Biochimica et Biophysica Acta 1807 (2011) 1398–1413

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Review

The cytochrome *bd* respiratory oxygen reductases

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ARTICLE INFO

Article history:
Received 23 May 2011
Received in revised form 23 June 2011
Accepted 24 June 2011
Available online 1 July 2011

Keywords:
Metabolism
Molecular bioenergetics
Oxidoreduction
Bacterial physiology
Microbe
Disease

ABSTRACT

Cytochrome *bd* is a respiratory quinol:O_2 oxidoreductase found in many prokaryotes, including a number of pathogens. The main bioenergetic function of the enzyme is the production of a proton motive force by the vectorial charge transfer of protons. The sequences of cytochromes *bd* are not homologous to those of the other respiratory oxygen reductases, i.e., the heme–copper oxygen reductases or alternative oxidases (AOX). Generally, cytochromes *bd* are noteworthy for their high affinity for O_2 and resistance to inhibition by cyanide. In *E. coli*, for example, cytochrome *bd* (specifically, cytochrome *bd*-I) is expressed under O_2-limited conditions. Among the members of the *bd*-family are the so-called cyanide-insensitive quinol oxidases (CIO) which often have a low content of the eponymous heme *d* but, instead, have heme *b* in place of heme *d* in at least a majority of the enzyme population. However, at this point, no sequence motif has been identified to distinguish cytochrome *bd* (with a stoichiometric complement of heme *d*) from an enzyme designated as CIO. Members of the *bd*-family can be subdivided into those which contain either a long or a short hydrophilic connection between transmembrane helices 6 and 7 in subunit I, designated as the Q-loop. However, it is not clear whether there is a functional consequence of this difference. This review summarizes current knowledge on the physiological functions, genetics, structural and catalytic properties of cytochromes *bd*. Included in this review are descriptions of the intermediates of the catalytic cycle, the proposed site for the reduction of O_2, evidence for a proton channel connecting this active site to the bacterial cytoplasm, and the molecular mechanism by which a membrane potential is generated.

1. Diversity of respiratory oxygen reductases

Respiratory oxygen reductases (terminal oxidases) are enzymes at the end of the respiratory chains of organisms which couple the oxidation of a respiratory substrate (one-electron donor, cytochrome *c*, or two-electron donor, quinol (QH_2)) to the four-electron reduction of O_2 to water. There are three families of oxygen reductases (Fig. 1).

1.1. Heme–copper family

The first, most extensively studied family comprises the heme–copper oxygen reductases. They have a binuclear O_2-reduction site composed of a high spin heme (a_3, o_3, or b_3) and a copper ion (Cu_B), and these enzymes generate a PMF via a "proton pump" mechanism [1–7]. The PMF is utilized for various biosynthetic activities (e.g., ATP production), solute active transport and mechanical movement (e.g., flagellar rotation). The heme–copper family of oxygen reductases includes both cytochrome *c* oxidases and quinol oxidases. Most of the heme–copper oxygen reductases are members of one of three distinct subfamilies: A, B, and C [8,9]. The A subfamily includes the mitochondrial cytochrome *c* oxidases as well as many prokaryotic cytochrome *c* oxidases and quinol oxidases. Enzymes in the A-subfamily utilize at least two proton pathways to deliver protons to the active site or for proton pumping. The B subfamily includes a number of oxygen reductases from extremophilic prokaryotes, such as the ba_3-type oxygen reductase from *T. thermophilus* [10]. The enzymes of the C subfamily are all cbb_3-type oxidases [11]. Recently, it has been shown that the enzymes from the B and C subfamilies utilize only one proton-conducting input pathway [10,12]. High-resolution X-ray crystal structures of the heme–copper oxidases from all three subfamilies have been reported [11,13–23].

1.2. Alternative oxidase (AOX) family

The second family of respiratory oxygen reductases comprises cyanide-resistant AOX found in mitochondria of higher plants, fungi and protists as well as in prokaryotes and some animal species [24]. In plants,

Abbreviations: AOX, alternative oxidase; CIO, cyanide-insensitive quinol oxidase; E_m, apparent midpoint redox potential; IC_{50}, the half maximal inhibitory concentration; PMF, proton motive force; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylendiamine; Q, quinone; QH_2, quinol; UQH_2, ubiquinol; MQH_2, menaquinol; PQH_2, plastoquinol; A^1, one electron-reduced O_2-bound species; A^3, fully reduced O_2-bound species; R^1, one electron-reduced species; R^3, fully reduced species; O, fully oxidized species; F, oxoferryl species; P, peroxide-bound species; $\Delta\mu_H^+$, transmembrane difference in the electrochemical H^+ potentials; τ, time constant reciprocal of rate constant ($t_{1/e}$)

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0005-2728/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2011.06.016

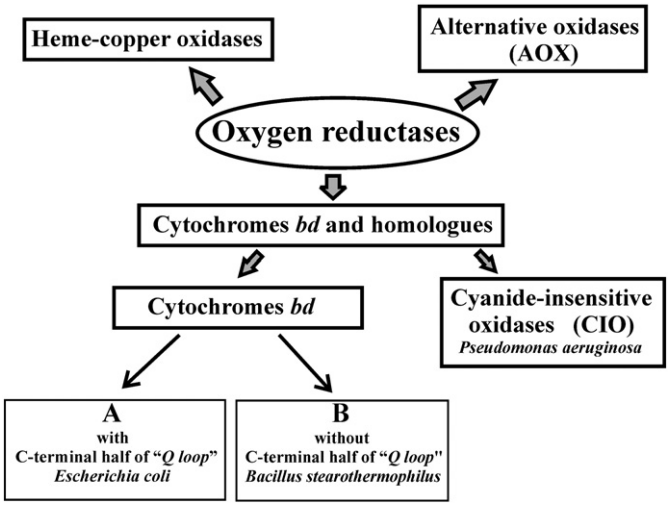

Fig. 1. Respiratory oxygen reductases. The *bd*-family is subdivided into the A-subfamily (long Q-loop), B-subfamily (short Q-loop) and the cyanide insensitive oxygen reductases (CIO). These are subdivisions based entirely on spectroscopic and structural observations and are not phylogentically defined clades.

this is a homodimeric enzyme associated with the matrix side of the inner mitochondrial membrane. AOX uses UQH_2, but not cytochrome *c*, as the electron donor, and contains a non-heme di-iron carboxylate active site for O_2 reduction.

AOX does not produce a PMF, and is not coupled to transmembrane charge transfer. However, AOX is responsible for heat generation in some tissues, and plays a role in the regulation of energy metabolism, facilitating turnover of the TCA cycle, protection against oxidative stress, and homeostasis. To date, no high-resolution AOX structure has been reported, but crystals that diffract to better than 3.0 Å have been described [25].

1.3. Cytochrome bd-family

The third family of oxygen reductases comprises cytochromes *bd*. These are quinol oxidases found in a wide variety of prokaryotes. They show no sequence homology to any subunit of heme–copper family members or AOX and do not contain any copper or non-heme iron [26–33]. This two-subunit integral membrane protein (subunits I and II) contains three hemes, b_{558}, b_{595} and *d*, and it is generally thought that hemes b_{595} and *d* form a di-heme site for the reduction of O_2 (Fig. 2) [34–43]. Unfortunately, no X-ray structure of any *bd*-type oxygen reductase has been reported. Cytochrome *bd* generates a PMF by transmembrane charge separation, but does so without being a "proton pump" [41,44–50]. In a number of organisms, the *bd* oxygen reductase is induced under O_2-limited conditions as well as under other growth conditions that can be considered stressful, such as Fe deficiency [51–54]. All known members of the *bd*-family of oxygen reductases are quinol oxidases, most commonly using ubiquinol or menaquinol as substrates.

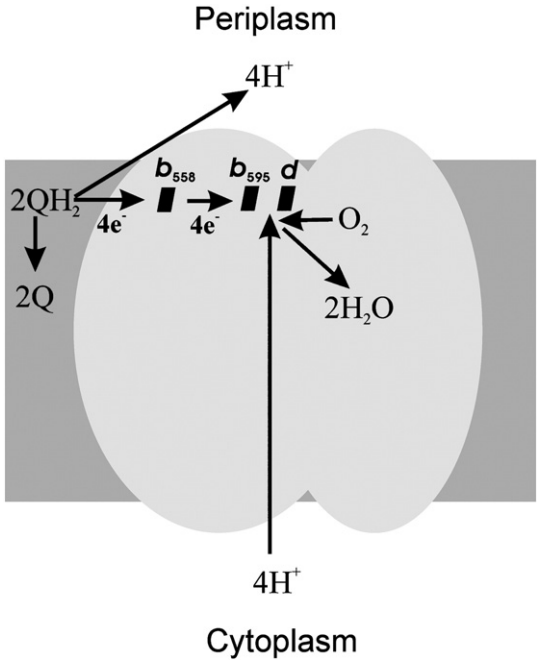

Fig. 2. Proposed cytochrome *bd* model.

Analysis of prokaryotic genomes shows that many aerobic prokaryotes do not contain any member of the *bd*-family, but contain only heme–copper oxygen reductases. However, there are a number of prokaryotes that encode more than one *bd*-family member, for example, two: *E. coli* [53,55], *Bacillus subtilis* [56]; three: *Vibrio cholerae* [57]; and as many as six *bd*-type oxygen reductases: some *Acidithiobacillus* strains. Organisms that express one or more *bd*-type oxygen reductases, usually also possess at least one heme–copper oxygen reductase. However in some cases (e.g., *Lactobacillus plantarum* [58], *Zymomonas mobilis* [59], the two *Thermoplasma* strains [60]) cytochrome *bd* is the only oxygen reductase.

