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ROLE OF REDOX-DEPENDENT PROTEINS IN REALIZATION OF CELLULAR REDOX REGULATION

UDC 577.151.63

Cytochrome *bd* as Antioxidant Redox Enzyme

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Received March 21, 2023; revised June 1, 2023; accepted June 1, 2023

Abstract—One of the main functions of enzyme complexes that constitute electron transport (respiratory) chains of organisms is to maintain cellular redox homeostasis by oxidizing reducing equivalents, NADH and quinol. Cytochrome *bd* is a unique terminal oxidase of the chains of many bacteria including pathogenic species. This redox enzyme couples the oxidation of ubiquinol or menaquinol by molecular oxygen to the generation of proton motive force, a universal energy currency. The latter is used by the organism to produce ATP, another cellular energy currency, via oxidative phosphorylation. *Escherichia coli* contains two *bd*-type oxidases, *bd*-I and *bd*-II, encoded by the *cydAB* and *appCB* operons, respectively. Surprisingly, both *bd* enzymes make a further contribution to molecular mechanisms of maintaining the appropriate redox balance in the bacterial cell by means of elimination of reactive oxygen species, such as hydrogen peroxide. This review summarizes recent data on the redox-modulated H_2O_2-scavenging activities of cytochromes *bd*-I and *bd*-II from *E. coli*. The possibility of such antioxidant properties in cytochromes *bd* from other bacteria is also discussed.

Keywords: redox enzyme, respiratory chain, cytochrome *bd*, heme, hydrogen peroxide, *Escherichia coli*, terminal oxidase, reactive oxygen species, oxidative stress

DOI: 10.1134/S0026893323060031

INTRODUCTION

Like most prokaryotes, *Escherichia coli* possesses a branched and flexible electron transport (respiratory) chain [1, 2]. The chain allows a bacterial population to grow and survive under changing environmental conditions, from oxic oxidized to anoxic reduced [3, 4]. It contains two NADH dehydrogenases (NDH-1 and NDH-2), succinate dehydrogenase (SDH), and three terminal oxygen reductases (*bd*-I, *bd*-II, and bo_3) [5] (Fig. 1). NDH-1 and NDH-2 oxidize NADH by ubiquinone. SDH oxidizes succinate by ubiquinone. *bd*-I, *bd*-II, and bo_3 oxidize ubiquinol and/or menaquinol by O_2. NDH-1, *bd*-I, *bd*-II, and bo_3 are primary generators of proton motive force [6–9] that is used for ATP production [10]. Energy conservation is one of the main functions of the chain. Another important function of the chain is maintaining cellular redox homeostasis. The latter is carried out through the oxidation of reducing equivalents, the NADH and ubiquinol/menaquinol pools.

A membrane-bound *bd*-type terminal oxidase is a key enzyme for bacterial adaptation to adverse environmental conditions, such as the presence of antibiotics [11], sulfide [12–16], nitric oxide [17–29], peroxynitrite [30, 31], ammonia [32], cyanide [33–35]. Cryogenic electron microscopy structures of both cytochrome *bd*-I [36, 37] and cytochrome *bd*-II [38, 39] were reported. A unified scheme of structural organization and catalytic function of the *E. coli bd* enzymes is shown in Fig. 2. The *bd*-I oxidase is composed of four subunits, CydA, CydB, CydX, and CydH (also called CydY). The *bd*-II enzyme is built by subunits AppC (homolog to CydA), AppB (homolog to CydB), and AppX (homolog to CydX), that is, one less. CydA/AppC carries a quinol binding site (named the Q-loop) and three hemes, one low spin, b_{558}, and two high spin, b_{595}, and *d* [40]. Heme b_{558} is located close to the Q-loop and directly involved in the oxidation of ubiquinol and/or menaquinol [33]. Heme *d* is the site at which O_2 is bound with a high affinity and reduced by four electrons to $2H_2O$ [41]. In *E. coli*, cytochrome *bd*-I and cytochrome *bd*-II are encoded by the *cydAB* and *appCB* operons, respectively [4].

Reactive oxygen species (ROS) include superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), and singlet oxygen ($^1O_2$). They are produced by the host immune system in response to microbial invaders. Bacteria can also generate H_2O_2 to eliminate their neighbors. *E. coli* cells growing exponentially produce H_2O_2 at rates of 9–22 μM/s [42].

