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RESPIRATORY OXIDASES are membrane enzymes that are ubiquitous among aerobic organisms. They catalyse the reduction of molecular oxygen to water and use the free energy available from this reaction to pump protons across the membrane. The respiratory oxidases are therefore active ion transporters, and contain within them a proton-conducting transmembrane channel. In eukaryotes the respiratory system is within the inner mitochondrial membrane, whereas in prokaryotes the analogous systems are found in the cytoplasmic membrane. The transmembrane proton- and voltage-gradient generated by the oxidase and by other components of the aerobic respiratory chain is converted directly to more useful forms by a number of membrane-bound energy-conserving systems, such as the ATP synthase and secondary active transport systems. The respiratory oxidases therefore play a crucial role in the physiology of virtually all aerobic organisms. Several questions remain concerning their function, and principal studies have focused on: (1) the mechanism by which oxygen is reduced to water; (2) the mechanism of proton translocation; and (3) the interrelationship between these two mechanisms.

In recent years, it has emerged that most respiratory oxidases from species

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The cytochrome oxidase superfamily of redox-driven proton pumps

Melissa W. Calhoun, Jeffrey W. Thomas and Robert B. Gennis

Most respiratory oxidases of eukaryotic and prokaryotic organisms are members of a superfamily of enzymes that couple the redox energy available from the reduction of molecular oxygen to the mechanism of pumping protons across the membrane. The recent applications of site-directed mutagenesis and of a variety of spectroscopic techniques have allowed major advances in our understanding of the structure and function of these proteins.

ranging from microbes to humans are members of a single superfamily, called the heme–copper oxidase superfamily[1-3]*. The members of this superfamily share important structural and functional features. These similarities are being exploited by applying site-directed mutagenesis to several of the bacterial enzymes, and analysing the resulting mutants by spectroscopic techniques developed for studying the mitochondrial oxidase[2]. These studies have resulted in a structural model of the catalytic core[2,4]. At the same time, powerful time-resolved spectroscopic techniques are yielding insights into the

mechanism of oxygen reduction, as well as providing information about the coupling of the redox chemistry to proton pumping[5-9].

The superfamily of respiratory oxidases

The heme–copper oxidase superfamily is defined by two criteria: (1) a high degree of amino acid sequence similarity within the largest subunit (subunit I); and (2) a unique bimetallic

*For older literature citations the reader is directed to Ref. 33. For the best single source of different perspectives on the current status, consult J. Bioenerg. Biomembr. (1993) 25, No. 2, which is entirely dedicated to articles on this topic.

active site, consisting of a heme and a closely associated copper atom, where dioxygen is reduced to water. Within the heme–copper oxidase superfamily there is substantial diversity, in terms of both the substrates utilized and the heme and copper components of the individual oxidases[2].

There are two main branches of the superfamily, which have distinct substrate specificities: the mitochondrial respiratory oxidases and many bacterial oxidases use cytochrome *c* as a substrate and, hence, are called cytochrome *c* oxidases. Bacteria, unlike most mitochondria, contain multiple respiratory oxidases. Many of the prokaryotic respiratory oxidases use membrane-bound quinol (ubiquinol or menaquinol) as a substrate rather than cytochrome *c*. By the criteria mentioned above, a number of these quinol oxidases have been shown to be members of the heme–copper oxidase superfamily and to pump protons as efficiently as the cytochrome *c* oxidases[1-3].

Structural characteristics. The eukaryotic cytochrome *c* oxidases all contain three mitochondrially encoded subunits (I, II and III) and up to ten nucleus-encoded subunits[3]. All prokaryotic members of the superfamily contain homologs of subunit I, and most also contain homologs of subunits II and III. Subunits homologous to the eukaryotic nucleus-encoded subunits have not been found in bacteria. The cytochrome *c* oxidases from *Paracoccus denitrificans*[10] and *Rhodobacter sphaeroides*[11] contain only three subunits, but they catalyse electron transfer reactions and proton pumping as efficiently as the 13-subunit bovine oxidase. The oxidase from *P. denitrificans* has also been isolated in a fully functional form that contains only subunits I and II, indicating that these two subunits are sufficient for both oxidase activity and proton pumping[10].

