Response of the Local Heme Environment of (Carbonmonoxy)hemoglobin to Protein Dehydration†

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ABSTRACT: The effects of protein dehydration upon the equilibrium and dynamic properties of the heme active site in human hemoglobin (HbA) have been probed by resonance Raman scattering. Spectra of equilibrium carbonmonoxy-HbA and the photolytic heme transient species generated within 10 ns of ligand photolysis have been obtained from thin films of protein in various stages of dehydration. These data provide detailed information concerning the response of the heme and its bonding interactions with both the proximal histidine and carbon monoxide as a function of protein hydration. For protein hydration levels of 0.4–1.0 g of H_2O/g of protein, our results indicate that the C=O stretching mode of carbonmonoxy-HbA is dramatically affected by protein hydration levels, thus corroborating the infrared results of Brown et al. [Brown, W. E., Sutcliffe, J. W., & Pulsinelli, P. D. (1983) *Biochemistry 22*, 2914–2923]. However, we find that both heme skeletal modes and the Fe–C bond strength are largely insensitive to dehydration. Moreover, the proximal pocket geometry (as reflected in the behavior of the Fe–proximal histidine stretching mode) immediately following ligand photolysis was found to be very similar to that of R-state solution hemoglobin. At protein hydration levels below the theoretical monolayer limit, small changes in the resonance Raman spectra of both equilibrium HbCO and the transient heme species generated subsequent to ligand photolysis are detected. These include broadening of the Fe–C stretching mode in equilibrium HbCO and a small shift to lower frequency of the Fe–His mode in the photolytic transient species. We conclude that the continuous dependence of the C=O stretching mode upon protein hydration level derives primarily from distal pocket effects and not from changes in either heme geometry or degree of proximal strain in the dehydrated protein.

In recent years various spectroscopic and theoretical studies have revealed that proteins are dynamic systems (Debrunner & Frauenfelder, 1982; Gurd & Rothgeb, 1979; Friedman et al., 1982; Karplus & McCammon, 1981; Frauenfelder et al., 1979). The ligand binding heme proteins hemoglobin (Hb) and myoglobin (Mb) have been shown to exhibit both fluctuational and kinetic behavior at the heme active site, which can be directly related to their ligand binding properties. In particular, these proteins possess internal motions that are strongly influenced by globin–solvent interactions. Changes in solvent viscosity and pH or dehydration of the solvent sphere surrounding the globin produce alterations in both the steady-state ligand conformation at the heme site and its ligand binding kinetics (Hassinoff & Chishti, 1983; Caughey & Shimada, 1982; Caughey et al., 1981; Brown et al., 1983). A series of extensive transient absorption studies by Frauenfelder and co-workers (Beece et al., 1980, 1982) demonstrated that O_2 and CO rebinding rates following photolysis are inversely proportional to solvent viscosity. Steady-state liganded Hb and Mb display a number of different heme–CO conformers that are interconvertible and can be spectroscopically identified by their distinct C=O bond strengths (vibrational frequencies) (Caughey & Shimada, 1982; Caughey et al., 1981). The relative stability of these conformers has been found to be quite solvent dependent. In particular, Brown et al. (1983) have demonstrated using reflectance infrared spectroscopy that removal of the globin hydration sphere profoundly affects the bound C=O frequency in both Hb and Mb.

We have employed resonance Raman spectroscopy to examine both the steady-state and kinetic behavior of the heme active site of Hb in various stages of dehydration. Resonance Raman scattering has been shown to be a highly specific probe of the heme protein active site (Spiro, 1983). The molecular specificity of this technique allows for the direct assessment of the local heme structure. Specifically, the Fe–C bond strength and the C=O vibrational frequency of bound CO can be determined (Tsubaki et al., 1982; Kerr et al., 1983). Moreover, by employment of time-resolved techniques, the response of the proximal heme pocket immediately subsequent to ligand photolysis can be monitored (Friedman et al., 1982a,b, 1983; Scott & Friedman, 1984; Findsen et al., 1985; Ondrias et al., 1983; Terner et al., 1981; Irwin & Atkinson, 1981).