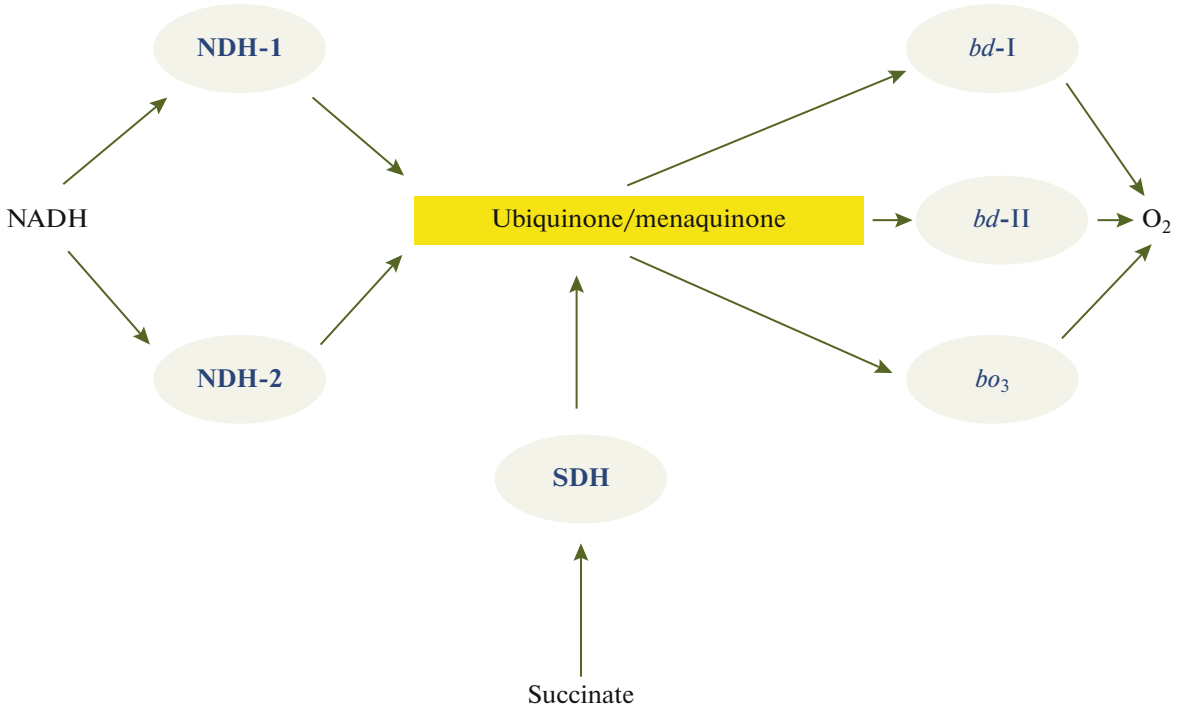

Fig. 1. Components of the respiratory electron transport chain of *Escherichia coli*. Two NADH dehydrogenases (NDH-1 and NDH-2) transfer electrons from NADH to ubiquinone/menaquinone. Succinate dehydrogenase (SDH) transfers electrons from succinate to ubiquinone/menaquinone. Three terminal oxygen reductases (*bd*-I, *bd*-II, and bo_3) oxidize ubiquinol/menaquinol by molecular oxygen to produce water. NDH-1, *bd*-I, *bd*-II, and bo_3 are primary generators of proton motive force. NDH-2 and SDH do not produce proton motive force.