The proton-pumping ubiquinol oxidase from *Escherichia coli*, cytochrome *bo*,

The amino acid sequence of subunit I from this quinol oxidase is more than 40% identical to that of bovine cytochrome *c* oxidase, illustrating the importance of many of the residues in this subunit[2]. Thus, the characteristics of subunit I unify the members of this enzyme superfamily, whereas it appears that differences within subunit II correlate with differences in substrate specificity.

Subunit I. This subunit binds to three metal prosthetic groups (two hemes and one copper atom), and contains the oxygen-binding site and most, if not all, of the proton-conducting channel(s). Spectroscopic studies have defined that histidines bind to all three metal atoms and have also defined the metal–metal distances[1-3]. One of the hemes is bound to two histidines and serves an electron-transfer role, passing electrons to the site where oxygen is reduced. The second heme, bound to a single histidine, is part of the heme–copper binuclear center and is the site where oxygen binds and is reduced to water. The open coordination site on the heme iron, where oxygen binds, also provides a binding site for exogenous ligands such as carbon monoxide and cyanide. The copper atom at the binuclear site is called Cu_B and is located approximately 3–5 Å from the heme iron. The two heme iron atoms are approximately 15 Å apart.

Three different heme types (Box 1) are found in a variety of combinations in members of the oxidase superfamily: heme B, heme O[12] and heme A. The chemical distinctions are indicated in Fig. 1. Heme B is the species found in globins, and recent data[13,14] have clearly demonstrated that this is biosynthetically converted, by addition of a farnesyl group, to form heme O, which can then be converted to heme A. Some organisms, such as *E. coli*, lack the enzymes required to convert heme O to heme A. Some of the variability in the heme and copper composition of the respiratory oxidases in the superfamily is illustrated in Fig. 2. An important point is that there is no correlation between the substrate used (quinol versus cytochrome *c*) and the heme species within subunit I.

The sequence of subunit I is the most highly conserved among the subunits
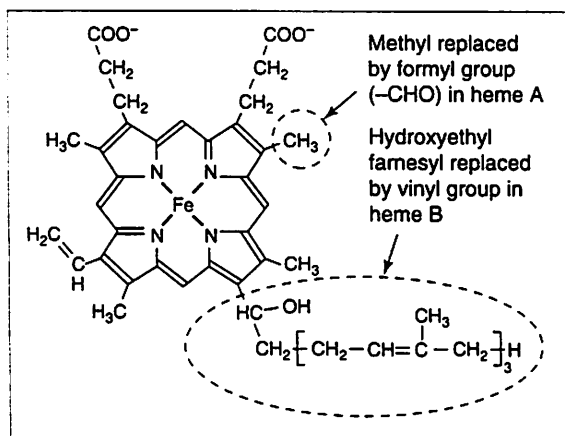
Figure 1

The structure of heme O (Ref. 12). This heme differs from heme B by the replacement of a vinyl group by a hydroxyethyl farnesyl group, as shown. The spectroscopic properties of heme O and of heme B are similar, and each confers a red color to the enzymes containing them (e.g., bo_3-type oxidases). Oxidation of the indicated methyl group of heme O to a formyl group results in heme A, the prosthetic group in all the eukaryotic cytochrome *c* oxidases. Heme A has spec-

for this subunit from over 75 different species. The hydrophobicity patterns indicate that all the subunits have a minimum of 12 transmembrane helices. Experimental evidence using gene fusions with the cytochrome bo_3 quinol oxidase from *E. coli*, as well as all the results from site-directed mutagenesis, are consistent with this 12-span model[2]. Figure 3 shows the membrane topology of subunit I from the aa_3-type cytochrome *c* oxidase from *Rb. sphaeroides*[15], illustrating the 12 membrane spans and highlighting some of the highly conserved residues whose functional importance has been examined by site-directed mutagenesis.

