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Structure and Function of Bacterial Cytochrome *c* Oxidase

So Iwata[1]

Uppsala University, Department of Biochemistry, Biomedical Center, BOX 756 S-75123 Uppsala, Sweden

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The crystal structure of cytochrome *c* oxidase from the soil bacterium *Paracoccus denitrificans* has been reported. This structure has provided a basis for understanding the mechanism of the redox-coupled transmembrane proton pump which is the key component of the respiratory chain in most aerobic organism. Over the past ten years, there have been many site-directed mutagenesis studies performed on bacterial oxidases. Structural features of *Paracoccus* oxidase have been summarized in the light of these mutagenesis studies and other structural works.

Key words: cytochrome *c* oxidase, membrane protein, quinol oxidase, three-dimensional structure, X-ray crystallography.

Respiratory oxidases are membrane enzymes that are ubiquitous among aerobic organisms. They catalyze the reduction of molecular oxygen to water and use the free energy change available from this reaction to pump protons across the membrane (Fig. 1a). The transmembrane proton- and voltage-gradient generated by oxidase and other components of the aerobic respiratory chain is directly converted to more useful energy forms by a number of membrane bound energy conserving systems.

Over the years, more and more structural knowledge on respiratory oxidases has been accumulated. The structure of the four-subunit cytochrome *c* oxidase (COX) has been reported from *Paracoccus denitrificans* (*1*) followed by the structure of bovine heart cytochrome *c* oxidase (*2, 3*). Very recently, the projection structure of the ubiquinol oxidase from *Escherichia coli* (*4*) and the 3D structure of the two-subunit oxidase from *P. denitrificans* (*5*), a subset of the four-subunit oxidase, have been determined.

The respiratory oxidases (from species ranging from microbes to humans) are members of a single superfamily, called the heme-copper oxidase superfamily (*6, 7*). There are two main branches of this superfamily which are characterized by their substrate specificities: (i) The mitochondrial respiratory oxidases and many bacterial oxidases use cytochrome *c* as a substrate, and hence are called cytochrome *c* oxidases. (ii) Some bacteria contain multiple respiratory oxidases and many of them use membrane-bound quinol as a substrate and are called quinol oxidases. Evolutionary investigations led to the conclusion that this superfamily may have been present before the level of atmospheric oxygen rose as a result of photosynthetic activities (*8, 9*). Subunit I of the heme-copper oxidases is related to the subunits of NO reductase (*8*) and N_2O reductase (*10*) in nitrate respiration, which existed earlier than oxygen respiration.

[1] Tel: +46-18-471-4451, Fax: +46-18-511755, E-mail: iwata@xray.bmc.uu.se

Abbreviations: COX, cytochrome *c* oxidase; su, subunit.

The sequences of subunits I and II are particularly well conserved throughout this superfamily. Subunit II of COX contains the binuclear Cu_A center which receives electrons from cytochrome *c* and transfers them to heme *a*, where they are finally transferred to the binuclear heme a_3-Cu_B center (*11*). The heme groups, whose chemical identity can vary (hemes A, B, or O have been found), and Cu_B are bound to subunit I. However, the Cu_A center is absent in ubiquinol oxidases but could be restored by protein engineering (*12*). The total number of subunits varies from two or three found in some bacteria to 13 present in mammalian mitochondria.

During the past ten years, many site-directed mutagenesis studies combined with spectroscopy has been performed to reveal the structure-function relationship in oxidases. These studies are mainly done on three bacterial oxidases; bo_3 ubiquinol oxidase from *E. coli*, cytochrome *c* oxidase from *Rhodobacter sphaeroides*, and cytochrome *c* oxidase from *P. denitrificans*. This review article summarizes the structural features of *Paracoccus* oxidase in light of recent results obtained from mutagenesis studies. A number of reviews and comments on the oxidase structures have appeared since the end of 1995 and are recommended to the interested reader (*13-19*, for more general reviews, see Refs. *7, 20,* and *21*).

