Determination of CO Orientation in Myoglobin by Single-Crystal Infrared Linear Dichroism

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One mechanism by which heme proteins control active site reactivity is through interaction of distal pocket residues with exogenous ligands.[2] In myoglobin (Mb), it is believed that energetically unfavorable steric interactions with distal pocket residues[3] reduce the binding affinity of CO, while bound O_2 is stabilized by a hydrogen bond with the distal histidine.[4] The distal pocket interactions in MbCO have particular interest, due to the existence of conformational substates that are functionally distinct[5,6] and can be identified by vibrational frequencies of the Fe–C–O group.[7,8] The relative populations of these substates can be controlled by experimental conditions including temperature, pressure, pH, and hydration.[5,7,9] Spectroscopic[10] and crystallographic[11a] evidence indicates that His64, which interacts with the bound ligand in the "closed" distal pocket states (A_1 and A_3), is displaced from the heme pocket toward solvent in the "open" pocket state (A_0).

The orientation of the bound CO in Mb remains an unresolved issue, despite several crystallographic studies.[11,12] According to earlier structural models,[12] bending of the Fe–C–O unit displaces the C–O bond by an angle θ = 40–60° from the heme normal, but a recent structure[11b] shows the Fe–C–O moiety deviating from linearity by only 13° in MbCO. Photoselection measurements[13] based on the IR dichroism of samples partially photolyzed with polarized visible light lead to substate-specific values for θ that range from 15° (for A_0) to 33° (for A_3). Here, we present the results of polarized IR measurements on single crystals of MbCO in order to characterize the CO orientation in the major conformational substates A_0, A_1, and A_3. We find that projected CO dipole moments for the A_0, A_1, and A_3 states of the protein lie within a few degrees of each other, suggesting that variations of the heme geometry among these states are much smaller than previously claimed.[13]

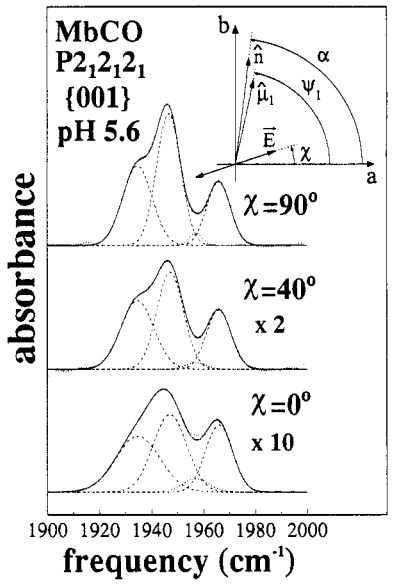

Figure 1. Polarized IR spectra recorded on transmission through the {001} face of orthorhombic ($P2_12_12_1$) MbCO crystals. The inset coordinate system shows the angle χ that the electric field $\vec{E}$ of the IR beam makes with the *a*-axis in the {001} plane. The CO stretch transition dipole $\hat{\mu}_1$ and the heme normal $\hat{n}$ do not lie in the {001} plane, and the angles ψ_1 and α, respectively, describe the orientations of their *projections* onto this plane. Crystals were immersed in 4 M phosphate solution between two CaF_2 windows separated by a 50-μm Teflon spacer on the sample stage of a Digilab UMA-500 IR microscope coupled to the output port of a Digilab FTS-60A FTIR spectrometer. A polynomial base line was subtracted from the absorption spectra, which were calculated as the ratio of transmission through the crystal to transmission through a nearby region of the salt solution.

Bands located at 1966, 1946, and 1934 cm^{-1} in IR spectra of MbCO crystals grown according to standard procedures[14] correspond to the CO stretching frequencies of the conformational substates A_0, A_1, and A_3 assigned in solution. When a polarizer is placed in the optical path, the absorbance of all three bands varies systematically with the polarizer orientation, as shown for orthorhombic ($P2_12_12_1$) crystals in Figure 1. By symmetry, the CO transition dipole of each protein in the unit cell contributes equally to the absorbances $\mathcal{A}_a$ and $\mathcal{A}_b$ measured parallel to the crystallographic *a*- and *b*-axes, and the expression $\tan^2\psi_i = (\mathcal{A}_b/\mathcal{A}_a)_i$ partially specifies the angle ψ_i that the dipole for the substate A_i (i = 0, 1, 3) makes with the crystallographic *a*-axis after projection onto the *ab*-plane. Values of the projected angles ψ_i calculated from this expression lie within a 4° range (Table 1).[15]

Such a result is unexpected in view of photoselection measurements[13b] at T = 10 K, which predict that the CO orientations in the A_0 and A_3 states differ by at least 18°. In principle, this could occur due to a fortuitous orientation of the protein in the orthorhombic crystal lattice that allows the CO bonds of A_0, A_1, and A_3 to lie in a plane orthogonal to {001}. However, additional measurements on {100} and {001} faces of monoclinic ($P2_1$) crystals (Table 1) demonstrate that the CO orientations for A_0, A_1, and A_3 lie within a range of 4° or less

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(15) Note that only a small change in orientation, from ψ_0 = 74.0° to ψ_1 = 77.8°, is sufficient to describe the 75% increase in the intensity of A_1 relative to A_0 as χ varies from 0° to 90°.

