RESONANCE RAMAN SPECTRA OF PHOTODISSOCIATED HEMOGLOBINS

Implications on Cooperative Mechanisms

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ABSTRACT Resonance Raman spectra at cryogenic temperatures of photodissociated hemoglobins and the corresponding deoxygenated preparations are compared and significant differences are found in modes with contributions from peripheral substituents of the heme as well as in the iron-histidine stretching mode. These differences in heme vibrational spectra reflect differences in the tertiary structure of the heme pocket between deoxyhemoglobin and the CO-bound form. An analysis of the effects of cooperative energy storage on the tertiary structure around the heme is made and used to interpret this resonance Raman data. The differences between the spectra of the deoxygenated preparations and the photoproducts provide evidence that a fraction of the free energy of cooperativity, ΔG, is located away from the heme. These data support models for cooperativity in which the cooperative energy is distributed over many bonds or is localized in protein bonds only, such as those at the subunit interface. In addition, the local changes in amino acid positions, which must occur following the change in the state of ligand binding, may drive the changes in the structural relationships of the subunits and hence form one of the initial steps for triggering the quaternary structure transition.

INTRODUCTION

The molecular mechanism of the control of oxygen binding in hemoglobin has been a topic of extensive study and much controversy. Many physical and biochemical techniques have been applied to the problem (1) and yet the pathway that links ligand binding at a heme to the subunit interface is unresolved and the location of the free energy of cooperativity, ΔG, the energy difference for binding oxygen in the high affinity (R) and the low affinity (T) structure has not been found. Some have proposed that ΔG is localized in a few bonds at the heme (2), while others have proposed that the energy resides in the protein either at the subunit interface (3) or is distributed over many bonds (4–6). No definitive experiments have been reported to clarify this question. Not only is this problem essential to solve in order to settle the question of the molecular basis for hemoglobin function, but the principles learned may apply to some of the many other biologically important heme proteins and may also relate to the general question of protein control of active site properties in enzymes.

The technique of resonance Raman scattering has been applied by many workers to the study hemoglobin (7, 8) and other heme proteins (9, 10). The basic properties of the heme Raman spectrum are becoming well characterized and several types of studies have been reported that relate directly to hemoglobin energetics (8). These include quaternary–structure-dependent studies of deoxy hemoglobins (11), studies of the protein effect on the ligand vibrational modes in six coordinate hemoglobins (12–14), determination of the time evolution of the resonance Raman spectra of transients following photodissociation of a photolabile ligand (15), and investigation of low-temperature metastable forms generated by ligand photodissociation (16). In spite of these many studies, the central question concerning the location of the free energy of cooperativity has not been answered. Here we present new data from low-temperature photodissociated forms of hemoglobin. We consider these results in the context of models for cooperative interactions in which the free energy is localized at the heme and those in which it resides in bonds of the protein. We find positive evidence for a contribution to the free energy of cooperativity delocalized from the heme.

In resonance Raman scattering, light is scattered from a chromophore yielding its vibrational frequencies (17). Thus, for heme proteins, by selecting laser frequencies that coincide with the optical transitions of the heme group, a very intense Raman spectrum of the porphyrin macrocycle and its associated ligands is obtained. The vibrational modes of the polypeptide do not appear in this resonance Raman spectrum because the optical absorption bands of the amino acids of the protein occur at much higher energies and therefore the incident laser frequencies are