Architecture of cytochrome *c* oxidase

The fully oxidized COX from *P. denitrificans*, complexed with the antibody F_V fragment 7E2, has been crystallized in the presence of 0.1% sodium azide (*22*). The structural model contains 6 protein subunits: subunits I, II, III, and IV of COX, and V_H and V_L chains of antibody F_V fragment. A general view of the *P. denitrificans* COX parallel to the membrane is presented in Fig. 1b. In this projection, the part integrated into the membrane has a trapezoidal appearance. The width at the bottom corresponds to the cytoplasmic surface and is ~90 Å; at the top, which represents the periplasmic surface, is ~75 Å. The height of the trapezoid is 55 Å. It comprises 22 membrane spanning helices. The globular domain of subunit II is attached to the trapezoid from the top resulting in a maximal height of 95

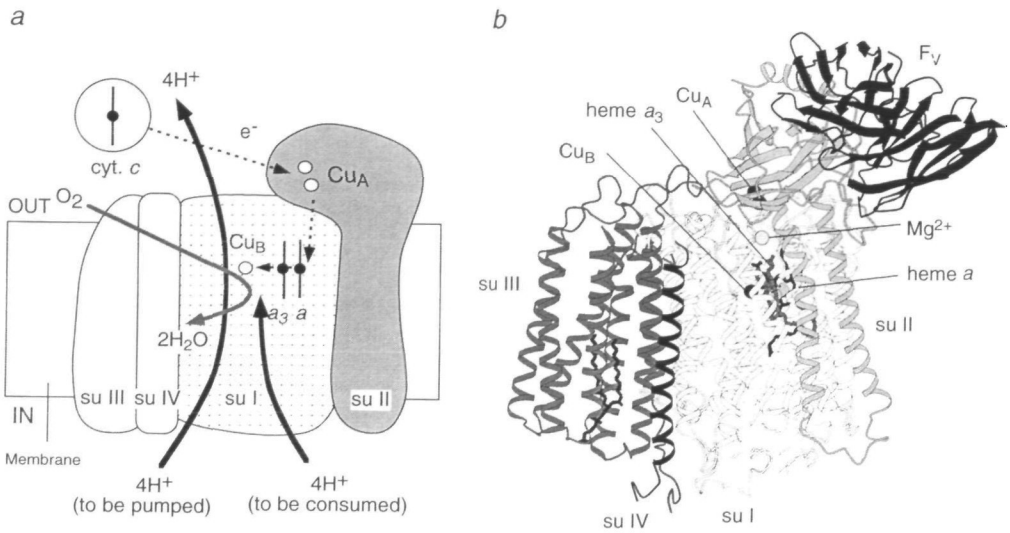

Fig. 1. **a: Schematic drawing of the subunit I, II, III, and IV of the COX complex from *Paracoccus denitrificans*.** The arrangements of the subunits in the membrane and the position of the metal centers are shown. The electron- and proton-transfer is shown by arrows. **b: The entire cytochrome *c* oxidase from *P. denitrificans* complexed with an antibody F_V-fragment viewed parallel to the membrane.** The subunits I, II, III, and IV and the F_V-fragment are shown.

Å. The central part of the complex is made up by subunit I which binds both hemes and Cu_B. Subunit I is associated with subunits II at one end and with subunit III at the other. Both the N- and C-terminus of subunit II protrude into the periplasmic space and form a globular domain containing Cu_A. This globular domain is the binding site of the antibody fragment. Subunit IV consists of one transmembrane helix. In a view down to the membrane this cytochrome *c* oxidase has an oval shape with largest dimensions of 90 and 60 Å.

Distribution of aromatic residues, which displays a clear difference between membrane and soluble proteins, are of general interest in the studies of membrane proteins. Tryptophan residues are preferentially located at the border between the hydrophobic and hydrophilic zones. Similar observations have been made for photosynthetic reaction centers and bacterial light-harvesting complexes. Tyrosine and phenylalanine residues are preferentially located in the polar and the non-polar regions, respectively. Interestingly, only a few aromatic residues are observed in the middle of the transmembrane section. This distribution of the residues can be related to their hydrophobicity.

Subunit IV

The function of this small subunit is unknown. Its presence in the *Paracoccus* cytochrome *c* oxidase has only recently been discovered (*23*). The subunit consists mainly of one transmembrane helix with the N-terminus on the cytoplasmic side (Fig. 1b). A number of additional lipids and detergent molecules are localized around this subunit. Despite of its small size, subunit IV is in contact with all of the other subunits. In the projection structure of quinol oxidase from *E. coli,* subunit IV was also found at the similar location (*4*). Some evidences suggest that this subunit is important for stability of Cu_B center in subunit I (*24*).

