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Structure of a bd oxidase indicates similar mechanisms for membrane-integrated oxygen reductases

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Abstract

The cytochrome bd oxidases are terminal oxidases that are present in bacteria and archaea. They reduce molecular oxygen (dioxygen) to water, avoiding the production of reactive oxygen species. In addition to their contribution to the proton motive force, they mediate viability under oxygen-related stress conditions and confer tolerance to nitric oxide, thus contributing to the virulence of pathogenic bacteria. Here we present the atomic structure of the bd oxidase from *Geobacillus thermodenitrificans*, revealing a pseudosymmetrical subunit fold. The arrangement and order of the heme cofactors support the conclusions from spectroscopic measurements that the cleavage of the dioxygen bond may be mechanistically similar to that in the heme-copper-containing oxidases, even though the structures are completely different.

Living in a reducing atmosphere more than 3 billion years ago, the ancestors of the cyanobacteria found away to extract electrons from water via oxygenic photosynthesis. The released oxygen can form reactive oxygen species, and partly reduced oxygen is highly toxic. To cope with this threat, microorganisms had to survive in anaerobic niches and remove oxygen by reducing it to water. Several enzymatic machineries are employed for the reduction of oxygen to water. Oxygen-tolerant methanogenic archaea use a soluble di-iron flavoprotein (1), whereas most other organisms use membrane-integrated enzymes,

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SUPPLEMENTARY MATERIALS

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primarily members of the heme-copper-containing oxidase (HCO) superfamily. Different HCOs use cytochromes or quinols as electron donors providing the electrons from the extracellular side, whereas the protons required for water formation are delivered from the intracellular side. This vectorial delivery of oppositely charged substrates also generates the electrochemical proton gradient used for energy-requiring processes such as adenosine triphosphate (ATP) synthesis.

The cytochrome bd oxidases form the other known family of membrane-spanning oxygen reductases of bacteria and archaea, unrelated to HCOs or the membrane-attached cyanide-resistant alternative oxidases (AOXs). The bd oxidases use quinols as electron donors from the extracellular side and protons from the intracellular side like HCOs, but do not pump protons (2). They possess a high oxygen affinity and thus play a role in protection against oxidative stress (3, 4) and the colonization of O₂-poor environments by pathogenic bacteria (5, 6). The bd oxidases also rapidly dissociate gaseous inhibitory nitrogen ligands and confer tolerance under nitric oxide stress conditions (7). This ability is crucial for the viability of pathogenic bacteria upon host infection (5, 8). The hypersensitivity of a bd oxidase deletion strain of *Mycobacterium tuberculosis* to bedaquiline, a tuberculosis drug that inhibits the F₁F₀-ATP synthase (9, 10), highlights the potential of bd oxidases as drug targets.

Crystal structures of representative members of all three HCO families (A, B, and C) are known, yet the structure of bd oxidases has remained elusive. The bd oxidases contain two individual protein subunits of ~45 kDa (CydA) and ~35 kDa (CydB) (11–13); those of the proteobacteria contain a third subunit consisting of a single transmembrane helix of ~4 kDa, which plays a crucial role in the activity of the enzyme (14, 15). The bd oxidases contain a low-spin hexacoordinated heme B (b₅₅₈) and a high-spin pentacoordinated heme B (b₅₉₅) and a chlorin-type heme D (2, 16, 17) as electron-transferring prosthetic groups. The quinol to be reduced is initially bound via a conserved water-exposed loop (a Q loop) located in the hydrophilic extracellular space connecting transmembrane helices 6 and 7 of CydA. Subsequently, the quinol is oxidized and heme b₅₅₈ acts as a primary electron acceptor, whereas two protons are released to the extracellular side. The electrons are transferred to the b₅₉₅/d high-spin active site, where oxygen binding and the reduction of oxygen to water take place (2, 18). No Cu cofactor is present in the active site of the bd oxidases.

Here we present the crystal structure of the bd oxidase from *G. thermodenitrificans* K1041, using crystals diffracting anisotropically to 3.1 to 4 Å resolutions (19). The prominent structural feature of the bd oxidase is the presence of 19 transmembrane helices in a nearly oval arrangement when viewed down to the membrane (Fig. 1A). CydA and CydB possess the same fold, sharing a nine-transmembrane helix topology, each composed of two four-helix bundles and an additional peripheral helix. CydA and CydB are related by an approximate twofold rotation axis perpendicular to the membrane. A structural alignment of both subunits yields a relatively low root mean square deviation value of 3.1 Å (Fig. 1B and fig. S2). Such a pseudosymmetrical structural arrangement is most likely the result of a gene duplication of a single ancestral gene that encoded a homodimeric protein and subsequent mutations. The situation resembles that of the photosynthetic reaction centers, where the core is constituted by two structurally similar protein subunits, only one of them being used for electron transfer (20, 21). The heterodimer formation is mediated by interactions of

hydrophobic residues at the interface between $\alpha 3$, $\alpha 4$, and $\alpha 9$ of CydA and the symmetry-related helices $\alpha 3$, $\alpha 4$, and $\alpha 9$ of CydB. The Q loop, a hydrophilic region between transmembrane helices 6 and 7 of CydA (segments 249 to 319) facing the extracellular space, possesses a largely irregular protein fold apart from a short helical stretch leading to an antiparallel β sheet (Fig. 1A). A potentially accessible pocket for the binding of the substrate quinol was identified near Glu²⁵⁷ and Lys²⁵² (Glu²⁵⁷ and Lys²⁵² in *Escherichia coli*) in the Q loop (Fig. 2C); both residues, which are found in the short helix (segments 249 to 258), have already been associated with the binding and oxidation of quinol by site-directed mutagenesis experiments (22). Lys²⁵² is in reasonable distance to form a polar contact with the O1/2A propionate group of heme b₅₅₈.