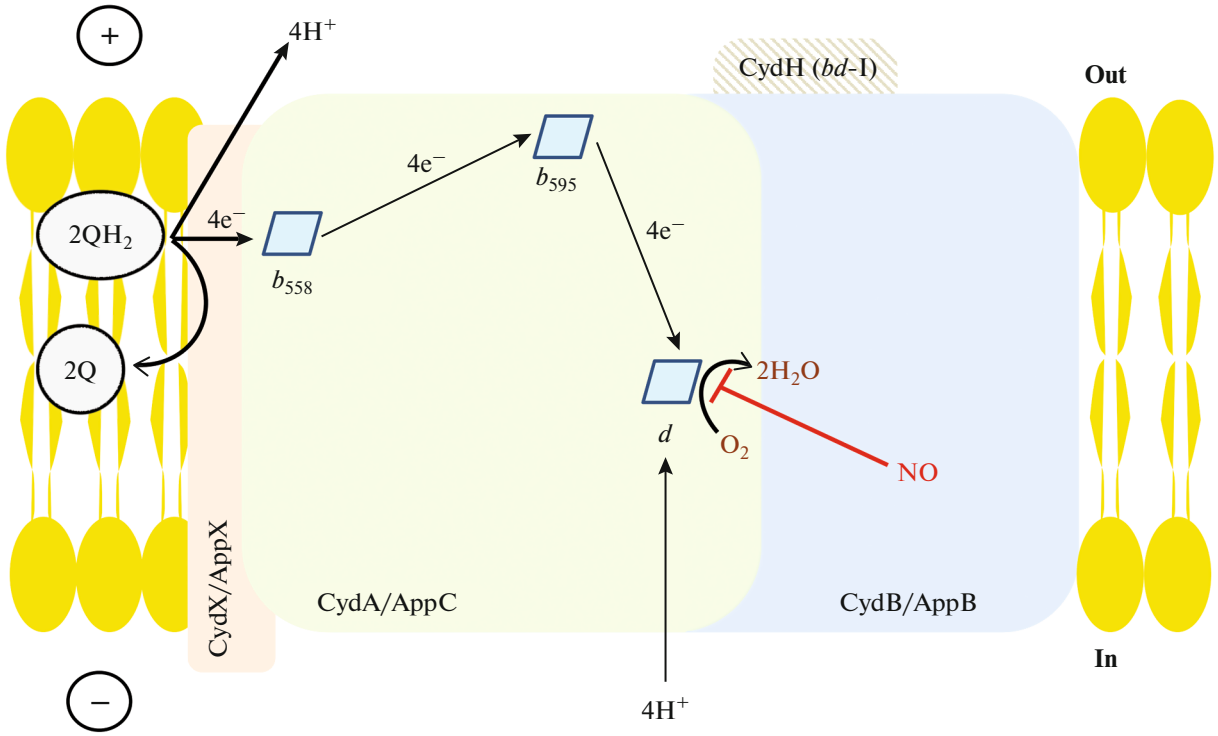

Fig. 2. Overview of structure and catalytic function of *E. coli bd* oxidase in the membrane bilayer. Cytochrome *bd*-I is composed of four subunits, CydA, CydB, CydX, and CydH. Cytochrome *bd*-II consists of three subunits, AppC, AppB, and AppX. CydA/AppC carries a ubiquinol/menaquinol (QH_2) binding site and three hemes, b_{558}, b_{595}, and *d*. Electrons are transferred from QH_2 to heme b_{558}, then to heme b_{595} and eventually to heme *d*. The latter is the active site at which O_2 is reduced to $2H_2O$ by four transferred electrons and four protons arrived from the inner side of the membrane via a proposed transmembrane proton half channel. The enzyme-catalyzed oxygen reduction reaction is inhibited by NO.

Increased levels of ROS disrupt the cellular redox homeostasis, cause the oxidative stress and severe damage to DNA, RNA, proteins and lipid membranes. Bacteria contain special enzymes, such as catalases, superoxide dismutases and peroxidases, which detoxify ROS [43]. *E. coli* for this purpose uses the KatG and KatE catalases [44], the NADH peroxidase AhpCF [45], and the cytochrome *c* peroxidase YhjA [46]. Recently, evidence has emerged that a *bd*-type terminal oxidase also contributes to the antioxidant defense machinery in *E. coli* cells.

CATALASE-LIKE ACTIVITY OF TERMINAL OXIDASES *bd*-I AND *bd*-II FROM *E. coli*

The polarographic studies showed that both cytochrome *bd*-I and cytochrome *bd*-II, being isolated from the *E. coli* membranes without tags, decompose H_2O_2 with concomitant evolution of O_2 [47–49]. The fact that O_2 formation is no longer observed after thermal inactivation of the cytochrome suggests that this activity is associated with a native protein rather than adventitious transition metals in the enzyme preparation. Approximately half a mole of O_2 is evolved per mole of H_2O_2 added. The reaction product, O_2, apparently does not inhibit the catalytic decomposition of H_2O_2 because the reaction rates under microaerobic and aerobic conditions are virtually identical. The rate of O_2 evolution increases linearly with enzyme concentration. The reaction also accelerates linearly with the H_2O_2 concentration until the latter reaches 0.5 mM. At higher concentrations of H_2O_2 the rate tends to saturate. The apparent k_{cat} and K_M are about 165 s^{-1} and 1.7 mM for cytochrome *bd*-I and 1030 s^{-1} and 3.9 mM cytochrome *bd*-II, respectively. Both enzymes retain the catalase-like activity when they are in turnover with the reducing substrate ubiquinol-1 and the terminal oxidant O_2. The fact that H_2O_2 does not inhibit the O_2 reductase activity in which heme *d* is directly involved indicates that the participation of this heme in the catalase-like reaction is unlikely.

