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Ligation and Quaternary Structure Induced Changes in the Heme Pocket of Hemoglobin: A Transient Resonance Raman Study[†]

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ABSTRACT: The extent to which ligation and quaternary structure modify the heme–heme pocket configuration is determined by generating and analyzing transient resonance Raman spectra from various photolyzed and partially photolyzed hemoglobins (Hb). From small frequency shifts in Raman band I ($\sim 1355\text{ cm}^{-1}$) it is determined that ligation induces a configurational change about the heme. The extent to which ligation modifies the heme pocket is influenced by the quaternary structure. With respect to the structural parameter responsible for variations in the π^* orbital electron density of the porphyrin, the degree of alteration of the heme pocket configuration relative to deoxy-Hb(T) follows the sequence: liganded Hb(R) > liganded Hb(R) + IHP > liganded

Hb(T) [α chain > β chain] > deoxy-Hb(R). This progression of configurations also forms a sequence with respect to the “retentiveness” of the heme pocket as reflected in the ligand dynamics associated with geminate recombination. The results indicate that the heme–heme pocket of the R-state Hb’s, relative to those of the T-state species, favors ligand retention in a dynamic, as well as thermodynamic, sense. The analysis of these and other related data implicates a ligation and quaternary structure modulated electronic and/or electrostatic interaction between the π system of the porphyrin and the surrounding heme pocket as the basis for this variation in ligand dynamics as well as for the energetics of cooperativity.

The binding of ligands to the hemes in Hb¹ is a highly localized perturbation that initiates a sequence of propagating structural events that culminates in a change in quaternary structure from the low-affinity deoxy T state to the high-affinity liganded R state (Perutz, 1970). Although these two equilibrium species have been well characterized, both the mechanism by which ligation triggers structural destabilization and the structural basis for the dynamics remain as yet undetermined. Moreover, these questions are interdependent—the dynamics being influenced by ligation-sensitive structures surrounding the heme. Understandably, it is of fundamental interest to probe those ligation-dependent interactions that affect the stability of the heme environment (heme pocket) and to establish the relationship between structural variations of this environment and kinetic phenomena.

In the equilibrium structures of Hb the functionally relevant energies associated with the coupling of ligation and quaternary structure need not be localized near the heme. However, in metastable species, generated immediately after ligation or deligation, these energies must be manifested, at least transiently at the interface between the binding site and the surrounding protein. Hence, appropriate time-resolved studies of these metastable species would enhance the likelihood of detecting these hitherto unidentified physiologically important heme–protein interactions. Photolysis of liganded Hb’s has been used extensively to prepare nonequilibrium populations

of Hb. In turn, transient absorption spectroscopy has yielded extensive data on the kinetics of these nonequilibrium species. Such studies (Shank et al., 1976; Noe et al., 1978; Reynolds et al., 1981; Greene et al., 1978; Chernoff et al., 1980) have shown that photodissociation and the subsequent recovery to the ground electronic state of deoxyheme occur on a picosecond time scale. However, transient absorption studies (Chernoff et al., 1980; Sawicki & Gibson, 1974; Lindquist et al., 1980) also reveal differences between the spectrum of the stable T-state deoxy-Hb and that of the photolyzed species. In all likelihood, differences in spectra observed on these time scales originate from environmental differences about the porphyrin chromophore. Absorption spectroscopy, unfortunately, cannot be used to determine the structural origins of these interactions because of the numerous potential contributions to a porphyrin absorption spectrum. In contrast, resonance Raman spectroscopy [for a detailed review, see Spiro (1981) and Asher (1981)] provides a more suitable probe of localized structure.

It has been established by Raman difference spectroscopy (Shelnutt et al., 1979a,b; Rousseau et al., 1981) that resonance Raman scattering is well suited as a probe of heme–protein interactions. Raman band I, which occurs at $\sim 1355\text{ cm}^{-1}$ for deoxyhememes and in the 1370s for liganded ferrous hememes, has been shown to display a sensitivity in its frequency (for deoxyheme) to variations in the π^* -electron distribution originating from perturbations occurring either through the iron

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¹ Abbreviations: Hb, hemoglobin; Mb, myoglobin; HbCO, carboxy-hemoglobin; HbNO, nitrosylhemoglobin; HbO₂, oxyhemoglobin; Hb(x), hemoglobin with the x (R or T) quaternary structure; Bis-Tris, 2,2-bis(hydroxymethyl)-2,2',2''-nitrotriethanol.

(Spiro & Burke, 1976) or through the periphery of the porphyrin (Tsubaki et al., 1980). The Raman difference studies of the small frequency shifts in band I for a variety of stable R and T deoxy-Hb's have revealed that the frequency of Raman band I is correlated without exception to quaternary structure stability for deoxy-Hb's.