not in resonance with these transitions. The vibrational modes of the heme are sensitive to the electronic structure and the conformation of the prosthetic group. In general, changes in frequency are directly proportional to changes in bond energies so that any protein structural change that affects the heme should be manifested through the frequencies of the vibrational modes detected in the Raman spectrum. The bonds at the heme that link the protein structure to the ligand binding site may also be detected and an assessment of the energetics may be made. In the low-frequency region (50–600 cm^{-1}) the resonance Raman spectra are dominated by vibrational modes involving the peripheral substituents (18–21). Although most resonance-enhanced normal modes are in-plane motions, in a recent analysis it was proposed that in this region there are significant contributions from out-of-plane motions as well (22). The precise assignment of the these normal modes awaits additional isotopic and theoretical studies, but it has become qualitatively clear that the modes in this region are very sensitive to protein structure. In the low-frequency region there are enormous variations in the spectrum from one heme protein compared with another (e.g., compare Hb to Mb) (12,16) and extreme variations in the spectrum within the same protein when changing oxidation state. The modes assignable to the iron-histidine stretching vibration as well as other iron-ligand modes are present in this low-frequency region. The iron-histidine (Fe-His) stretching mode in hemoglobin has been convincingly identified (23) in the five coordinate case as a strong line (with blue excitation) in the 200–240 cm^{-1} range. It has also been detected in myoglobin (12, 24), cytochrome oxidase (25), and horse radish peroxidase (26). Unfortunately, a definitive assignment for this line has not been made in six coordinate heme proteins although iron-exogenous ligand modes have been identified. Owing to the sensitivity of the low-frequency modes to the protein environment, the present study focuses on the behavior of the low-frequency hemoglobin modes.

A direct comparison between deoxyhemoglobin stabilized in the R state and the native deoxy protein measures the perturbation on the deoxy heme resulting from the quaternary structure-induced changes in the heme pocket. The most notable change in such a comparison occurs in the Fe-His stretching mode that is at 216 cm^{-1} in native deoxyhemoglobin and 222 cm^{-1} in R state deoxyhemoglobin. There are several changes in frequency and intensity of other low-frequency lines as well (11). The behavior of the Fe-His stretching mode with variation in protein structure has revealed several important poperties. In particular, it was found that for human hemoglobins changes in frequency of the iron-histidine stretching mode correlate with changes in the porphyrin skeletal modes sensitive to electron density (11). Furthermore, this concerted response correlates with the equilibrium constant, K_1, for the first step in the oxygenation of hemoglobin (27). Thus, changes in the deoxy heme monitor the functional properties of the molecule. In contrast when comparing the native R-state liganded hemoglobin to a mutant or fish hemoglobin that may be stabilized in the T-quaternary structure while remaining liganded, no large frequency changes have been detected. In oxyhemoglobin the Fe—O_2 stretching mode at 570 cm^{-1} and in carbonmonoxy hemoglobin the Fe-CO mode at 505 cm_{-1} are unaffected by changing the quaternary structure (8, 12–14). Thus, the Raman data have demonstrated that the deoxy heme has sensitivity to functional properties but that the liganded heme is insensitive to them, leaving the question of the origin of the cooperative mechanism unanswered.

HEMOGLOBIN ENERGETICS

The thermodynamic properties of hemoglobin can be well understood by the two-state allosteric model (28). In this model there are but two quaternary structures for the protein, the high-affinity (R) structure and the low-affinity (T) structure. The binding of oxygen in the T structure may be defined as g and that in that R structure is $g + \Delta G$, where ΔG is referred to as the free energy of cooperativity. In the absence of oxygen or other exogenous ligands the T structure has lowest energy, and when ligand bound the R structure has the lowest energy.

To determine the molecular basis of ΔG, it is necessary to examine the heme in each of the four possible states: T and R deoxy and T and R liganded. For deoxyhemoglobin (d) in the T state we may write (8, 11) the total energy E as follows

$$E_d^T = P_d^T + H_d^T + L_{O_2}, \tag{1}$$

where P_d^T is the chemical bond energy of all the bonds in the protein. H_d^T is the chemical energy of all the bonds in the heme including any heme-protein interactions. L_{O_2} is the bond energy of the free dioxygen molecule. A similar expression may be written for the R structure

$$E_d^R = P_d^R + H_d^R + L_{O_2}. \tag{2}$$

For ligand bound (ℓ) hemoglobins

$$E_\ell^T = P_\ell^T + H_\ell^T + B^T \tag{3}$$

and

$$E_\ell^R = P_\ell^R + H_\ell^R + B^R \tag{4}$$

In this expression, B^R is the chemical energy of the bound oxygen including the heme-oxygen and protein-oxygen interactions. The free energy of cooperativity, ΔG_{RT}, is the difference between binding ligand in the R structure and the T structure. So

$$\Delta G_{RT} = (E_\ell^R - E_d^R) - (E_\ell^T - E_d^T) \tag{5}$$

or

$$\Delta G_{RT} = (E_\ell^R - E_\ell^T) - (E_d^R - E_d^T). \tag{6}$$

By making the appropriate substitutions and by using a condensed notation [$P_\ell^R - P_\ell^T = P_\ell^{RT}$] we obtain

$$\Delta G_{RT} = [P_\ell^{RT} - P_d^{RT}] + [H_\ell^{RT} - H_d^{RT}] + B^{RT}. \quad (7)$$