Subunit II. The structural distinctions between the quinol and cytochrome *c* oxidases appear to reside primarily in subunit II (Fig. 2). In the cytochrome *c* oxidases, this subunit contains the binding site for cytochrome *c* and a redox-active copper center, Cu_A, which is the immediate electron acceptor from cytochrome *c*. Recent spectroscopic data suggest that Cu_A is a mixed valence (Cu^{2+}–Cu^+) binuclear copper center[16,17], and the amino acids that are ligands have also been identified in a recent study[18].

In most species, subunit II contains two transmembrane helical spans and has a large carboxy-terminal hydrophilic domain that is located on the outside of the bacterial membrane (equivalent to the mitochondrial inter-membrane space) and contains both the cytochrome *c*-binding site and Cu_A. In some variants, the hydrophilic domain also contains covalently bound heme C. In the quinol oxidases, subunit II does not contain the cytochrome *c*-binding site, Cu_A or heme C[1–3].

The stepwise reduction of oxygen to water

Time-resolved absorption and resonance Raman spectroscopies have been most valuable in elucidating the reaction sequence of oxygen with the mammalian aa_3-type cytochrome *c* oxidase[5–9,19,20]. The reaction scheme in Fig. 4 shows the two metal components of the binuclear center (such as heme a_3 iron and Cu_B) and the sequence of oxygen

intermediates as dioxygen is reduced to two water molecules. Oxygen binds to the enzyme only after the metals in the binuclear center have been reduced[21], and then proceeds through a series of observable intermediates to form two water molecules. The entire cycle takes of the order of 2 ms to complete. Time-resolved resonance Raman spectroscopy has clearly identified several of the species shown in Fig. 4 and measured the rates of their appearance and subsequent disappearance during the reaction sequence. The important point to note is that the reaction is seen to occur as a sequence of discrete steps.

Proton movements during oxidase turnover

These reaction steps are associated not only with the transfer of electrons, but also with the movement of protons of two types. 'Chemical' protons are required in the formation of water, and 'pumped' protons are those that cross the membrane bilayer from the interior (bacterial cytoplasm or mitochondrial matrix) to the exterior (bacterial periplasm or mitochondrial intermembrane space). The chemistry of forming water requires the timely delivery of protons at specific steps in the reaction. These protonations are indicated in Fig. 4, although the exact timing of proton delivery and the protonation states of the intermediates are not

known exactly. It is clear, however, that the overall reaction cannot proceed without these chemical protons. Proton pumping appears to be coupled primarily to the addition of the third and fourth electrons to the binuclear center[22], as shown in Fig. 4. These steps convert the peroxy form of the enzyme to two water molecules and regenerate the oxidized form of the enzyme. Changes in protein conformation associated with these two steps in the reaction cycle somehow drive protons across the membrane and convert free energy available from the reaction to a transmembrane proton-electrochemical gradient. Under some circumstances, it is possible to uncouple proton pumping from the oxidase activity[23,24].