In previous transient Raman studies it was observed (Friedman & Lyons, 1980) that, relative to its frequency in T-state deoxy-Hb, the frequency of band I is lower by at least 2 cm^{-1} for the metastable deligated species occurring within 10 ns of the photolysis of HbCO. A subsequent pulse probe Raman experiment (Lyons & Friedman, 1981) revealed that for photolyzed (10 ns) HbCO there are two distinct time scales associated with the relaxation of the shifted band I: a deligation-induced tertiary structure relaxation on an $\sim 1\text{ }\mu\text{s}$ time scale and a tertiary structure relaxation on a longer time scale that is associated with the change in quaternary structure. These transient Raman results help delineate the time scales associated with the R-T switch and the structural changes about the heme.

In view of the potential importance of band I, we have examined the role played by ligation and quaternary structure in determining its frequency. Because changes in the state of ligation or of oxidation produce large shifts in the Raman spectrum of the heme, it is necessary to maintain the hemes in identical states in order to compare the more subtle effects of protein environments. These comparisons are made by generating and comparing the resonance Raman spectrum of stable deoxy-Hb's and metastable deligated Hb's produced within 10 ns after photodissociating a particular liganded species. Since the electronic state relaxation subsequent to photodissociation occurs on a picosecond time scale (Shank et al., 1976; Noe et al., 1978; Reynolds et al., 1981; Greene et al., 1978; Chernoff et al., 1980) and the structure of the protein does not respond to deligation until well after 10 ns (Lindquist et al., 1980; Lyons & Friedman, 1981; Terner et al., 1980, 1981), we are comparing the structure of two electronically relaxed deligated (deoxy) hemes; in one case, the structure of the surrounding protein is at equilibrium whereas in the other, the structure of the surrounding protein is still that characteristic of a liganded heme and is thus metastable. Differences in the two spectra can reflect both transient and static differences in the environments of the deligated hemes.

Materials and Methods

Transient resonance Raman spectra were generated by using the output of a nitrogen laser (Molelectron UV 24) pumped dye laser operating at 10 Hz. For those experiments in which both high yields of photolysis and maximum resonant enhancement of Raman band I were required, the excitation frequency was chosen to be in the vicinity of $4200\text{ }\text{\AA}$ (Bis MSB) whereas $4800\text{ }\text{\AA}$ (coumarin 481) was used in the HbNO experiments that required resonant enhancement of Raman band VI ($\sim 1640\text{ cm}^{-1}$). The excitation light was focused (a 500- or an 800-mm focal length lens for $4200\text{ }\text{\AA}$ and a 25-mm lens for $4800\text{ }\text{\AA}$) into a temperature-controlled cuvette maintained at 2°C . No difference in spectra was observed between static or recirculated samples. The 90° scattered light was collected by an off-axis elliptical mirror and dispersed by 1-m F/8 J. Y. Ramanor HG-23 spectrometer fitted with an RCA C31034-02HQ photomultiplier tube. The output of the photomultiplier tube was gated (1 ns) and averaged with a PAR no. 163 box car integrator. The resulting signal was stored and processed with a Nicolet 1174 signal averager, which allowed for repetitive scans of a given spectrum (backlash $<0.2\text{ cm}^{-1}$).

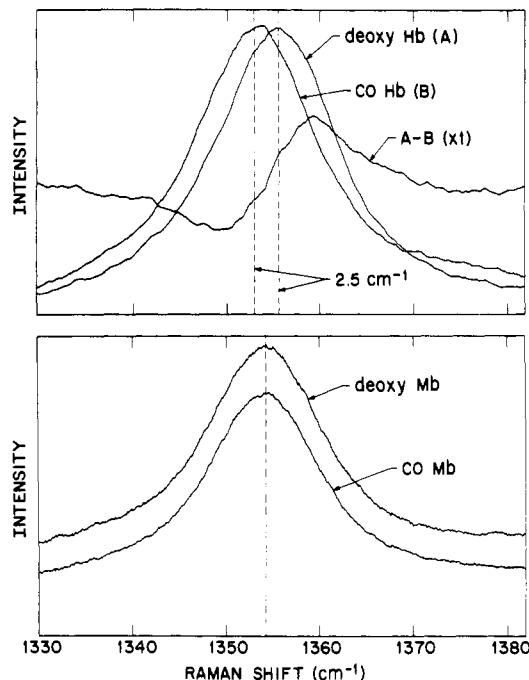


FIGURE 1: Comparison between Hb (human adult) and Mb (whale) with respect to the shifting of Raman band I in going from the stable deoxy form to the metastable species occurring within 10 ns of the photolysis of the corresponding carboxy form.

Spectrometer and laser drift were minimized by maintaining a nearly constant room temperature. Drift was checked for by recording a reference spectrum (same frequency region) before and after each transient spectrum. Peak positions and amplitudes for band I were determined by performing a nonlinear least-squares analysis for a given spectrum over the spectral region between 1300 and 1400 cm^{-1} . The sensitivity of this technique with respect to measuring small frequency differences between different Hb's was checked by comparing frequency differences of stable Hb's obtained by this approach with those obtained by Raman difference spectroscopy (Shelnutt et al., 1979b; Rousseau et al., 1981). The frequency difference agreed to within 0.2 cm^{-1} for each of the three comparisons made (deoxy-Hb vs. deoxy- β^{SH} , deoxy- α^{SH} , and deoxy-Mb).