Remarkably, the catalase-like activity of both cytochromes in the presence of excess ubiquinol-1 is no longer observed after they consume all O_2 in the measuring chamber and are converted into the fully reduced state. If a commercial catalase is then added to the chamber as a control, the O_2 production is observed again. Thus, this activity is sensitive to the enzyme redox state. This finding can exclude the possibility that the observed activity is due to impurities in the enzyme preparations which contain an *E. coli* catalase. It is unlikely that the contaminant catalase, if present, would be redox-sensitive. It is known that usually dithionite, a very potent reductant, cannot reduce a catalase [50], even if a redox mediator is added [51]. Furthermore, the catalase-like activity is also observed in vivo [47]. There are significant rates of O_2 evolution following the addition of H_2O_2 to respiring *E. coli* UM2 cells which are deficient in the KatG and KatE catalases but overexpressing cytochrome *bd*-I. If the *bd*-I enzyme is not overexpressed, the activity is not observed.

It should be noted that neither the isolated hexahistidine-tagged *bd*-I enzyme and nor membrane fractions with Strep-tagged cytochrome *bd*-I lacking CydH reveal a catalase-like activity [52, 53]. For production of the His_6-tagged *bd*-I protein, a *cydABX* pACYC177 overexpression vector modified by addition of six histidine triplets at the 3'-end of *cydA* (C-terminal His_6-tag) was used [52]. For production of the Strep-tagged cytochrome *bd*-I, a pET17bp*cydABX* vector with a linker and streptag-II sequence down-stream of *cydX* was used [53]. The data reported in [52, 53] are inconsistent with our results on the untagged *bd*-type oxidases [47–49]. This discrepancy could be due to the differences between the enzyme forms, untagged vs tagged, and other experimental conditions. Additional work is required to resolve the discrepancy.

To try to identify a specific site in the enzyme that is associated with the catalase-like activity, we checked a few inhibitors with different mode of action. We found that both CO and NO, which were reported to target the ferrous heme *d* [17, 54], do not inhibit the activity. This further suggests that heme *d* is not responsible for the reaction. *N*-ethylmaleimide was found to be ineffective to inhibit the activity meaning that a sulfhydryl group is not involved in the activity. Antimycin A and the oxidized ubiquinone-1 also do not affect this activity suggesting that the quinol binding site of the enzymes does not participate in the reaction. Cyanide and azide, on the contrary, turned out to inhibit the catalase-like activity of both cytochrome *bd*-I and cytochrome *bd*-II. Both usually target a high-spin ferric heme. The apparent IC_{50} for NaCN are 2.5 and 4.5 μM for the *bd*-I and *bd*-II, respectively. 100 μM NaN_3 was reported to inhibit 98% and 35% of the activity of the *bd*-I and *bd*-II, respectively. Cyanide and azide are much poorer inhibitors of the O_2 reductase activity of the enzymes and most likely do it via binding to the high-spin heme *d* [4, 33, 55]. In view of the latter fact, much higher sensitivity of the catalase-like activity to these poisons points to the involvement of a high-spin heme, different from heme *d*, in the reaction. Since a *bd*-type oxidase contains only two high-spin hemes, b_{595} and *d*, the former could be a reasonable candidate (Fig. 3). In this regard, it is interesting to note that the reduced-minus-oxidized difference absorption spectrum of heme b_{595} is similar to those of catalases and peroxidases which contain protoheme IX as a cofactor [56]. The addition of 50 μM NaCN, completely inhibiting the catalase-like activity, gives rise to a red shift in the Soret absorption band in both enzymes. The magnitudes of the induced absorption change suggest that the inhibitor binds to a high-spin *b*-type heme in a small fraction of the cytochrome population [47, 49].