1.3.1. The Q-loop

The hydrophilic region of subunit I connecting transmembrane helices 6 and 7, facing the outside of the prokaryotic cell, has been implicated as part of the quinol binding site [61–66], and this is referred to as the "Q loop". Some of the *bd*-family oxygen reductases have an insert in the C-terminal portion of the Q-loop and, hence, have a "long Q-loop", e.g., enzymes isolated from *Escherichia coli* and *Azotobacter vinelandii* [67,68]. The majority of *bd*-type oxygen reductases have a "short Q-loop", e.g., the enzyme isolated from *Bacillus stearothermophilus* [67–69]. It is not clear what the functional consequences are, if any, from this difference in the size of the Q loop.

1.3.2. Cyanide insensitive oxidases (CIO)

An anecdotal observation is that some of the "short Q-loop" oxygen reductases appear to have an altered heme content, in which the amount of heme *d* is significantly reduced (or totally missing) and is replaced by a heme *b*. This appears to be the case for a *B. subtilis* cytochrome *bd* [70]. When these enzymes, with a low content of heme *d*, have been characterized in bacterial membranes, respiration continues even in the presence of 1 mM KCN [71], but the membranes do not have the spectroscopic signature of heme *d* (a peak in the reduced form near 630 nm) [71–75]. As a result, these enzymes have been called *c*yanide *i*nsensitive *o*xidases (CIO) [73]. Examples are *P. aeruginosa* [71–73,76], *P. putida* [77], *P. pseudoalcaligenes* [74], *Staphylococcus carnosus* [78], *C. jejuni* [75], and *Z. mobilis* [59]. On the contrary, using low temperature absorption spectroscopy, EPR and mass spectrometry, Mogi et al. [79] reported that CIO in the membranes from *G. oxydans* has the same heme contents present in a classical cytochrome *bd*, although reveals unique spectroscopic and ligand-binding properties. Whether the CIO heme composition is strain- and/or growth-specific, or the heme spectral features were not detected due to a very low enzyme concentration in the tested membranes remains to be studied. It is now clear that CIOs are *bd*-family oxygen reductases.

cioA and *cioB* genes which encode CIO in *P. aeruginosa* and *P. pseudoalcaligenes* were sequenced [73,74]. They comprise the *cio* operon. CioA and CioB are homologous to subunits I and II of cytochrome *bd*-I from *E. coli* and the *bd*-oxidase from *A. vinelandii* [73]. Histidine and methionine residues identified in cytochrome *bd*-I from *E. coli* as the axial ligands to heme b_{558} and heme b_{595} are conserved [73]. It was proposed that the slight differences in sequence and structure of the CydB subunit are responsible for cyanide resistance

[78]. It is of interest to note that cytochrome *bd* of the cyanobacterium *Synechocystis* sp. PCC 6803 appeared to be structurally related to CIO [80]. To date, no CIO has been purified and characterized, primarily because these enzymes appear to be particularly labile. At low O_2 tensions, the opportunistic pathogen *P. aeruginosa* synthesizes HCN as a metabolic product at concentrations of up to 0.3 mM [81]. Under these conditions, the heme–copper oxidases are inhibited. CIO likely has a role in allowing aerobic respiration under cyanogenic and microaerobic growth conditions [71,73,82]. Cyanide can be found in tissues infected with *P. aeruginosa* [83] that is consistent with the conclusion that CIO is required for full pathogenicity of *P. aeruginosa* in the cyanide-mediated paralytic killing of nematodes [84]. Mutation or overexpression of the *cioAB* genes of *P. aeruginosa* leads to temperature sensitivity for growth, difficulty exiting stationary phase, abnormal cell division and multiple antibiotic sensitivity [85].

There is no distinguishing feature in the sequences of the genes that allows one to differentiate CIO from other cytochrome *bd* family members. It is not yet clear whether the "short Q-loop" is a requirement for having the CIO phenotype or under what conditions such enzymes may or may not contain a stoichiometric content of heme *d*.

2. Physiological functions

The bioenergetic function of cytochrome *bd* is to conserve energy in the form of $\Delta\mu_H^+$ [41,45–50], although the H^+/e^- ratio is 1, half the value of the A-subfamily heme–copper oxygen reductases such as the mitochondrial cytochrome *c* oxidase or cytochrome bo_3 from *E. coli* because the *bd*-type oxygen reductases do not pump protons [45,49,50].

Apart from PMF generation, cytochrome *bd* endows bacteria with a number of vitally important physiological functions. Cytochrome *bd* facilitates both pathogenic and commensal bacteria to colonize O_2-poor environments [86–89], serves as an O_2 scavenger to inhibit degradation of O_2-sensitive enzymes such as nitrogenase [90–98], and support anaerobic photosynthetic growth [99]. It is of interest to note that *bd*-type oxygen reductases predominate in the respiratory chains of bacteria that cause such diseases as bacillary dysentery [100], brucellosis [88,101], tuberculosis [87], pneumonia, life-threatening sepsis, meningitis [102], as well as *Salmonella* [103,104], *Bacteroides* [86], and *Listeria monocytogenes* [105] infections. There is a positive correlation between virulence of bacterial pathogens responsible for these diseases and level of cytochrome *bd* expression. Cytochrome *bd* enhances bacterial tolerance to nitrosative stress [106–111], contributes to mechanisms of detoxification of hydrogen peroxide in *E. coli* [112–114], suppresses extracellular superoxide production in *Enterococcus faecalis* [115], and is involved in the degradation of aromatic compounds in *Geobacter metallireducens* [116]. The *A. vinelandii* cytochrome *bd* might be directly involved in energizing Fe-siderophore transport or in reduction of Fe (III)-chelates and, thus, metal liberation in the cytoplasm [117]. As a source of oxidizing power, cytochrome *bd*-I in *E. coli* can support disulfide bond formation upon protein folding catalyzed by the DsbA–DsbB system [118], as well as the penultimate step of heme biosynthesis, the conversion of protoporphyrinogen IX into protoporphyrin IX, catalyzed by protoporphyrinogen IX oxidase [119].

The expression and membrane content of cytochrome *bd* in *E. coli* increase not only at low O_2 concentrations [120–122], but also under other stressful conditions, such as alkalization of the medium [123], high temperature [124,125], the presence of poisons in the environment (for instance, cyanide [126,127]), uncouplers-protonophores [123,128,129] and high hydrostatic pressure [130,131]. *E. coli* mutants defective in cytochrome *bd* are sensitive to H_2O_2 [125], zinc [127,132] and a self-produced extracellular factor that inhibits bacterial growth [133,134]. *E. coli* mutants that cannot synthesize cytochrome *bd* are also unable to exit from the stationary phase and resume aerobic growth at 37 °C [135,136].

Since cytochrome *bd* is found only in prokaryotes, including a number of human pathogens, the enzyme may be of interest as a drug target. A search for specific inhibitors of the *bd*-type oxygen reductases, which could be used in clinical practice, has been started [137,138]. An alternative, "positive" potential use of cytochrome *bd* might be for a therapy of respiratory chain deficiencies. It is known that mutations in genes encoding structural subunits of cytochrome bc_1 complex and cytochrome *c* oxidase can lead to severe neuromuscular and non-neuromuscular human diseases [139,140]. At the same time, it was reported that mixing purified cytochrome *bd*-I from *E. coli* with myxothiazol-inhibited bovine heart submitochondrial particles restores up to half of the original NADH oxidase and succinate oxidase activities in the absence of exogenous ubiquinone analogs [141]. Respiration bypassing the bc_1 complex is saturated at amounts of added *bd*-oxidase similar to that of other natural respiratory components in submitochondrial particles. Bacterial cytochrome *bd*-I tightly binds to the mitochondrial membrane and functions as an intrinsic component of the chimeric respiratory chain [141]. Thus, cytochrome *bd*, as well as AOX [142–144], might compensate for respiratory chain deficiencies in human cells.

3. Inhibitors

Table 1 shows the effect of different inhibitors on the respiratory activity of cytochrome *bd* from some bacteria. Quinol oxidase inhibitors can be divided into two groups: Q-like compounds acting at the Q binding site and heme ligands (e.g., cyanide, azide or NO) acting at the O_2 binding/reducing site. A specific feature of cytochrome *bd* is that it is much less sensitive to cyanide and azide than a heme–copper oxygen reductase like cytochrome bo_3 [27]. The lower sensitivity of cytochrome *bd* to anionic heme ligands may be a result of an elevated electron density on the central ion of iron due to breaking the conjugate π-electron structure in the *d*-type porphyrin ring and/or may point to a more hydrophobic environment of the O_2-reducing site. It was reported that cytochrome *bd*-I in *E. coli* is a bacterial membrane target for a cationic cyclic decapeptide gramicidin S (IC_{50} ~ 5.3 μM, Table 1), although it has been generally accepted that the main target of gramicidin S is the membrane lipid bilayer rather than the protein components [145]. This finding can provide a new insight into the molecular design and development of novel gramicidin S-based antibiotics. The effect of gramicidin S on cytochrome *bd*-I and some other membrane-bound proteins could be the alteration of the protein structure through binding to its hydrophobic protein surface [145].