Results

Effect of Ligation: Hb vs. Mb. The steady-state resonance Raman spectra of the liganded and deoxy forms of Hb and Mb reveal that the electronic response of the porphyrin to ligation is very nearly identical for these two proteins. Consequently, for these two proteins, a comparison of the frequency shifts of Raman band I between the deoxy form and the metastable species ($\leq 10\text{ ns}$ after photolysis) should provide a direct measure of the responsiveness of the structure of the respective heme pockets to ligation for Hb and Mb. Figure 1 depicts the results of such a spectral comparison. In the upper portion of the figure is seen the previously noted difference between deoxy-Hb and photolyzed HbCO. Also shown is the computer-subtracted difference between the two bands. Both a nonlinear least-squares analysis and a computation based on the shape of the difference curve (Rousseau, 1981) reveal that the Raman band I of the metastable species is 2.5 cm^{-1} lower in frequency than that of the stable deoxy form. The lower portion of the figure reveals that there is a much smaller shift between the corresponding two spectra in Mb. The nonlinear least-squares analysis indicates that there is a barely detectable ($\approx 0.25\text{ cm}^{-1}$) shift for Mb that is in the same

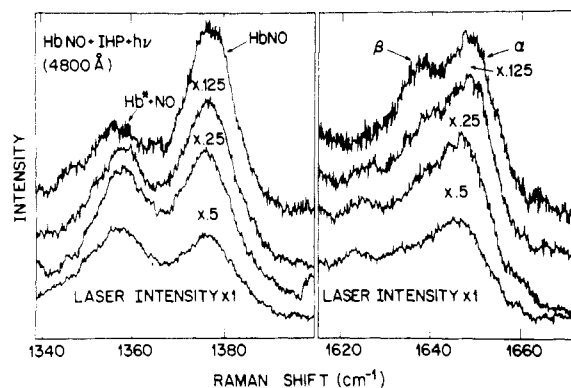


FIGURE 2: Transient resonance Raman spectra of partially photolyzed HbNO(T) are presented in order of decreasing laser intensity from top to bottom. For each excitation intensity, Raman band I (~ 1353 cm^{-1}) for the photolyzed unliganded Hb* and ~ 1375 cm^{-1} for the unphotolyzed HbNO is shown on the left, and the subunit-specific doublet associated with HbNO(T) is shown on the right. Band I is used to monitor the yield of unliganded heme generated by the single 10-ns excitation pulse while the spectra on the right indicate the chain-specific parentage of the remaining liganded hemes. The excitation source (which also generates the Raman spectra) is at 4800 Å (coumarin 481). The number of scans for each of the above spectra was varied in order to maintain approximately constant signal to noise for each intensity setting. The pH 6.5 Bis-Tris buffered HbNO solution (50 μM heme, 1 mM IHP) was prepared by flushing and evacuating an O_2 -free fresh HbCO solution with NO gas that has been washed in a concentrated KOH solution.

direction as for Hb. To determine whether the reduced response in Mb is related to it being monomeric and/or noncooperative, we generated a similar set of spectra from the separated and isolated subunits of Hb. Both the isolated β^{SH} chains (tetrameric and noncooperative) and α^{SH} chains (monomeric and noncooperative) manifest a 1.6-cm^{-1} shift to lower frequency in a comparison of the deoxy form and the metastable species.

Influence of Quaternary Structure. In the previous section a comparison was made between deoxy-Hb that has the T structure and photolyzed HbCO that has the R structure; therefore, that result does not determine the extent to which the shift in band I is a result of ligation or of change in quaternary structure. To resolve this question, we generated band I and analyzed it from both R and T photolyzed liganded Hb's. In order to obtain a frequency from a T-state photolyzed carboxy species, it was necessary to use a mutant Hb, Hb_{Kansas} + IHP (pH 6.5). It was observed that for photolyzed carboxy-Hb_{Kansas}(T), band I is $\sim 0.6\text{ cm}^{-1}$ higher in frequency than for photolyzed HbCO(R). Other R-state carboxy-Hb's derived from Hb_{Kempsey} (pH 6.5 and 9) and Hb_{Kansas} (pH 9) yielded the same frequency as photolyzed HbCO (pH 6.5 and 9). The addition of IHP to HbCO shifted band I a few tenths of an inverse centimeter to higher frequency.

HbNO is of particular interest in this study because unlike HbCO or HbO₂ it can provide a stable isolated population that has either the R or the T structure (Perutz et al., 1976) without having to resort to mutant or chemically modified forms of Hb. An R vs. T comparison of the Raman spectrum of the metastable species occurring within 10 ns of photolysis of HbNO reveals that over a wide range of pulse powers (20 μJ –0.5 mJ) and frequencies (4100–4800 Å), the T-state HbNO exhibits a significantly greater amount of deliganded heme. Moreover, a chain specificity may be demonstrated in the photolysis of HbNO.

