guaiacol peroxidation starts to fall quite rapidly with time, i.e. the hydrogen peroxide-induced inhibition is observed (not shown), whereas at guaiacol concentration of 1.3 mM the reaction rate remains constant for ~20 min. This observation indicates that guaiacol protects

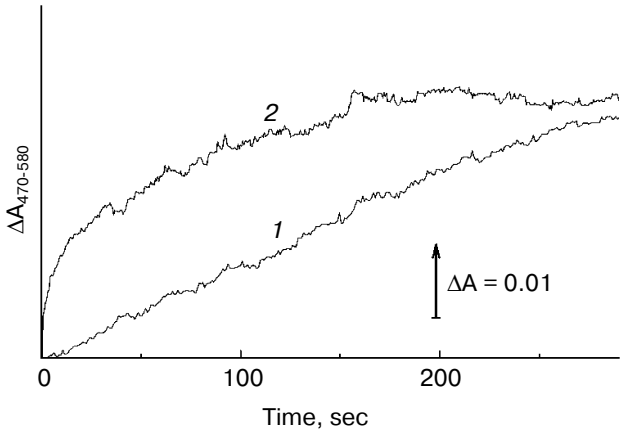

Fig. 5. Kinetics of peroxidase reaction at different concentrations of hydrogen peroxide: 2.5 mM (curve *1*) and 25 mM (curve *2*). The reaction was initiated by addition of hydrogen peroxide. Conditions as in Fig. 4.

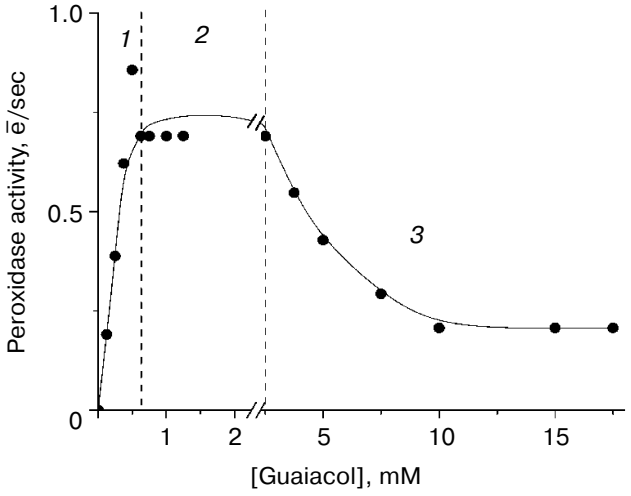

Fig. 6. Dependence of rate of peroxidase reaction on concentration of guaiacol. The concentration dependence is arbitrarily divided by dotted lines into three sections (see "Results"). The reaction was initiated by addition of hydrogen peroxide. Conditions as in Fig. 2, but the concentrations of cytochrome *bd* and hydrogen peroxide are 0.06 μM and 1.5 mM, respectively.

the enzyme from inhibition by hydrogen peroxide. This is also evidenced by the fact that the increase in the minimum at 419 nm, which corresponds to destruction of hemes by hydrogen peroxide, is slowed in the presence of guaiacol. The protection by substrates against inhibition by hydrogen peroxide is known for many peroxidases [67].

According to the preliminary data, the peroxidase activity of cytochrome *bd* depends on pH of the medium, revealing a maximum value at pH = 7 that is quite consistent with the data of Lorence et al. [58] on the pH dependence of the quinol oxidase activity of the purified solubilized cytochrome *bd* from *E. coli*.

Effect of inhibitors on peroxidase activity of cytochrome *bd*. The effect of inhibitors of the oxidase activity of the enzyme on its peroxidase activity was studied. The results are shown in the table and Fig. 7. It can be seen that the effect of inhibitors on the peroxidase and oxidase activity appeared to be similar. Potassium cyanide is known to be an inhibitor of cytochrome *bd*, which being bound to heme *d* blocks the operation of the enzyme active site. According to Kita et al. [64], 2 mM cyanide inhibits the oxidase activity of the enzyme by 50%. In this work, we showed that potassium cyanide at a concentration of 3 mM inhibits the peroxidase activity of cytochrome *bd* by 50%. The residual activity (~30%) is not inhibited by cyanide with increasing the inhibitor concentration up to 40 mM (Fig. 7). One reason for the incomplete inhibition might be heterogeneity of the preparation, which is known in the literature [1, 4], but other explanations are also possible.

