The Orientation of CO in Carbonmonoxy Cytochrome Oxidase and Its Transient Photoproducts. Direct Evidence from Time-Resolved Infrared Linear Dichroism

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Cytochrome oxidase (cytochrome aa_3 or CcO) mediates the facile reduction of O_2 by cytochrome *c* in all plants and animals and numerous lower organisms and conserves the energy of this reaction as a transmembrane proton gradient.[1] The crucial early steps in the activation of O_2 involve its binding to the heme/copper site, denoted "cytochrome a_3/copper B".[2-4] The coordination chemistry and structure of this site are essential, but poorly understood, functional features of the enzyme. Infrared study of CcO–CO and other ligated forms of the enzyme have revealed significant information regarding the active site and its surroundings.[5-7] Quite recently, we reported the first application of time-resolved infrared (TRIR) spectroscopy to the study of CcO–CO photodynamics.[8] We demonstrated clearly that photodissociated CO binds to Cu_B^+ as a microsecond transient at ambient temperature. In this report we have applied time-resolved infrared linear dichroism (TRIRLID) to the CO complex of fully reduced CcO from bovine heart and have measured directly the orientation of the C–O bond axis with respect to the heme normal of cytochrome a_3, both in Fe_{a3}^{2+}–CO and in the Cu_B^+–CO phototransient. This approach shows great promise in the characterization of specific structural features of ligand binding and protein dynamics in this complex enzyme and other systems.

The $\pi-\pi^*$ electronic transitions of hemes are polarized in the plane of the macrocycle.[9] Thus, if polarized light is employed to photodissociate heme–CO, the dissociated molecules will be a photoselected population having heme planes preferentially oriented parallel to the electric vector of the photodissociating light. This photoselection results in induced linear dichroism effects, which persist until the orientation is randomized by rotational diffusion. In particular, absorption of polarized infrared light at the C–O stretching frequency will be bleached preferentially if the infrared **E** vector is perpendicular to that of the photodissociating light. The polarization ratio $R(t) = (\Delta A_\perp)/(\Delta A_\parallel)$ (where the subscripts refer to infrared polarization perpendicular (θ = 90°) and parallel (θ = 0°) to that of the photodissociation pulse) at $t = 0$ is related to the angle α between the normal to the heme plane and the C–O bond axis by expressions that have been developed in detail.[10,11] This approach has recently been used by Frauenfelder and co-workers to determine heme–CO angles in static experiments on MbCO in frozen glasses[11] and by Hochstrasser and co-workers in picosecond time-resolved experiments on HbCO, MbCO, and iron porphyrin carbonyls in fluid solution.[10,12,13] The present study is unique in the application of TRIRLID under ambient conditions to a redox rather than a ligand transport protein and in our ability to determine the orientation after photodissociation of CO bound to a non-heme metal (viz., Cu_B^+).