It has been shown (Nagai et al., 1980) that in T-state HbNO, the high- and low-frequency components of the split Raman band VI (1637 and 1645 cm^{-1}) are assignable to the

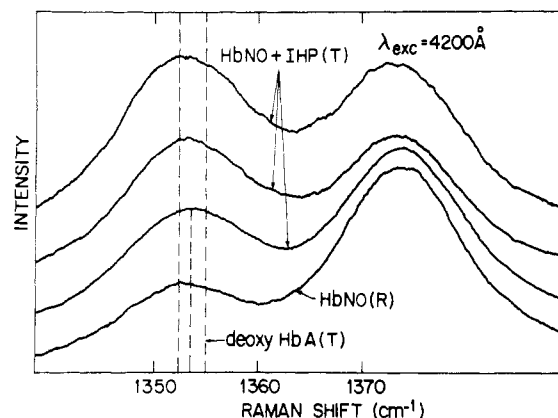


FIGURE 3: Representative transient (10-ns) Raman spectra (band I) used in the preparation of Figure 4. Similar experimental conditions as in Figure 2 except for the focusing optics which now consists of an 800-mm focal length lens and for the excitation frequency which has been changed to 4200 Å (Bis-MSB) for increased resonance enhancement of band I.

nitrosylhemes of the β and α chains, respectively. Using this information, it is possible to monitor the chain-specific populations of NO hemes as a function of photolysis. The results of such an experiment are shown in Figure 2. The doublet on the left is band I; the higher frequency ($\sim 1375\text{-cm}^{-1}$) component is from the liganded hemes while the lower frequency ($\sim 1355\text{-cm}^{-1}$) component is from the deliganded hemes. The Raman spectrum on the right is the chain-specific Raman doublet discussed above. A given spectrum on the left provides a measurement of the extent of photolysis while the matching spectrum on the right yields the identities of the remaining liganded chains. Starting at the top, where it is observed that there is already a small amount of photolysis, both α and β peaks are evident. As the amount of photolysis increases, the peak associated with the liganded β heme disappears first to the extent that it appears possible to generate a T-state $\alpha^{\text{NO}}\beta^{\text{deoxy}}$ hybrid by photolysis. It is also observed that under the corresponding range of excitation conditions, the R-state HbNO undergoes considerably less photolysis.

Chain-specific photolysis makes it possible, by monitoring the shift in band I as a function of percent photolysis, to establish whether there are chain-specific responses to ligation at the level of the heme-heme pocket interaction. Although the frequency of Raman band I associated with the photolyzed hemes of R-state HbNO is shifted to lower values² ($\sim 3\text{ cm}^{-1}$) with respect to deoxy-Hb, there is minimal variation in the peak position as a function of the amount of photolysis. However, T-state HbNO displays a progressively larger shift toward lower frequencies with increasing photolysis, which indicates that the frequency of band I for the photolyzed β subunit is higher than that of the α subunit. Representative spectra are shown in Figure 3. To extract accurate values for the shifts, it was necessary to deconvolute the contribution of the liganded peak to the band shape by using a nonlinear least-squares curve-fitting procedure. The analysis reveals that the R vs. T results for photolyzed HbNO are qualitatively similar to those obtained for the HbCO study. However, the shift to lower frequency for photolyzed HbNO(R) relative to deoxy-Hb is 0.6 cm^{-1} larger than that for photolyzed HbCO. For the T-state HbNO, the photolyzed β hemes have a higher

² In these studies it was not possible to photolyze the R-state HbNO fully; consequently, it is possible but unlikely that the difference between HbNO and HbCO is related to the incomplete photolysis. Photolyzed HbCO and HbO₂ at pH 6.5 and 9 appear to have the identical frequency.

frequency than the composite species in which both α and β subunits are photolyzed.

Discussion

Response of the Heme Pocket to Ligation and Quaternary Structure. The shifts in the π electron sensitive Raman frequencies that we observe are small ($\leq 3 \text{ cm}^{-1}$). However, because of the stability of the distribution of π electrons in the porphyrin ring, it is anticipated that even major changes in the heme environment might produce only small alterations in the electronic makeup of the porphyrin. Thus small shifts in the frequency of the π -sensitive Raman lines could reflect major changes in the contacts and interactions between the heme and its environment. As a first step in determining whether in fact these shifts are a measure of functionally relevant structural changes, we examine whether there is a systematic variation in frequency with both ligation and quaternary structure.