Our results indicate that the frequency of the C=O stretching motion of bound CO is markedly affected by dehydration and thus corroborate the previous IR studies. However, the Fe–C mode and heme skeletal mode frequencies are largely insensitive to these effects. The spectra of photolytic transients of dehydrated Hb are quite similar to solution spectra, leading us to conclude that the initial proximal heme pocket geometry following photolysis is not highly dependent upon extent of hydration. Thus, the observed dependence of the C=O stretching mode on dehydration down to ~0.1 g of H_2O/g of protein must derive primarily from distal pocket effects and not from changes in the degree of proximal heme pocket strain or heme geometry.

Materials and Methods

HbA was purified from fresh whole cells following a modified procedure of Antonini and Brunori (1971). Samples were dialyzed against 50 mM Tris, pH 7.4, and then concentrated

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Table I: Resonance Raman Active Modes of Equilibrium and Photolytic Transient Species of HbCO

	solution (R state)		dehydrated (0.4 g of H_2O/g of protein)		dehydrated (0.1 g of H_2O/g of protein)	
mode[a]	HbCO	Hb*	HbCO	Hb*	HbCO	Hb*
$\nu_{C_\alpha-C_\beta}$	~1620[b]	1619	~1620	~1618	~1620	~1617
ν_2	1584	1566	1582	1565	1583	1565
ν_{11}	~1560	1527	~1560		~1560	
ν_3	1499	1472	1501	1470	1501	1470
ν_4	1374	1355	1374	1355	1374	1355
ν_7	676		676		676	
ν_{Fe-C}	505		505		505	
$\delta_{C_bC_\alpha C_\beta}$		408		407		407
γ_{C_b-S}		366		367		367
ν_8				~345		~347
$\gamma_{C_mC_a}$ / $\delta_{C_bC_\alpha C_\beta}$		307		306		~303
		230		229		227
ν_{Fe-His}						
$\nu_{C=O}$	1954		1952/1975		1952/1975	

[a] Mode assignments follow Choi and Spiro (1983). C_a, C_b, and C_m designate the a, b, and methine carbons of the porphyrin macrocycle, respectively, while C_α and C_β designate the carbons of the vinyl substituents. The nomenclature ν, δ, and γ denotes in-plane stretching, in-plane bending, and out-of-plane bending motions, respectively. [b] All frequencies are accurate to ±2 cm^{-1} except ν_4 and ν_7, which are accurate to ±1 cm^{-1}.

to 22–25% protein by using an Amicon PM-30 membrane. About 100 μL of this solution was spread on a cuvette surface and allowed to air-dry to an appropriate weight. The cuvette was quickly purged with CO_2-free carbon monoxide. The sample in the stoppered cuvette was equilibrated for approximately 30 min and purged with carbon monoxide again. The cuvette was then stoppered and reweighed. This procedure routinely yielded thin films (<1 mm thick) of dehydrated HbCO with a uniform consistency and appearance. The quantification of the extent of dehydration was found to be reproducible to ±0.1 g of water/g of protein.

Resonance Raman spectra were collected with the instrumentation described, in detail, elsewhere (Findsen, 1986) using a nitrogen/dye laser system for an excitation source. Spectra of equilibrium-dehydrated carbonmonoxy-Hb were obtained with a defocused laser beam at 406 nm (0.2 mJ/pulse). Laser pulses of 440 nm (0.3 mJ/pulse) were used to generate the photolyzed transient data. These pulses were tightly focused with a cylindrical lens. Each pulse served both to photolyze the HbCO and to generate a transient Raman spectrum. Thus, the transient spectra are representative of the average population of the heme species generated within the laser pulse width (~10 ns) subsequent to photolysis. All data were obtained in a backscattering geometry. The samples were prepared on one face of an optical cuvette, which was placed in a configuration such that the laser beam first penetrated the window opposite the sample before striking the sample itself. Thus, all spctra were gathered from the side of the dehydrated film that was exposed to internal CO atmosphere (~1 atm) of the cell and not the quartz cuvette face. This eliminated any potential interfering effects from the glass itself or from anomalous glass–protein interactions. Individual scans of each spectrum were monitored to assure that there were no time-dependent changes in the sample properties (i.e., sample degradation).

