Structure of Cytochrome c Oxidase, Energy Generator of Aerobic Life

Robert Gennis and Shelagh Ferguson-Miller

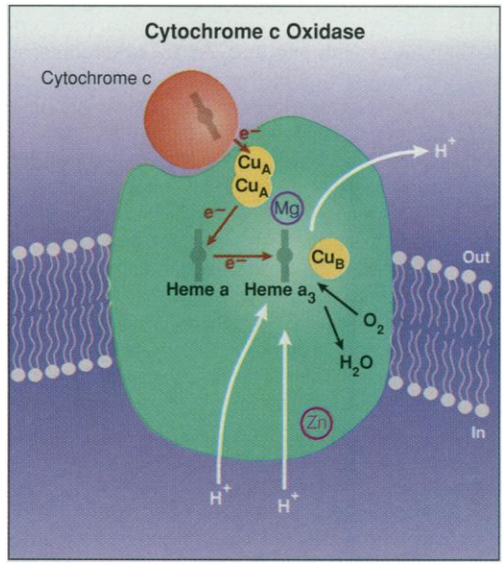
A tooth, a chicken, or a potato? Initial glimpses of cytochrome c oxidase revealed its shape to be tooth-like by low-resolution analysis of two-dimensional crystalline arrays, and later descriptions were of a more elongated molecule rather resembling a dead chicken (*1*). At atomic resolution, this key protein in the generation of cellular energy looks more like a potato. In this issue of *Science*, Yoshikawa and his colleagues now report the x-ray analysis of three-dimensional crystals of the bovine oxidase (*2*).

The achievement of Yoshikawa and his colleagues began 20 years ago, in a collaboration with W. Caughey, then at Colorado State University at Fort Collins. In Caughey's lab, during efforts to make highly concentrated cytochrome oxidase for infrared spectral analysis, crystallization was initially achieved. But that was only the beginning of a 15-year effort to obtain atomic-level resolution. The winning crystals were produced when Yoshikawa switched from a polyoxyethylene ether detergent to decyl maltoside. Since then, it has taken the Japanese group less than 1 year to solve the complete structure.

With 13 different subunits, the mammalian cytochrome c oxidase is by far the most complex membrane protein to yield a defined structure. Although analysis of all the data is not yet complete, the basic features of the protein and a detailed picture of the metal binding sites are now available. The arrangement of the metal centers is in remarkable agreement with mutagenesis studies (*3*–*5*) and with the recently reported structure of another member of the large superfamily of heme-copper oxidases, the homolgous but simpler (four-subunit) cytochrome c oxidase from the bacterium *Paracoccus denitrificans*, presented by H. Michel at the meeting of the Protein Society in Davos, Switzerland, in June 1995.

The main function of cytochrome c oxidase, the terminal enzyme in the respiratory chain, is energy conservation. Situated in the inner mitochondrial membrane in eukaryotic organisms, cytochrome c oxidase is a proton pump that uses redox chemistry to drive protons from the mitochondrial matrix across the membrane. The electrochemical potential thus generated causes protons to flow back into the matrix via the F_1F_0 adenosine triphosphate (ATP) synthase: The inward flux of protons is coupled to the synthesis of ATP from adenosine diphosphate (ADP) and inorganic phosphate (P_i). In this way, cytochrome c oxidase provides the driving energy for ATP synthesis.

Electron transfer into cytochrome oxidase is initiated by binding of cytochrome c to subunit II on the external side of the membrane (see figure). This subunit contains the Cu_A center, which is shown in the x-ray structure to be a bimetallic Cu-Cu site. Most studies on the electron transfer indicate a linear sequence of events, with the electrons proceeding from cytochrome c to Cu_A, then to heme a, and on to the heme a_3–Cu_B center (see figure) (*6*). Heme a, heme a_3, and Cu_B are all ligated to residues within subunit I of the oxidase, and the crystal structure shows they are at approximately the same depth in the membrane. This feature, also predicted from mutagenesis studies (*4*, *5*), is in accord with the finding that the electron transfer between the hemes does not per se generate a transmembrane electric potential. Oxygen binds to heme a_3 and is reduced to water through a series of short-lived, elusive intermediates, yet to be fully characterized by various time-resolved spectroscopies (*7*–*9*). The new structural information helps to define the chemistry of the environment in which these reactions occur.



How does cytochrome c oxidase couple the oxygen reduction process to the proton pump? The location of the metal centers toward the outside of the membrane dictates that the enzyme provide channels to facilitate access of protons to the heme a_3–Cu_B center to form water (one chemical proton per electron) and to be pumped across the membrane (one pumped proton per electron). Indeed, two possible channels are suggested in the structure of the bacterial oxidase.

The story of cytochrome c oxidase started to take shape in 1925 when D. Keilin at Cambridge University described a series of pigment bands in cells and tissues—visible with a hand spectroscope, sensitive to oxygen, and present in 86 different cell types, both eukaryotic and prokaryotic (*10*). He named these bands "cytochromes" (cell colors) and recognized that they were the missing clue that would resolve the long-standing controversy between O. Warburg's "Atmungsferment" and Weiland's dehydrogenase regarding the source of energy in living cells. He had discovered the electron transport chain. However, it was not until 1938 (*11*) that the bands associated with cytochrome a_3 were identified as the elusive cytochrome oxidase, Warburg's "Atmungsferment."

In the 20 years that followed, enormous progress was made in understanding the aerobic energy–generating process, but a major difficulty in analyzing the individual components arose from their membrane-embedded nature. The full significance of the membranous character of the system only became apparent when Mitchell (*12*) proposed the chemiosmotic hypothesis of energy transduction. Then the role of the electron transfer chain in generating a separation of charge across the membrane was clarified, and the use of the electrochemical gradient to drive ATP synthesis was understood. Nevertheless, the molecular mechanism of generating and utilizing the membrane potential remains to be solved.