A pattern associated with the shifts of band I with respect to ligation and quaternary structure can be ascertained from a comparison of the above transient Raman results with those obtained from deoxy-Hb's. Qualitatively, the R-T differences observed for the liganded species are mimicked in the deoxy forms. A Raman difference study (Shelnutt et al., 1979b; Rousseau et al., 1981) of an extensive series of deoxy-Hb's including chemically modified forms that are stable in the R-state configuration revealed that the π marker bands for the R-state deoxy-Hb's have lower frequencies ($\leq 1.6 \text{ cm}^{-1}$ for band I) compared to those for the T state. A comparable difference was detected between deoxy-Hb and the metastable species occurring after a 1- μ s relaxation subsequent to the photolysis of HbCO (Lyons & Friedman, 1981). This metastable species, which still has an R quaternary structure but has undergone a local structural relaxation in the heme pocket, can be thought of a quasi-stable deoxy-Hb(R). Hence, it is not surprising that the frequency of Raman band I for this quasi-stable deoxy-Hb is shifted from that of deoxy-Hb by the same amount as in the stable but chemically modified NES-des-Arg-deoxy-Hb, which also has the R quaternary structure (Shelnutt et al., 1979b; Rousseau et al., 1981).

These data are represented schematically in Figure 4. The vertical axis represents the frequency with respect to deoxy-Hb(T). Descending the vertical axis corresponds to a lowering of the frequency of band I. The left side of the figure contains the T-state Hb's whereas the right side contains the R-state Hb's along with some monomeric species. A comparison of the right and left sides of Figure 4 reveals the differing response of Hb to ligation as a function of quaternary structure. In every instance examined [including Hb_{Kempsey} at pH 6.5 and 9 (S. R. Simon, M. Ondrias, D. L. Rousseau, and J. M. Friedman, unpublished results) and Hb_{Kansas}(R), which are not shown in the figure], the metastable deoxyheme associated with the photolyzed R quaternary structure exhibits a larger shift to lower frequency than those of the corresponding T structure. Addition of IHP to HbCO(R) resulted in a small shift toward the regime associated with the metastable T structures. In each instance the porphyrin is assumed to be in the same zero-order unliganded state; therefore, the ordering with respect to the decreasing frequency of band I (deoxy T-deoxy R-liganded T-liganded R) suggests that a single parameter may be used to characterize the heme-heme pocket response to both ligation and quaternary structure change. From these data, it follows that with respect to this parameter (i.e., the structural determinant of the frequency shifts of band I) the heme environment in Mb is considerably more rigid than in either the Hb's or the isolated α and β chains. These

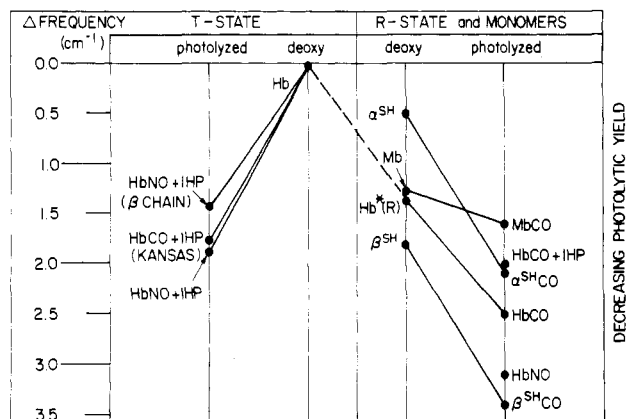


FIGURE 4: The points along the vertical lines represent the relative frequencies of Raman band I for deligated (deoxy) ferrous hemes in a variety of stable and metastable protein configurations. The frequencies are referenced to that of deoxy-Hb(T) (1355 cm^{-1}). Points below the zero indicate lower frequencies (e.g., $2.0 = 1355 - 2.0 \text{ cm}^{-1}$). The points designating liganded proteins such as HbCO and HBNO refer to the frequency associated with the deoxyheme in the metastable protein occurring within 10 ns of photolysis of the corresponding liganded Hb. Protein configurations that decrease the frequency of deoxyheme band I are associated with porphyrin macrocycles that have a higher π^* -electron density. Not shown are the points for photolyzed Hb_{Kansas}CO(R) and Hb_{Kempsey}CO(R), both of which occur within 0.1 cm^{-1} of HbCO. The Mb is from the skeletal muscle of whale. Except for the Hb_{Kansas} ($>100 \mu\text{M}$), the heme concentrations for the above solutions were $\approx 50 \mu\text{M}$. The frequency used for deoxy-Hb in the R state [Hb(R)] is derived from two independent experiments. One measurement, derived from a Raman difference study (Shelnutt et al., 1979b; Rousseau et al., 1981), is associated with NES-des-Arg-deoxy-Hb(R), a chemically modified Hb that remains stabilized in the R structure when deoxygenated. This same frequency is observed (Lyons & Friedman, 1981) for the transient deoxy-Hb species occurring a few microseconds after photodissociating HbCO with a 10-ns pulse. At this temporal point, the heme pocket has undergone a deligation-related relaxation ($T \approx 1 \mu\text{s}$), leaving the system in a deoxy R-state configuration, which subsequently decays ($T \gg 1 \mu\text{s}$) to the stable deoxy T structure.

findings suggest that the structural responsiveness of Hb to ligation originates from the inherent responsiveness of the configuration of the heme pocket within the subunits that comprise the tetramer. However, on the basis of Figure 4, the specific range of accessible configurations (heme pocket) for a deoxy or a liganded species is determined by the overall quaternary structure.