Results

Spectra of equilibrium-dehydrated HbCO, taken under conditions of minimal ligand photolysis and maximum enhancement of scattering from liganded heme sites, reveal the bond strengths of the Fe—C=O group and the effects of dehydration upon the liganded heme vibrations. Table I summarizes the frequencies of the Raman modes observed for the equilibrium species.

Figure 1 displays the C=O stretching mode of the bound CO. This mode is only weakly apparent in resonance Raman

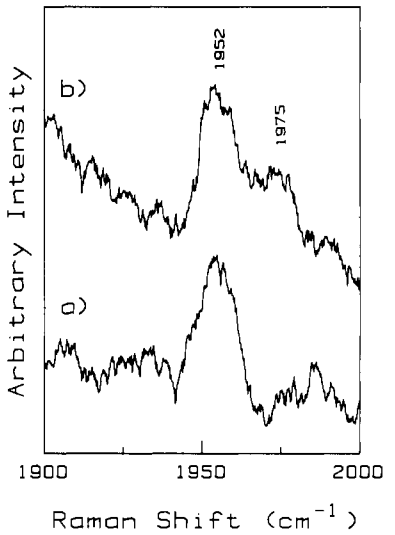

FIGURE 1: Spectra of $\nu_{C=O}$ region of solution (trace a) and dehydrated (0.3 g of H_2O/g of protein) samples (trace b). The HbCO dehydrated sample was prepared as described in the text. The solution sample was 25 μM in HbCO (100 μM in heme) in 0.05 M Tris, pH 7.5. Excitation at 406 nm was employed as described in the text. Spectral band pass was ~8 cm^{-1}, and spectra are the unsmoothed sums of 8–10 scans at 4 s cm^{-1}.

spectra of COHb and COMb, but even at poor signal-to-noise ratios, it is apparent that at dehydration levels of 0.8 g of H_2O/g of protein and below there are two C=O stretching modes. Their frequencies (~1952 and ~1975 cm^{-1}) compare well to the frequencies measured via reflectance infrared spectroscopy (Brown et al., 1983) (1951/1968 cm^{-1}) and are contrasted to the single mode observed at ~1954 cm^{-1} with solution HbCO. Rehydration of the solvent-depleted samples resulted in a single C=O mode at ~1954 cm^{-1}. The existence of two C=O stretching modes in our dehydrated sample is convincing confirmation that we are indeed observing HbCO in a state of dehydration that has depleted part of its immediate solvent sphere.

Surprisingly, the degree of protein hydration has little or no effect upon the frequency of the mode assigned as the Fe–C stretching motion. Figure 2 demonstrates that varying degrees of hydration (ranging from 0.1 to 1.0 g of H_2O/g of protein) all yield the solution value of 505 cm^{-1} for the peak position for this mode. At dehydration levels below the theoretical limit (Halle et al., 1981) for the first hydration monolayer of the protein (0.25 g of H_2O/g of protein), definite broadening of

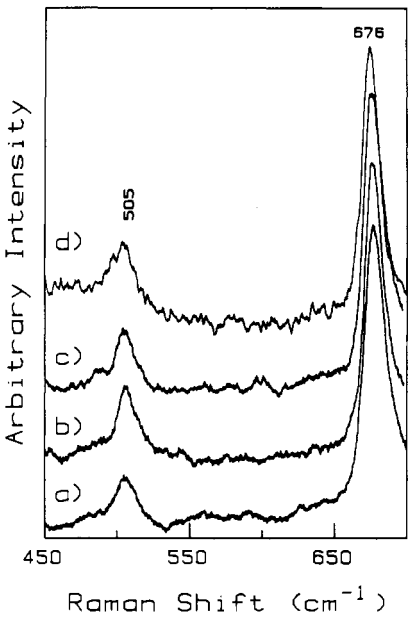

FIGURE 2: Spectra of ν_{Fe-C} region of equilibrium HbCO at successive levels of dehydration: (trace a) solution; (trace b) 0.8 g of H_2O/g of protein; (trace c) 0.4 g of H_2O/g of protein; (trace d) 0.1 g of H_2O/g of protein. Conditions and protocol were the same as in Figure 1 except spectra are the unsmoothed sums of 2–5 scans.