Subunit III

Subunit III possesses seven transmembrane helices which are arranged in a less regular manner than those found in subunit I due to the long loops between helices (Fig. 1b). The seven transmembrane helices are divided into two bundles, one formed by the first two helices and the other by the last five helices. The two bundles are separated by a large V-shape cleft. At the bottom of the cleft, one firmly bound lipid molecule is found. In some members of the oxidase superfamily, including quinol oxidase from *E. coli*, the first two helices of subunit III is assumed to be fused into subunit, thereby leaving only five helices in subunit III. The association of subunit III with subunit I is weak (*25*), and removal of subunit III can be accomplished using LDAO. Subunit III is likely not to be directly involved in the proton pumping of the oxidase, because the enzyme remains fully functional without subunit III. Instead, it is probably involved in the assembly of the complex as shown by a subunit III deletion mutant failing to properly assemble oxidase in the membrane (*26*). Moreover, the role of subunit III might be to prevent blockage of the oxygen channel entrance by other proteins. The structure of the bacterial oxidase indicates a possible channel for diffusion of oxygen leading from the cleft of subunit III to the binuclear center (*27*).

Subunit II and Cu_A center

Subunit II is composed of three segments: an N-terminal loop, two transmembrane helices and a C-terminal globular domain which contains the Cu_A center (Fig. 2). The N-terminal loop and C-terminal domain interact tightly on the periplasmic side. Both are located above pore B of subunit I (see Fig. 3). The C-terminal globular domain contains a 10-stranded β-barrel. The folding of this domain is similar to that of class I copper proteins like plastocyanin and azurin. The Cu_A center of the quinol oxidase is assumed to have been lost during the evolution. This can be restored by mutational studies at the site as demonstrated for the ubiquinol oxidase from *E. coli*. The structure of a water soluble fragment of the quinol oxidase with this re-engineered Cu_A center has been determined at 2.3 Å resolution (*12*). The Cu_A center is a mixed-valence [Cu(1.5)-Cu(1.5)] complex (*28–31*, Fig. 2). The ligands of Cu_A-1 atom are the residues Cys216, Cys220, His181, and Met227, and those of Cu_A-2 atom are Cys216, Cys220, His224, and the carbonyl oxygen of Glu218. The two cysteine residues bridge the two copper atoms. Distances between the two

copper atoms are 2.6 Å in the crystal structure of COX and 2.5 Å in the re-engineered *bo* oxidase (*12*).

Subunit I

Subunit I contains 12 transmembrane helices. This subunit shows an unexpected approximate threefold rotational symmetry (Fig. 3). When viewed from the periplasmic side, the 12 segments appear to form three symmetrically related arcs. The order of the helices is sequential in an anticlockwise manner. Helices XI, XII, I, and II form the first arc, III–VI the second, and VII to X the third. The arcs together with the last helix of the previous arc have a pore-like appearance. In such a description segments II–VI, VI–X, and X–XII, I and II would form "pores" A, B, and C, respectively. These "pores" are blocked; pore A by preferentially conserved aromatic residues, pore B by heme a_3 and Cu_B, and pore C by heme *a*. In the interface region between subunit I and II, a non-redox-active metal can be assigned. A Mg site was modeled in the bovine oxidase structure (*2*). In the bacterial oxidase, the site was identified as a Mg/Mn site (*1, 5, 32, 33*). The function of this metal center is still unclear.

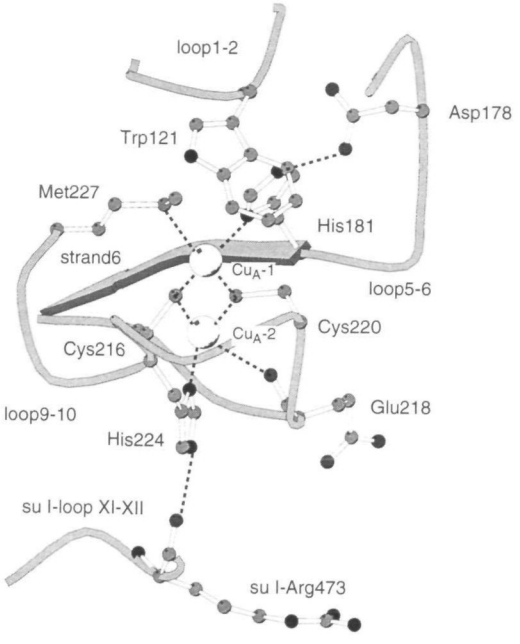

Fig. 2. **The Cu_A center of subunit II.** The loops providing ligands to the copper atoms are shown, and named according to the β-strands that they connect.