The members of other classes of inhibitors, such as pentachlorophenol, zinc ions, and HOQNO, suppress the quinol oxidase activity of cytochrome *bd* by disrupting the operation of the ubiquinol binding site. As shown in the table, pentachlorophenol at a concentration of 1.5 mM, suppressing the oxidase activity of cytochrome *bd* by 61%, completely inhibits the peroxidase activity of the enzyme. HOQNO at a concentration of 100 μM also inhibits the peroxidase activity more strongly (by 70%) than the oxidase activity (by 44%). However, zinc ions as an inhibitor of the oxidase activity had no effect on the peroxidase activity of the enzyme even at a concentration of 150 μM. The reason why zinc ions do not inhibit the peroxidase activity is not clear yet. One can suggest that zinc ions bind to histidine residues or carboxyl groups of the protein, which form hydrogen bonds with two hydroxy groups of ubiquinol upon its specific binding in the active site. Guaiacol has only one hydroxy group that is partially shielded by the methoxy group in *o*-position; therefore, its binding in the quinol dehydrogenase site is likely to have mainly hydrophobic character and be insensitive to zinc-induced blockage of groups forming hydrogen bonds with OH-groups of ubiquinol.

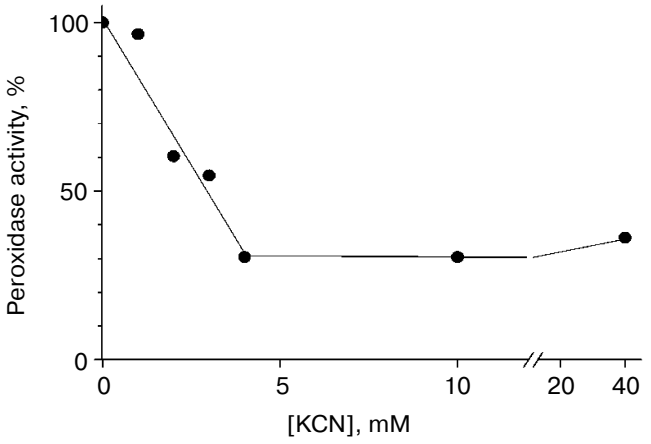

Fig. 7. Inhibition of cytochrome *bd*-catalyzed peroxidase activity by cyanide. The 100% rate of the reaction (in the absence of inhibitor) under these conditions corresponds to 1.12 electrons per second ($\bar{e}$/sec). The medium as in Fig. 2, but the concentrations of cytochrome *bd*, guaiacol, and hydrogen peroxide are 0.12 μM, 1.3 mM, and 2.5 mM, respectively.

Effect of guaiacol on the enzyme preparation. Figure 8 shows the effect of guaiacol on the absorption spectrum of the purified solubilized cytochrome *bd*. The cytochrome *bd* preparation pre-incubated under aerobic conditions ("air-oxidized") contains several forms of the enzyme. The main fraction is in the oxygenated form (~60-70%); there are also fractions of the oxo-ferryl (~20-

Comparison of effect of inhibitors of oxidase activity of cytochrome *bd* from *E. coli* on its peroxidase activity

Inhibitor	Peroxidase activity		Oxidase activity	
	concentration of inhibitor	residual activity, %	concentration of inhibitor	residual activity, %
Potassium cyanide	3 mM	50	2 mM	50 [64]
Pentachlorophenol	1.5 mM	0	1.5 mM	39
HOQNO	100 μM	30	100 μM	56
Zinc ions	150 μM	100	60 μM	50 [64]

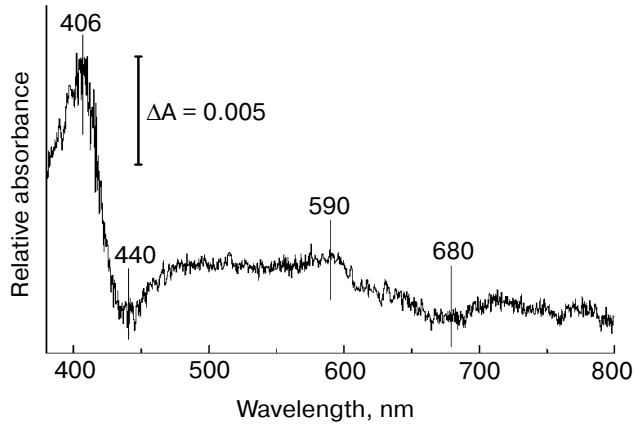

Fig. 8. Interaction of guaiacol with cytochrome *bd* preparation. The difference spectrum of the "air-oxidized" enzyme (90 min incubation with guaiacol *minus* 90 min incubation without guaiacol) is shown. The medium as in Fig. 2, but the concentrations of cytochrome *bd* and guaiacol are 3 μM and 1.3 mM, respectively.