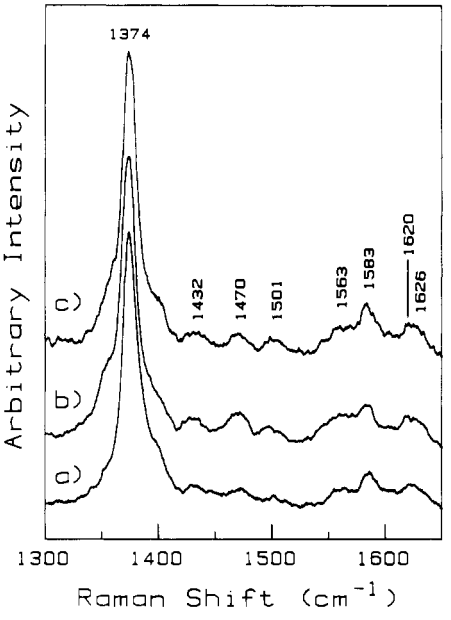

FIGURE 3: High-frequency spectra of equilibrium HbCO: (trace a) 0.8 g of H_2O/g of protein; (trace b) 0.4 g of H_2O/g of protein; (trace c) 0.1 g of H_2O/g of protein. Conditions and protocol were the same as in Figure 2.

this mode to lower frequency is apparent.

The higher frequency skeletal heme modes of HbCO also appear to be insensitive to dehydration (Figure 3). The prominent modes ν_4 (~1373 cm^{-1}), ν_3 (~1505 cm^{-1}), ν_{28} (~1475 cm^{-1}), ν_2 (~1585 cm^{-1}), and ν_{11} (~1560 cm^{-1}) and the vinyl group stretching (~1620 cm^{-1}) and bending (~1430 cm^{-1}) modes all appear at frequencies that are the same (±2 cm^{-1}) as those of the fully hydrated protein. Slight changes in the intensity of ν_4 relative to the higher frequency modes and in the position of the vinyl stretching mode (~1620 cm^{-1}) are seen at very low hydration (0.1 g of H_2O/g of protein) levels.

Photolysis of CO from the heme results in a metastable heme protein geometry that either rebinds the ligand or evolves

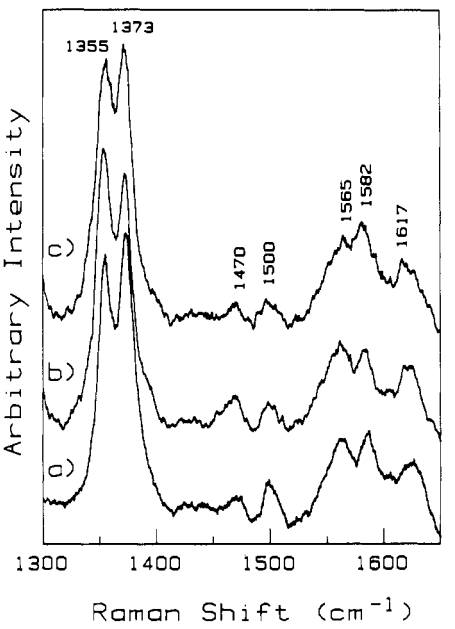

FIGURE 4: High-frequency spectra of photolytic transients of dehydrated HbCO: (trace a) 0.8 g of H_2O/g of protein; (trace b) 0.4 g of H_2O/g of protein; (trace c) 0.1 g of H_2O/g of protein. Excitation at 440 nm was used both to photolyze the HbCO and to generate a resonance Raman spectrum within 10 ns of photolysis as described in the text. Other conditions were the same as in Figure 2.

to the steady-state unliganded (deoxy) geometry (on a 1–10-μs time scale). Figure 4 depicts the resonance Raman spectra of the transient heme species generated within 10 ns of CO photolysis from dehydrated hemoglobins (0.8 to 0.1 g of H_2O/g of protein). The high-frequency spectra are complicated by the fact that a significant amount of scattering from liganded steady-state COHb is also present. This is due to an experimental restriction on the amount of laser power that could be employed without damaging the hemoglobin film. In practice, the beam was defocused to the point where only ~30% photolysis was achieved. This is clear from the relative intensities of the liganded (~1373 cm^{-1}) and deoxy (~1355 cm^{-1}) ν_4 peaks in the spectrum. The spectra of dehydrated transients are quite similar, differing only in degree of photolysis and hence the relative intensities of the COHb modes at ~1373 (ν_4), ~1500 (ν_3), ~1585 (ν_2), and ~1630 cm^{-1} (ν_{10} and vinyl stretch). Moreover, the positions of the transient deoxy heme modes [at ~1355 (ν_4), ~1472 (ν_3), and ~1565 cm^{-1} (ν_2)] in the spectra of dehydrated Hb agree to ±2 cm^{-1} with those obtained from COHb solutions.