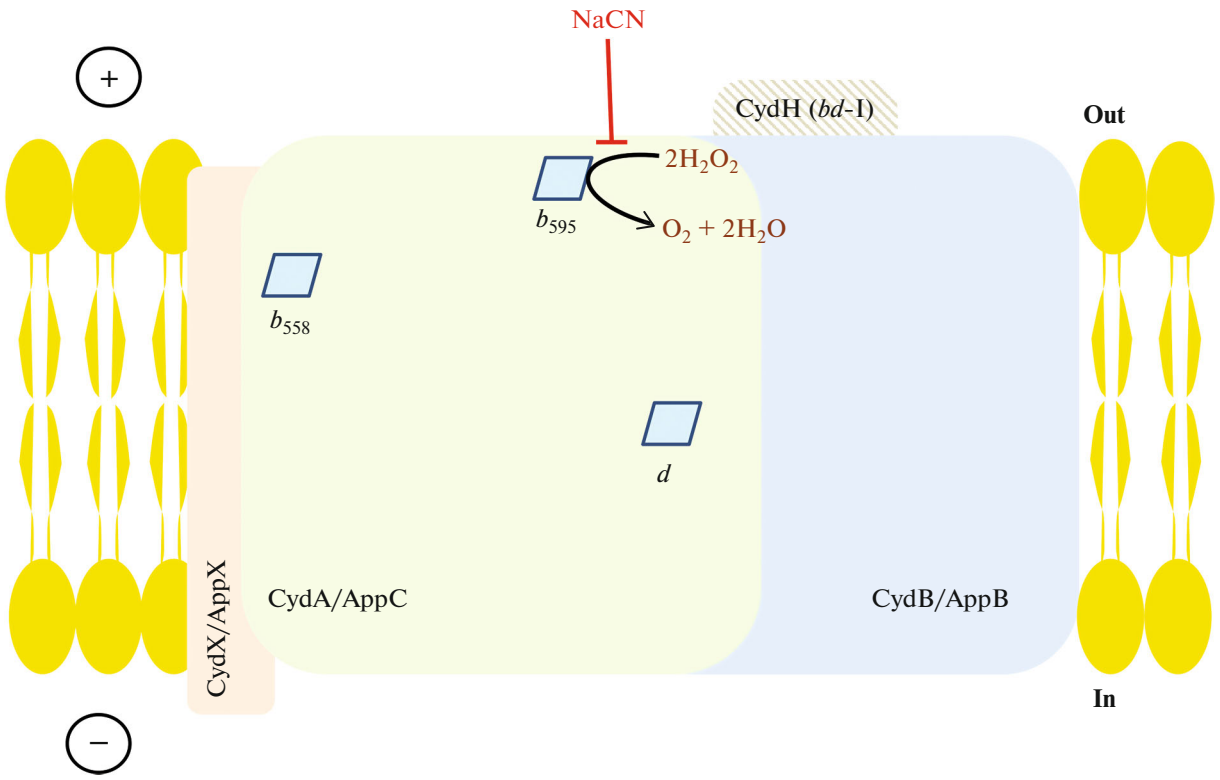

Fig. 3. Schematic representation of proposed mechanism of catalase-like reaction catalyzed by *E. coli* oxidases *bd*-I and *bd*-II. The catalase-like reaction is possibly associated with heme b_{595}. The reaction is inhibited by NaCN.

This in turn might indicate that only a minor part of the enzyme macromolecules participates in the reaction. Further experiments are required to test this hypothesis.

PEROXIDASE-LIKE ACTIVITY OF TERMINAL OXIDASE *bd*-I FROM *E. coli*

The *E. coli* cytochrome *bd*-I is also able to scavenge H_2O_2 via peroxidase-like mechanism that was first observed with the untagged purified enzyme [57]. The electron donors, such as guaiacol, ferrocene, benzohydroquinone, and ferrocyanide (at the ratio of ferrocyanide to ferricyanide of 1 : 10) were shown to be oxidized by the enzyme under aerobic conditions following the addition of H_2O_2. The effect of several inhibitors of the O_2 reductase activity on the peroxidase-like activity of cytochrome *bd*-I was examined using guaiacol as the substrate. Cyanide, 2-*n*-heptyl 4-hydroxyquinoline-*N*-oxide (HQNO), and pentachlorophenol appeared to inhibit the enzyme-mediated peroxidation of guaiacol [57]. This finding suggests that guaiacol reacts with the enzyme at the level of the quinol binding site and donates electrons to heme *d* via heme b_{558} located close to the site and probably heme b_{595}. The electrons reduce the H_2O_2 bound to heme *d* to form water (Fig. 4). In these experiments, the rate of the peroxidase-like reaction was quite low, ~4 s^{-1}. Nevertheless, it was suggested that the activity should be significantly higher in vivo conditions in which the endogenous ubiquinol-8 and/or menaquinol-8 serves as the substrate [31].