30%) and fully oxidized (~5-10%) species. We decided to see which of the enzyme species interacts with guaiacol. It was found that the addition of guaiacol induces selective disappearance of the oxo-ferryl form of the enzyme (the development of a trough at 680 nm). A small peak around 590 nm and a blue shift of the Soret band can also be observed. A small increase in extinction at 590 nm could be due to partial reduction of heme b_{595}. The changes in the Soret band corresponding to this reduction (the expected narrow maximum at ~440 nm) can be the reason for the asymmetry of the observed blue shift mainly belonging to heme *d*, where a minimum at 440 nm is much smaller than a maximum at 406 nm. The oxygenated and oxidized forms of the enzyme do not interact with guaiacol. The fact that guaiacol influences the cytochrome *bd* preparation is useful from the methodological point of view since it enables removal of the admixture of the oxo-ferryl form from the aerobic oxygenated enzyme preparation, making the latter more homogeneous.

DISCUSSION

Why does oxidation occur via the peroxidase mechanism but it does not occur via the oxidase mechanism? The main result of our work is that cytochrome *bd* under aerobic conditions is capable of oxidizing various substrates in the peroxidase reaction, although they cannot be oxidized by the enzyme with any noticeable rate in the oxidase reaction. This fact can be explained by thermodynamic reasons. The midpoint potential values (E_m) for the substrates used are higher than the E_m values for the heme centers of cytochrome *bd* in the oxygenated and oxidized states. The E_m values for ferrocene and benzohydroquinone are ~ +400 mV [68] and +285 mV [69], respectively. The E_m values for ferrocyanide at the ferrocyanide/ferricyanide ratio of 1 : 10 is ~+500 mV [54]. The oxidation of guaiacol is an irreversible process accompanied by polymerization of the products, and its E_m value cannot be given. However, the first step of one-electron oxidation of the guaiacol molecule leads to the formation of a quite unstable free radical and, accordingly, this step should correspond to a sufficiently high redox potential (for example, guaiacol cannot be directly oxidized with ferricyanide). In the oxidized form of cytochrome *bd* from *E. coli*, the E_m values for the redox centers do not exceed +265 mV [10, 41, 70]. The E_m value for the d^{2+}-O_2/d^{3+}-OOH couple was not determined experimentally, but judging from the presence of the peroxidase activity, it is lower than the E_m values for the substrates of the peroxidase reaction tested. At the same time, the oxo-ferryl form of the enzyme has a much higher E_m value, and the participation of this form as a strong electron acceptor allows the reaction to take place. A midpoint potential for the transition of the oxo-ferryl form of cytochrome *bd* into the oxidized species was not determined experimentally, but based on the data obtained with a large number of hemoproteins, it is in the range from +0.8 to +1 V [71, 72]. A decisive role of the higher oxidation state of the heme iron in the catalysis of the peroxidase reaction is supported by the fact that guaiacol selectively reacts with the oxo-ferryl intermediate in the "air-oxidized" cytochrome *bd* preparation (Fig. 8).

It should be noted that a slight peroxidase activity of cytochrome *bd* with respect to a substrate such as *o*-dianisidine was observed in [73], but the authors did not pay much attention to this fact assuming that the peroxidase activity of the enzyme is due to heme b_{595}, which resembles the heme of cytochrome *c* peroxidase in its spectral and electrochemical properties.