The low-frequency spectra (see Figure 5) of photolyzed HbCO are less complicated since there are no prominent liganded (unphotolyzed) heme modes below 375 cm^{-1}. Of particular interest is the behavior of the mode at ~230 cm^{-1}, which has been assigned as the Fe–proximal histidine stretching mode (Nagai et al., 1980). Here again there is very little indication of sensitivity to dehydration in the 1.0–0.4 g of H_2O/g of protein range. The position of the Fe–His mode in these dehydrated samples (~230 cm^{-1}) is indicative of a strengthened Fe–His bond (relative to solution deoxy-Hb) comparable to that exhibited by photolytic transients of R-state hemoglobins in solution (229 ± 3 cm^{-1}) (Friedman et al., 1982). Other characteristics of solution hemoglobin transients are a shift of the ~300-cm^{-1} mode to ~307 cm^{-1} and the disappearance of a band at ~340 cm^{-1}. Spectra of the dehydrated samples display a similar shift in the 300-cm^{-1} mode; however, at hydration levels below 0.5 g of H_2O/g of protein the mode at ~340 cm^{-1} is clearly evident, while the relative

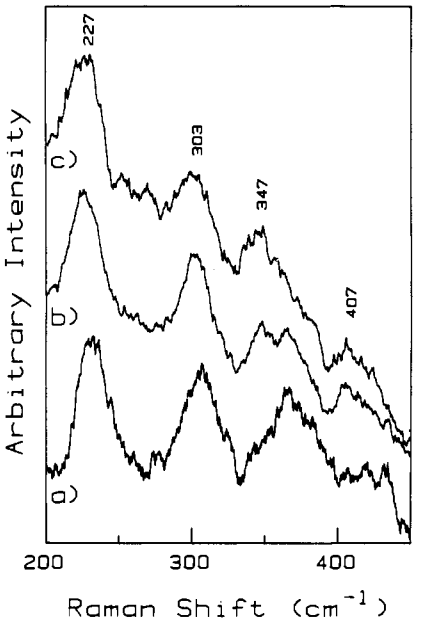

FIGURE 5: Low-frequency spectra of photolytic transients of dehydrated HbCO: (trace a) 0.8 g of H_2O/g of protein; (trace b) 0.4 g of H_2O/g of protein; (trace c) 0.1 g of H_2O/g of protein. Conditions and protocol were the same as in Figure 4.

intensity of the band at ~365 cm^{-1} is diminished. In addition, small shifts (~2 cm^{-1}) are apparent in the ~307-cm^{-1} and Fe–His modes at extreme dehydration levels (0.1 g of H_2O/g of protein). Table I summarizes the frequencies of the vibrational modes of the hemoglobin species examined.

DISCUSSION

The resonance Raman data presented above can be used to analyze the effects of dehydration upon the equilibrium and kinetic characteristics of the local heme environment in hemoglobin. The properties of the distal pocket (when CO is bound), the heme skeletal modes, and the dynamics of the proximal pocket subsequent to ligand photolysis can all be monitored. In general, we find that dehydration most readily affects the distal heme pocket and the vibrational properties of the bound CO. Only small changes in heme skeletal modes, Fe–CO bond strength, and proximal pocket geometry are indicated by our data, and these are observed only at hydration levels below the theoretical monolayer level.