4. Genetics

4.1. Genes in E. coli encoding the protein subunits and assembly factors

Of the *bd* family, the best studied oxidase is cytochrome *bd*-I from *E. coli*. The two subunits of cytochrome *bd*-I are encoded by the *cydAB* operon [28,146,147] located at 16.6 min on the *E. coli* genetic map [146,148]. It was cloned [149] and sequenced [28]. The molecular weights of subunit I (CydA) — 57 kDa, and subunit II (CydB) — 43 kDa, determined by sodium dodecyl sulfate-polyacrylamide-gel electrophoresis [26], are consistent with those of 58 and 42.5 kDa based on DNA sequence [28]. The enzyme subunits carry three hemes: b_{558}, b_{595}, and *d* [34,150]. Heme b_{558} is located on subunit I (CydA), whereas hemes b_{595} and *d* are likely to be in the area of the subunit contact [151]. CydA can be expressed and purified without CydB using mutant strains defective in *cydB* [152]. The purified CydA retains heme b_{558} but lacks hemes b_{595} and *d* [152]. In addition to the *cydAB* operon, the two other genes, *cydC* and *cydD* of the *cydCD* operon located at 19 min on the *E. coli* genetic map [132,153,154], are essential for the assembly of cytochrome *bd*-I [153–156]. CydC and CydD however are not subunits of cytochrome *bd*-I. It was shown that *cydCD* encodes a heterodimeric ATP-binding cassette-type transporter that is a glutathione transport system [157]. An orphan

Table 1
Effect of inhibitors on respiratory activity of cytochrome *bd*.

Inhibitor	Bacterium			
	E. coli[a]	*B. stearothermophilus*	*A. vinelandii*	*Photobacterium phosphoreum*
KCN or NaCN	2 mM[b] [27]	0.5 mM[e] [68]	–	62 μM[b] [288]
NaN_3	400 mM[b] [27]	8.2 mM[e] [68]	–	40 mM[b] [288]
H_2O_2	120 mM[b] [27]	–	–	–
2-*n*-heptyl-4-hydroxyquinoline N-oxide (HOQNO)	7 μM[b] [27]	–	5–20 μM[d] [249]	8.2 μM[b] [288]
$ZnSO_4$ or $ZnCl_2$	60 μM[b] [27]	200 μM[e] [68]	–	2.7 μM[b] [288]
Piericidin A	15 μM[b] [27]	–	–	–
Antimycin A	50 μM, 80%[c] [285]	–	11 μM[d] [279,286]	–
Undecylhydroxydioxobenzothiazole (UHDBT)	20 μM, 18%[c] [285]	–	20 μM[d] [279,286]	–
(1,5-Dimethylhexyl)quinazolinamide	100 μM, 88%[c] [285]	–	–	–
(1-Methyldecyl)quinazolinamide	100 μM, 85%[c] [285]	–	–	–
Stigmatellin	200 μM, 14%[c] [285]	–	–	–
Nigericin	100 μM, 44%[c] [285]	–	–	–
Dibromothymoquinone	100 μM, 38%[c] [285]	–	–	–
Aurachin A	700 μM, 27%[c] [285]	–	–	–
Aurachin C	214 nM, 90%[c] [285]	–	–	–
Aurachin D	400 nM, 93%[c] [285]	–	–	–
decyl-aurachin D	–	–	13 nM[d][249]	–
p-benzoquinone	–	120 μM [e] [68]	–	–
2,6-Dimethyl-*p*-benzoquinone	–	65 μM [e] [68]	–	–
Nitric oxide (NO)	100 nM[d] [106]	–	100 nM[d][106]	–
Carbon monoxide (CO)	–	–	0.5–1 mM, 80% [g] [287]	–
Pentachlorophenol (PCP)	200 μM[d] [32]	–	–	–
2-Thenoyl trifluoroacetone (TTFA)	1 mM, 35%[f] [26]	–	–	–
Gramicidin S	5.3 μM[b] [145]	–	–	–

[a] Data are referred to cytochrome *bd*-I.
[b] IC_{50} for ubiquinol-1 oxidase activity of the purified enzyme.
[c] Concentration and % inhibition of duroquinol oxidase activity of cytochrome *bd*-containing membranes.
[d] Inhibition constant (K_i) for ubiquinol-1 oxidase activity of the purified enzyme.
[e] IC_{50} for duroquinol oxidase activity of the purified enzyme.
[f] Concentration and % inhibition of ubiquinol-1 oxidase activity of the purified enzyme.
[g] Concentration and % inhibition of ascorbate-2,6-dichlorophenolindophenol oxidase activity of cytochrome *bd*-containing particles.

protein, YhcB, was proposed to be a third subunit of cytochrome *bd*-I [158], but this was later shown not to be the case [159].

In *E. coli*, a second cytochrome *bd* (*bd*-II) encoded by *cyx*AB genes (also named *app*BC or *cbd*AB) was identified [160]. The *cyx*AB genes, located at 22 min on the *E. coli* genetic map, are upstream from pH 2.5 acid phosphatase (*app*A) gene [160]. The *cyx*AB and *app*A genes constitute the complex operon. The *cyx*A and *cyx*B genes encode 58.1 kDa and 42.4 kDa integral membrane proteins, respectively. The deduced amino acid sequences of *cyx*A and *cyx*B genes reveal homologies of 60 and 57%, respectively, to subunit I (CydA) and subunit II (CydB) of cytochrome *bd*-I [160].

4.2. Regulation of gene expression in E. coli and other bacteria

Cytochrome *bd*-I is expressed by *E. coli* when the O_2 tension is low [120–122,161,162]. The expression of the *cydAB* operon is controlled by the two global transcriptional regulators, Arc and Fnr [121,161,163–169]. Arc is a two-component regulatory system that includes ArcA, a cytosolic response regulator, and ArcB, a transmembrane histidine kinase sensor. ArcA controls several hundred genes [170] and responds to the oxidation state of the Q pool which is sensed by ArcB [171]. ArcB is activated in response to the transition from aerobic to microaerobic growth and remains active during anaerobic growth. Upon stimulation, ArcB autophosphorylates and then transphosphorylates ArcA [171,172]. Under microaerobic conditions (i.e., O_2 tension of 2 to 15% of air saturation), the increased level of phosphorylated ArcA activates the *cydAB* operon [173]. Another global regulator, Fnr (an O_2-labile transcription factor regulating hundreds of genes), controls induction of anaerobic processes in *E. coli* [174,175]. The Fnr protein has a Fe–S cluster which serves as a redox sensor. The levels of the Fnr protein are similar under both aerobic and anaerobic conditions [165,176], but the protein is active only during anaerobic growth. The active Fnr protein represses *cydAB* operon during the transition to anaerobic conditions (i.e., O_2 tension of less than 2% of air saturation) [167,168,176].

Expression of *cyx*AB-*app*A operon (coding for cytochrome *bd*-II in *E. coli*) is induced by phosphate starvation and entry into a stationary phase [177]. The *cyx*AB genes can also be induced by anaerobic growth and this induction is controlled by transcriptional regulators AppY and ArcA but independent of Fnr, in contrast to *cyd* operon [177,178]. Cytochrome *bd*-II is likely to function under even more-O_2-limiting conditions than cytochrome *bd*-I [178]. Cytochrome *bd*-II has been partially purified [179], and contains two subunits by SDS-PAGE with apparent molecular weights 43 kDa (subunit I) and 27 kDa (subunit II). These subunits show no cross-reactivity to subunit-specific polyclonal antibodies directed against the subunits of cytochrome *bd*-I [179]. The spectral properties of cytochrome *bd*-II closely resemble those of cytochrome *bd*-I. Of the quinols tested as substrates, cytochrome *bd*-II utilizes menadiol as the preferred substrate (although ubiquinol-1, the most efficient in vitro substrate for cytochrome *bd*-I, was not tested). TMPD oxidase activity of cytochrome *bd*-II is much more sensitive to cyanide than that of cytochrome *bd*-I [179]. It was reported that though the electron flux through cytochrome *bd*-II can be significant, the enzyme does not contribute to the generation of the PMF [180]. Shepherd et al. [181] proposed that under conditions of an apparently fully uncoupled mode, *E. coli* can create PMF by means of consumption of intracellular protons in synthesis of γ-aminobutyric acid (GABA) and the generation of a pH gradient via uptake of glutamate and export of GABA by glutamate/GABA antiport.

In *A. vinelandii*, regulation of cytochrome *bd* expression is achieved by CydR (an Fnr homologue), which represses transcription of the *cydAB* genes [182]. The *cydABCD* operon coding for cytochrome *bd* in *B. subtilis* was reported to be activated by ResD and repressed by YdiH (Rex) and CcpA regulators [183–185]. Rex is also a repressor for the *cydABCD* operon in *Streptomyces coelicolor* [127]. ResD may activate the *cydA* gene

in *L. monocytogenes* [105]. In *Rhodobacter capsulatus*, expression of cytochrome *bd* is likely controlled by RegA regulator [186].

