ern human society these brain actions may often be maladaptive; now we need prefrontal cortex regulation to act appropriately. These neurochemical changes may explain why the stress of an initial error can cause an athlete to lose concentration and thus lose a competition, or why children in stressful home environments (for example, undergoing divorce) can exhibit behaviors resembling attention deficit hyperactivity disorder, a disorder of prefrontal cortex function. Further research on these important neurochemical mechanisms may help us to elucidate why prefrontal cortical deficits are so prominent in many mental illnesses that are exacerbated by stress (*15*) (affective disorder, schizophrenia) and to develop better treatments for these devastating disorders. And finally, this understanding may allow us to be more compassionate with our own failings in response to life's stressors.

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PROTEIN STRUCTURE

Cytochrome c Oxidase: One Enzyme, Two Mechanisms?

Robert B. Gennis

The cytochrome c oxidases, along with other members of the superfamily of "heme-copper oxidases" (*1*, *2*), are responsible for nearly all aerobic respiration on Earth. These critical enzymes recently provided the occasion for a wonderful—and unexpected—achievement in membrane structural biology. In a single week in the summer of 1995, two independent x-ray structures were reported in *Science* and *Nature*: the 13-subunit enzyme from bovine heart mitochondria (*3*), and the four-subunit enzyme from the soil bacterium *Paracoccus denitrificans* (*4*). Now on page 1723 (*5*) of this issue, Yoshikawa *et al.* report the refinement of the structure of fully oxidized bovine cytochrome c oxidase to 2.30 Å plus the structural changes that occur upon full reduction of the enzyme's metal atoms and upon the binding of ligands, azide, and CO. This major achievement yields a much closer look at this energy-transducing membrane protein and reveals interesting differences between the mammalian and bacterial oxidases that prompt the authors to propose a radically different mechanism of proton pumping for the bovine enzyme.

Cytochrome c oxidase reduces dioxygen (O_2) to water in a way that conserves the considerable free energy made available from this highly favorable reaction (*6*, *7*). This free energy, once harnessed, is then used for a wide variety of energy-requiring biological and biochemical functions, notably ATP synthesis. The oxidase accomplishes this by coupling the redox chemistry (dioxygen reduction to water) to proton pumping, thereby generating transmembrane voltage and proton gradients that supply the proton motive force. For every turnover of the enyzme (dioxygen reduction), eight protons are taken from the inside aqueous compartment, four protons being used to make two molecules of water, and the remaining four protons are pumped to the opposite side of the membrane, about 50 Å away. Understanding how oxidase functions is largely a matter of defining how, when, and where protons move during the catalytic cycle. But knowing the protein architecture of a static structure or set of structures may not be sufficient to deduce mechanism and dynamics.

Cytochrome c oxidase must clearly have more than one extended proton-conducting channel or pathway, but what do such things look like? Net translocation of protons can occur over a long distance through a protein by hopping between pairs of hydrogen-bonded donor and acceptor residues (*8*–*10*). A string of such residues connected by hydrogen bonds can be thought of as a "proton wire" (*9*). Not all hydrogen bond networks can function as a proton wire, because a certain rotational mobility of the component parts of the wire is required. Water is an excellent component of a proton wire (*9*, *10*), but if the internal water molecules are disordered or mobile, or if the x-ray diffraction data are limiting, then these functionally important components of the proton wire will not be apparent. Furthermore, there is no need to have a stable, long-lived continuous hydrogen-bonded chain as a proton wire. Such structures could well be transient, not observed in the static structure, and still carry out their function. In short, finding extended proton-conducting pathways from the x-ray structures is neither sufficient nor necessary to define a "proton wire." Needless to say, this adds some uncertainty to the interpretation of structural data.

Generally, the structures of the bacterial and mammalian oxidases have proven remarkably similar, including significant structural details that have emerged since the original reports (*5*, *11*, *12*). But in their new work, Yoshikawa *et al.* report several differences in their structure of the bovine enzyme. Briefly,

1) The refined structure of the fully oxidized bovine oxidase shows a peroxide molecule at the heme-copper center. This surprising finding will certainly provoke further experiments to demonstrate chemically that the so-called "resting" oxidized form of the enzyme (*13*, *14*) is, in fact, a peroxide adduct. Until spectroscopic and biochemical studies on the crystals can better define which of the several "resting" forms is present, there is no way to know how to relate the unexpected peroxide-containing structure to previous studies of the enzyme in solution. How important this will ultimately be is not clear, because even the authors do not believe that this species is involved in the catalytic cycle.

2) The azide complex of the oxidized bovine enzyme has the same ligation of Cu_B as does the enzyme in the absence of azide. This is in contrast to the "missing" or disordered histidine ligand that was reported in the original structure of the azide adduct of the bacterial oxidase (*4*). The importance of this observation has nothing to do with azide, but rather the implication that the ligands to Cu_B might labile and vary in different states of the enzyme. It has been proposed (*4*, *15*)

