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The Origin of Stark Splitting in the Initial Photoproduct State of MbCO

Karin Nienhaus,[‡] John S. Olson,[§] Stefan Franzen,[⊥] and G. Ulrich Nienhaus*,[‡,||]

University of Ulm, Department of Biophysics, Albert-Einstein-Allee 11, 89081 Ulm, Germany, Rice University, Department of Biochemistry and Cell Biology and the W. M. Keck Center of Computational Biology, Houston, Texas 77005-1892, North Carolina State University, Department of Chemistry, Raleigh, North Carolina 27695, and University of Illinois at Urbana-Champaign, Department of Physics, 1110 West Green Street, Urbana, Illinois 61801

Received June 5, 2004; E-mail: uli@uiuc.edu

A microscopic structural description of the deceptively simple process of ligand photodissociation and recombination in myoglobin (Mb) has begun to emerge with the application of low-temperature[1−5] and time-resolved[6−9] X-ray crystallography. Within picoseconds after photolysis, the ligand settles into an initial docking site B[10] on top of the heme group, parallel to pyrrole C,[1−10] where it resides for several nanoseconds before either rebinding or escaping. Electron density maps of the initial B state photoproduct reveal only a single location for the dissociated CO, whereas two major spectrally and kinetically distinct states, B_1 and B_2, are observed in time-resolved and low-temperature infrared spectra.[10−14] Lim et al.[10] proposed that the CO molecule adopts two opposite orientations in these states. They assigned the high frequency (∼2130 cm^{-1}) B_1 state to the conformer with the O atom pointing back toward the heme iron atom and the low frequency (∼2120 cm^{-1}) B_2 state to the other orientation, on the basis of the kinetics of the appearance of the ν_{CO} bands after photolysis and the effects of isotopic substitution on the rate of recombination.[14] Recently, we have made the opposite assignments on the basis of mutagenesis data.[15]

In view of this discrepancy, we have reexamined the effects of mutating His64 and Val68 on the CO infrared stretching bands associated with the B_1 and B_2 photoproduct states. Wild-type, H64L, V68F, and H64L−V68F MbCO were selected for experimental and theoretical analyses. Fourier transform infrared (FTIR) spectroscopy and density functional theory (DFT) calculations were combined to examine the effects of the electrostatic environment on the B state photoproduct bands.

Free CO gas[16] absorbs at 2143 cm^{-1}. Within the protein matrix, the stretching frequencies ν_{CO} of both heme-bound and dissociated CO are shifted by the internal electric field acting on the CO dipole.[17−22] Hydrogen bonding between the CO and a donor side chain can be considered equivalent to an externally applied electric field, with the X−H dipole (X = N, O, C) causing the Stark shifts.[19] The C−O bond length is altered by electrical and mechanical anharmonic interactions with the dipole moment of the X−H group.[31] Quantitative models of the ν_{CO} shifts of heme-bound CO in many MbCO mutants indicate a significant effect of hydrogen bonding on ν_{CO}.[19] Using similar models, we have studied the interactions that cause the observed splitting of the B state bands and have related CO orientation with ν_{CO}.

At neutral pH, wild-type and V68F MbCO display similar heme-bound CO spectra, with contributions from bound states A_1 and A_3 (Figure 1a).[15,23] In the absence of the distal histidine (H64L and H64L−V68F MbCO), a single A_0 band is visible. After photolysis of wild-type MbCO at 3 K,[13] the major B_1 and B_2 photoproduct bands are at 2131 and 2119 cm^{-1}. The small B_0 population at 2149 cm^{-1} is associated with the minor A_3 state.[24] The Stark splitting between B_1 and B_2 increases from H64L < H64L−V68F < wild-type < V68F Mb (Figure 1b).

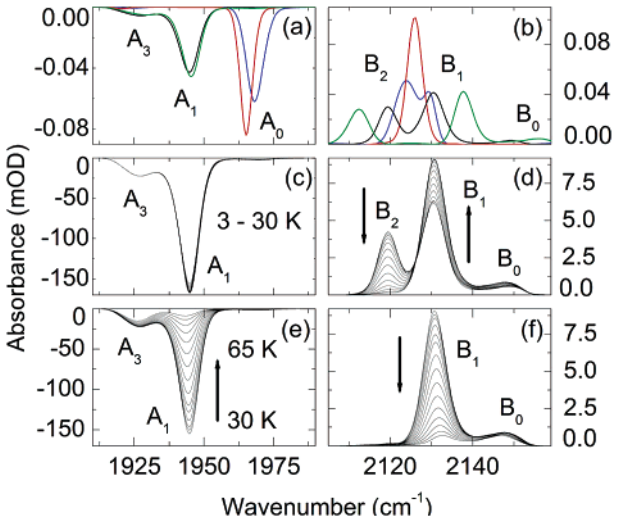

Figure 1. (a, b) FTIR photolysis difference spectra of wild-type MbCO (black) and mutants H64L (red), V68F (green), and H64L−V68F (blue), calculated from transmission spectra collected before and after 1-s illumination at 3 K (pH 7.5, areas normalized to 1 OD cm^{-1}). FTIR spectra of wild-type MbCO during a temperature ramp (5 mK/s) from (c, d) 3−30 K and (e, f) 30−65 K.