A comprehensive study of the C–O vibrational properties of CO bound to hemoglobin and myoglobin in various stages of dehydration has recently been conducted by Pulsinelli and co-workers (Brown et al., 1983) using infrared spectroscopy. They found that dehydration of the proteins favored a Fe–CO conformer with $\nu_{CO} \approx 1968$ cm^{-1} over the predominate conformer in fully hydrated proteins ($\nu_{CO} \approx 1951$ cm^{-1} for HbCO) and concluded that the former species possessed a less sterically hindered linear-perpendicular Fe–CO bonding arrangement that resulted from a more open distal heme pocket and a more tightly bound CO. They further postulated that the more open distal pocket was accompanied by an increase in deoxy-like steric hindrance in the proximal heme pocket. Our results corroborate the data obtained by this group but indicate that the major perturbation of the heme–CO system engendered by general protein dehydration is largely confined to the distal heme pocket.

Distal Heme Pocket and Fe–C–O Response to Dehydration. Our data clearly show that the existence of two ν_{CO} modes (at 1952 and ~1975 cm^{-1}) is completely dependent upon the degree of protein hydration and is reversible upon rehydration of the protein. However, one cannot make a direct quantitative assessment from the resonance Raman data of the fraction of heme–CO sites that are in each configuration. The resonance Raman activity of ν_{CO} (and any axial ligand mode) is predicated upon the degree to which that mode couples with the heme electronic transition that is resonant with the incident photon energy. This coupling can be greatly affected by local geometry, and thus the intensity of ν_{CO} for each conformer is only a relative measure of its concentration. Nevertheless, it is obvious that we detect significant ν_{CO} intensity from the $\nu_{CO} \approx 1975$ cm^{-1} conformer at dehydration levels where it is signficantly present (>30% of the heme–CO sites) in the infrared spectra. At ~0.3 g of H_2O/g of protein, $\nu_{CO} = 1975$ cm^{-1} remains less intense than its 1952-cm^{-1} counterpart while it is the major component in the infrared spectra at that hydration level. Apparently, the relative enhancement of the $\nu_{CO} \approx 1975$ cm^{-1} conformer is less than that of the $\nu_{CO} \approx 1952$ cm^{-1} species, indicating that it couples less effectively into the π–π^* transitions of the heme, which are resonant with our laser wavelengths. This is not unexpected since the putative linear, perpendicular geometry of the high-frequency conformer would reduce the interactions between the π systems of the heme and the CO.

In hemoglobin, myoglobin, and a variety of heme model complexes, an increase in ν_{CO} of bound CO is accompanied by a decrease in the Fe–C stretching frequency (Kerr et al., 1983). Figure 2 effectively demonstrates that this trend is not followed by dehydrated HbCO. On the basis of the existence of two C=O stretching modes, two Fe–C stetching frequencies would be expected, one at ~505 cm^{-1} (the value for solution HbCO) and one at lower frequency. This is clearly not the case. Only a single ν_{Fe-C} is apparent at 505 cm^{-1}. The shoulder at ~490 cm^{-1} is a weak heme mode present in deoxy-Hb spectra. Its intenstiy is strongly dependent upon the small amount of photolysis present in our samples. This behavior cannot be a result of preferential CO photolysis of a linear conformer during the experiment, since the intensity of this mode increases with increasing extent of photolysis. Thus, despite the perturbation of ν_{CO} resulting from dehydration, the bond strength of Fe–CO remains remarkably constant. Moreover, the line width of the 505-cm^{-1} bond is quite similar to that of fully hydrated HbCO, giving no evidence for additional heterogeneous broadening. It is only at hydration levels well below the theoretical monolayer that ν_{Fe-C} broadens to lower frequency.

Heme Skeletal Modes. The spectra displayed in Figures 2 and 3 indicate that the heme skeletal modes are virtually unaffected by protein dehydration. The modes ν_2 (1584 cm^{-1}), ν_{11} (1556 cm^{-1}), ν_4 (1373 cm^{-1}), and ν_7 (676 cm^{-1}) and the peripheral vinyl stretching mode (1620 cm^{-1}) all appear at frequencies within ±2 cm^{-1} of their positions in fully hydrated protein. The frequencies of these modes have been empirically related to porphyrin π electron density (ν_4), heme core size (ν_{11}, ν_3), and the behavior of the heme vinyl substituents (ν_7, vinyl stretch) by numerous studies of heme proteins and model complexes [see Spiro (1983) and references cited therein]. The insensitivity of all these modes to dehydration demonstrates that, for equilibrium HbCO, the heme itself is not dramatically affected by globin–solvent interactions.