Heme *a* and heme a_3-Cu_B binuclear center in subunit I

Heme *a* is a low-spin heme with two axial histidine ligands. It transfers electrons from Cu_A to the binuclear center. Heme *a* as well as heme a_3 are type A hemes which are characterized by being non-covalently bound to the protein. Also, a formyl group and a hydrophobic hydroxyethyl farnesyl groups are present at C18 and C3, respectively. The axial ligands of heme *a* are His98 (helix II) and His413 (helix X). The formyl group forms a hydrogen bond with the side chain of Arg54. The side chains of arginines 473 and 474 form ion pairs with one propionate group, the other is hydrogen bonding with the mainchain carbonyl group of Arg474 and the side chain of Trp87.

The binuclear center formed by heme a_3 and Cu_B is the O_2 reduction site (Fig. 4). Its binding site is located in pore B. The Fe atom of heme a_3 appears to be fivefold coordinated with His411 (helix X) as the only axial protein ligand. The Fe is *ca.* 0.7 Å out of the heme plane. The heme a_3-Cu_B binuclear center is the catalytic core for O_2 reduction (*34*). The free coordination site of the iron points towards the free coordination site of Cu_B which is further coordinated by three histidine residues, His276, His325, and His326.

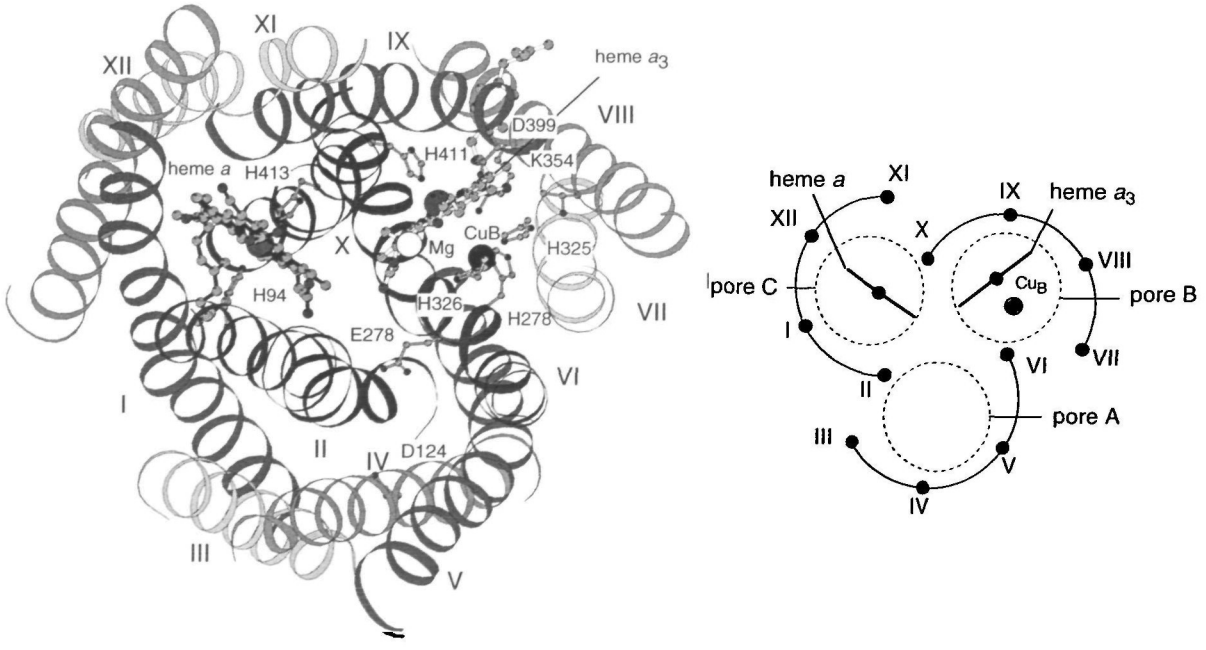

Fig. 3. **View of subunit I along the membrane normal from the periplasmic side.** Only helices are shown together with some important residues. The icon shows schematically the arcs of helices and the pores A, B, and C formed by them.

