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Functional Aspects of Ultra-rapid Heme Doming in Hemoglobin, Myoglobin, and the Myoglobin Mutant H93G*

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Heme iron out-of-plane displacement following ligand dissociation in hemoglobin, myoglobin, and the proximal cavity mutant H93G is shown to be as rapid as the heme iron out-of-plane vibrational period by sub-picosecond time-resolved resonance Raman spectroscopy. The results demonstrate that the effect of steric repulsion initiated by the spin change of the iron gives rise to heme doming independent of covalent attachment of the proximal ligand to the protein. It is concluded that the protein plays a passive role in the initial ultrafast heme iron motion toward the out-of-plane position observed in the deoxy structure of hemoglobin and myoglobin. The results suggest that the spin change of the heme iron is the primary cause of rapid heme doming and that steric repulsion of the proximal ligand with the heme plays a secondary role in forcing the iron out of the heme plane.

The study of cooperative ligand binding among the four subunits of the protein hemoglobin has occupied a central role in the understanding of allosteric transitions of enzymes (Monod *et al.*, 1965). The role of the heme iron out-of-plane displacement, or doming, as the trigger for structural changes leading to the cooperative transition in hemoglobin has been suggested based on an observed correlation between the extent of iron out-of-plane motion and the free energy of cooperativity (Perutz, 1979), as well as on quantum chemical calculations (Olafson and Goddard, 1977) and molecular dynamics simulations (Henry *et al.*, 1985; Kuczera *et al.*, 1990; Gibson *et al.*, 1992). Cooperative interactions among the subunits of hemoglobin are induced by the breaking of a chemical bond between the heme iron and a diatomic ligand that leads to displacement of the heme iron by ≈0.4 Å out of the heme plane (Baldwin and Chothia, 1979). To function as a trigger for the cooperative transition, heme doming must be the first conformational change to occur following ligand dissociation.

Both time-dependent absorption (Sawicki and Gibson, 1976; Hofrichter *et al.*, 1983; Martin *et al.*, 1983) and resonance Raman spectroscopies have been used to evaluate the time scale of heme doming in hemoglobin (Findsen *et al.*, 1985; Franzen *et al.*, 1994a). In the present paper, we describe application of sub-picosecond time-resolved resonance Raman as a probe of the dynamics of the heme iron. The results presented here suggest that the heme-(histidine)-imidazole complex undergoes an ultrafast reaction to which the protein matrix reacts on longer time scales analogous to rapid formation of a dipolar excited state in a polar solvent. We substantiate this view by demonstrating ultra-rapid heme doming in the myoglobin mutant H93G(Im) in which the proximal histidine is replaced by glycine, and exogenous imidazole (Im)[1] is not chemically bonded to the protein but occupies the cavity created by the H93G mutation and is bonded to the heme iron (Barrick, 1994).

EXPERIMENTAL PROCEDURES

The time-dependent resonance Raman signal was observed using a two color experiment (Petrich *et al.*, 1987). The pump wavelength was 570 nm and had a duration of 100–150 fs as determined from an autocorrelation measurement. The pump beam energy was about 50 μJ, which was sufficient to photolyze 50% of the hemes in the sample. The Raman beam at 435 nm, which is in resonance with the Soret band of all species observed, was generated by using the frequency-doubled output of a chain of three amplifiers using laser dye LDS867. The frequency bandwidth of the Raman beam was fixed using a bandpass filter with a 6-Å bandwidth after a cuvette containing H_2O, in which a spectroscopic continuum was generated. The LDS867 amplifiers were pumped by the output of a frequency-doubled injection-seeded Nd:YAG (neodymium: yttrium-aluminum-garnet) laser at 30 Hz and functioned in a regime saturated in the femtosecond pulse (Migus *et al.*, 1980). This apparatus leads to a consistently stable 435-nm beam over nearly an order of magnitude change in the intensity of the input pulse from the continuum and permits signal averaging for many hours with little drift. After frequency doubling to 435 nm, the bandwidth of the Raman probe pulse was 25 cm^{-1}, and the pulse duration was 700 fs. Raman probe pulse energies were 500 nJ/pulse giving rise to no detectable photodissociation of HbCO by the probe pulse alone. The pump and probe pulses were made collinear using a dichroic mirror and had a cylindrical form (100 μm × 400 μm) at the sample. The scattered light was collected in a 90 degree geometry and dispersed onto a microchannel plate (EG&G) using a 1 m Jobin-Yvon monochromator.