5. Distribution and evolution

The *bd*-family of oxygen reductases has a wide phylogenetic distribution with homologs found in at least one sequenced member of 18 bacterial phyla: Acidobacteria, Actinobacteria, Aquificae, Bacteroidetes, Chlamydiae, Caldithrix, Chlorobi, Chloroflexi, Chrysiogenetes, Cyanobacteria, Deferribacteres, Firmicutes, Nitrospirae, Planctomycetes, Proteobacteria, Thermi, Thermodesulfobacteria and Verrucomicrobia. To date no *bd*-family homologues have been detected in the following 12 bacterial phyla: Dictyoglomi, Elusimicrobia, Fibrobacteres, Fusobacteria, Gemmatimonadetes, Lentisphaerae, Poribacteria, Synergistetes, Thermotogales, and candidate phyla NC10, TM7 and WWE1. A number of Archaea also encode *bd*-family homologues, with members of the family found in Crenarchaeota, Euryarchaeota [60] and Korarchaeota. Cytochrome *bd*-type oxygen reductases are very common is some phyla, such as the Proteobacteria and Actinobacteria, and sporadically distributed in others. Interestingly, *bd*-family homologues have been detected in many species described as strict anaerobes such as *Methanosarcina barkeri*, *Methanosarcina acetivorans* [60], *Bacteroides fragilis* [86], *Desulfovibrio gigas* [187–189], *Desulfovibrio vulgaris* Hildenborough [190], *Geobacter metallireducens* [116], *Moorella thermoacetica* [191] and *Chlorobaculum tepidum* [192].

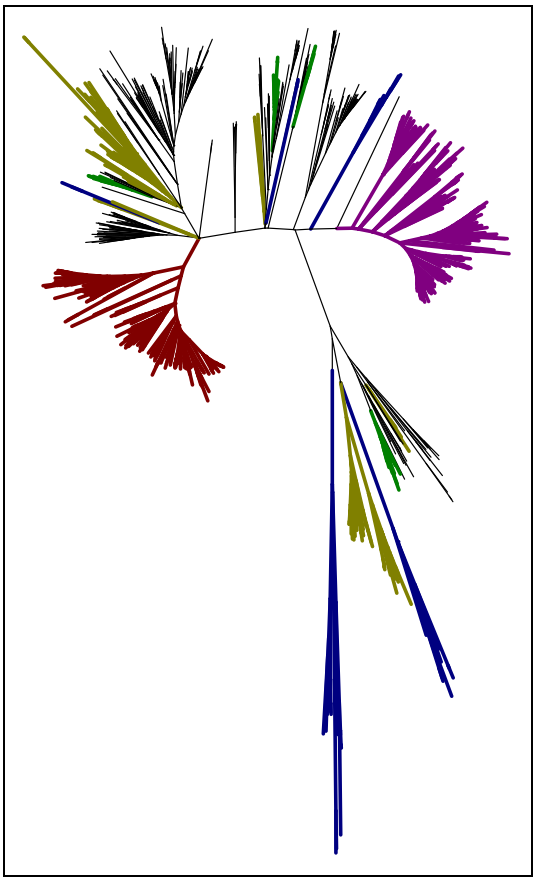

Fig. 3. The *bd*-family of oxygen reductases. An unrooted phylogenetic tree showing the relationships between 815 sequences of cytochrome *bd* oxidases. Members with the Q-loop insertion (long Q-loop) are shown in red. All other members of the family have the "short Q-loop". A number of members from the purple clade have been classified as cyanide insensitive oxidases (CIO) with a low content of heme *d*. Cytochromes *bd* from Archaea are shown in blue and form two related clades. In contrast, cytochrome *bd*-type oxygen reductases from the Firmicutes (yellow) and Bacteroidetes (green) are highlighted to demonstrate the sporadic distribution of enzymes within these phyla which resulted from horizontal gene transfer.

Early work suggested that the *bd*-family of oxygen reductases is an ancient innovation, already present in the ancestor of both Bacteria and Archaea [193]. However it was recently reported that the family may have originated in Bacteria and was later acquired by Archaea via horizontal gene transfer [60,194]. Phylogenetic analysis of the *bd*-family showed that horizontal gene transfer plays a significant role in the distribution of the family, with many phyla acquiring cytochrome *bd* genes multiple times independently (Fig. 3).

Sequence analysis has demonstrated that subunits I and II have different rates of evolution, with subunit II evolving 1.2 times faster than subunit I [194]. The biological relevance of this asymmetrical evolution is currently unknown.

6. Membrane localization

Cytochrome *bd* is embedded in the prokaryotic cytoplasmic (plasma) membrane. It was reported that in *E. coli*, cytochrome *bd*-I is not evenly distributed within the plasma membrane, being concentrated in mobile (on the subsecond time scale) patches, of the order of 100 nm in diameter [195,196]. These clusters contain variable numbers of cytochrome *bd*-I tetramers [196]. Cytochrome *bd* in cyanobacteria [197–203] has been reported to also be located in the thylakoid membrane [200,201,203–207], though this has been disputed [208–211]. The presence of a *bd*-type PQH_2 oxidase in cyanobacterial thylakoid and/or cytoplasmic membranes may depend on culturing conditions and the light regime [201,206].

7. Cofactors and substrates

7.1. Quinones

The nature of the quinols used by cytochrome *bd* as an electron donor is species-specific. For instance, in *A. vinelandii* and *E. coli* the cytochrome *bd* enzyme can oxidize ubiquinol (UQH_2), in *B. stearothermophilus*, the substrate is menaquinol (MQH_2). In *E. coli*, cytochrome *bd*-I can also oxidize MQH_2 [212,213], which replaces UQH_2 upon change of growth conditions from aerobic to anaerobic [166]. There is evidence that in cyanobacteria cytochrome *bd* is active as a plastoquinol (PQH_2) oxidase [200,201,203–206], although some reports have questioned this conclusion [208–210]. The presence or absence of bound Q in solubilized cytochrome *bd*-I from *E. coli* depends on the purification protocol. In some preparations of the purified enzyme, there is no apparently bound quinone [26,27,46,214] whereas others clearly contain bound quinone [41,215]. A stable semiquinone radical has been observed in the *E. coli* cytochrome *bd*-I [216,217].

7.2. Hemes

The two subunits of *E. coli* cytochrome *bd*-I carry three metal-containing redox-centers, two protoheme IX groups (hemes b_{558} and b_{595}) and a chlorin molecule (heme *d*) which are in 1:1:1 stoichiometry per the enzyme complex. The enzyme contains no Fe–S cluster and no copper ion [218–222]. Heme b_{558} is clearly located within subunit I. Both subunits are required for the assembly of heme b_{595} and heme *d*, suggesting that these two hemes may reside at the subunit interface [151]. Heme b_{595} appears to be oriented with its heme plane at ~55° to the plane of the membrane [223]. The millimolar extinction coefficients used commonly for the determination of the cytochrome *bd* concentration in *E. coli* and *A. vinelandii* are listed in Table 2.

7.2.1. Heme b_{558}

Heme b_{558} has been shown to be located within subunit I by expressing subunit I (*cyd*A) in the absence of subunit II (*cyd*B) and showing that the isolated subunit I contains heme b_{558} [152]. Antibodies directed against subunit I [61,63], as well as selective proteolysis of this subunit [62,64], inhibit UQH_2 oxidase activity of cytochrome *bd*-I. These

Table 2
Extinction coefficients used for determination of cytochrome *bd* concentration in *E. coli* and *A. vinelandii*.

Absorption spectrum	Heme	Wavelength pair (nm)	Δε ($mM^{-1}\,cm^{-1}$)	Reference
E. coli (cytochrome *bd*-I)				
Difference	*d*	628–607	10.8	[37]
Reduced minus	*d*	628–651[a]	27.9	[36]
'as prepared'	*d*	628–649[a]	18.8	[27]
	b_{558}	561–580	21	[36]
	b_{595}	595–606.5	1.9	[36]
	All	429–700[b]	303	[36]
CO/reduced	*d*	642–622	12.6	[27]
minus reduced	*d*	643–623	13.2	[48]
Absolute				
Reduced	*d*	628–670	25	[41]
'As prepared'	All	414–700[b]	223	[36]
A. vinelandii				
Difference				
Reduced minus	*d*	628–605	9.5	[241]
'as prepared'	*d*	629–608	12	[257]
	d	629–650[a]	27	[257]
CO/reduced	*d*	622–642	18	[257]
minus reduced				

[a,b]These values cannot be recommended for determination of cytochrome *bd* concentration since.
[a] The 'as prepared' enzyme contains varying amounts of the ferrous heme *d*-oxy complex that absorbs at 649–651 nm.
[b] The intensity of the Soret band is variable depending on the purity of the preparation.

findings suggest that heme b_{558} is associated with subunit I and is involved in QH_2 oxidation. The α- and β-bands of the reduced heme b_{558} at room temperature reveal maxima at 560–562 and 531–532 nm, respectively (Table 3) [150,224,225]. The maximum and minimum of the γ-band in the "reduced *minus* oxidized" difference absorption spectrum are 429.5 and 413 nm, respectively (Table 3) [225]. Heme b_{558} is low-spin hexacoordinate [37], and amino acid residues His186[1] and Met393 of subunit I (*E. coli* cytochrome *bd*-I) have been identified as its axial ligands [226–228]. The location of heme b_{558} is predicted to be near the periplasmic surface [67,229].