Measurements of proton uptake by the oxidase demonstrate that both the chemical and the pumped protons originate from the interior. Site-directed mutagenesis experiments (see next section) strongly indicate, however, that the binuclear center (where oxygen is reduced) is located near the exterior surface[2,4]. Hence, the protein must provide pathways to facilitate the movement of the chemical protons from the interior aqueous bulk phase to the site where oxygen is reduced, and the pumped protons from the interior aqueous bulk phase to the exterior aqueous phase. The extent to which the
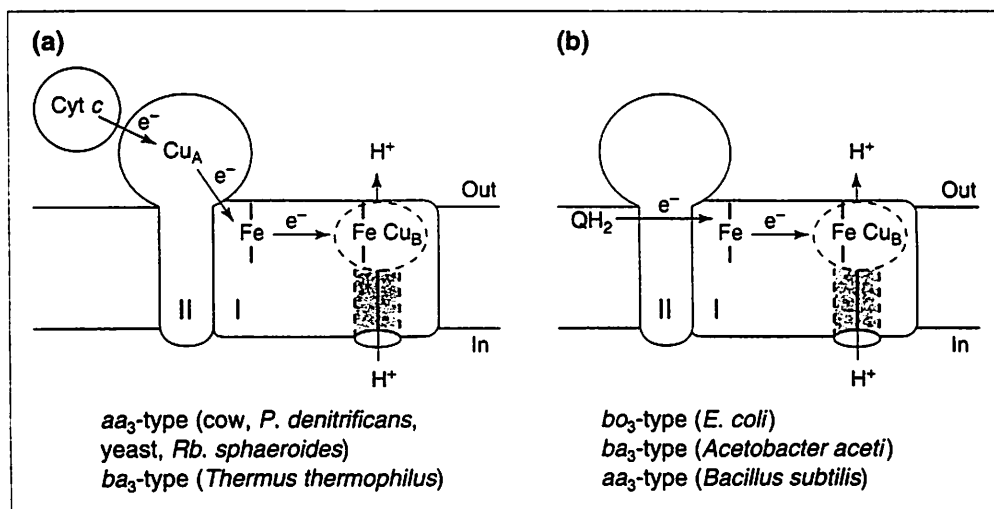

aa₃-type (cow, *P. denitrificans*, yeast, *Rb. sphaeroides*)
ba₃-type (*Thermus thermophilus*)

bo₃-type (*E. coli*)
ba₃-type (*Acetobacter aceti*)
aa₃-type (*Bacillus subtilis*)

Figure 2
Some of the variations among the heme–copper oxidase superfamily[34]. The two hemes in subunit I are pictured as seen edge-on with the iron shown. For simplicity, the binuclear center is shown in association with a single, shared proton channel to convey both the chemical and the pumped protons. The hemes associated with the oxidases of the indicated species are shown[1]. **(a)** Cytochrome *c* oxidases, which do not have any heme associated with subunit II. **(b)** Quinol oxidases. Not shown are the cytochrome *c* oxidases, which have heme *c* covalently linked to subunit II, and the recently reported cytochrome *c* oxidases that lack both Cu_A and the traditional subunit II. In place of subunit II and Cu_A these cbb_3-type oxidases have two membrane-bound cytochrome *c* molecules[35].
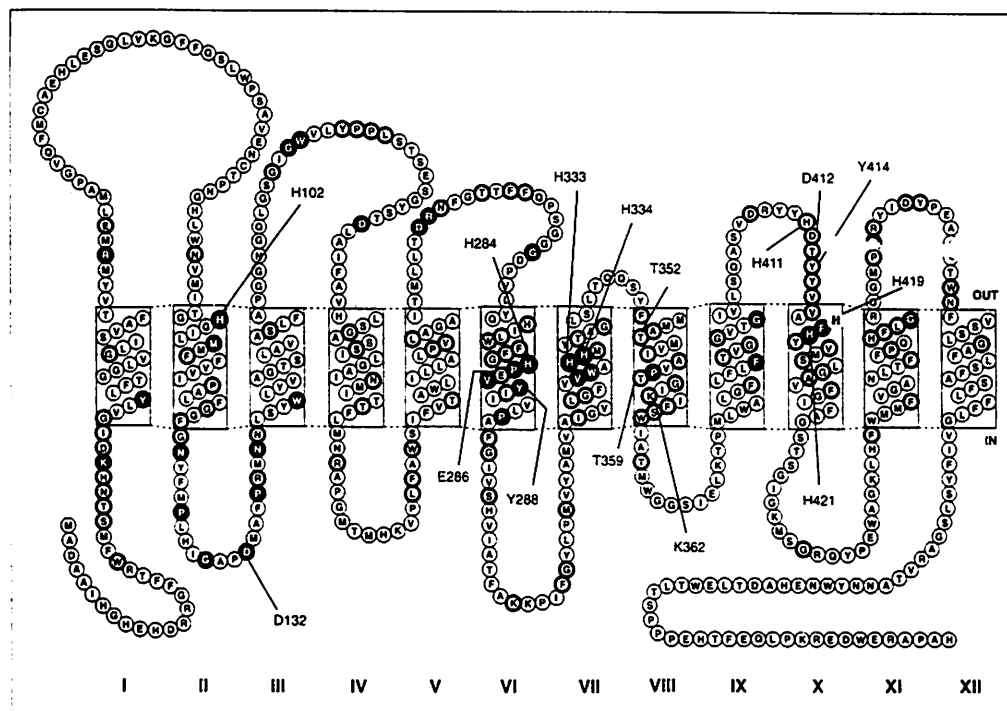
Figure 3