The samples were the carbon monoxide (CO) complexes of HbCO, horse heart myoglobin (MbCO), and the H93G(Im)CO mutant of sperm whale myoglobin (200–400 μM in heme). The preparation of hemoglobin and horse heart myoglobin has been described elsewhere in detail (Franzen *et al.*, 1994b). The H93G(Im) mutant of sperm whale myoglobin was prepared from an *Escherichia coli* strain containing the mutant plasmid grown in medium containing 10 mM imidazole. The protein purification procedure has been discussed in detail elsewhere (DePillis *et al.*, 1994).

RESULTS

Fig. 1 shows three significant changes in the equilibrium HbCO vibrational spectrum upon photolysis. The iron-His F8 out-of-plane vibration, ν(Fe-His), appears at ≈225 cm^{-1} (shifted relative to the ≈212 cm^{-1} in equilibrium deoxy Hb) along with an unassigned mode at ≈304 cm^{-1}. The iron-ligand stretching vibration, ν(Fe-CO), at ≈505 cm^{-1} decreases in intensity upon photolysis due to the departure of the CO ligand (Tsubaki *et al.*, 1982). A considerable amount of experimental effort has been devoted to assigning ν(Fe-His) and showing that

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[1] The abbreviations used are: Im, imidazole; HbCO, human hemoglobin; MbCO, horse heart myoglobin; RR, resonance Raman.

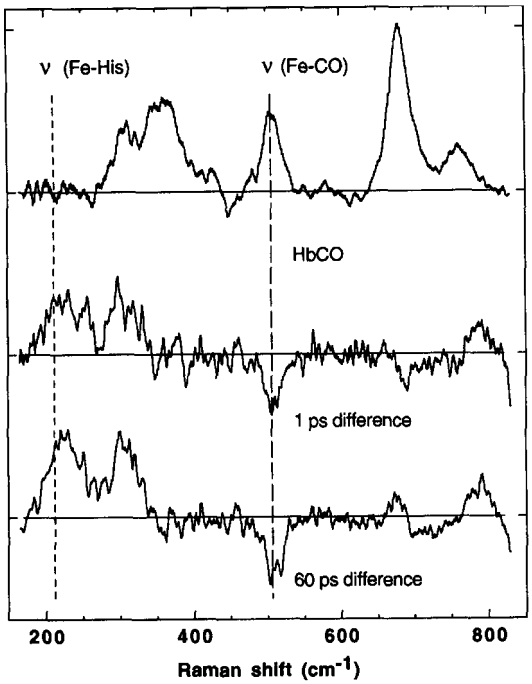

FIG. 1. **Time-resolved resonance Raman spectra for HbCO.** Equilibrium HbCO Raman spectra were produced using probe pulses, which arrive at the sample 5 ps prior to the pump pulse. Difference Raman spectra consist of subtraction of the HbCO spectrum from a Raman spectrum in which the 435-nm probe arrives at the sample after the 575-nm pump.

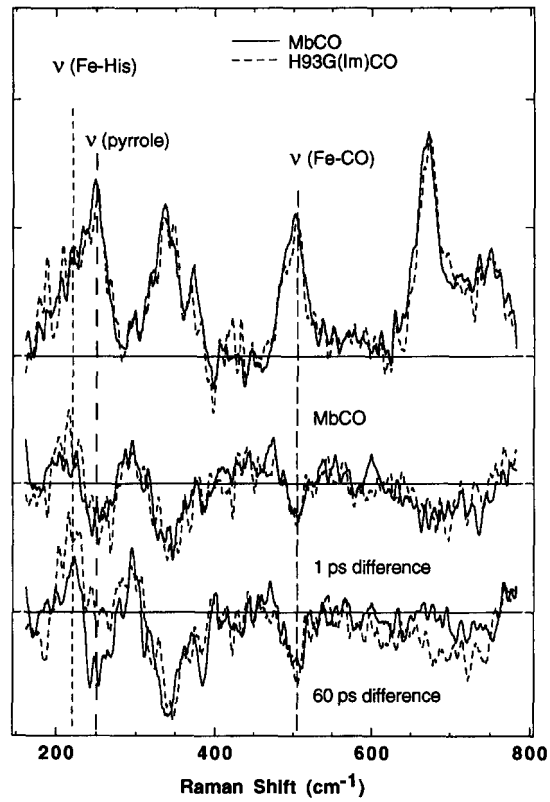

FIG. 2. **A comparison of equilibrium Raman spectra and Raman difference spectra at 1 and 60 ps is shown for MbCO and H93G(Im)CO.** The spectra are overlaid to show their overall similarity. The difference in the intensity of the lowest frequency peaks is due to the differences in the deoxy resonance Raman spectra of the respective species.