Relationship between Structure and Dynamics: Geminate Recombination.³ If each frequency point on the vertical axis of Figure 4 does indeed represent a different configuration of the heme-heme pocket unit, then those dynamic processes that depend upon this configuration might be expected to systematically vary or respond to changes in the frequency of band I. Geminate recombination is one such dynamic process that involves the ligand dynamics that are dictated by the innermost barriers associated with the heme and the heme pocket. The decrease in geminate recombination with increasing temperature observed in previous works (Friedman & Lyons, 1980; Duddell et al., 1979) suggests that for a given ligand, it is the barrier [for a full discussion of these barriers, see Frauenfelder (1981)] controlling the escape from the heme pocket rather than recombination which determines the rate associated with geminate recombination in the temperature range between 0 and 40°C . Furthermore, the variation with protein structure of the quantum yield of photolysis for a given ligand at a given

³ Geminate recombination is a protein cage effect in which the photolyzed ligand rebinds instead of diffusing into the solvent.

temperature is a result of differences in the amount of geminate recombination (Duddell et al., 1980; Friedman & Lyons, 1980).

An examination of Figure 4 reveals a striking correlation between geminate recombination and the frequency of band I for a photolyzed species. If, for a given ligand, the yield of geminate recombination is compared for two different metastable species, it is found that the protein exhibiting the lower frequency for band I invariably has the higher yield of geminate recombination (lower quantum yield of photolysis). For example, the quantum yield of photolysis (Sawicki & Gibson, 1979; Saffron & Gibson, 1977) as well as the observed yield of geminate recombination (Friedman & Lyons, 1980; Duddell et al., 1979) for R-state HbCO is ~50% at room temperature. However, as the population becomes more T-like either by the addition of IHP (Saffron & Gibson, 1977) or by reducing the concentration of CO (Sawicki & Gibson, 1979), the quantum yield of photolysis increases (~100% for T-state HbCO) (Sawicki & Gibson, 1979). In Figure 4 it is seen that these changes are mirrored in the corresponding sequence of increasing frequencies of band I in going from HbCO(R) to HbCO + IHP to HbCO(T). It follows that in contrast to the T-state configurations, *the more R-like heme-heme pocket configurations favor ligand retention*. Similarly, it is seen from this study that in going from HbNO(R) to the α chain of HbNO(T) to the β chain of HbNO(T) the frequency of band I increases while the yield of photolysis increases.⁴ To the extent that spontaneous deligation can be considered an adiabatic process with respect to the response of the heme-heme pocket configuration, these geminate recombination results have a direct and important bearing on the structural origin of the macroscopic off rates.

Structure and Raman Band I: Implications for Cooperativity. A full understanding of the relationship between structure and frequency for band I is of importance not only because of the connection to geminate recombination but also because of the link to cooperativity. In many of the numerous X-ray crystallographic studies of these heme proteins, there have been attempts to correlate the observed stereochemistry with function. It is of interest that the magnitude of the shift of band I can be readily correlated with the observed change in tertiary protein structure between liganded and the relaxed deoxygenated forms of the different species. Despite these correlations it is still quite difficult from the X-ray work to identify the specific stereochemical features responsible for the shifts in band I. This difficulty is in part due to the fact that band I is sensitive to changes in the electron density of the heme whereas the X-ray studies focus on the average nuclear coordinates. Thus it is important to consider how the various known stereochemical changes in the heme pocket affect the electron density of heme. For instance, it is known that in myoglobin the heme propionates form strong hydrogen bonds with basic groups of the protein, which make the heme pocket rigid. Changes in tertiary structure on ligand binding are much smaller than in hemoglobin [compare changes in myoglobin described by Takano (1977) with those pictured by Baldwin & Chothia (1979) in hemoglobin]. Similarly, the exaggerated shift of band I in HbNO might reflect an exaggerated R-type tertiary structure that is a result of the weakened or lengthened Fe-N_e bond of the nitrosylhemes