7.2.2. Heme b_{595}

The spectrum of heme b_{595} is similar to that of catalases and peroxidases containing pentacoordinate (high-spin) protoheme IX [150]. Heme b_{595} has an α-band at 594–595 nm and β-band at 560–562 nm in the difference absorption spectrum (Table 3) [150,224,225]. A trough at 643–645 nm in the difference spectrum of heme b_{595} is indicative of the disappearance in the reduced heme b_{595} of an absorption feature due to charge transfer from the Fe to the ligand, characteristic of oxidized high-spin heme *b*, as in the case of peroxidases. The γ-band of ferrous heme b_{595} is characterized by a maximum at ~440 nm as clearly revealed by femtosecond spectroscopy [38]. The maximum and minimum of the γ-band in the difference "reduced *minus* oxidized" absorption spectrum are 439 and 400 nm, respectively (Table 3) [225]. Heme b_{595} is high-spin pentacoordinate [37], ligated by His19 of subunit I [230] and located near the periplasmic surface [67,229]. The role of heme b_{595} remains obscure. It is proposed that heme b_{595} participates in the reduction of O_2 forming, together with heme *d*, a di-heme O_2-reducing site, somewhat similar to the heme/Cu O_2-reducing site in heme–copper oxidases [35–41,43,231]. In favor of this hypothesis is the finding that the CD spectrum of the reduced wild type cytochrome *bd* in the Soret band shows strong excitonic interaction between ferrous hemes *d* and b_{595} [42]. Modeling the excitonic interactions in the absorption and CD spectra yields an estimate of the Fe-to-Fe distance between heme *d* and heme b_{595} to be about 10 Å [42]. In the opinion of some, the function of heme b_{595} is limited to transferring an electron from heme b_{558} to heme *d* [232,233], whereas

[1] Here and below — amino acid numbering refers to cytochrome *bd*-I from *E. coli*.

Table 3
Spectral properties of cytochrome *bd*-I from *E. coli*. Shown are wavelengths (nm) and extinction coefficients (in parentheses, $mM^{-1}\,cm^{-1}$) for "reduced-*minus*-oxidized" difference absorption spectra. Data are taken from reference [225].

	Heme b_{558}	Heme b_{595}	Heme *d*
Maxima	429.5 (90), 531.5 (5.8), 561 (17.2)	439 (113), 561.5 (8.2), 594 (5.3)	430 (30), 629 (18)
Minima	413 (−40), 497 (−4.3), 545 (~0)	400 (−37), 500 (−3.6), 643 (−1.18)	405 (−23), 468 (−6.3), 657.5 (−2.7), 739 ± 2 (−2.4)
Isosbestic points	421, 450, 518, 573	422, 457, 535, 613	418.5, 449, 602, 648

others have postulated that heme b_{595} can form a second site capable of reacting with O_2 [218,234].

7.2.3. Heme *d*

Heme *d* is a chlorin-type molecule [235]. The α-band of the reduced heme *d* in the absolute absorption spectrum of *E. coli* cytochrome *bd*-I shows a peak at 628–630 nm. However, upon isolation of the enzyme, heme *d* is in the stable oxygenated (O_2-ligated ferrous) form, which is characterized by an absorption band with a maximum at 647–650 nm in the absolute absorption spectrum [236–239]. The affinity of ferrous heme *d* for O_2 is indeed high, showing the $K_{d(O2)}$ values of 0.28 μM and 0.5 μM for the enzymes from *E. coli* and *A. vinelandii*, respectively [240,241]. The maximum and minimum of the γ-band in the difference "reduced *minus* oxidized" absorption spectrum are 430 and 405 nm, respectively (Table 3) [225].

Remarkably, the spectral contribution of heme *d* to the complex Soret band is much smaller than those of either hemes *b* [225]. Heme *d* is predicted to be located near the periplasmic surface [67,229], and is the site for capturing and, subsequently, reducing O_2 to H_2O. In the absence of external ligands, heme *d* is in the high-spin state with an open coordination site for binding O_2. The nature of the axial ligation of heme *d* to the protein, or even whether there is an axial ligand provided by the protein, is unclear. It has been claimed that the reduction of cytochrome *bd* is associated with binding of an endogenous protein ligand to heme *d* [242]. The oxidized heme *d* may or may not be ligated to an endogenous protein substituent. Resonance Raman and ENDOR studies indicate that the ligand is not histidine, cysteine or tyrosinate, but that the single axial ligand is either a weakly coordinating protein donor or a water molecule [230,243,244]. In contrast, EPR studies indicated that the heme *d* axial ligand is histidine in an anomalous condition or some other nitrogenous amino acid residue [245]. Finally, it has been suggested that Glu99 of subunit I is a prime candidate for such a role [214,246].

7.3. Heme redox potentials

The apparent values for the midpoint redox potentials of hemes b_{558}, b_{595} and *d* for the *bd* enzymes solubilized in *n*-dodecyl-β-D-maltoside at pH 7.0 (E_m) are respectively +176, +168, and +258 mV (*E. coli bd*-I) and +166, +251, and +310 mV (*A. vinelandii*) [241]. These are within the range of the values reported earlier for *E. coli* [219,220,224,247,248] and *A. vinelandii* [249]. Notably, the E_m value of heme b_{558} can depend on the detergent used for solubilization [248]. In particular, octylglucoside and cholate cause a large decrease in the E_m value of heme b_{558}, and this correlates with the reversible inactivation of the enzyme [248]. The E_m values of all three heme components of cytochrome *bd* are sensitive to pH between pH 5.8 and 8.3 with a $\Delta E_m/\Delta pH$ of −61 mV for heme *d* and −40 mV for hemes b_{558} and b_{595}, indicating that reduction of cytochrome *bd* is accompanied by enzyme protonation [248]. A recent study [225] revealed a significant redox interaction between heme b_{558} and heme b_{595}, whereas the interaction between heme *d* and either both hemes *b* appears to be rather weak.

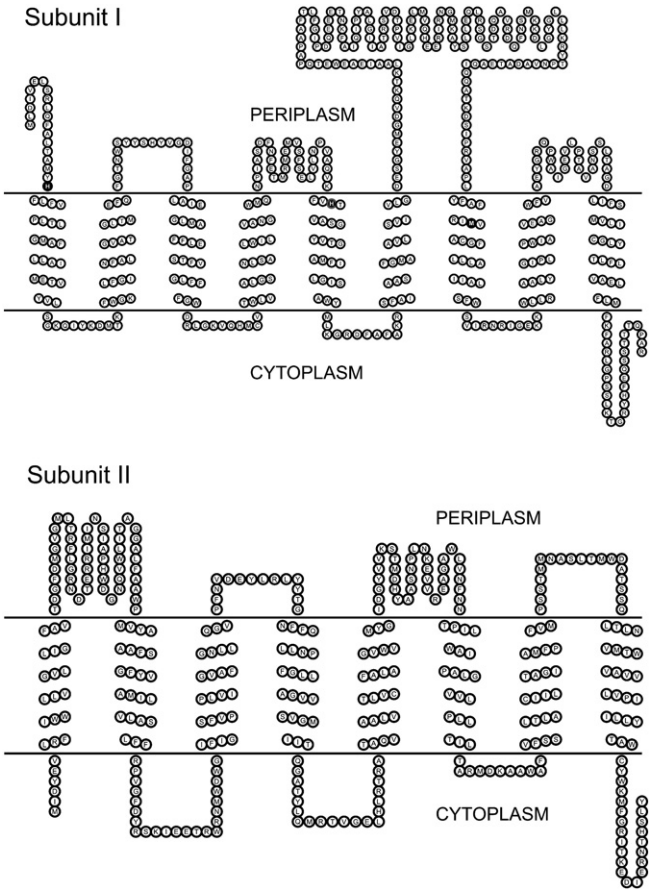

Fig. 4. Proposed topology of subunits I and II of cytochrome *bd*-I from *E. coli*. The axial ligands of heme b_{595} (H19) and heme b_{558} (H186 and M393) in subunit I are highlighted. The model is based on the data reported in [67,213,229].

However, the presence of heme *d* itself decreases the much larger interaction between the two hemes *b* [225].

8. Proposed structure

The X-ray structure of cytochrome *bd* has not been determined. Conventional studies of the protein topology in the membrane suggest that all three hemes are located near the periplasmic side of the membrane [67,229], although an alternative view also exists [250,251]. Fig. 4 shows topological models of subunits I (CydA) and II (CydB) of cytochrome *bd*-I from *E. coli* [213]. Both subunits are integral membrane proteins. Subunit I consists of nine transmembrane helices with the N-terminus in the periplasm and the C-terminus in the cytoplasm [67]. Subunit II is composed of eight transmembrane helices with both N- and C-termini in the cytoplasm [67]. The Q-loop in subunit I connects transmembrane helices 6 and 7, and is directly involved in QH_2 binding and oxidation [61–66]. Thus the QH_2-oxidizing site in cytochrome *bd* is located on the periplasmic side of the membrane. Cytochrome *bd*-I from *E. coli* is proposed to contain a single site for the binding and oxidation of quinol [65,66,252]. However, evidence for a second quinone binding site in cytochrome *bd* from *Corynebacterium glutamicum* has also reported [69].

Using a set of 815 sequences of genes encoding cytochrome *bd*, a number of residues in subunit I are totally (>99%) conserved [213]. These residues include those which are identified as ligands to the heme components of the enzyme. In addition, since the active site of O_2 reduction is located near the periplasmic surface and protons for H_2O production are taken from the bacterial cytoplasm, there must be at least one transmembrane proton-conducting pathway to convey protons from the cytoplasm to the heme b_{595}/heme *d* site [41,46,48,67] (Fig. 5). Several polar or ionizable residues that are highly conserved in the *bd*-family have been postulated to be a part of this putative proton channel.