this mode is the signature of a domed heme iron, thus correlating the resonance Raman signal with a change in structure (Kitagawa *et al.*, 1979). From the time-resolved RR difference spectra shown in Fig. 1, one can follow the breaking of the iron-ligand bond by observing the reduction in scattering intensity of the Fe-CO stretch ν(Fe-CO) at $\approx$505 cm^{-1}. This serves as both an internal calibration for the extent of photolysis and a clock for structural changes. The ν(Fe-His) vibration and $\approx$304 cm^{-1} mode appear together in less than 1 ps following photolysis with a rise in intensity, which tracks the bond-breaking event. The mode ν(Fe-His) appears with a band shift of 12 $\pm$ 3 cm^{-1} to higher energy in photolyzed HbCO with constant frequency out to 60 ps (Findsen *et al.*, 1985; Franzen *et al.*, 1994b).

Evidence for ultrarapid heme doming can be found in the time-resolved RR spectra of MbCO and in H93G(Im)CO as shown in Fig. 2. At a delay of 1 ps, there is a clear increase in intensity of ν(Fe-His) at $\approx$220 cm^{-1} and of an unassigned mode at $\approx$300 cm^{-1} (as seen in the hemoglobin spectrum in Fig. 1), indicating that the same modes appear in myoglobin and hemoglobin on the picosecond time scale. Both the equilibrium MbCO and H93G(Im)CO difference RR spectra are very similar between 1 and 60 ps within the spectral resolution of the 700-fs Raman pulse. The intensity of the iron-histidine out-of-plane mode is larger in H93G(Im) myoglobin than in horse heart.[2] This is observed in the continuous wave Raman spectrum as well, and this intensity difference can be understood in terms of the larger intensity of the ν(Fe-Im) mode in deoxy H93G(Im) when compared with ν(Fe-His) in wild type deoxy Mb (see below). The iron-histidine out-of-plane mode is more symmetrical in deoxy H93G(Im) than in deoxy wild type horse heart or sperm whale myoglobin;[2] however, the level of resolution required to observe the line-shape difference is much higher than that attainable in a sub-picosecond time-resolved resonance Raman experiment.

One significant difference between myoglobin and hemoglobin is the frequency of the ν(Fe-His) mode relative to the equilibrium deoxy spectrum. The approximate 12-cm^{-1} shift that is found in hemoglobin appears to be entirely absent in myoglobin. The error in determining the peak position is inherently larger in myoglobin due to the mode at $\approx$250 cm^{-1}. This mode, which appears in the MbCO but not the HbCO RR spectra, is thought to be a heme pyrrole out-of-plane tilt (Choi and Spiro, 1983). Regardless of origin, this mode is observed in MbCO but not in deoxy Mb or any Hb RR spectrum (Tsubaki *et al.*, 1982). This accounts for the negative feature that appears in the RR difference spectrum of photolyzed MbCO and H93G(Im)CO (Fig. 2) but not HbCO (Fig. 1).

DISCUSSION

The stability of the iron-histidine (Fe-Nϵ) bond is important both for the biological relevance of flash photolysis as a technique and the properties of ligand binding in the physiological function of myoglobin and hemoglobin. The observation of the ν(Fe-His) out-of-plane mode at the earliest times in both myoglobin and hemoglobin demonstrates that the Fe-Nϵ bond is not broken on the femtosecond time scale. The fact that the iron-histidine bond remains intact on the picosecond time scale is important for the validity of conclusions about ligand rebinding on a fast time scale based on flash photolysis experiments. NO rebinding occurs on the 10-ps time scale in hemoglobin, and the most rapid phase of the CO and O_2 ligand recombination reactions could also be affected by proximal ligand dynamics if photolysis occurred. In the H93G mutant of myoglobin, the proximal imidazole is no longer covalently attached to the

[2] S. Franzen, S. Decatur, G. DePillis, S. G. Boxer, R. B. Byer, and W. H. Woodruff, submitted for publication.

protein, and even in this cavity mutant, the iron-histidine bond appears to remain intact on the picosecond time scale. The results in Figs. 1 and 2 demonstrate that there is no detectable proximal ligand photolysis on the time scale relevant to any of the ligand binding processes studied.