Eq. 7 is written so as to separate the possible regions of the molecule where the free energy of cooperativity may reside. The term in the first set of brackets is the contribution to the free energy of cooperativity in the protein-protein interactions, for example, contributions from the distributed energy model as described by Hopfield (6) and others (4, 5) or from energy storage at the subunit interface (3). The second set of brackets identifies the contribution to ΔG localized in the heme bonds or in direct interactions between the heme and the protein. Finally, the term B^{RT} describes any energy storage involving the bound ligand.

To interpret the energetics of cooperativity it is useful to consider the structure in a schematic way also. Furthermore, the structural considerations allow order to be placed on the various types of resonance Raman experiments. We present the structure by a series of diagrams (Fig. 1) in which we distinguish the state of ligand binding of the heme, the tertiary environment around the heme, and the quaternary structure of the tetramer.

In Fig. 1 the large square represents the T-quaternary structure and the large circle represents the R-quaternary structure. The smaller squares, diamonds, circles, etc., represent the tertiary structure surrounding the central heme depicted as being nonplanar and five coordinate in the deoxy case and planar in the liganded case. No significance is associated with the particular shapes that represent the tertiary structures, other than the fact that they are different.

The tetramer in the upper left of Fig. 1 represents native T-state deoxy hemoglobin. The tertiary structure surrounding the heme corresponds to a deoxy heme in a molecule having a T-quaternary structure and is designated d_T. The tetramer, R', in the upper right is still deoxy but has the R-quaternary structure. (For example this could be a mutant hemoglobin such as Kempsey or a chemically modified hemoglobin such as NES des-Arg HbA.) In this case the tertiary structure around the deoxy heme must be energetically compatible with the R-quaternary structure so it is labeled d_R. The tetramer on the bottom right depicts native liganded hemoglobin and therefore has the R-quaternary structure. The tertiary environment must now accommodate a liganded heme and the R-quaternary structure, so it is labeled as ℓ_R. The tetramer, T', in the lower left is fully liganded but has the T-quaternary structurer. (For example this might be the mutant hemoglobin Kansas in the presence of IHP.) In this case the tertiary environment, labeled ℓ_T, around the liganded heme must be compatible with the T-quaternary structure.

The two central tetramers T* and R* are photodissociated molecules. In this case, as shown in Fig. 2, a