Later, the peroxidase-like activity of the isolated hexa-histidine-tagged *bd*-I enzyme under anaerobic conditions was detected [52]. In this work, decylubiquinol was used as the substrate. The k_{cat} and K_M for H_2O_2 were determined to be 101 s^{-1} and 6.6 mM, respectively, yielding a specificity constant, k_{cat}/K_M, of 1.5×10^4 M^{-1} s^{-1}. Unlike the catalase-like activity [47–49], peroxidation of decylubiquinol is quickly and reversibly inhibited by NO [52]. The latter further suggests that heme *d* is directly implicated in the peroxidase-like mechanism. Thus, the peroxidase-like reaction requires decylubiquinol as the electron donor and likely occurs at heme *d* [52]. HQNO at the concentrations of 10–15 μM causes 50% inhibition of the decylubiquinol peroxidase activity. Hence, in this reaction decylubiquinol donates electrons directly at the quinol binding site of the enzyme.

The ability of the *bd*-type terminal oxidase to degrade H_2O_2 at high rates may play a role in the *E. coli* physiology by protecting the microbial cells against oxidative stress and maintaining a balanced redox status.

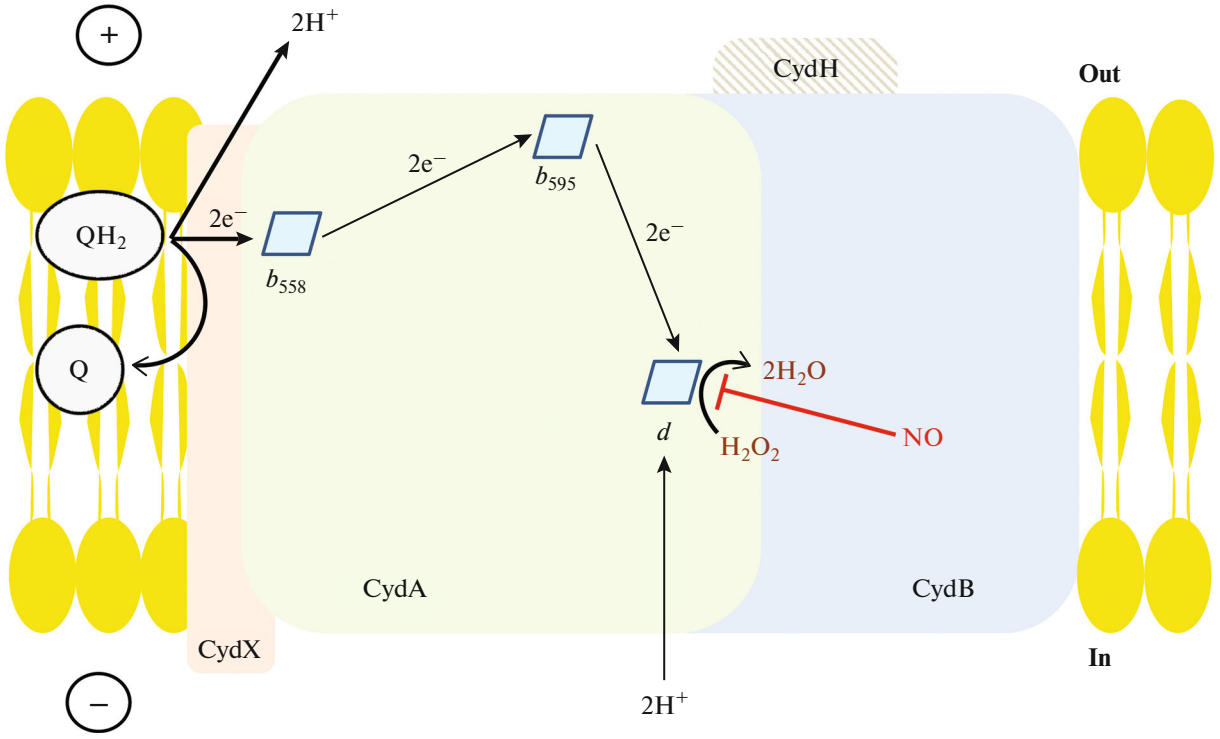

Fig. 4. Schematic representation of proposed mechanism of peroxidase-like reaction catalyzed by *E. coli* oxidase *bd*-I. The peroxidase-like reaction requires decylubiquinol (QH_2) as the electron donor and likely occurs at heme *d* [52]. The reaction is inhibited by NO.

ARE *bd*-TYPE TERMINAL OXIDASES FROM OTHER BACTERIA CAPABLE OF H_2O_2 DETOXIFICATION?

To date the H_2O_2-scavenging activities were clearly demonstrated only for the *bd* enzymes of *E. coli*. But do cytochromes *bd* from other bacteria have these activities? For now, this question remains open. Nonetheless, there is some evidence that such a protective function of the *bd* oxidase is possibly not limited to *E. coli*. It was reported that low H_2O_2 concentrations are significantly more toxic to the *Azotobacter vinelandii* mutant lacking cytochrome *bd* than to the wild-type [58]. *Brucella abortus* mutants deficient in cytochrome *bd* activity also reveal heightened sensitivity to H_2O_2 [59]. The sensitivity is reversed in transformants provided with the plasmid pSEK102 having the entire *cydAB* operon. Overexpression of Cu^{+2}/Zn^{+2} superoxide dismutase and catalase is sufficient to alleviate the loss of cytochrome *bd* suggesting similar antioxidant functions of these enzymes in *B. abortus*. The transcriptome analysis of the cellular response of *Staphylococcus aureus* to H_2O_2-induced