After photolysis at 3 K and slow heating (5 mK/s) to 30 K, very little CO rebinds to Mb, as judged from the small change in spectral area (Figure 1c). However, there is a net conversion from the B_2 to the B_1 conformer (Figure 1d). The barrier against this exchange is small (3−4 kJ/mol)[13] compared to that against recombination at the heme iron, ∼10 kJ/mol.[11] The B_2 to B_1 transition appears to involve a simple rotation of the ligand about the center of the C−O bond. Warming to ∼50 K causes the B_1 peak to disappear with concomitant reformation of the A_1 state (Figure 1 e, f). Thus, rebinding occurs primarily from B_1.

Here we propose that the ligand carbon is directed toward the heme iron in B_1 (Figure 2). Both theory and experiment support the presence of the N_ϵ−H tautomer of His64 in the A_1 and A_3 states.[19,21,25,26] Upon photodissociation at 3 K, a change to the N_δ−H tautomer is unlikely to occur in the frozen protein. Consequently, our assignment implies a direct hydrogen bonding interaction between the N_ϵ−H atom of His64 and the CO ligand. The N_ϵ−H···C−O interaction should increase the bond order and ν_{CO} of unbound CO, whereas the N−H···O−C interaction should decrease both. In H64L MbCO, the Stark splitting is absent, although both CO orientations are likely to occur in the photoproduct (Figure 1b).

[‡] University of Ulm.
[§] Rice University.
[⊥] North Carolina State University.
[||] University of Illinois at Urbana-Champaign.

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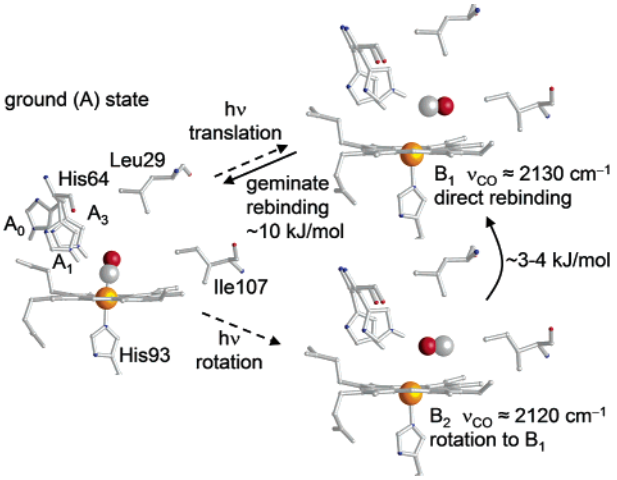

Figure 2. (Left) Schematic representation of heme-bound states A_0, A_1, and A_3. The O and C atoms of the ligand are red and white, respectively. (Right) Photoproduct states B_1 and B_2, with the His64 imidazole farther away from dissociated ligand, as in the 40 K Mb*CO photoproduct (PDB code 1ABS), and closer, as in deoxyMb (PDB code 2MGL), and most electron density maps of photodissociated MbCO at room temperature.[1,7]

Table 1. Infrared Bands of the Initial Photoproduct of MbCO

	ν_{CO} (cm^{-1})		
sample/model	B_2	B_1	B_0
wild-type MbCO (exp)	2119 (37)[a]	2131 (58)	2149 (5)
Im−H···OC, CO (porph.)	2122	2129	
H64L (exp)		2126 (100)	
H64L−V68F (exp)	2124 (28)	2130 (72)	
V68F (exp)	2112 (39)	2138 (53)	2156 (8)

[a] In parentheses: percentage of the total absorbance.

This observation suggests strongly that proton donation by His64 plays a predominant role in the wild-type protein. The V68F mutation enhances the Stark splitting significantly (Figure 1b, Table 1). The large benzyl side chain fills the back of the distal pocket, sequesters the photodissociated CO closer to the His64 side chain, and provides an additional electrostatic field.[15,27] The latter effect of the phenyl ring can be seen in the H64L−V68F double mutant as a small splitting of the B state peaks (~6 cm^{-1}, Figure 1 and Table 1).

To test the feasibility of the proposed B_1 and B_2 assignment, calculations of the expected vibrational frequencies of photodissociated CO were carried out on a series of simple model systems using DFT methods (DMol3, Accelrys, Inc.).[19,28−31] Stretching frequencies at 2158 and 2152 cm^{-1} are obtained for single hydrogen-bonding interactions in Im−N_ϵ−H···CO and Im−N_ϵ−H···OC (N_ϵ···ligand distance: 3.5 Å). Earlier studies have indicated marked contributions from the porphyrin ring and other side chains in the distal pocket.[32,33] More complex, energy-minimized Im−H···CO···H−CH_3 and Im−H···OC···H−CH_3 models including two interactions predict stretching frequencies of 2179 and 2151 cm^{-1}, where the methane molecule mimics the interaction of the CO with the methyl groups of Leu29 or Ile107 (Figure 2). When the CO was placed above a simple porphine macrocycle as a more realistic model, the ν_{CO} values of Im−H···CO and Im−H···OC were calculated to be 2129 and 2122 cm^{-1} (Table 1, Supporting Information).

The relative frequency shifts for all the models are in agreement with the assignments of the experimentally observed B_1 and B_2 bands in Figure 2, although the magnitudes vary. Our interpretation of the B states is also consistent with DFT estimates of the energies of Im−H···CO versus Im−H···OC interactions which show that hydrogen-bond donation to the C atom produces a ~7 kJ/mol lower energy state than the alternative orientation, accounting for the B_2 to B_1 interconversion between 3 and 30 K (Figure 1d).

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Supporting Information Available: Stretching frequencies and bond lengths obtained from DFT calculations of selected model systems, including coordinates. This material is available free of charge via the Internet at http://pubs.acs.org.

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