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Table 1. Infrared Dichroism Measurements on MbCO Crystals[a]

	orthorhombic	monoclinic	
	{001}	{001}	{100}
ψ_0	74.0° ± 1.0°	−20.6° ± 2.2°	111.7° ± 1.0°
ψ_1	77.8° ± 0.5°	−21.6° ± 3.7°	111.2° ± 1.0°
ψ_3	78.3° ± 1.0°	−24.6° ± 7.0°	114.5° ± 1.7°
α[b]	82.9°	−24.0°	95.0°
β[b]	75.4°	77.5°	23.5°
γ[b]	119.5°	−40.2°	38.8°
ϕ_1[c]	69.2° ± 2.0°	45.2° ± 7.7°	348.4° ± 2.7°
	229.2° ± 2.3°	216.3° ± 6.1°	143.9° ± 0.9°
	21.4° ± 6.4°	*d*	278.8° ± 1.7°
	289.6° ± 7.6°	*d*	136.9° ± 1.7°

[a] Orthorhombic crystals were immersed in 4 M phosphate buffer, pH 5.6. Monoclinic crystals were immersed in saturated ammonium sulfate solution buffered to pH 4.8 with phosphate. [b] The Euler angles (α, β, γ) position the heme relative to the crystallographic axes. [c] Calculated assuming the value[13b] $\theta_1 = 28° \pm 2°$. [d] Only two solutions for $\theta \leq 44.2°$.

after projection onto three independent directions.[16] Furthermore, it is unlikely that ψ_1 would lie within 5° of the projected direction of the heme normal (listed as α in Table 1) for both {001} measurements if the angular displacement of the CO bond from the heme normal were as large as[13b] $\theta_1 = 28°$.

As a critical test for consistency with the photoselection measurements, we use the orientation of the heme known from the X-ray structures[12a,17] to calculate all values of the azimuthal angle ϕ_1 in the molecular coordinate system[12a] that are consistent with our experimental values for $\tan^2 \psi_1$, assuming that the polar angle $\theta_1 = 28° \pm 2°$ derived from the 10 K photoselection measurements[13b] is correct. With this constraint on θ_1, we fail to find consistent values of ϕ_1 among the single-crystal measurements, as shown in the lower rows of Table 1. For monoclinic crystals, solutions are found only in the first and third quadrants of the molecular coordinate system for the {001} measurements, while the {100} results lead to solutions in the second and fourth quadrants. This situation is not improved by taking the value[13a] $\theta_1 = 20°$ found from room temperature photoselection measurements. Similar problems occur when analyzing the results for A_0 and A_3.

These results strongly suggest that structural changes of the protein among the A_0, A_1, and A_3 states have little effect on the average CO orientation relative to the heme. Although structural differences between crystal and solution could in principle account for discrepancies with photoselection results, we note that the band frequencies are not perturbed by crystallization. Thus, the minimal differences in CO orientation observed here suggest that frequency shifts among conformational substates of Mb are not primarily determined by heme–C–O distortion, but are probably more sensitive to changes in distal pocket polarity.[18,19] Furthermore, the present results on $P2_1$ and $P2_12_12_1$ crystals are consistent with the nearly linear CO orientation recently reported for the *P*6 crystal form, which contains more water and has fewer intermolecular contacts.[11b]

In principle, the data in Table 1 provide sufficient information for a direct, independent determination of the CO orientation (θ,ϕ). As we will show elsewhere,[20] a complete analysis yields self-consistent solutions with $\theta < 10°$ for A_0, A_1, and A_3. This analysis requires corrections for small errors in ψ_i arising from structural disorder of the CO[21] as well as from beam convergence in the microscope,[22] both of which cause an apparent shift of ψ_i away from the nearest crystallographic axis. The convergence correction is significant when the absorbing dipole has a large component parallel to the average IR beam direction, and may account for the larger deviation between ψ_i and α observed in the monoclinic {100} measurement, where the angle β between the heme normal and the beam direction is only 23.5° (see Table 1). While it is important to consider these small corrections for an accurate determination of the absolute CO orientation, they apply systematically to all three bands and thus do not alter the basic conclusion that differences in CO orientation among conformational substates of MbCO are minimal. We note that the interpretation of IR photoselection measurements[13] is also affected by CO disorder,[21] as well as requiring additional assumptions (planar absorption by the heme, uniform photolysis rates for all hemes that contribute to a given IR band) used to calculate the angular distribution of photolyzed hemes.

The present results are not inconsistent with some steric control of CO binding, since the small changes in CO orientation among substates are accompanied by relatively small variations in CO binding affinity. We have determined from pH-dependent kinetic and spectroscopic measurements[6] that the CO association and dissociation rates in the "open" pocket (A_0) state are enhanced by factors of 11 ± 2 and 6.4 ± 0.6, respectively, relative to the "closed" pocket states (A_1 and A_3). This corresponds to a 40% reduction in CO affinity in the closed pocket or a free energy difference of 0.3 ± 0.1 kcal/mol. If we take 0.72 mdyn·Å/rad^2 as the force constant,[18a] 4° variations in the tilt of the Fe–C–O unit relative to the heme normal are sufficient to account for energy differences of this magnitude.

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(16) For the {100} measurements, ψ_i is measured from the *c*-axis and $\tan^2 \psi_i = (\mathcal{A}_b/\mathcal{A}_c)_i$.

(17) Only the structure of metMb has been determined for orthorhombic crystals (W. Hendrickson, unpublished results). We have assumed that the relative orientation of the hemes in MbCO (ref 12a) and metMb (Takano, T. *J. Mol. Biol.* **1977**, *110*, 537–568) is the same in orthorhombic and monoclinic crystals.

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