oxidative stress showed that the *cydAB* genes are strongly induced upon exposure to this ROS, pointing to the role of cytochrome *bd* in oxidative protection processes [60]. It was found that in *Mycobacterium tuberculosis* inactivation of the thioredoxin-like protein Rv3673c, required for heme insertion in cytochrome *c*, results in both increased expression of the *bd* oxidase and increased resistance to H_2O_2 [61]. This finding suggests that the *M. tuberculosis* cytochrome *bd* may contribute to enzymatic antioxidative defense mechanisms via direct detoxification of H_2O_2 [62]. Consistently, *Mycobacterium smegmatis* mc2155 mutant Δ*cydA*::kan shows the hypersensitivity to exogenous H_2O_2, indicating that cytochrome *bd* plays a protective role during oxidative stress in this bacterium [63]. Exponentially growing Δ*cydbd* mutant strain of *Porphyromonas gingivalis* was reported to demonstrate the increased susceptibility to H_2O_2 compared to the wild-type [64]. The complementation of the mutant with the native *cydAB* genes partially restores the resistance to this ROS. Similarly, Δ*cydbd* mutant strain of *Alishewanella* sp. WH16-1 appeared to be more sensitive to H_2O_2 than the wild-type, suggesting that the *bd* oxidase could catalyze the decomposition of H_2O_2 [65]. Consistently, in the case of *Xanthomonas oryzae* pv. *oryzicola* strain BLS256, *cydA*- and *cydAB*-knockout mutants display a higher sensitivity to H_2O_2 along with a reduced bacterial virulence compared to the wild-type [66]. Summarizing the above, it can be assumed that the *bd* enzymes in the above-mentioned bacteria also contribute to the molecular mechanisms of protection against ROS-induced oxidative damage through the direct elimination of H_2O_2.

CONCLUSIONS

Studies of cytochromes *bd* at the molecular level suggest that the enzymes from *E. coli* and possibly other bacteria enable the microbial cell to maintain a balanced redox status in two different ways: via (i) the oxidation of the membrane-bound quinol molecules and (ii) ROS detoxification.

FUNDING

This work was supported by the Russian Science Foundation (project no. 22-24-00045, https://rscf.ru/en/project/22-24-00045/).

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare no conflicts of interest. This article does not contain any research involving humans or animals as research objects.

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ADDITIONAL INFORMATION

The text was submitted by the author(s) in English.

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