(Scheidt, 1977). Given the traditional emphasis placed on the role of the position and the motion of the heme iron in determining Hb reactivity and cooperativity, it is reasonable to consider whether variation in the displacement of the iron or in Fe-His bonding interactions might be responsible for the shifts in band I. It has been shown (Shelnutt et al., 1979b; Rousseau et al., 1981) that the frequency of the anomalously polarized porphyrin core size Raman marker band (~1555 cm⁻¹ for deoxy-Hb) does not shift under circumstances that result in major shifts in band I. Furthermore, the response times of the two Raman bands subsequent to photolysis differ by at least a factor of 10 (T. G. Spiro, private communication), which indicates that the two bands are not directly linked together. Since the frequency of this core-size marker band can be related (Spaulding et al., 1975; Warshel, 1977) to the displacement of iron from the porphyrin plane, movement of the iron cannot be readily invoked to account for the shifts in band I. More recently it has been suggested (Baldwin & Chothia, 1979) that the variation in the tilt of the proximal histidine with respect to the plane of the porphyrin is responsible for localization of the free energy of cooperativity at the iron-histidine bond. It has been shown (Ondrias et al., 1982) that the shift in frequency (Raman) of the iron-histidine stretch correlates with the shifts in Raman band I in a considerable number of deoxy-Hb derivatives. This correlation appears to extend to photolyzed R and T species as well (J. M. Friedman, unpublished results). However, in frozen solutions of Hb it is observed (Ondrias et al., 1981) that the Fe-His stretching frequency is significantly modified whereas that of band I is not. Thus while there is a common mechanism linking the shifts of the two Raman bands, the decoupling observed upon freezing indicates that the change in porphyrin π^* density as reflected in band I may not be caused by changes at the iron-proximal histidine bond. Nonetheless, until there is a comparable study involving the temperature dependence of the ligand affinities, it is not possible to state whether one or both of these Raman bands are directly linked to the thermodynamics of ligation.

The inability to clearly assign the frequency shifts of band I to these specific structural changes at the heme leads to the consideration of a general reorganization of the heme-heme pocket configuration. Tilting of the porphyrin with respect to the protein has been considered theoretically (Gelin & Karplus, 1977) and has been observed experimentally (Makinen & Eaton, 1974; Makinen, 1975; Pulsinelli, 1973; Anderson, 1973; Heidner et al., 1976; Moffat et al., 1979). Of course other modifications within the heme pocket in response to ligation and change in quaternary structure have also been observed (Pulsinelli, 1973; Anderson, 1973; Heidner et al., 1976; Moffat et al., 1979). The magnitude of these changes seems to scale with the shifts in band I. For example, upon ligation the heme in Mb tilts considerably less than in Hb (Makinen, 1975), and the T-state heme appears more constrained with respect to tilting than in the R state (Pulsinelli, 1973; Anderson, 1973). These reduced responses of Mb and T-state Hb are mimicked in the response of band I as seen in Figure 4. The tilt angle of the porphyrin with respect to the amino acids lining the heme pocket could be the structural parameter responsible for the correlation between the frequency of the Fe-His stretch and the π^* -sensitive Raman band I. The change in the porphyrin contacts upon tilting can account for a redistribution of π density in the porphyrin ring. Similarly, the tilt of ring would perturb the Fe-His bond to a degree modulated by the rigidity of the F helix, which is quaternary structure sensitive. Although further elaboration

⁴ The large protein structure dependent variation in the time-averaged yield of photolysis at 10 ns for the HbNO system indicates that NO like O₂ but unlike CO (Chernoff et al., 1980) undergoes a subnanosecond geminate recombination.

of these specific structural details will require additional experiments such as time-resolved UV resonance Raman studies involving the amino acids, the data shown in Figure 4 provide some insight into the interactions responsible for the coupling of ligation to structural change, i.e., the trigger for the R-T switch.

Evidence for an Electronic Trigger. Traditionally, gross molecular movements have been the basis for models describing how ligation induces the structural rearrangements leading to a change in quaternary structure. Together, the steady-state (Shelnutt et al., 1979b; Rousseau et al., 1981) and the transient studies (Lyons & Friedman, 1981; Friedman & Lyons, 1980) indicate that the frequency shifts of band I are related to ligation-sensitive interactions that trigger or initiate the destabilization of a quaternary structure.

An intriguing mechanism by which ligation could engender these delocalized responses is suggested by the direction of the shifts in band I upon ligation and the observed electronic rearrangements that occur upon ligation. It is clear, from the shift in resonance Raman spectra (same as oxidation), that ligation induces a reorganization of π density that leaves the π^* orbital deficient of negative charge with respect to the deoxyheme (Shelnutt et al., 1979b; Rousseau et al., 1981; Warshel & Weiss, 1981). Because of the time scales (picoseconds) associated with the electronic relaxation of the photodissociative state (Shank, 1976; Noe et al., 1978; Reynolds et al., 1981; Greene et al., 1978; Chernoff et al., 1980) and the much longer time scale for the relaxation of the protein structure of the photolyzed liganded Hb (Sawicki & Gibson, 1974; Lindquist et al., 1980; Lyons & Friedman, 1981), the points on the vertical axis of Figure 4 represent a "solvation" effect of the particular liganded protein structure upon the π system of a relaxed deoxy iron porphyrin macrocycle. All the points associated with the photolyzed liganded proteins are at a lower frequency than those of the relaxed deoxy species, indicating that the heme-heme pocket configuration of the photolyzed liganded Hb's results in higher π^* -electron densities on the deliganded porphyrin than for any of the relaxed or equilibrium deoxy proteins. Therefore, relative to the deoxy proteins, these ligation-induced configurations might tend to stabilize the electron-deficient π^* systems of the liganded hemes. The lower the frequency of band I (the lower the point on the vertical axis), the greater the electronic stabilization of the liganded heme by the heme pocket. From this analysis and from Figure 4, it is apparent that the influence of the quaternary structure upon the configurational response of the heme pocket to ligation results in greater stabilization of the R-state liganded heme. If the absolute π^* -electron density of the deoxyheme is sufficiently high, then configurations falling on the extreme upper portion of Figure 4 should contribute to the stabilization of the deoxyheme-heme pocket. The lower ligand affinity of T-state Hb would then arise not only from decreased stabilization of the liganded heme but also from a protein (deoxy T)-induced stabilization of the electronic structure of the unliganded ferrous heme. As has been suggested earlier in connection with the electronic interaction model (Shelnutt et al., 1979b; Rousseau et al., 1981) and the more recent electrostatic model (Warshel & Weiss, 1981), this ligation- or deligation-induced change in electronic density in the porphyrin macrocycle should destabilize the heme-heme pocket configuration, hence acting as a trigger for tertiary changes localized about the heme. As the heme pocket relaxes, there should be a concurrent change in the properties of the effective barrier (height, distribution, or fluctuations), limiting the conversion between quaternary