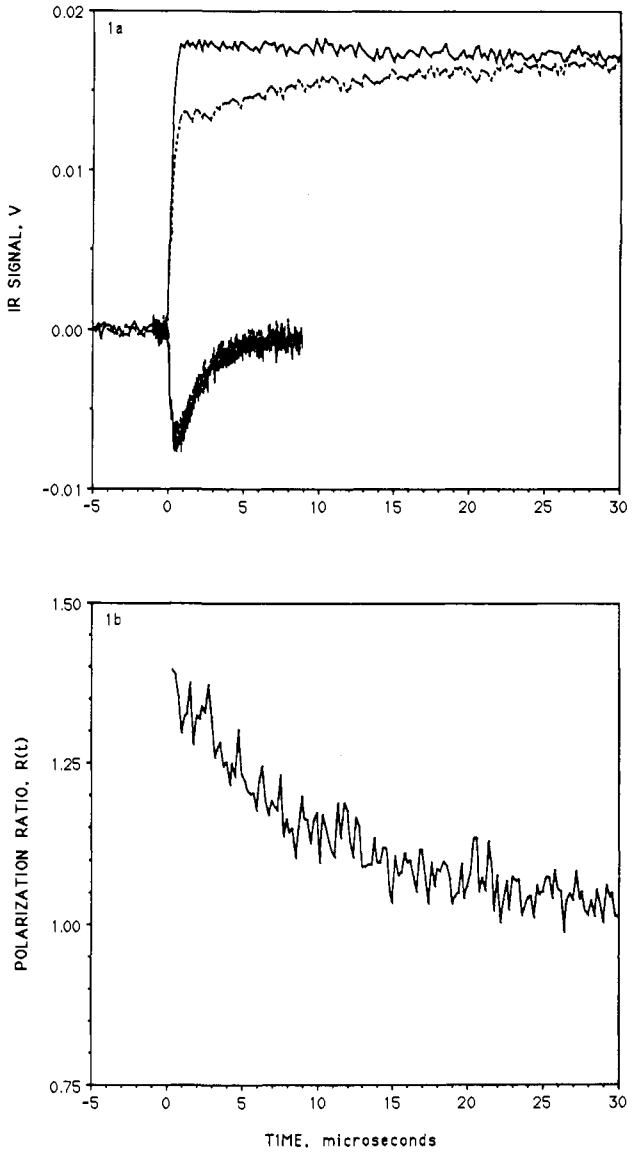

Figure 1. (a) Time-resolved infrared linear dichroism signals (raw detector signal; positive displacement is bleaching) of carbonmonoxy cytochrome oxidase between −5 and 30 μs with respect to the time of the photodissociation pulse. The upper and lower pairs of traces are for Fe–CO and Cu–CO, respectively. The polarization of the infrared probe light with respect to that of the photodissociation pulse is indicated on the figure; the solid traces indicate parallel polarization, and the dashed traces perpendicular. The fraction of photodissociation is 30%, and the amplitude of the Cu–Cd transient is exaggerated by a fraction of 5. See text for additional details. (b) The time-dependent infrared polarization ratio $R(t) = (\Delta A_\perp)/(\Delta A_\parallel)$, calculated from the Fe–CO data in part a.

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Bovine heart cytochrome oxidase was isolated and the CO complex prepared by procedures reported elsewhere.[8,15] The apparatus used was similar to the one described previously for our TRIR experiments.[8] It is based upon photodissociation by laser pulses at 532 nm (7-ns duration, 10-Hz repetition rate) and infrared probing with a CW tunable diode laser. CaF_2 was substituted for sapphire optics, and a half-wave plate in the YAG beam allowed polarization vector orientation. Infrared transient signals (typically 2000 averaged transients) were recorded as a function of YAG vs IR E-vector orientation and also as a function of photodissociation fraction (f) between 10% and 67%. Values of α were determined from the equation $R = [4 - \langle\sin^2\alpha\rangle]/[2 + 2\langle\sin^2\alpha\rangle]$,[12] with a value of $R(t=0)$ obtained by linear extrapolation to $f = 0$. The preceding equation assumes x,y degeneracy of the heme absorbance at 532 nm.

Figure 1a shows typical results of the TRIRLID experiments for heme-CO (1963 cm^{-1}) and Cu-CO (2061 cm^{-1}). The heme-CO data show the transient over the first 30 μs after photodissociation, during which period the recombination of CO with the heme is negligible.[15] The results for Cu_B^+-CO are similar except that the Cu-CO transmittance transient, which is formed in less than 200 ns,[8] is negative rather than positive and persists only for a few microseconds, the lifetime of the Cu_B^+-CO complex.[8] Transient linear dichroism is clearly evident in the Fe-CO results; the short-time bleaching at $\theta = 90°$ is substantially greater than at $\theta = 0°$. The dichroism disappears with time as a consequence of rotational diffusion of the protein, with a half-life of approximately 6 μs under these conditions (T = 300 K, 1 mM CcO, 50% glycerol solution).