Peroxidase activity of cytochrome *bd* is low compared to its oxidase activity. The peroxidase activity of the enzyme with respect to guaiacol appeared to be quite low. At $[H_2O_2]$ = 7.5 mM, the maximum enzyme turnover number observed was 3.6 (electrons) per second, whereas the oxidase activity of cytochrome *bd* with respect to ubiquinol-1 under similar conditions can reach up to 1100 per second [74]. The cytochrome *bd* turnover number in the peroxidation of the compounds tested is also not compatible with the specific activity of peroxidases for which the catalysis of such a reaction is the main function. One of the rate-limiting factors for the peroxidase reaction of cytochrome *bd* is the rate of the interaction of the enzyme with hydrogen peroxide. Based on the value of the second-order rate constant for the interaction of hydrogen peroxide with cytochrome *bd* (the formation of the oxo-ferryl complex of heme *d*) of 600 $M^{-1}\cdot sec^{-1}$ [53] (whereas for peroxidases this value can reach ~10^7 $M^{-1}\cdot sec^{-1}$), at $[H_2O_2]$ = 7.5 mM, the turnover number of about nine (electrons) per second can be expected. Thus, the peroxidase activity observed at this concentra-

tion of hydrogen peroxide is not so far from the maximum possible under these conditions. Similarly, the peroxidase activity (turnover number) of cytochrome *c* oxidase with respect to potassium ferrocyanide is 0.5 per second [54], which is three orders of magnitude lower than its oxidase activity. Interestingly, the peroxidase activity of cytochrome *c* oxidase measured recently [55] with respect to a number of aromatic substrates appeared to be about 1000 times lower than the specific rate of peroxidation of guaiacol by cytochrome *bd* measured in our work. Cytochrome *c* oxidase does not oxidize guaiacol at all [55]. Such differences are likely to be due to differences in the structure of the "input" centers in these two terminal oxidases. The "input" center of cytochrome *c* oxidase (Cu_A) is hydrophilic and therefore interacts poorly with relatively hydrophobic aromatic substrates. On the contrary, cytochrome *bd*, having a hydrophobic "input" (ubiquinol oxidase) center, catalyzes rather efficiently the peroxidation of the aromatic compounds such as guaiacol, ferrocene, and benzohydroquinone.

Mechanism of the peroxidase reaction. Some views on the mechanism of the peroxidase reaction catalyzed by cytochrome *bd* can be gleaned from the data on the effect of the inhibitors on this reaction. The inhibition of guaiacol-peroxidase activity by the inhibitors that block the ubiquinol-binding site suggests that guaiacol actually interacts with the enzyme at the same center as the natural donor (ubiquinol), at subunit I, and passes an electron through heme b_{558} into the di-heme oxygen-reducing site. The potassium cyanide-induced inhibition suggests that the reaction involves heme *d* that can interact with hydrogen peroxide, according to published data [53, 53]. This is possibly also the case for ferrocene and benzohydroquinone. How the oxidation of a substrate such as potassium ferrocyanide proceeds is less clear. Ferrocyanide is very hydrophilic, whereas the ubiquinol-binding site in cytochrome *bd* is hydrophobic, as mentioned above. Indeed, it is known that ferricyanide is a poor oxidant for cytochrome *bd* [75]. It is possible that ferrocyanide donates electrons to the enzyme like N,N,N′,N′-tetramethyl-*p*-phenylenediamine, bypassing the coenzyme Q-binding site.

The existence of peroxidase activity in bacterial cytochrome *bd* indicates that this partial reaction of the oxidase activity might be universal for respiratory oxidases. The presence of the peroxidase activity in cytochrome *bd*, analogous to that detected earlier in the mitochondrial heme–copper-containing oxidase, suggests that this reaction is linked to heme rather than to copper atoms. The identification of the peroxidase activity of the enzyme is essential for understanding the mechanism of the reaction of cytochrome *bd*.

Possible physiological and pathophysiological significance of peroxidase activity of cytochrome *bd*. It is likely that the peroxidase activity of cytochrome *bd* occurs under physiological conditions. In this case, an important function of cytochrome *bd* might be to provide the cell with detoxification of hydrogen peroxide. This assumption is evidenced by the fact that the *E. coli* mutants unable to synthesize cytochrome *bd* are sensitive to H_2O_2 [17]. In addition, a possible pathophysiological significance of this reaction for humans and animals cannot be ignored. Cytochrome *bd* present in *E. coli* is probably able to oxidize various aromatic compounds (including pharmacological agents) in the intestines of mammals and humans, leading thus to the formation of toxic products.

The authors are grateful to Prof. R. Gennis for the strain of *E. coli* GO105/pTK1.

This work was supported in part by grants from the Russian Foundation for Basic Research (project 08-04-00093-a) and Howard Hughes Medical Institute (project 55005615).

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