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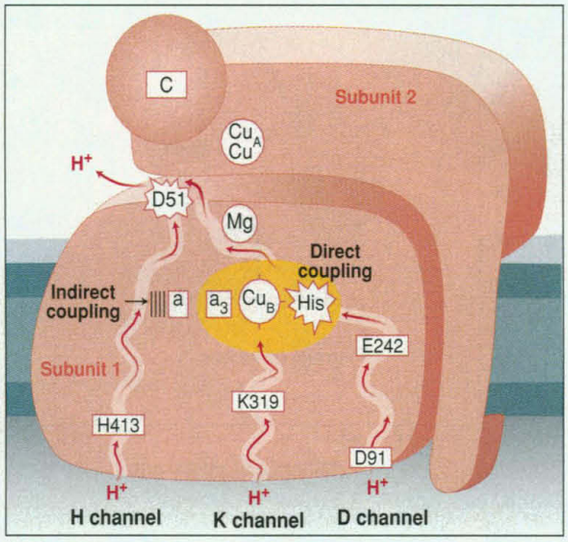
that one of the Cu_B ligands might cycle on and off the metal during enzyme turnover, thus providing a mechanism to shuttle protons across the membrane. Thus, any evidence of changes of the Cu_B ligation is comforting to those favoring such a model for proton pumping.

3) Upon full reduction of the metals, a conformational change is observed in the vicinity of D51, located at the interface between subunits I and II in the bovine oxidase. This observation has been extrapolated by the authors into a model of proton pumping. In the fully oxidized enzyme, D51 is postulated to be protonated and in protonic equilibrium with the aqueous phase on the opposite side of the membrane (the matrix), about 50 Å away, via a "proton wire" (5). The conformational change that is observed upon reduction of the metals results in changing the hydrogen bond pattern of D51 so that it no longer has access to the proton wire leading to the matrix side of the membrane, and D51 can lose a proton on the cytosolic side of the membrane. This shifting accessibility of D51 is proposed to be a central feature of the proton pump in the bovine oxidase. However, the fully reduced enzyme is not found during normal steady-state turnover (6, 7), so there is no guarantee that changes accompanying full reduction are relevant to the proton-pumping mechanism.



Pumping protons. Three proposed proton-conducting channels in subunit I of the cytochrome c oxidase. An indirect coupling mechanism via residue D51 and the H channel has been proposed for the bovine oxidase, whereas a direct coupling mechanism via a histidine ligand to Cu_B and the D channel is proposed for the bacterial oxidases.

There are other problems with this hypothesis. As the authors point out, D51 is not a conserved residue among the cytochrome c oxidases. Generally, this residue has no equivalent in the oxidases from plants, lower eukaryotes (such as yeast), or bacteria (including *P. denitrificans*). Hence, this specific gating mechanism for a proton channel must be restricted to a limited set of oxidases from animals. Not surprisingly, the putative proton-conducting pathway is largely inferred rather than observed. The pathway includes a cavity that is large enough to contain several disordered water molecules as well as a long, narrow canal where water must be postulated to exist transiently during protein fluctuations. The proposed pathway for proton conduction also requires a tautomerization of a peptide bond, the kinetics of which is certainly debatable. The question of how energy is provided to move protons against a gradient through this pathway (coupling) is not specifically addressed; but this pathway is distant from the heme-copper center, and thus the coupling must be allosteric and not directly involve the chemical events at the active site. This is a key element in the new proposal because it cleanly separates the "pumped" protons from the "chemical" protons, which are delivered to the active site through a totally separate channel. This proposal is certainly thought provoking. Unfortunately, the most convenient systems to test the model are the bacterial oxidases (*1*), which are presumed not to use this proton-conducting "pumping" pathway.

In the case of the bacterial oxidase, a model of proton pumping has been extrapolated from the observation that one of the histidine ligands to Cu_B in the heme-copper center is not observed in the azide adduct of the fully oxidized enzyme (*4*). This, at least, suggests lability of the Cu_B ligands and lends weight to the concept that the mechanism by which the chemistry at the active site is coupled to the proton pump is by one of the Cu_B ligands cycling on and off the metal during the catalytic cycle ("histidine cycle" models) (*4*, *15*). This kind of model requires that the pathway for pumped protons passes through the heme-copper center. The structure proposed to fill this role is called the D channel, which runs from an aspartate (D91 in bovine oxidase; D124 in *P. denitrificans*) near the surface of the protein on the matrix side (bacterial cytoplasm) to a glutamic acid buried within the enzyme (E242 in bovine oxidase; E278 in *P. denitrificans*), a distance of about 25 Å. Yoshikawa *et al.* (*5*, *12*) see this channel ending at the glutamic acid, essentially a dead end with no channel function. However, site-directed mutagenesis studies clearly support an important role for residues within the D channel in several of the bacterial oxidases (*16*–*21*). Elimination of the carboxyl moiety at either position in the D channel (equivalent to D124 and E278 in *P. denitrificans*) substantially reduces turnover of the enzyme, and when residual activity is measurable, there is no proton pumping. Although not seen in the structures, it has been proposed that three or four internal water molecules could complete the proton wire (10 Å) to the heme-copper center (*22*). If the D channel functions this way, then the sorting of "pumped" and "chemical" protons becomes important to address. Although less likely, the mutagenesis results could indicate that the acidic residues in the "D channel" are required for an allosteric coupling mechanism to a proton-conducting channel not yet located in the bacterial oxidases.

In summary, we have two remarkably similar structures in the mammalian and bacterial cytochrome c oxidases, and no indication from biochemical or biophysical studies that they operate by different mechanisms. The interpretation of the new structures by Yoshikawa *et al.* (*5*) that the animal oxidases have a unique proton-pumping mechanism or proton-conducting pathway may or may not hold up to closer scrutiny. Nevertheless, the model will certainly generate experimental efforts that will move the field forward.

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