The HCOs generally exhibit a more compact and globular packing within the membrane than the bd oxidase. The conserved catalytic core subunits (subunit 1 or CcoN) of the HCO-family enzymes generally contain 12 transmembrane helices with a threefold rotational symmetric arrangement, resulting in the generation of three pores that are formed by semicircular arrangements of four helices each (23). Neither the overall topology of CydA with its nine transmembrane helices nor the heme organization indicates any evolutionary relation of any functional motif. Comparison with other structures in the Protein Data Bank (PDB) (24) reveals the uniqueness of the overall protein structure and the redox active site organization. The four-helix bundle motif as an individual structural unit is found in a few other bioenergetically important membrane proteins, such as cytochrome b₆₅₁ (PDB accession number 4O7G) and the transmembrane part of a polysulfide reductase (PsrC) (PDB accession number 2VPX).

A third subunit, which we designate CydS, consisting of only one single transmembrane helix, was identified in the electron density map. CydS, a short peptide of 33 amino acid residues, could be purified and sequenced by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) (fig. S3). We located its gene immediately downstream of the *cbdB* gene in the *Geobacillus* genome. Positioned at the peripheral interface of $\alpha 5$ –6 of CydA, CydS may stabilize the heme b₅₅₈ harboring a four-helix bundle ($\alpha 5$ –8 of CydA) during potential structural rearrangements of the Q loop upon binding and oxidation of quinol (Fig. 1A). Although conserved in the order of Bacillales (fig. S5), there is no sequence similarity between CydS and the proteobacterial single transmembrane helix subunits. However, because a recent de novo blind structure prediction for the *E. coli* bd oxidase proposes an almost identical location of CydX, CydS and CydX might play analogous roles (25).

The three hemes are found in a triangular arrangement (Fig. 2A) and not in the form of a linear chain in the order b₅₅₈, b₅₉₅, and then d as expected (2). The low-spin heme b₅₅₈ is located within the membrane core, with its plane tilted by $\sim 110^\circ$ to the membrane plane. The vertical distance from the heme iron to the extracellular surface is ~ 18 Å. It is in close proximity to the Q loop (Fig. 2, A and C). As inferred from mutagenesis experiments and electron paramagnetic resonance data (16, 26), His¹⁸⁶ and Met³²⁵ (His¹⁸⁶ and Met³⁹³ in *E. coli*) are the axial ligands of heme b₅₅₈ (Fig. 2C). The terminal electron acceptor heme d is located closer to the extracellular membrane surface, with its plane tilted by $\sim 60^\circ$ to the membrane plane. The vertical distance from the heme iron to the P side of the membrane is

~13.5 Å. Heme b₅₉₅ is coordinated by the invariant axial ligand His²¹ (His¹⁹ in *E. coli*) on one side. On the opposite side, Glu¹⁰¹ (Glu⁹⁹ in *E. coli*) appears to be ligated to the heme iron. Originally proposed to be ligated by Glu¹⁰¹ (27), heme d seems to possess another glutamate ligand, namely Glu³⁷⁸ (Glu⁴⁴⁵ in *E. coli*), which has been postulated to be a key residue taking up a charge-compensating proton, when the high-spin hemes become reduced (Fig. 2D) (28). Our MALDI-MS analyses indicate that heme d is present as a cis-heme d hydroxychlorin γ -spirolactone in the *Geobacillus* bd oxidase (fig. S4). This result is supported by the electron density map, which moreover implies a bent conformation of heme d.

Despite their close proximity, the arrangement of the two high-spin hemes does not support the existence of a binuclear active center (see the supplementary materials). The hemes are in van der Waals contacts, with edge-to-edge distances around 3.5 Å and a distance between the central Fe atoms of 11.6 Å. The highly conserved (>99%) Trp³⁷⁴ residue may be important for the electron transfer between heme b₅₅₈ and heme d. Conserved Trp residues are often found between intermediate electron acceptors in biological electron transfer chains, such as in the active branch of the photosynthetic reaction centers from purple bacteria and of photosystem II between a (bacterio)phaeophytin and a quinone acceptor (20), or between the high-spin heme and the low-spin heme of the HCOs. In addition, Trp residues themselves can donate electrons and form Trp radicals. In particular, Trp³⁷⁴ could donate an electron to compound I (F⁺ state)—a catalytic intermediate of heme d forming an oxoferryl-porphyrin radical during the splitting of the dioxygen bond by a single four-electron transfer reaction (29)—and then receive an electron from heme b₅₅₈.