The similarity of the time-resolved resonance Raman signal in photolyzed Mb*CO and H93G(Im)*CO is not entirely expected. There are large structural differences between the position of imidazole ring in the ligated H93G mutant and in ligated wild type myoglobin based on a comparison of the aquo-met myoglobin crystal structures (Barrick, 1994). The angle between the imidazole ring and the *N*(pyrrole)-Fe-*N*(pyrrole) line on the heme is nearly 0° in wild type ligated myoglobin and is 40–45° in the aquo-met form of the H93G mutant. The difference in angle of the imidazole obtained in the aquo-met structure is preserved in the cyano-met form based heme-methyl NMR hyperfine shift patterns.[3] The available evidence from NMR studies of the CO complex suggests that the imidazole ring is similarly rotated.[4] Given these observations, a strong hydrogen bond formed between the imidazole nitrogen and OH of serine 92 seen in the aquo-met x-ray crystal structure may be the origin of the rotation angle of the imidazole in H93G(Im). These considerations lead us to suggest the hydrogen bond and hence the rotation angle of the imidazole are preserved in the deoxy structure as well.

As a consequence of the greater freedom for the rotated histidine to approach the heme, the Fe-Nϵ bond is 1.85 Å in H93G, as opposed to 2.17 Å in the aquo-met Mb form. The origin of the longer bond length in wild type myoglobin is likely to be steric repulsion of the histidine by the pyrrole nitrogens of the heme. If this difference in bond length is preserved in the deoxy, Mb should result in an altered ν(Fe-Im) frequency when compared with ν(Fe-His) in wild type. The similarity of the frequency of the ν(Fe-His) mode in the RR spectrum of the photolyzed species suggests that the iron-histidine bond lengths are not significantly different in Mb*CO and H93G(Im)*CO. However, there is at present no structural evidence from NMR or x-ray diffraction on deoxy species to substantiate this suggestion. In principle, resonance Raman should be sensitive to small changes in rotation angle and bond length; however, detailed analysis of the structural implications of small changes in linewidth and frequency require high spectral resolution continuous wave resonance Raman experiments. Preliminary continuous wave resonance Raman spectra show that the frequency of ν(Fe-Im) in H93G(Im) is at 226 cm^{-1},[2] which is shifted <2 cm^{-1} from 224 cm^{-1}, the weighted average of the two components (80% at 220 cm^{-1} and 20% at 240 cm^{-1}), which comprise the asymmetric ν(Fe-His) mode in wild type horse heart (or sperm whale) myoglobin (Bangcharoenpaurpong *et al.*, 1984). Based on the continuous wave resonance Raman spectra, the intensity of the ν(Fe-Im) mode in H93G(Im) is greater by nearly a factor of two than ν(Fe-His) in wild type Mb,[2] which explains the difference in intensity of this mode in the difference Raman spectrum shown in Fig. 2.

The results in Figs. 1 and 2 confirm the hypothesis of significant sub-picosecond heme iron doming based on the appearance of a deoxy-like species in the time-resolved absorption spectrum within 300 fs following photo-dissociation in HbCO and MbCO (Petrich *et al.*, 1988). The 300-fs iron doming time corresponds to one-half period of the 50 cm^{-1} Fe-heme out-of-plane vibration frequency in deoxy myoglobin (Zhu *et al.*, 1994). Our data show the separation of time scale between heme doming and the subsequent large conformational changes, which can be viewed as a rapid chemical reaction followed by slower solvent (*i.e.* protein) relaxation. The fact that ultrafast heme doming is observed even in a heme-imidazole complex in the H93G(Im) mutant proves that the forces responsible for this reaction do not arise from the protein matrix that holds the heme-imidazole complex in place. Nor do the steric repulsive forces between the proximal ligand (histidine in wild type) and the heme appear to be responsible, as previously suggested, based on molecular orbital calculations (Olafson and Goddard, 1977). Rather, these results suggest that the spin change of the heme iron forces the iron out of the heme plane. The lack of a frequency shift in ν(Fe-His) further suggests that the motion of the accompanying proximal histidine occurs on a similarly rapid time scale, thereby allowing the protein structure on the proximal side to evolve rapidly toward the deoxy configuration without dissipation of the energy of heme doming. The time-resolved resonance Raman data, together with other experiments (Franzen *et al.*, 1994b), suggest that a significant part of F-helix motion associated with the iron out-of-plane displacement occurs on the picosecond time scale in myoglobin. In hemoglobin, a similar set of changes occurs within each subunit as tertiary structure evolves following ligand dissociation. In this cooperative protein, the alterations in tertiary structure that capture the energy of heme doming, such as the initial phase of F-helix motion, are crucial to the formation of intersubunit interactions. The specificity of these motions allows contact to be made on the surface of each subunit on the microsecond time scale, which gives rise to the R-T conformational switch. The rapid protein response to the out-of-plane motion of the heme iron is responsible for the communication of the rupture of the iron-ligand bond over a distance of >25 Å, which leads to cooperative interactions in hemoglobin.

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[3] S. Decatur and S. G. Boxer, submitted for publication.

[4] S. Decatur, unpublished results.

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