structures and affecting the R-T switching times in response to the degree of ligation.

Conclusions. The findings presented in this paper indicate that there is an induced fit response to ligation at the binding site. The degree of response is sensitive to the particular protein (Hb or Mb) and the quaternary structure. It is observed that the ligation-sensitive heme-heme pocket configurations are related both to the stability of the quaternary structure and to the fast religation dynamics subsequent to photolysis. These results suggest that the extreme heme-heme pocket configuration of the liganded R-state protein favors ligand retention both thermodynamically and kinetically, whereas the corresponding T-state configuration favors escape of the ligand from the heme pocket. The structural changes associated with these configuration-related properties do not appear to be highly localized within any one bond; instead, they appear to originate from an interaction between the ligand responsive π system of the porphyrin and the R-T sensitive arrangement of amino acids surrounding the porphyrin.

Added in Proof

We recently concluded a detailed transient Raman study of the behavior of the Fe-His (F8) stretching mode in an extensive series of photolyzed Hb's. Our findings in conjunction with results from stable deoxy species (Ondrias et al., 1982) indicate that the inverse correlation between the frequency of Raman band I and the frequency of the Fe-His stretching mode is far more pervasive and certain than originally thought. We also observe that in photolyzed Hb's the time evolution is the same for the two Raman bands, which makes it almost certain that the π^* -electron density of the porphyrin is being modulated by perturbations involving the proximal histidine.

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Complete Amino Acid Sequence of Porcine Heart Citrate Synthase[†]

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ABSTRACT: The detailed proof of the 437-residue amino acid sequence (M_r 48 969) of porcine heart citrate synthase (EC 4.1.3.7) is described. The S-carboxymethylated protein has been cleaved at methionine (cyanogen bromide) and arginine (trypsin digest of citraconylated enzyme) residues to yield 14 and 17 major peptides, respectively. Peptides were initially fractionated by gel filtration, and those useful for sequence analysis were purified by high-performance liquid chroma-

tography. Sequence analyses were performed on these primary peptides and on subpeptides generated by cleavage with the bromine adduct of 2-[(2-nitrophenyl)sulfonyl]-3-methylindole, *Staphylococcus aureus* V8 protease, trypsin, chymotrypsin, or acid. The overall sequence was confirmed by analyzing products of cleavage by hydroxylamine, acid, and subtilisin. A novel feature of the sequence is the identification of trimethyllysine at residue 368.

Citrate synthase [citrate oxaloacetate-lyase [coenzyme A (CoA)¹ acetylating]; EC 4.1.3.7] catalyzes the aldol-type condensation of oxaloacetate and acetyl-CoA. The analysis of this enzyme is closely linked to landmarks in the history of biochemical studies such as the discovery of the cyclic nature of the citric acid cycle [reviewed by Ochoa (1954)] and the identification of "active acetate" as acetyl-CoA (Lynen & Reichert, 1951; Lynen et al., 1951; Stern et al., 1951). The

enzyme was first isolated in crystalline form from pig heart by Ochoa and his collaborators (Stern et al., 1950; Ochoa et al., 1951) and has subsequently been purified from a wide variety of sources (Weitzman & Danson, 1976). The most common form of the enzyme has a molecular mass of 90 000-100 000 and is made up of two identical subunits (Singh et al., 1970; Srere, 1975). The stereochemical course of the reaction mechanism has been elucidated in a number of elegant studies which have been comprehensively reviewed (Spector, 1972; Srere, 1975). In addition, enzyme activity is

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¹ Abbreviations: BNPS-skatole, bromine adduct of 2-[(2-nitrophenyl)sulfonyl]-3-methylindole; CM, S-carboxymethyl; HPLC, high-performance liquid chromatography; NaDodSO₄, sodium dodecyl sulfate; Tos-LysCH₂Cl, 1-1-chloro-3-(tosylamido)-7-amino-2-heptanone; Pth, phenylthiohydantoin; CoA, coenzyme A.