The residues that are totally conserved within the entire *bd*-family include His19 (the heme b_{595} axial ligand [230]), His186 and Met393 (the heme b_{558} axial ligands [226–228]), Lys252 and Glu257 (involved in QH_2 binding [66]), Arg448 (unknown function), and Glu99, Glu107, and Ser140 (proposed to be components of a proton channel [48,67] and important for heme binding in the heme *d*/heme b_{595} di-heme site [213,214]). Slightly less conserved (95–99%) are Glu445 (required for charge compensation of the b_{595}/d O_2-reducing site upon its full reduction by two electrons [41]), Asn148 (plausible component of a proton channel), and Arg9 (unknown function) [213]. Somewhat less conserved (~85%) are Arg391 (stabilizes the reduced form of heme b_{558} [253]) and Asp239 (unknown function), however these residues are totally conserved within the A subfamily of cytochromes *bd* [213]. Other conserved residues are glycines, prolines, phenylalanines, or tryptophans, which may play structural roles. There is only one totally (>99%) conserved residue (Trp57) in subunit II [213]. Within the subfamily of *bd*-type oxygen reductases which have the "long Q-loop", Arg100, Asp29, and Asp120 of subunit II are totally conserved and Asp58 (subunit II of *E. coli* cytochrome *bd*-I) is either an aspartate or glutamate [213]. The N-terminal portion of subunit II has been suggested to be involved in the binding of heme *d*/heme b_{595} [213,254].

Fig. 3 shows an unrooted tree showing the relative sequence relationships of 815 sequences of cytochrome *bd* from the genomes of Bacteria and Archaea. It is seen in Fig. 3 that the "long Q-loop" members form a phylogenetic clade distinct from the other members of the family. This is most likely due to an insertional event within the Q-loop. This subfamily contains many, but not all, of the cytochrome *bd* oxygen reductases from Proteobacteria (including *E. coli*). Also shown in Fig. 3 are two clades that define the *bd*-family members found in Archaea. In contrast, the *bd*-family oxygen reductases found in Firmicutes or Bacteroides are distributed widely among the phylogenetic groups shown in Fig. 3. This illustrates the large role played by horizontal gene transfer in the distribution of the *bd*-type oxygen reductases.

9. Binding of ligands (other than O_2)

Since hemes *d* and b_{595} in cytochrome *bd* are in the high-spin pentacoordinate state, they could potentially bind ligands. One may

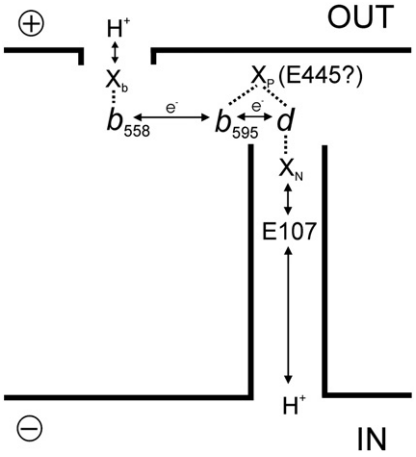

Fig. 5. Scheme for electron and proton transfer pathways in cytochrome *bd*-I from *E. coli*. There are two protonatable groups, X_P and X_N redox-coupled to the heme b_{595}/heme *d* active site. A highly conserved E445 was proposed to be either the X_P group or the gateway in a channel that connects X_P with the cytoplasm or the periplasm [41]. A strictly conserved E107 is a part of the channel mediating proton transfer to X_N from the cytoplasm [48].

anticipate that the enzyme in the reduced state binds electroneutral molecules like O_2, CO, and NO, whereas the oxidized cytochrome *bd* prefers ligands in the anionic form such as cyanide and azide. Heme *d* binds ligands readily whereas the ligand reactivity of heme b_{595} is minor despite the fact that this is a high spin heme [37,39,255]. Heme b_{558}, although a low-spin hexacoordinate, may also bind ligands to some extent (e.g., CO or cyanide) [37,255]. Such a marginal reactivity is possibly due to weakening the bond of the methionine axial ligand (Met393) to heme b_{558} iron caused by the isolation procedure and/or protein denaturation [255].

9.1. Carbon monoxide

Addition of CO to the three-electron reduced form of cytochrome *bd*, denoted as R^3, causes a red shift of the 628 nm heme *d* band and the increased absorption around 540 nm in the visible, as well as a distinctive W-shaped difference spectrum in the Soret region [37,39,150,255–257]. The W-shaped feature is due to a small bandshift of unligated heme b_{595} induced by CO interaction with the nearby heme *d* [38,40,43]. Only a small fraction of heme b_{595} (<5%) in cytochrome *bd* binds CO at room or low temperature [37,39]. The apparent K_d for the CO-heme *d* complex with the fully reduced (R^3) cytochrome *bd*-I from *E. coli* was determined to be ~80 nM [255]. The R^3 cytochrome *bd* can form a photosensitive heme *d*-CO complex [258]. Flash photolysis of CO bound to heme *d* at cryogenic temperatures results in a redistribution of CO such that as much 15% of heme b_{595} is bound to CO, showing the proximity of these two hemes [35]. Following flash-photolysis of the heme *d*-CO complex in the fully reduced enzyme (R^3) at room temperature, CO recombines with ferrous heme *d* proportionally to the external CO concentration with a second order bimolecular rate constant of $10^8\ M^{-1}\ s^{-1}$ (Table 4) [43,222,249,259].

The one-electron reduced form of the enzyme (R^1) can also be examined. Since heme *d* has a substantially higher midpoint potential than the other two heme components, heme *d* is the only heme reduced in the R^1, or mixed-valence, state of the 'as prepared' enzyme. Upon reaction with CO, one gets the CO-heme *d* adduct ($b_{558}^{3+}b_{595}^{3+}d^{2+}$-CO) [38,40,43,48,107,249]. After flash photolysis of the R^1-CO complex, a substantial fraction of the CO flashed off heme d^{2+} gets trapped inside the protein and undergoes geminate recombination with heme d^{2+} on the pico- and nanosecond time scale [38,43]. The data indicate that the redox state of heme b_{595} controls the pathway for ligand (CO) transfer between heme *d* and the bulk phase, which is open when heme b_{595} is reduced but closed when heme b_{595} is oxidized [38,43,107].

9.2. Nitric oxide and other nitrogen-containing ligands

A number of small nitrogen-containing molecules can react with R^3 cytochrome *bd* from *E. coli* and *A. vinelandii*. NO_3^-, NO_2^-, $N_2O_3^{2-}$ (trioxodinitrate), NH_2OH and NO, when added to membranes containing cytochrome *bd* or the purified enzyme, give rise to decrease in amplitude and shift of the 630 nm peak of ferrous heme *d* to 641–645 nm [31,37,106,107,218,245,257,260–264]. It appears that all of these ligands result in chemical reactions, forming the same or a very similar heme–nitrosyl compound [31], e.g., heme d^{2+}-NO adduct. It has also been suggested that a heme b_{595}^{2+}-NO adduct can be observed upon adding nitrite to cytochorme *bd* in membranes [218].

Cytochrome *bd* can also produce a stable complex with NO in the R^1 state, in which ligand bound heme *d* is reduced while the *b* hemes are oxidized [107,245]. The rates of NO dissociation from heme d^{2+} in both R^3 and R^1 states of cytochrome *bd* were determined [107]. In the R^3 state, NO dissociates from heme d^{2+} at an unusually high rate, $k_{off} = 0.133\ s^{-1}$ [107], which is ~30-fold higher than the off-rate measured for the ferrous heme a_3 of the mitochondrial cytochrome *c* oxidase ($k_{off} = 0.004\ s^{-1}$ [265]). These data are consistent with the proposal that, in the heme–copper oxidases, Cu_B acts as a gate controlling ligand binding to the heme in the active site [266]. Another remarkable feature of NO dissociation from cytochrome *bd* is that the k_{off} value in the R^1 state ($0.036\ s^{-1}$), although still quite high, is significantly lower than that measured with the R^3 enzyme [107] (Table 4). These data show that the redox state of heme b_{595} controls the kinetic barrier for ligand dissociation from the active site of cytochrome *bd*, similar to the observations with CO dissociation from ferrous heme *d* [38,43,107]. The unusually high NO dissociation rate from cytochrome *bd* may explain the observation [106] that the NO-poisoned cytochrome *bd* recovers respiratory function much more rapidly than a heme–copper oxygen reductase. It is postulated that expression of *bd*-type, instead of heme–copper-type oxygen reductase, enhances bacterial tolerance to nitrosative stress, thus promoting colonization of host intestine or other microaerobic environments [107,108]. It was reported that, apart from ferrous heme *d*, NO can also react with the oxoferryl and ferric state of heme *d*, yielding the oxidized nitrite-bound heme *d* and the nitrosyl adduct, respectively [110,111].

9.3. Cyanide

Reaction of 'air-oxidized' cytochrome *bd* with KCN causes the decay of the ferrous heme *d* oxy-complex [267–273]. Cyanide-induced changes to the EPR-spectrum include a low-spin signal and,

Table 4
Kinetic and thermodynamic parameters for reaction of cytochrome *bd* with gaseous ligands at room temperature.

