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Oriented X-ray Absorption Spectroscopy of Membrane bound Metalloproteins

Graham N. George[1], Roger C. Prince[1], Terrence G. Frey[2] & Stephen P. Cramer[3]

1. Exxon Research & Engineering Co., Annandale, NJ 08801
2. Biology Dept., San Diego State University, San Diego, CA 92182
3. Schlumberger-Doll Research, Ridgefield CT 06877

Many important enzymes are associated with lipid bilayer membranes. Such membranes, complete with their integral proteins, may be aligned by partial dehydration. These samples possess order along the membrane normal, and are eminently suitable for polarization spectroscopy. We have used polarized X-ray absorption spectroscopy to study the local structure and geometry of the metal sites of the two major actors in the biological oxygen cycle; the chloroplast water-splitting enzyme and mitochondrial cytochrome oxidase.

For oriented systems, the angular variation of the EXAFS amplitude is to a first approximation $\cos^2\beta$, where β is the angle between the X-ray e-vector and the absorber-backscatterer (a-b) vector. Approximating the effects of sample disorder by a Gaussian distribution in orientations, the angular dependance of the EXAFS amplitude, $F_{ab}(\theta)$, is :

$$F_{ab}(\theta) \propto 3 \frac{\int_{\phi=0}^{\pi} \left(\frac{1}{2} \sin^2\theta\sin^2\phi + \cos^2\theta\cos^2\phi \right) \sin\phi \; e^{-(\log_e 2)(\phi-\phi_{ab})^2/\Omega^2} \, d\phi}{\int_{\phi=0}^{\pi} \sin\phi \; e^{-(\log_e 2)(\phi-\phi_{ab})^2/\Omega^2} \, d\phi} \qquad [1]$$

where θ is the experimentally varied angle between the membrane normal and the X-ray e-vector, ϕ_{ab} is the angle between the a-b vector and the membrane normal (Fig. 1A), and Ω is the half-width of the Gaussian distribution of ϕ_{ab}. Ω was estimated from computer simulations of EPR spectra of paramagnetic sites within the membrane (eg. [1]) as 15°-20°. Fig. 1B shows a series of amplitudes calculated from equation 1, for Ω=17°, with various values of ϕ_{ab}. Note that equation 1 also describes the angular variation in intensity of dipole-allowed bound state transitions, in which case ϕ_{ab} is the angle between the membrane normal and the transition dipole operator. EXAFS data were quantitatively analyzed by fitting the data measured at various values of θ (using model compound total amplitude and phase-shift functions) and fitting the angular variations in amplitude thus obtained to equation 1. For sites with more than one ligand of a given type at similar distances, it is generally not possible to obtain the values of the individual ϕ_{ab}'s. Nevertheless an average value $\langle\phi\rangle$ can be obtained, defined by equation [2], in which n_b is the number of indistinguishable a-b interactions, and the summation is over b.

$$\cos\langle\phi\rangle = \left[\frac{\sum n_b \cos^2\phi_{ab}}{\sum n_b} \right]^{1/2} \qquad [2]$$

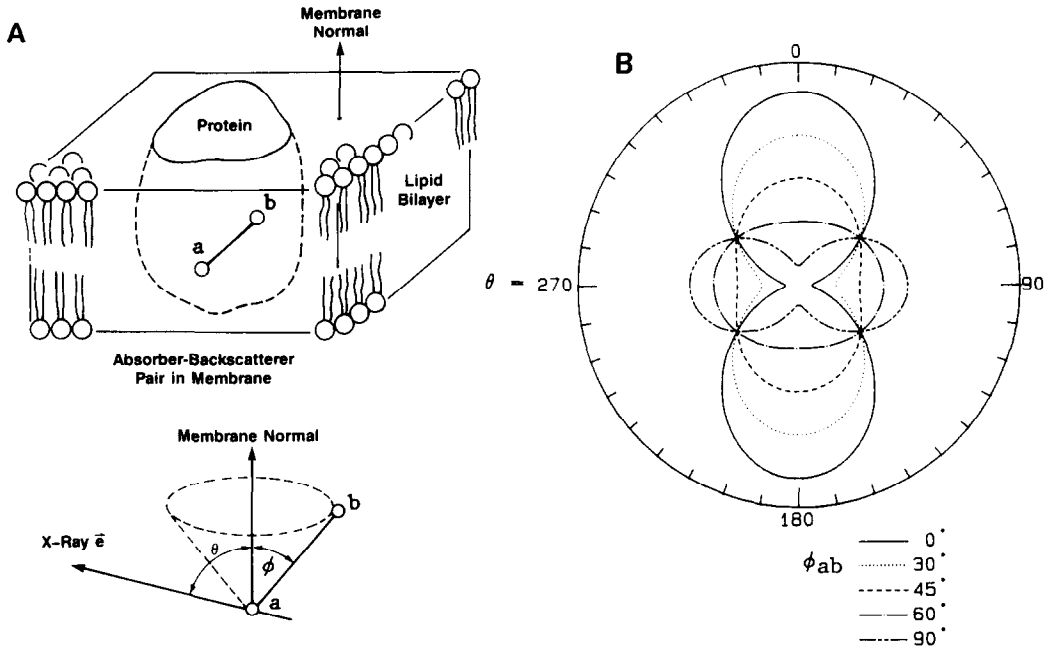

Figure 1A. **Coordinate system for oriented membranes.** *B.* **Polar plot of calculated EXAFS amplitudes (equation 1), for Ω = 17°.**