A two-dimensional 12-span model of subunit I of the heme–copper oxidases. The boxed regions indicate putative transmembrane helices. The 'in' side corresponds to the bacterial cytoplasm or the mitochondrial matrix. Several of the members of the superfamily have additional transmembrane spans in subunit I (Ref. 2), but none have fewer than the 12 indicated. The specific sequence shown is from the aa_3-type oxidase from *Rb. sphaeroides*[15]. Highly conserved residues that have been the targets of site-directed mutagenesis experiments discussed in the text are indicated. The proposed ligands to heme a (red), heme a_3 (yellow) and Cu_B (blue) are shown. Other very highly conserved (solid) and less highly conserved (heavy circles) residues are designated.

changes with different amino acid substitutions, without the potential perturbations resulting from detergent solubilization and isolation. Hence, specific amino acids can be placed in a three-dimensional model with some confidence. The results from all the studies on the two different bacterial oxidases are generally consistent and are the basis of the model shown in Fig. 5.

Metal ligands. Spectroscopic studies have implicated histidines as metal ligands of all three metals in subunit I (Refs 1–3). Only six histidines are totally conserved in all the sequences, and these are all within putative transmembrane regions; using the *Rb. sphaeroides* sequence as in Fig. 3, these are: H102 (helix II), H284 (helix VI), H333 and H334 (helix VII), and H419 and H421 (helix X). All substitutions for these residues result in inactive oxidases that, in most cases, are assembled and can be characterized[2,4,24,29]. The results are consistent with the following assignment: heme a is bound to H102 and H421; heme a_3 is bound to H419; and Cu_B is bound to H333, H334 and H284. Perturbations owing to substitutions for Y288 suggest that this residue may also be a Cu_B ligand[30]. These data provide constraints to organize helices II, VI, VII and X in a three-dimensional arrangement, as indicated in Fig. 5.

There are several important implications of this model. (1) All three metal centers are near the top of the membrane, so electron transfer between the two hemes within subunit I is essentially parallel ·to the membrane surface and should not be voltage generating *per se*. (2) The two hemes are ligated by histidine residues on opposite faces at the top of helix X, and are separated by only a single residue, so rapid electron transfer via covalent bonds from heme a to the oxygen-binding heme a_3 is easily rationalized[27]. (3) The location of the heme–copper

chemical and pumped protons share a common channel is not known.