The triangular arrangement of the hemes and the distances between them suggest a direct electron transfer from heme b₅₅₈ to heme d, because the edge-to-edge distance and the Fe-Fe distance between these hemes are significantly shorter (5.9 and 15.2 Å) than the respective distances between the two b hemes (8.5 and 19.4 Å). Finally, the electron would equilibrate between heme d and heme b₅₉₅. For the breakage of the dioxygen bond, four electrons are required. In the reduced enzyme, two of them could be supplied by the heme d iron by the formation of an oxoferryl state, the third one from heme b₅₉₅, and [according to (29)] the fourth one from the macrocycle of heme d. Therefore, the bd oxidases and the HCOs appear to use the same principle to avoid the formation of superoxide anions and of peroxides (29), namely a very rapid or simultaneous transfer of four electrons onto dioxygen, leading to breakage of the dioxygen bond. The main functional difference appears to be that one electron is provided by an amino acid side chain (tyrosine) in the case of the HCOs and by the heme d macrocycle in the case of the bd oxidases (29). The analogy goes further: The oxygen-binding heme (d or a₃ for the canonical heme aa₃ HCOs) is located between a low-spin heme (b₅₅₈ versus a) and the donor of the third electron (heme b₅₉₅ versus Cu_B). There is even a Trp residue between the low-spin heme and the dioxygen-binding high-spin heme in both the bd oxidases and the HCOs.

Although the bd oxidase doesn't pump protons across the membrane, the existence of proton transfer pathways from the cytoplasm is crucial for the access of protons to the oxygen-binding site and potentially also for protons involved in compensating for the negative charge of the electrons used for heme reduction. We identified two potential pathways

through which protons could pass from the cytoplasm to the high-spin heme site. One pathway is formed inside of the four-helix bundle $\alpha 1-4$ of CydA and the second one in the symmetry-related $\alpha 1-4$ four-helix bundle of CydB. Therefore, we call these potential routes for proton transfer CydA and CydB pathways (Fig. 3 and fig. S7). The location of Glu¹⁰⁸ (Glu¹⁰⁷ in *E. coli*) in our structure, together with previous mutagenesis experiments and Fourier transform infrared spectroscopy data, supports the proposal that this glutamate residue is a redox state-dependent mediator of proton transfer to a charge compensation site (30). With the CydA pathway leading to Glu¹⁰¹, this residue might be the hypothetical protonatable group used for charge compensation upon heme b₅₉₅ reduction (28). It remains to be investigated whether protons entering the CydA pathway can be transferred from Glu¹⁰¹ to Glu³⁷⁸ for compensating the negative charge of a second electron used to reduce the two high-spin hemes. Protonation of Glu³⁷⁸ could alternatively also be accomplished by a proton accessing from the extracellular side. Currently it remains unclear whether the CydA pathway is solely providing protons for charge compensation or whether Glu¹⁰⁸ can be a branching point that is able to pass protons via the heme b₅₉₅ propionates to the oxygen-binding site. With both channels leading to heme b₅₉₅, there must be an additional proton transfer step from heme b₅₉₅ to heme d in order to deliver protons to the oxygen reduction site (Fig. 3). Based on the current structure, we can exclude the possibility of direct proton transfer between the two high-spin hemes. It seems more plausible that the final proton transfer from heme b₅₉₅ to heme d is facilitated by connecting unresolved water molecules. The heme propionates of heme b₅₉₅ are in a hydrophobic environment without any charge compensation. They are thus most likely protonated and could supply protons for water formation. These protons would be replenished via the proton pathways (Fig. 3 and fig. S7).

HCOs contain oxygen channels lined by hydrophobic residues leading from the membrane interior to their binuclear heme-Cu active site (31). Our bd oxidase structure does not indicate the presence of such voluminous pathways; instead, molecular oxygen may access heme d laterally from the alkyl chain interface with the membrane over a short distance (Fig. 2B). Hence, oxygen dissolved in the membrane could rapidly bind to heme d without traveling through any tunnel-like protein cavity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Overall structure of the bd oxidase from *G. thermodenitrificans*
 (A) Ribbon model of the bd oxidase consisting of the subunits CydA (blue), CydB (red), and CydS (beige). (B) Structural superposition of CydA and CydB. The dashed line indicates the border between the hydrophobic and hydrophilic layers of the membrane.

Fig. 2. The prosthetic groups in the bd oxidase

(A) Picture of the heme groups represented by stick models bound to CydA (blue). Heme b₅₅₈ and b₅₉₅ are depicted in yellow and heme d in brown. (B) Surface representation of CydA showing a pocket formed between $\alpha 1$ and $\alpha 8$ that could enable dioxygen to directly access heme d. (C) The initial electron acceptor heme b₅₅₈ in proximity to the Q loop. (D) The high-spin heme site composed of hemes b₅₉₅ and d.

Fig. 3.
The potential proton transfer pathways in the bd oxidase.