Cu_B is 5.2 Å away from the heme iron. In the bacterial oxidase, one of the three histidines, His325, seems to be disordered (*1*). Spectroscopic evidence suggests that a bridging ligand may exist between the Fe and Cu_B atoms of the binuclear center (*35, 36*). We could see electron density at this position. However, due to the lack of resolution, the identification of this density is unclear.

The planes of both heme groups are perpendicular to the membrane and their interplanar angle is 108°. The shortest distance between heme *a* and heme a_3 is only 4.7 Å. The electronic coupling between heme *a* and the binuclear center is very strong, and fast electron transfer between these two redox centers has recently been used to explain the high apparent oxygen affinity of oxidase by kinetic trapping of bound oxygen (*37*).

Possible proton pumping mechanism

For cytochrome *c* oxidase, the most important question to be answered is how oxygen reduction is coupled to proton translocation. It is very likely that the endergonic proton pumping reaction is directly coupled to the exergonic redox reaction at the binuclear center (*38*). Many models and hypotheses have been proposed to explain the proton pumping mechanism (*39-47*). Two possible proton transfer pathway have been observed in the protein structure (Fig. 5). These show good agreement with the result of site directed mutagenesis experiments for bacterial oxidases (*48-52*). The first pathway (K-channel) leads along the conserved polar face of transmembrane helix VIII to the binuclear center. It also includes the hydroxyl group of the hydroxyethyl farnesyl side chain of heme a_3, Tyr280 and firmly bound water molecules. Replacement of the residues of the polar face of transmembrane helix VIII leads to an inactive enzyme (*48, 52*). All of these residues are, in principle, connected by hydrogen bond. The exception is Lys354 which does not form hydrogen bonds to either Ser291 or Thr351. Because the environment of this lysine residue is completely hydrophobic, it should be deprotonated. In other possible conformations it may form hydrogen bonds to Ser291 or Thr351.

The second pathway (D-channel) starts at the conserved Asp124 located at the entrance of the pore formed by helices II-VI and leads into a large polar cavity containing several water molecules. The conserved glutamate residue Glu278 is found at the end of the cavity. Beyond this glutamate, the proton pathway becomes rather speculative. It is most probably connected to Cu_B center or histidine ligands of Cu_B through water molecules which are disordered in the structure. This idea is supported by mutagenesis and spectroscopic studies (*53*). Replacing Asp124 with asparagine or two subsequent asparagine residues (Asn113, Asn131) with hydrophobic residues abolishes proton pumping but not oxygen reduction (*49-51*). These findings suggest at a first glance, that the first pathway is for protons to be consumed for water formation whereas the second pathway is for protons to be pumped. However, the characteristics of the Lys354 mutants raise some questions about the assignment of these channels (*52*). The two channel might be working at the different stages of the oxygen reduction cycle. Further studies are needed to confirm the roles of these channels.

Two papers (*44, 47*) are of particular relevance in formulating a mechanism for proton pumping. In the first paper, Rich (*44*) postulates electroneutrality of redox changes around the heme-copper center, which means that