Several proposals have been made that link the energy-producing steps of oxygen reduction to the energy-requiring proton translocation. Proposed mechanisms mostly invoke changes in the ligation of one or more of the metal centers as their redox states change during turnover of the oxidase[21,25–28]. Assuming a common mechanism for proton pumping, it can be concluded that neither the formyl group of heme A nor the Cu_A redox center is required for proton pumping, since some members of the oxidase superfamily that lack these groups can pump protons (Fig. 2). One possibility is that ligand changes (either amino acids or oxygen intermediates) at the heme–copper binuclear center 'gate' the movement of protons across the membrane[21,26–28]. Site-directed mutagenesis has provided a guide in defining a structural framework for subunit I, and in identifying a set of residues that may be directly involved

The contribution of site-directed mutagenesis

Alignment of the sequences of subunit I reveals a set of residues that are either totally or very highly conserved; these residues have been the primary targets for site-directed mutagenesis. Site-directed mutants have been made at over 60 different positions in subunit I of two different bacterial oxidases: *E. coli* cytochrome bo_3 (quinol oxidase) and *Rb. sphaeroides* cytochrome aa_3 (cytochrome c oxidase)[2,4,24,29]. These mutants have been evaluated using a large array of techniques, such as steady-state and single-turnover kinetics for the separate evaluation of oxidase activity and proton pumping kinetics, and spectroscopic techniques based on the enzyme metal centers. These latter techniques include resonance Raman, Fourier transform infrared, electron spin resonance and UV/visible absorption spectroscopies, as well as other biophysical techniques. A substantial amount of the spectroscopy can be per-

facilitate the movement of the chemical protons (required to make water), which come from the opposite side of the membrane.

Interhelical IX–X connection. Several mutations suggest that residues in this region form a cap over the three metal centers[2]. Examples are Y414F which, although fully active, alters the absorption spectrum of heme a in the *Rb. sphaeroides* oxidase, and D412N, which perturbs the stretching frequency of the bond between the heme a_3 iron and the nitrogen of histidine. The importance of this region of subunit I to the integrity of the binuclear center is also indicated by the analysis of second-site revertants of yeast cytochrome c oxidase[31].

Residues likely to facilitate the movement of protons. The essential proton-conducting channel(s) leading to the binuclear center is likely to be built around a select number of polar amino acid sidechains as well as bound water molecules. Helix VIII is particularly interesting in this regard, since it is amphiphilic and contains highly conserved polar residues that fall on the same face of the helix. Nonpolar substitutions for T352, T359 and K362 each result in the severe loss of oxidase activity[29]. Furthermore, spectroscopic analysis indicates that T352 is probably at or very near the binuclear center[29]. The results are easily rationalized if the delivery of protons required to form water is impeded in these mutants. Helix VIII can be easily modelled to place these residues in positions to help convey protons from the bacterial cytoplasm to the binuclear center (Fig. 5).

By analogy with bacteriorhodopsin, acidic residues might be expected to play critical roles in facilitating proton movement across the bilayer. Bacteriorhodopsin (from *H. halobium*) is a proton-pumping protein, the structure of which is known and the mechanism of which is understood in considerable detail[32]. Its proton pump is driven by the events following the absorption of light by a retinal molecule bound in the middle of the protein. The subsequent protonation/deprotonation of aspartic acid residues is critical to facilitate the movement of protons through the proton-conducting channels above and below the retinal. However, in the respiratory oxidases, there is only one highly conserved acidic residue that can be reasonably placed within the membrane-spanning helices: E286 in helix VI. However, this residue can be substituted by its corresponding amide (E286Q) without affecting the proton pumping of the cytochrome bo_3 quinol oxidase[24].

More interesting are substitutions for a highly conserved aspartic acid in the interhelical connection between helices II and III (D135 in the *E. coli* oxidase and D132 in the *Rb. sphaeroides* oxidase, see Fig. 3). These mutations uncouple proton pumping from electron transfer. The D135N mutant in the *E. coli* oxidase, for example, has about 40% of the oxidase activity, but does not pump protons[24]. This residue might be located near the cytoplasmic opening of a proton-conducting channel specific for pumped protons, although additional experiments will be required to examine this.