The insensitivity of heme modes to dehydration is not terribly surprising. Previous studies by Rousseau et al. (1984) of carbonmonoxy mutant and fish hemoglobins revealed very little difference in the heme vibrational modes as a result of R/T quaternary structural changes in the globin. Their studies also revealed that ν_{Fe-C} of the bound CO was invariant with

respect to quaternary structure. This strongly suggests that very little conformational freedom exists at the six-coordinate heme site of HbCO.

Brown et al. (1983) have noted changes in heme absorption properties as a function of protein dehydration. Absorption changes of a similar magnitude result from HbCO quaternary stuctural transitions (Perutz et al., 1976; Giardina et al., 1975) and appear to be correlated to small (<1-cm^{-1}) shifts in the ν_4 heme mode (Rousseau et al., 1984). Our data (with an accuracy of ± 1 cm^{-1} for this mode) cannot determine whether similar shifts in ν_4 (and by inference porphyrin π^* electron density) exist in the dehydrated protein. However, the quaternary structure-dependent shifts in ν_4 are not coupled to ν_{CO} and ν_{Fe-C} in the fully hydrated protein (Rousseau et al., 1984).

Proximal Heme Pocket/Heme Dynamics following Photolysis. The spectra obtained from the transient heme species generated within 10 ns of CO photolysis lend insight into the dynamics of heme–protein interactions. Dehydration of the protein evidently has little effect upon the initial Fe–histidine bond strength immediately subsequent to photolysis. It does, however, have some influence upon the local heme environment as evidenced by the behavior of the modes that are dependent upon peripheral substituent motion.

Spectra of photolyzed, dehydrated HbCO show an increase in the relative intensity of the mode at ~340 cm^{-1} with decreasing hydration level (see Figure 5). This mode has been assigned as a substituent in-plane bending mode, ν_8 (Spiro, 1983). It is characteristically absent in spectra of photolyzed HbA under a wide variety of solution conditons. Its reappearance (on a microsecond time scale) in the spectra of photolyzed solution HbA can be ascribed to relaxation of the heme pocket, perhaps involving the heme tilt within the pocket. In a study of the dynamics of a variety of hemoglobins, the transient behavior of this mode was found to vary widely from species to species but could not be correlated to ligand binding parameters such as on-rate or affinity (Carson, 1985). Other changes in the spectra of the photolytic transients of dehydrated HbCO (relative to solution species) are observed in the relative intensities or positions of modes at ~305, ~365, and ~410 cm^{-1}. At extreme dehydration levels, a weak mode appears at ~250 cm^{-1}. All of these modes have been assigned as having significant contributions from motions of vinyl peripheral substituents or out-of-plane heme deformations (Choi & Spiro, 1983). Collectively, the behavior of these modes in the spectra of dehydrated HbCO transients within 10 ns of photolysis indicates a change in the protein tertiary structure about the heme relative to solution conditions. The functional significance of these differences (if any) remains uncertain.

A more direct measure of the sensitivity of the proximal heme pocket to environmental effects is the behavior of the Fe–His stretching mode. However, while this mode is quite prominent in spectra of deoxy-Hb (both equilibrium and transient), it is not seen in liganded hemoglobin spectra. Thus, the steady-state properties of the proximal heme pocket must be inferred from the vibrational properties of the heme and distal ligand. As described above, neither ν_{Fe-C} nor the heme modes are sensitive to dehydration. This insensitivity argues for a lack of significant differences in the proximal bonding as a function of dehydration. In particular, the frequency of ν_{Fe-CO} has been found to be a sensitive indicator of the degree of proximal strain. In a study of heme model complexes with sterically hindered and unhindered substituted imidazole axial ligands, Kerr et al. (1983) detected up to 7-cm^{-1} shifts in ν_{Fe-CO}, which presumably result from increased proximal strain. Thus, the lack of a dehydration sensitivity of ν_{Fe-CO} argues strongly for an absence of any corresponding changes in proximal strain.