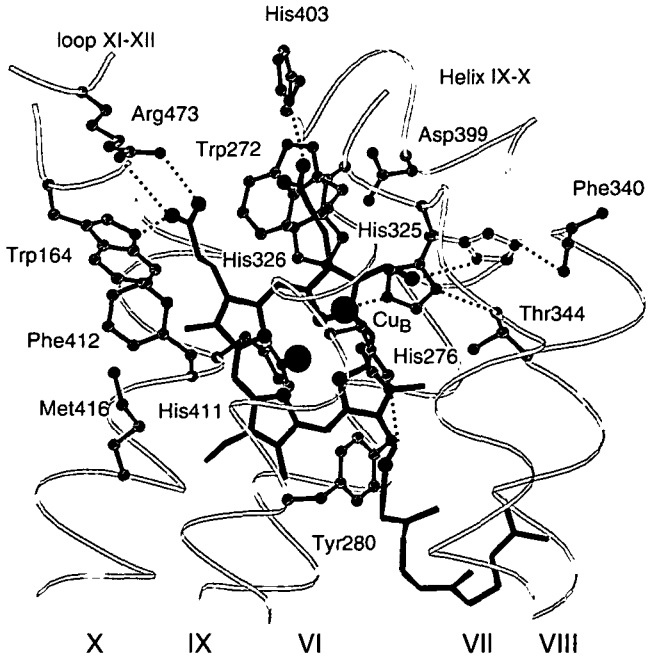

Fig. 4. **The binuclear center of heme a_3 and Cu_B.** The side chain of His325 may possess two alternative conformations, in one conformation (black) the histidine forms a covalent bond to Cu_B and a hydrogen bond to Thr344 and in the other (white) it makes hydrogen bonds to the formyl group of heme a_3 and the carbonyl oxygen of Phe340.

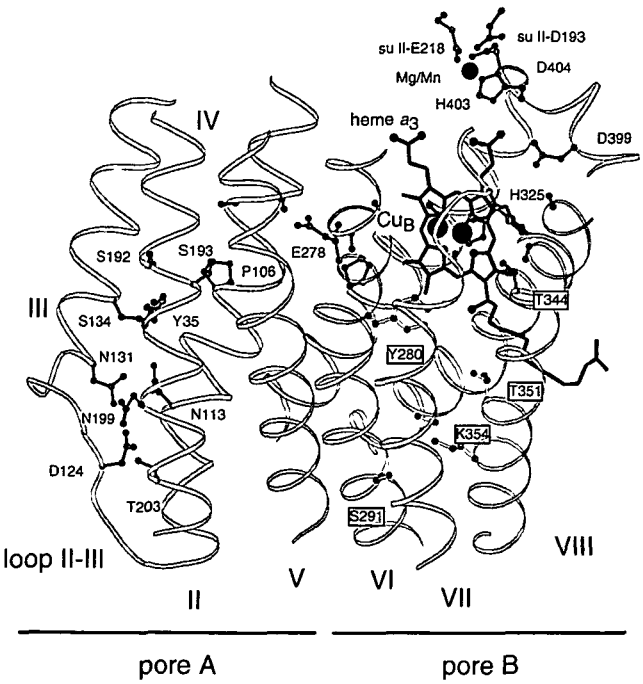

Fig. 5. **The proton pathways in subunit I.** Residues in the possible pathways for protons to be consumed in water formation and for protons to be pumped are labeled with and without boxes, respectively. Two alternative conformations of His325 are shown.