The structure presented by Tsukihara *et al.* in this issue (*2*) focuses on the location of the metals in the enzyme. As anticipated, two hemes A, three coppers, one magnesium, and one zinc atom are resolved. The heme a and heme a_3–Cu_B centers are located 13 Å into the membrane, close to the side where cytochrome c reacts. Three histidines on two transmembrane helices ligate Cu_B, with two of the histidines being adjacent residues in the sequence. This unusual arrangement was predicted from mutagenesis studies (*4*, *5*); but a tyrosine, proposed

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as a possible alternative ligand to Cu_B (*4*, *13*), is instead packed close to one of the histidine ligands and is hydrogen-bonded to the hydroxyl of the hydroxyethylfarnesyl side chain of heme a_3. This provides an interesting and unsuspected linkage between Cu_B and heme a_3 in this critical oxygen-reducing center.

Although the arrangement of the metals within the heme a_3–Cu_B center is similar to that predicted by spectral analysis, with an iron-copper distance of 4.5 Å, there are some surprises: The Cu is displaced upward from the axis normal to the heme by about 1 Å, and no bridging ligand is resolved between these metal atoms. This latter finding conflicts with some EXAFS studies, which concluded that a chloride (or sulfur) bridged the two metals in the oxidized form of the enzyme (*14*, *15*), and with the fact that the heme a_3–Cu_B center is EPR (electron paramagnetic resonance) silent. This latter finding, as well as magnetization studies, can be reasonably explained only by magnetic coupling between the two metals, and at a separation of 4.5 Å, this would require some sort of bridge (*16*). Perhaps water or another ligand (or both) are present in a disordered state. Cytochrome oxidase adopts several spectroscopically distinct forms in solution, making conformational heterogeneity a major problem (*17*). Thus, full characterization of the spectral properies of the crystal form of the enzyme will be critical to resolving this issue.

The ligation of the two heme components, heme a and heme a_3, was also correctly predicted from mutagenesis and spectroscopic studies (*4*, *5*). Heme a is bound by two histidines that are located in two different transmembrane spans (helices II and X) in subunit I. Heme a_3 is also ligated by a histidine in helix X, putting the two hemes on the same helix separated by a single residue. As predicted, the heme planes are perpendicular to the plane of the membrane, but what could not be predicted is the angle between the hemes (104 degrees), which is closer to perpendicular than parallel. This brings the heme edges to within about 5 Å at the top corners, suggesting a possible conduit for rapid electron transfer between the two hemes. Whether the electron transfer is through bonds or through space between metal centers or between the heme edges will certainly be a topic for the future. In any event, the structure does suggest that the heme a–heme a_3–Cu_B unit forms a functional "trinuclear" center. Time-resolved kinetic studies have already demonstrated that electron transfer from heme a to heme a_3 in some circumstances can occur more rapidly than transfer from Cu_B to heme a_3 (*7*, *8*).

The structure of the Cu_A center has been hotly contested over the past few years, but has now been clearly resolved. The amino acid ligands within subunit II were correctly predicted on the basis of homology to type-1 "blue" copper proteins and, subsequently, by mutagenesis studies (*18*). However, unlike the single-Cu blue copper proteins, the Cu_A locus contains two Cu atoms, which are 2.7 Å apart, perhaps close to the distance of a Cu–Cu bond, recently predicted from EXAFS studies (*19*), but bridged by two cysteine residues. A similar structure has also been observed in the bacterial oxidase and in the recently reported structure of the reconstructed Cu_A-containing soluble domain of the homologous cytochrome bo_3 from *Escherichia coli* (*20*).

The Cu_A locus is near the interface of subunits I and II and is almost equidistant from the two heme irons. Hence, the preferred pathway for electron transfer from Cu_A may not be determined by distance alone.

It was a relatively recent observation that mammalian cytochrome c oxidase contains not only copper and iron, but also magnesium and zinc (*21*). The initial observation was borne out by analysis of numerous oxidases, showing that eukaryotic enzymes have Mg and Zn in stoichiometric amounts, but bacterial cytochrome c oxidases have only Mg (*22*). However, little evidence is available as to the structural or functional significance of these additional, non–redox active metals. Several studies had placed Zn in one of the nuclear-encoded peptides in a cysteine-rich environment (*23*, *24*), and its resemblance to a zinc finger motif has been postulated (*25*). The x-ray structure reveals that these conclusions were correct in many respects, but further, that the site is at a distance from the active site on the matrix (inner) side of the membrane.

On the other hand, a central location of the Mg ion was predicted from analysis of site-directed mutants of a bacterial enzyme, in which Mn substitution gave it visibility (*26*, *27*). This finding is confirmed and emphasized by the crystal structure. At the interface of subunits I and II (with ligands from both), directly between Cu_A and heme a_3, the Mg center is clearly where the action is. A variety of structural and functional roles can be envisaged, including the strengthening of the subunit-subunit interaction, the control of direction of electron transfer, or even a direct facilitating role in proton transfer.

It is a stroke of luck that two crystal structures of cytochrome c oxidase have been reported almost simultaneously. A number of different conformers of oxidase are surmised to exist from spectral and kinetic studies; thus, if only one structure of one form of the enzyme were available, it would be difficult to discern what features are of fundamental importance. Given the structures of the two enzymes in two different states, the common features become highly significant. Indeed, with the wealth of structural information now available, not only for cytochrome c oxidase but also for the ATP synthase (28), the field of bioenergetics has become a gold mine of knowledge relevant to the structure and function of complex, multisubunit, membrane-associated proteins, which may well move many fields ahead.

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