Outlook

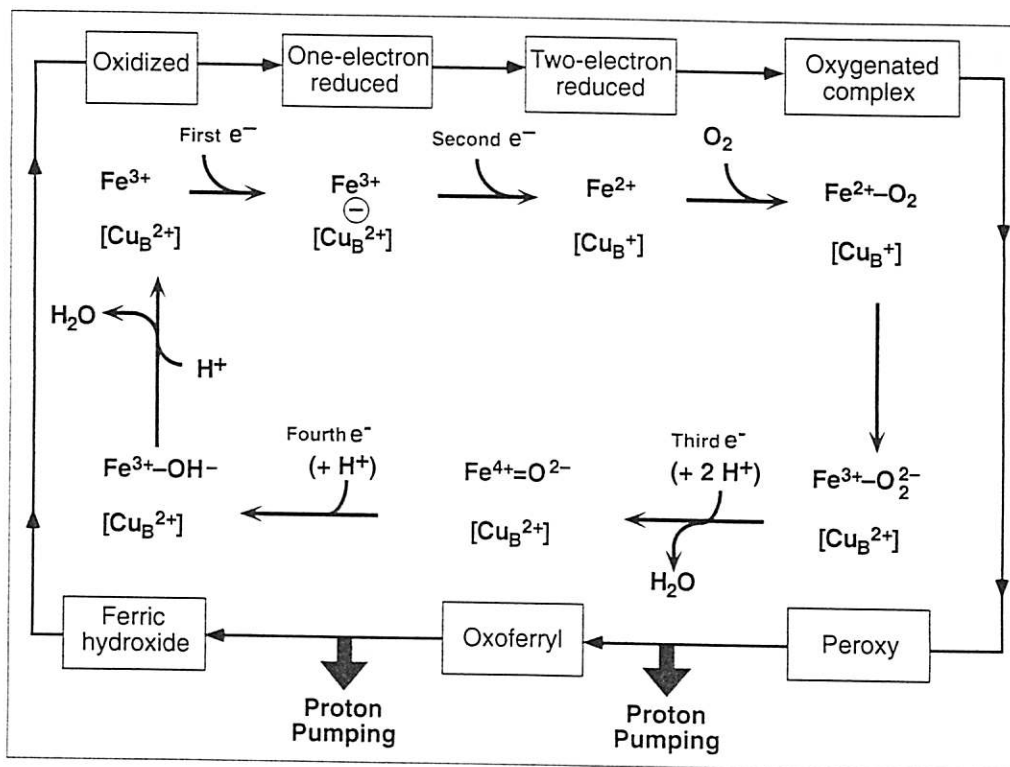Most eukaryotic and prokaryotic respiratory oxidases are members of a