Cytochrome oxidase is the terminal electron carrier in mitochondrial respiration, reducing oxygen to water in a four electron process. The enzyme contains two different coppers, Cu_A and Cu_B, two heme *a* groups, *a* and *a3*, and a single zinc. Heme *a3* and Cu_B form a magnetically coupled binuclear site thought to be the site of oxygen reduction, while heme *a* and Cu_A are more electronically independent; the role of the zinc is unclear. We have examined the angular dependence of the iron, copper and zinc X-ray absorption spectra of membranous cytochrome oxidase [2]. Curve fitting analysis of the copper EXAFS data indicates the presence of two readily discernible types of Cu-S (or Cu-Cl) interactions, at 2.3Å and 2.6Å, plus Cu-N (or Cu-O) interactions at 1.97Å. Analysis of their orientation dependence (Fig. 2A) indicates that there is ~1 2.6Å Cu-S bond per two coppers, oriented along the membrane normal ($<\phi>=0°$), 2-3 2.3Å Cu-S with $<\phi>=46°$, and 1-2 1.97Å Cu-N with $<\phi>= 36°$. Two alternative interpretations are that the long Cu-S is a ligand of Cu_A, or that it is the bridging ligand between Cu_A and heme *a3*. While the latter interpretation is consistent with Fe-S interactions in the iron EXAFS [2], and is qualitatively similar to the conclusions of other workers [3], the former is consistent with the EPR and MCD properties of the enzyme.

The other enzyme system which we have studied using these methods is the photosynthetic water-splitting enzyme of green plants. This enzyme, as the originator of the oxygen in our atmosphere, is arguably the most important enzyme in the world (at least for aerobic organisms). The active site of water oxidation is thought to be a multinuclear manganese cluster, and we have examined the Mn EXAFS of the enzyme in the dark adapted S_1 state [4]. Curve fitting analysis indicates a nearly isotropic oxygen or nitrogen coordination, with an average Mn-O,N distance of 1.9Å, presumably with contributions from bridging ligands plus bonds from the protein. We detect no very short (1.75Å) Mn-O interactions [5]. Two different Mn--Mn interactions can be discerned;

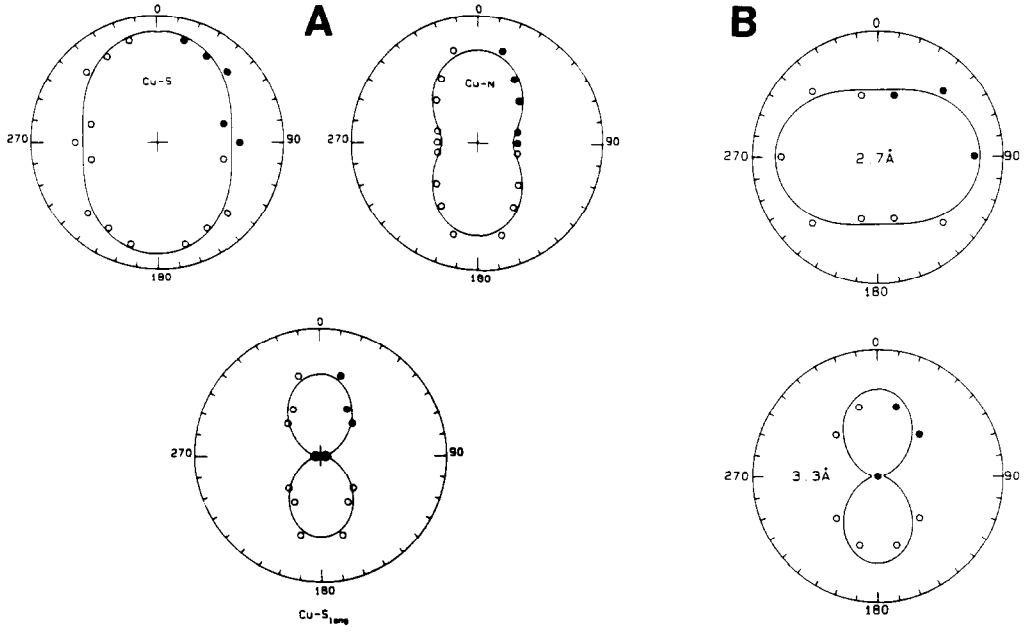

Figure 2. **Polar plots of EXAFS amplitudes of *A* Copper in cytochrome oxidase, and *B*. Manganese in the water-splitting enzyme.** Experimental points are shown as solid symbols, which have been mirrored for clarity as open symbols. The lines are the best fit to equation 1 with the parameters discussed in the text.

2.1±1.0 at 2.7Å with $\langle\phi\rangle$=62°, and 0.8±0.3 at 3.3Å with $\langle\phi\rangle$=0° (Fig. 2B). Such interactions probably originate from bridged atoms, since EXAFS from unconnected atoms should be damped by thermal and static disorder. The 2.7Å Mn--Mn interaction is symptomatic of more than one single atom bridge (eg. μ_2 or μ_3 oxo or hydroxo groups) between the Mn concerned, while the 3.3Å distance is suggestive of carboxylate bridges. The presence and stoichiometry of the 2 Mn--Mn distances indicates that the Mn cluster contains >2, and most probably 4, Mn atoms.

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