	E. coli (cytochrome *bd*-I)						*A. vinelandii*		
	O_2		CO		NO		O_2	CO	
	R^1-O_2	R^3-O_2	R^1-CO	R^3-CO	R^1-NO	R^3-NO	R^3-O_2	R^1-CO	R^3-CO
k_{on} ($M^{-1}\ s^{-1}$)		2×10^{9}[a,b]		8×10^{7}[a,c]			2×10^{9}[d] 2.8×10^{8}[f]	1×10^{8}[e]	1.5×10^{8}[e]
k_{off} (s^{-1})	78[g]		4.2[g]	6[g]	0.036[g]	0.133[g]			
K_d (nM)	280[h]			80[i]					

[a] [222].
[b] [47].
[c] [43].
[d] [241].
[e] [249].
[f] [278].
[g] [107].
[h] [240].
[i] [255].

after prolonged incubation, a second weak low-spin signal that may indicate some interaction of cyanide with heme b_{595} [220,257,274]. A simple and fast method for conversion of the oxygenated enzyme into the O form with the use of lipophilic electron acceptors [239] allowed us to study the interaction of cyanide with the homogenous oxidized preparation of cytochrome *bd* [37]. The MCD spectrum of the O cytochrome *bd*-I from *E. coli* is dominated by an asymmetric signal in the Soret. Submillimolar cyanide has no effect on the initial MCD spectrum. 50 mM KCN induces minor changes of the MCD signal in the Soret band, which can be modeled as transition of a part of the low-spin heme b_{558} (15–20%) to its low-spin cyano-complex [37]. There is no evidence of the interaction of high-spin ferric heme b_{595} with the ligand [37]. On the contrary, based on the EPR spectra, Tsubaki et al. [36] proposed that the treatment of 'air-oxidized' cytochrome *bd* with cyanide results in a cyanide-bridging species with a "heme d^{3+} – C═N – heme b_{595}^{3+}" structure. However the authors [36] did not account for the electron released from heme *d* upon cyanide binding to 'as prepared' cytochrome *bd*. Resonance Raman studies suggest that heme *d* is in the high-spin pentacoordinate state when it is compounded with cyanide [230,275]. This would require either that the endogenous axial ligand to heme *d* is displaced by cyanide, maintaining a high-spin pentacoordinate state, or that there is no endogenous axial ligand to heme *d* in the fully oxidized form of the enzyme.

9.4. Hydrogen peroxide

Addition of excess H_2O_2 to *E. coli* membranes containing cytochrome *bd*-I [276] and the purified enzyme in the 'as prepared' [231,237] or the O [46,231,277] states gives rise to an absorption band at ~680 nm. The reaction of H_2O_2 with the O cytochrome *bd* also induces a red shift of the γ-band [231,277]. H_2O_2 binds to ferric heme *d* with an apparent K_d value of 30 μM, but it seems not to interact with heme b_{595} [231,277]. The O cytochrome *bd* reacts with H_2O_2 with a second order rate constant of 600 M^{-1} s^{-1}. The decay of the H_2O_2-induced spectral changes upon addition of catalase ($k \sim 10^{-3}$ s^{-1}) is about 20-fold slower than expected for dissociation of H_2O_2 from the complex with heme *d* assuming a simple reversible binding of peroxide [277]. This suggests that the interaction of H_2O_2 with cytochrome *bd* is essentially irreversible, giving rise to the F state of heme *d* [277]. The assignment of the compound 680 to the F state of heme *d* is confirmed by resonance Raman spectroscopy data [221]. Heme *d* in the F state is suggested to be high-spin pentacoordinate [275].

10. Proposed catalytic mechanism

As discussed above, under physiological conditions cytochrome *bd* from different prokaryotes likely oxidizes UQH_2, MQH_2 or PQH_2. In vitro a *bd*-type oxygen reductase can also utilize short chain ubiquinols, menadiol, duroquinol, and artificial electron donors such as TMPD. Of the in vitro substrates, ubiquinol-1 (plus excess dithiothreitol) shows the highest turnover numbers [248,278]. The activity of the purified oxidase depends on the nature of the detergent in which the enzyme is solubilized. Cytochrome *bd*-I from *E. coli* is inactive in octylglucoside or cholate but shows high activity in Tween-20, Triton X-100 [248] or *N*-lauroyl-sarcosine [106]. The ubiquinol-1 oxidase activity of cytochrome *bd*-I has a broad optimum above pH 7.5 but decreases at more acidic pH values [248]. Cytochrome *bd* possesses three distinct active sites — for QH_2 oxidation, TMPD oxidation and O_2 reduction. All the three sites seem to be located at or close to the periplasmic surface of the membrane. Electrons donated from QH_2 transfer to heme b_{558} and then to the b_{595}/d di-heme site, whereas electrons donated from TMPD transfer directly to the b_{595}/d site bypassing the QH_2-binding site and heme b_{558} [62,279].

10.1. Mechanism of generation of the proton motive force

Cytochrome *bd* from *E. coli* and *A. vinelandii* was reported to generate a transmembrane electric potential both in single turnover [41,46–48] and under multiple turnover [27,44,280] conditions ($H^+/e^- \sim 1$ [34,45,49,50]; $q/e^- \sim 1$ [281]). When reconstituted into liposomes, cytochrome *bd* generates an uncoupler-sensitive transmembrane voltage difference with a value of 160–180 mV (negative inside) [27,44]. The QH_2 molecule generated by the dehydrogenases of the respiratory chain can diffuse laterally within the bilayer, finding its way into the QH_2 oxidizing site located near the outer side of the membrane. Upon oxidation of QH_2, two protons are released into the periplasmic space, and two electrons are transferred through heme b_{558} to the b_{595}/d O_2-reducing site, also located near the periplasmic surface of the membrane. The four protons used for O_2 reduction are taken up from the cytoplasm. Single-turnover electrometric experiments show that the generation of the membrane potential is associated with electron transfer from heme b_{558} to the b_{595}/d active site [41,46–48]. However,

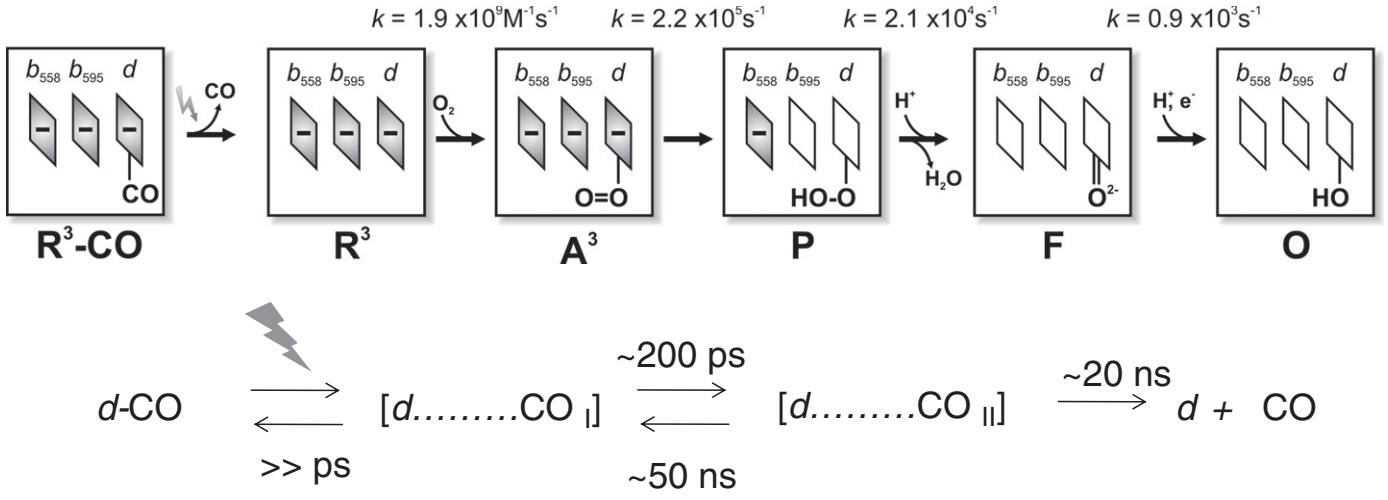

Fig. 6. *Top:* Scheme for reaction of fully reduced cytochrome *bd* with O_2. The three *rhombuses* represent hemes b_{558}, b_{595}, and *d*, respectively. The *minus sign* denotes that the heme is in the ferrous state. *Bottom:* Photolysis of CO from heme *d* in the fully reduced enzyme. Two different configurations of dissociated CO in the enzyme ($d \ldots CO_i$, $i = I, II$) are proposed [43]. The state ($d + CO$) denotes a state where CO escaped from the enzyme.

since all of the three hemes are likely located close to the periplasmic side of the membrane [67,229], the electron transfer itself is expected to be parallel to the membrane surface and, therefore, cannot be electrogenic [46]. Rather, it is proposed that electron transfer from heme b_{558} to the b_{595}/d active site is coupled to vectorial proton transfer from the cytoplasm towards the active site on the opposite (periplasmic) side of the membrane [41,46–48]. The latter implies that there must be a proton-conducting channel connecting the cytoplasm to the b_{595}/d active site [41,46,48] (Fig. 5). The transmembrane potential originates primarily from protons moving from the cytoplasm to the O_2-reducing site on the opposite side of the membrane, and this accompanies electron transfer from heme b_{558} to the b_{595}/d active site. As shown in Fig. 5, it is proposed that near the b_{595}/d active site there are two protonatable sites (X_P and X_N) that are accessible to the cytoplasm *via* a proton-conducting channel.