Figure 4

The reaction sequence of bovine cytochrome c oxidase. The names of different intermediate species are shown in the outer boxes. Under each name is shown the redox status of the two metals of the heme–copper binuclear center along with the nature of the bound oxygen species. The sequence starts in the upper left corner with the fully oxidized form of the enzyme. Oxygen is bound only after both metals at the binuclear center are reduced, requiring two electrons. Oxygen probably first encounters Cu_B upon entering the active site (not shown), but is then rapidly transferred to the iron of heme a_3. This initial ferrous-heme-a_3-oxygenated complex has a resonance Raman spectrum similar to those of oxygenated globins, and the appearance and subsequent decay of this initial complex can be observed spectroscopically[5,6]. The bound oxygen in this complex has substantial superoxide character (O_2^-), indicating partial oxidation of the heme a_3 iron. Electron transfer from heme a, if it is reduced, generates a two-electron-reduced oxygen adduct called the peroxy intermediate. If heme a is oxidized, the electron comes from Cu_B (shown here). Addition of the third electron to the heme a_3–oxygen complex results in dioxygen-bond cleavage and the formation of one equivalent of water (or OH$^-$). The remaining oxygen atom remains in the form of an oxoferryl adduct with heme a_3. The oxoferryl species is familiar from studies of other heme-containing enzymes (such as peroxidases), and has a well-defined resonance Raman spectrum[5,7]. The transfer of the fourth electron to the binuclear center results in the generation of the second molecule of water (or OH$^-$) and regenerates the ferric (Fe^{3+}) form of heme a_3. The appearance of the ferric heme hydroxide is also observable by resonance Raman spectroscopy[5,7]. The reaction of the fully reduced form of the bovine oxidase with dioxygen is rapid, and the reaction cycle is complete in approximately 2 ms.
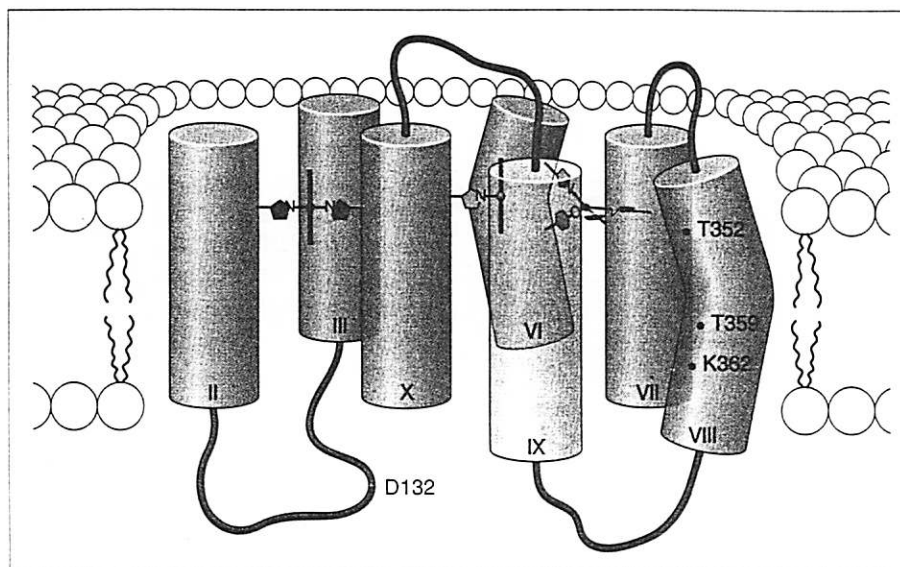
Figure 5

A model based on the results of site-directed mutagenesis experiments showing the three-dimensional arrangement of 7 of the 12 transmembrane helices of subunit I of the heme–copper oxidases[2]. Also shown are the two hemes (viewed edge-on) and Cu_B. Note that all three metals are near the outside surface and are at approximately the same level in the membrane. Helix X provides a covalent connection for rapid electron transfer between the two hemes. Helices VI, VII and VIII form a pocket around the Cu_B component of the binuclear center. Cu_B is proposed to bind to four residues: H333 and H334 in helix VII, and H284 and Y288 in helix VI. Four residues that could be important in facilitating proton movement during turnover of the oxidase are also indicated (T352, T359, K362 and D132). The numbering refers to the *Rb. sphaeroides* sequence.

superfamily of enzymes that all contain a heme–copper bimetallic site where oxygen is reduced to water. Time-resolved spectroscopic studies have identified key intermediates in the step-wise four-electron reduction of dioxygen to water. The delivery of the third and fourth electrons to the oxygen site are the steps that appear to be coupled primarily to the active transport of protons across the membrane. Site-directed mutagenesis and spectroscopic characterization have been used to identify residues that are the likely metal ligands, as well as residues that may be part of the proton-conducting channel(s) required for function. A plausible structural model that has empirical support provides a basis for the design of future experiments, as well as a solid platform for speculations about how this fascinating enzyme couples the redox chemistry to the active transport of protons across the membrane.

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Reflections

Reflections contains articles that not only consider historical events in biochemistry, but also developments in molecular biology and genetics, comparing the differences between working in the early days of molecular biology with the present. We also hope to include articles that reflect the roots of discovery of much of the molecular information and the techniques used by molecular biologists in the 1990s.

If there is a topic that you think may be a suitable contribution to the *reflections* column, please contact:

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