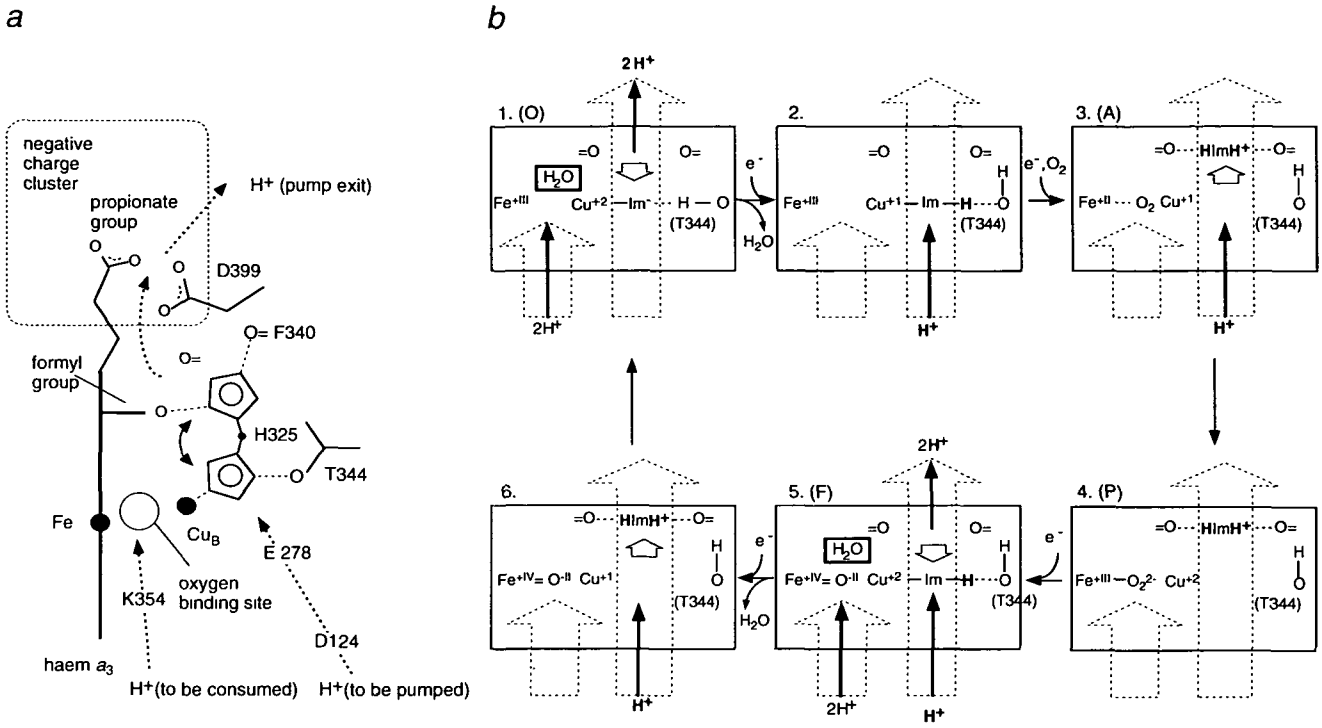

Fig. 6. **A possible proton pumping mechanism. a: Schematic representation of the proton pathways and the histidine shuttle mechanism.** The thick broken lines represent possible proton pathways. **b: Histidine shuttle mechanism for coupling of proton pumping to oxygen reduction to water molecule.** The symbols are follows: O, fully oxidized; A, the compound A; P, the peroxy; and F, the oxoferryl states. Protons to be pumped enter the boxes at the bottom right and leave at the top along the broken arrows. They are indicated in bold. Protons to be consumed enter from the lower left. Movements of the His325 side chain are indicated by the short white arrows in the box.

upon a single reduction of one of the metals, one proton is taken up for charge compensation, and two protons for double reduction. These protons are then released to the periplasmic side by electrostatic interactions, while two other protons are taken up for the formation of water further on in the oxygen reduction cycle. This paper does not present chemical details.

In the second paper, Wikström *et al.* proposed that a histidine ligand of Cu_B cycles between the imidazolate, imidazole, and imidazolium states twice upon the reduction of one molecule of O_2 (*47*). In this model, the protons being pumped are those of the imidazolium. In addition, the uptake of the protons to be consumed leads to the release of the protons to be pumped. The histidine shuttle mechanism proposed in Ref. *1* is based on this histidine cycle mechanism and strictly obeys the electroneutrality principle as well (Fig. 6). In the mechanism, the key residue is His325 which shows disorder in the electron density and can be modeled in two conformations. This histidine would be a Cu_B ligand in the imidazolate and imidazole states but not in the imidazolium state. In the latter, it might assume a position suited for proton transfer to the periplasmic side and the proton transfer is initiated upon the arrival of protons at the binuclear center needed for water formation. If a hydroxide were a Cu_B ligand at the oxidized state [a special case of the finding of one oxygen with exchangable proton(s) as a Cu_B ligand (*35*)], however, the first proton taken up upon reduction of the binuclear center would unavoidably lead to the formation of water. In this situation, the histidine cycle/shuttle mechanism would have to be reformed.

What is clearly needed is the determination of precise structures for the intermediates (*38*) involved in the redox reactions of the heme-copper oxidases. This is especially true in light of the recent paper by Fabian and Palmer (*54*), which raises doubts on some of the assignments (*38*) of spectroscopically identifiable intermediates for chemical structures during the redox reactions. Whether these doubts are justified remains to be seen. The high resolution studies of bovine heart oxidase and two subunit COX are in progress in Japan and Germany. Thus, there is a good reason to believe that the structures of these intermediates can be determined by X-ray crystallography in the near future.

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