Further evidence for a lack of significant modulation of HbCO proximal heme pocket geometry by dehydration is found in the spectra of heme transients generated within 10 ns of CO photolysis. Time-resolved resonance Raman studies of the metastable heme transients subsequent to ligand photolysis have been conducted with a large variety of hemoglobins [see Scott and Friedman (1984) and references cited therein] and other heme proteins (Alden & Ondrias, 1985; Findsen & Ondrias, 1984). It was found that the frequency of the Fe–proximal histidine mode in the photolytic transients of hemoglobin was invariably higher than that of the stable deoxy species. Both the position of ν_{Fe-His} and the rate at which the proximal heme pocket relaxed to a stable deoxy configuration were found to be modulated by pH, allosteric effectors, and protien quaternary structure. Moreover, the frequency of ν_{Fe-His} in both deoxy and transient species was inversely related to the degree of proximal histidine tilt (in its own plane) relative to the heme normal. These effects are absent in Mb and the monomeric hemoglobin of *Glycera dibranchiata* (Carson et al., 1986), neither of which shows any shift (relative to the steady-state deoxy species) in ν_{Fe-His} of their photolytic transients.

The dynamic behavior of the Fe–histidine bond of the dehydrated hemoglobins largely parallels that of fully hydrated, R-state HbCO. The invariance of ν_{Fe-His} as a function of hydration level (from solution to 0.4 g of H_2O/g of protein) strongly suggsts that there is no increase in the degree of proximal "strain" in the metastable photolytic transients (and by inference in the stable liganded species) of dehydrated hemoglobins. At the lowest hydration levels (0.1 g of H_2O/g of protein), ν_{Fe-His} becomes broadened and shifts slightly to lower frequency (from 229 to 227 cm^{-1}), but its position still easily falls within the range of frequencies of solution R-state hemoglobins. Thus, we find no evidence in our data of any significant strain (on the order of that produced by R/T quaternary structural changes) introduced into the proximal heme pocket by protein dehydration. This observation is consistent with the results of Frauenfelder and co-workers (Beece et al., 1980, 1982), who found that while ligand rebinding kinetics in Hb and Mb were strongly influenced by solvent viscosity, the final barrier to heme–CO recombination was not. This barrier most likely originates from the movement of the Fe atom into the heme plane during ligand binding. The results of picosecond time-resolved Raman studies make it extremely unlikely that processes exist that relax the proximal heme pocket geometry on a faster than 1-ns time scale (Findsen et al., 1985). Thus, we are forced to conclude that the proximal heme pocket geometry immediately following photolysis is quite similar in solution and dehydrated HbA.

The dynamics of the relaxation of this metastable geometry are, of course, still an open question. Recent theoretical studies have suggested that the relaxation of ν_{Fe-His} to its equilibrium value is tightly coupled to the movement of an "allosteric core" of the protein involving the F helix and the $\alpha\beta$ subunit interface (Gelin et al., 1983). Thus, the time course and energetics of heme pocket relaxation may be significantly modulated by solvent even though the initial proximal heme pocket geometry apparently is not.

Summary

The resonance Raman data presented here confirm that globin dehydration markedly affects the frequency of ν_{CO} in HbCO. We do not, however, find compelling evidence for significant dehydration effects at the heme itself in equilibrium

HbCO. To the contrary, both heme skeletal modes and ν_{Fe-C} are quite insensitive to protein dehydration although the latter does display some broadening at extreme dehydration levels. Our transient Raman results also reveal no pronounced dehydration effects at the heme. At extreme dehydration levels, some changes in the heme peripheral environment can be inferred. The iron–proximal histidine bond strength immediately subsequent to ligand photolysis is largely independent of hydration level, suggesting that little or no additional proximal strain exists in the liganded, dehydrated samples. We anticipate that the rate of relaxation of the proximal pocket after photolysis will be sensitive to hydration level.

In light of these findings, we conclude the continuous variability of ν_{CO} as a function of HbCO dehydration arises form specific distal effects that are not propagated to the heme itself. Studies are currently under way to accurately determine the geometry of the bound CO within the distal pocket of dehydrated HbCO and to probe the effects of globin dehydration upon the rate of relaxation of the proximal heme pocket subsequent to CO photolysis.

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Registry No. HbCO A, 9072-24-6; heme, 14875-96-8.

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