Figure 1b shows the dependence of the Fe-CO infrared polarization ratio upon time at $f = 30\%$. These data were measured at various values of f and extrapolated to $f = 0$, yielding $R = 1.71$ for heme-bound CO, whence $\alpha = 21°$ (±2°). For Cu_B^+-CO, $R = 1.07$ and $\alpha = 51°$ (±3°). These conclusions are model-dependent. For heme-CO, a discrete value of α is assumed rather than a distribution of angles.[10] This should be a good assumption for CcO in particular, because its CO IR peak is more narrow than that of any heme-CO protein examined to date (the C-O stretching frequency is sensitive to α; therefore a distribution in α leads to inhomogeneous broadening of the CO IR peak: see, for example, ref 5 and 12). The same assumption is made for Cu_B^+-CO with the same justification; the narrow line width of the copper-bound C-O stretch is good evidence that CO is not randomly oriented, despite almost isotropic linear dichroism, but instead is oriented at nearly the "magic angle" to the heme normal. In addition, it is assumed that the C-O axis of Cu-CO is approximately coplanar with the heme normal.

A number of workers have noted an empirical correlation between the frequency of the C-O stretch in CO-hemes and constraints that force the ligand to be oriented off the normal to the heme plane.[16-18] These constraints may "bend" the Fe-C-O angle away from 180°, or "tilt" the Fe-C-O structure as a unit away from the heme normal, or both. A high CO frequency has been taken as an indication that the CO is more nearly upright. In view of this, it has often been presumed that the high C-O frequency in CcO suggests that the ligand is nearly perpendicular to the heme plane. Our measurements indicate that CcO-CO (ν_{CO} = 1963 cm^{-1}) has $\alpha = 21°$, almost the same as the 1944-cm^{-1} MbCO conformer (20° at 300 K) and HbCO (18°, 1951 cm^{-1}).[12] The value of α is the sum of the bend and tilt angles, assuming that the two distortions are in the same plane. Rousseau and co-workers[20] estimated the bend angle in CcO-CO to be 175° (±5°), using equations that have been developed[17,19] for calculating this quantity from vibrational frequencies. They suggest from other arguments that CO is significantly tilted as well. We can quantitatively test conclusions, given our present work and related results on other proteins. The bend angle of 175° requires a 16° tilt of the Fe-C axis to achieve $\alpha = 21°$. Values of the Fe-C tilt between 10° and 14° have been estimated for HbCO and MbCO;[12] thus our value of α and the calculated bend angle[20] are plausibly consistent. The bend angle calculations from vibrational data embody assumptions that limit their general validity and therefore should be viewed with caution.[21] Nevertheless, it seems clear that the simple correlation of ν_{CO} with either bend angle or α is not valid. MbCO and CcO-CO apparently have very similar values of bend angle (174°, 175°) and α (20°, 21°), yet their ν_{CO}'s differ by 20 cm^{-1}.

The structural interpretation of the α value for Cu_B^+-CO is not as clear as for the heme because the 51° C-O vector of Cu-CO may point either toward or away from the heme plane, and also toward or away from the heme normal. Cu_B is thought to be 5 Å or less from the heme a_3 iron in CcO,[22] but no evidence exists as to its location with respect to the heme normal. Finally, we note that both the heme and Cu_B α values are cone half-angles, and we have no evidence as to the angular orientations of the (Fe)C-O and (Cu)C-O vectors on their respective cone surfaces. Experiments designed to clarify these structural issues are in progress.

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Ultrafast Studies of Transition-Metal Carbonyl Reactions in the Condensed Phase: Solvation of Coordinatively Unsaturated Pentacarbonyls

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Recently ultrafast studies on the photodissociation dynamics of transition-metal carbonyls have drawn considerable attention.[1] In the gas phase, electronically excited $Cr(CO)_6$ molecules lose CO, depending on the initial energy content. In liquids, on the other hand, only $Cr(CO)_6 \rightarrow Cr(CO)_5 + CO$ occurs due to fast vibrational relaxation.[2] A solvent molecule (S) is then expected to enter the vacant metal site of $Cr(CO)_5$ to form a complex. The absorption spectrum of $Cr(CO)_5S$ critically depends on the solvent property as its maximum is known to reflect the bond strength between the metal and the solvent.[3] While the absorption maxima

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