10.2. Reaction of the fully reduced enzyme (R^3) with O_2

The reaction of the R^3 cytochrome *bd* with O_2 has been studied using the flow-flash method [282] by means of spectroscopic and electrometric techniques [41,46–48,222]. Recording absorption spectra and membrane potential development with 1 μs time resolution resolves the sequence of the catalytic intermediates and establishes which catalytic steps are linked to electric potential generation [47]. The scheme for this reaction is presented in Fig. 6 (top panel). The initial complex of R^3 cytochrome *bd* with CO (R^3-CO) is photolyzed (the photolysis details are shown in Fig. 6, bottom panel) in the presence of O_2. The unliganded R^3 enzyme, generated by the CO-photolysis, binds O_2 very rapidly, forming the ferrous heme *d* oxy species (A^3). The $R^3 \rightarrow A^3$ transition is not electrogenic and its rate is proportional to $[O_2]$ ($k_{on} = 1.9 \times 10^9\ M^{-1}\ s^{-1}$ [47,222]). The A^3 formation is followed by electron transfer from heme b_{595} to form state P. The $A^3 \rightarrow P$ transition occurs with $\tau = 4.5$ μs and is also nonelectrogenic [47]. Thus, electron transfer from heme b_{595} to heme *d* is not coupled to membrane potential generation [41,47]. It is proposed that P is a peroxy complex of ferric heme *d* [47]. If this is the case, the bound peroxide is likely not to be in the anionic form but at least singly protonated. The proton may come from one of two postulated protonatable groups, X_P and X_N, near the b_{595}/d di-heme active site upon oxidation of the hemes [41]. P is further converted into F upon electron transfer from heme b_{558} with $\tau = 48$ μs. Formation of F is coupled to generation of a membrane potential [41,46–48] due to the accompanying proton transfer through the proposed proton channel (Fig. 5). At the F stage, the *b*-type hemes are in a ferric state and heme *d* in an oxoferryl state. When cytochrome *bd* contains bound QH_2, the reaction proceeds further to form the O enzyme. The $F \rightarrow O$ transition occurs with $\tau = 1.1$ ms and is electrogenic as well [41,47] since this also involves electron transfer from heme b_{558} to the b_{595}/d active site with the accompanying proton transfer.

Cytochrome *bd* can bind O_2 being in the R^1 state. Remarkably, in this reaction, the dependence of the rate of O_2 binding on $[O_2]$ is hyperbolic thus revealing a saturation behavior. This is not observed for O_2 binding to the R^3 enzyme [241]. It is speculated that the R^1 enzyme exists in the two different conformations in equilibrium, but only one of these forms binds to O_2. When in the "closed" conformation, cytochrome *bd* provides no access for O_2 to heme d^{2+}, whereas in the "open" conformation, O_2 binds easily. The R^3 enzyme is always in the open conformation [241].

10.3. Catalytic cycle

Several relatively stable forms of cytochrome *bd* corresponding to the intermediates of the catalytic cycle have been identified. Under aerobic conditions, cytochrome *bd* is predominantly in the one-electron-reduced state bound to O_2 (A^1), with lesser amounts of the F and O forms. Under anaerobic conditions, the reduced forms of the enzyme lacking an O_2 ligand with one (R^1) and three (R^3) electrons can be generated and examined. A short-lived complex of the three-

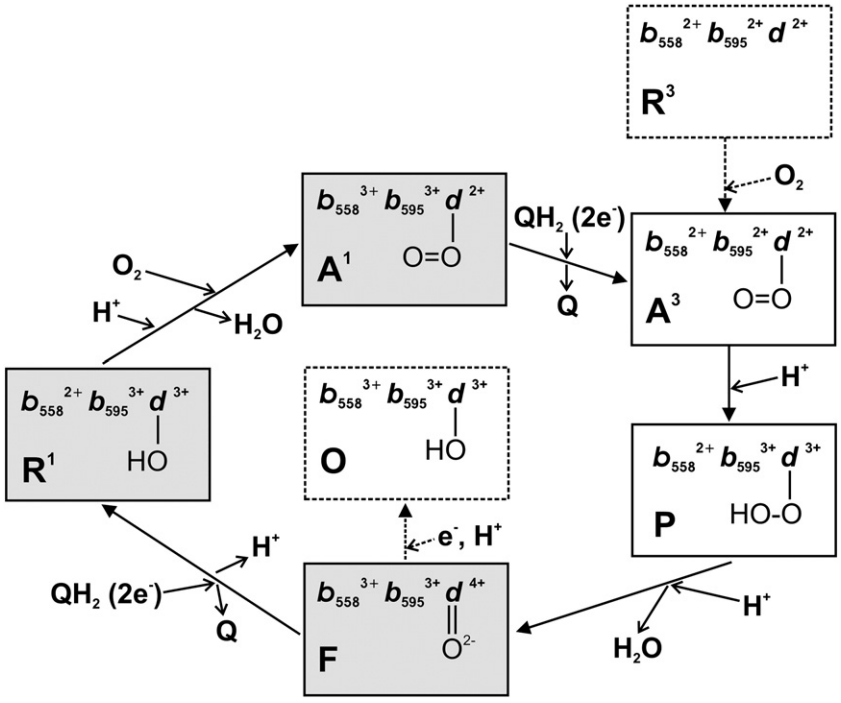

Fig. 7. Cytochrome *bd* catalytic cycle. The scheme is based on the reports of Jünemann et al. [278], Kavanagh et al. [289], Matsumoto et al. [252], Belevich et al. [47], Yang et al. [290], and Borisov et al. [283]. Solid arrows show the natural catalytic reaction pathway. Dotted arrows indicate transitions that are not being part of the catalytic cycle can be observed experimentally. The O form of the enzyme is most likely not to be an intermediate of the catalytic cycle [290]. Intermediates populated at steady-state [283] are highlighted in gray.

electron reduced cytochrome *bd* with O_2 (A^3) [46,47,222,241], an "peroxide" intermediate P [47] and an oxoferryl compound F [46,47,222] can be sequentially formed (Fig. 6). Turnover intermediates of *E. coli* cytochrome *bd*-I detected at steady-state are A^1 and F species (~40% each) and, to a lesser extent (~20%), a species with ferric heme *d* and possibly one electron on heme b_{558} (R^1) [283]. These data differ from those obtained with mammalian cytochrome *c* oxidase, in which oxygenous intermediates were not found to be populated at detectable levels under similar conditions [284]. A plausible scheme of the catalytic cycle of cytochrome *bd* is shown in Fig. 7.

10.4. Role of heme b_{595}

Exogenous ligands added to cytochrome *bd* bind to heme *d* but do not bind to a majority of the heme b_{595} population [31,37,39,255]. Heme b_{595}, although in the high-spin pentacoordinate state, is resistant to interaction with the classical ligands of high-spin iron-porphyrin complexes. It cannot be ruled out that despite the high-spin pentacoordinate state of the iron-porphyrin group, the specific features of the protein environment are such that this redox cofactor is protected from interaction with ligands. In such case, the participation of heme b_{595} in O_2 reduction in cooperation with heme *d* is unlikely and its role would be limited to the transfer of an electron to heme *d*. A more likely explanation is the following: (1) both heme b_{595} and heme *d* potentially can bind ligands; (2) the hemes are located close to each other forming a di-heme active site; (3) the spatial proximity of hemes b_{595} and *d* results in steric restrictions allowing the di-heme site to bind only one ligand molecule; and (4) heme *d* has a higher affinity for ligands than heme b_{595}, in which case the final result observed upon addition of a ligand will always be the ligand binding to heme *d*, whereas heme b_{595} will remain mainly in the unliganded state [37,39,231,255]. The data on the redox coupling of the two hemes to the same ionizable groups [41], and the migration of CO within the protein from heme *d* to heme b_{595} at cryogenic temperatures [35] are in agreement with this proposal. Modeling the excitonic interactions in absorption and CD spectra of cytochrome *bd* yields an estimate of the Fe_d-to-Fe_{b595} distance of about 10 Å [42]. This is markedly larger than that for the Fe/Cu_B pair in heme–copper oxidases (4–5 Å). If this is the case, heme b_{595} cannot be a functional analog of Cu_B. A possible role of heme b_{595}, apart from electron delivery to heme *d* and/or to an oxygenated intermediate form of heme *d*, would be as a binding site for hydroxide produced from heme *d*-bound O_2 upon reductive cleavage of the O – O bond [42].

11. Conclusion

There are at least two reasons why cytochromes *bd* may be of interest. First, they are found in many pathogenic bacteria and there is growing evidence for a positive correlation between the virulence and the level of cytochrome *bd* expression. We hope that our knowledge on the structure and function of the *bd* enzymes will provide new tools to combat diseases caused by pathogens, for instance, by using a bacterial *bd*-type respiratory oxygen reductases as a drug target. Second, it would be useful to know what are the common features and the differences between the mechanisms of O_2 reduction to H_2O by cytochromes *bd* and heme–copper oxidases. Such a comparison could allow us to gain further insight into the elements essential for proton pumping coupled to the redox reaction inherent in heme–copper oxidases.

Acknowledgments

Studies in our groups were supported by the Russian Foundation for Basic Research, grant 11-04-00031-a (to V.B.B.), the National Institutes of Health, grant HL16101 (to R.B.G.), and the Biocentrum Helsinki, the Sigrid Jusélius Foundation, and the Academy of Finland (to M.I.V.).

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