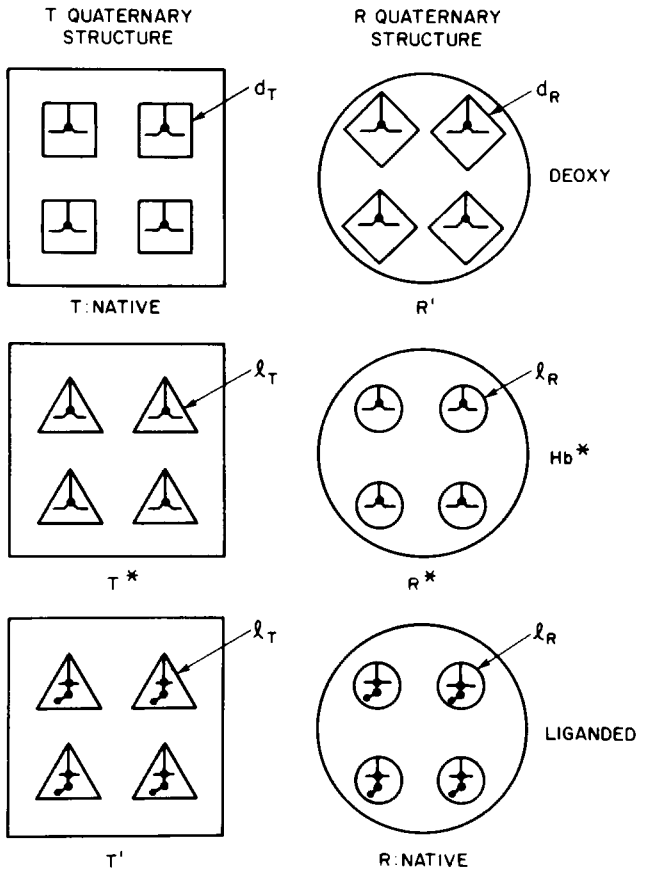

FIGURE 1 Schematic representation of hemoglobin structures. The top two structures represent deoxy hemoglobin in its native T conformation on the *left* and in its modified high-affinity (R') conformation on the *right*. Similarly on the bottom the two structures represent the liganded conformations, with the native high-affinity form on the *right* and the modified low-affinity (T') form on the *left*. The two structures labeled Hb* in the center are photodissociated forms of the corresponding six coordinate preparations. The large square and the large circle represent the T- and R-quaternary structures, respectively. The smaller squares, diamonds, triangles, and circles represent tertiary structures around the central heme, which is represented as being nonplanar and five coordinate for the deoxy and Hb* cases and planar and six coordinate in the liganded cases. The tertiary environment around the heme is designated by the type of heme (deoxy, d, or liganded, ℓ) and the quaternary structure (T or R subscript). Note that in the photodissociated structures, Hb*, the tertiary environment is characteristic of a liganded heme because it has not been allowed to relax to the tertiary environment of a deoxy heme.

liganded molecule is photodissociated producing a metastable species in which the ligand has been removed. However, the tertiary environment around the heme has not yet relaxed so that the deoxy heme finds itself in the tertiary environment of the liganded heme. Such metastable species may be examined by transient spectroscopy or they may be stabilized by going to low temperature. Returning to Fig. 1, the tertiary environment around the heme for such a metastable species is the same as that of the parent species, i.e., the tertiary environment of photodissociated native hemoglobin, R*, is ℓ_R and that of photodissociated T-state hemoglobin, T*, is ℓ_T. These diagrams are intended

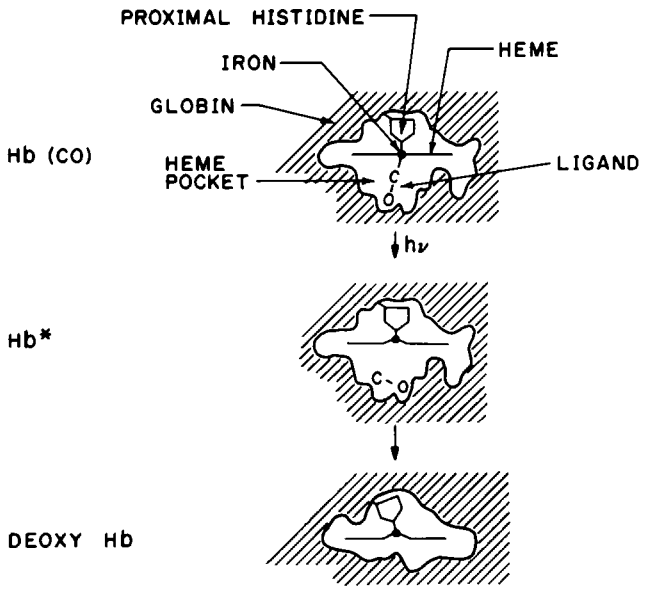

FIGURE 2 Schematic representation of the changes that occur upon photodissociation. The starting point at the top is carbon monoxide-bound hemoglobin. Immediately after photodissociation, in the middle drawing, the iron-carbon bond has been severed and the iron moves to an out of plane position. However, the tertiary environment of the heme has not yet relaxed from the configuration its adopted for the liganded heme. In a transient experiment the relaxation to the deoxy conformation (at the bottom) take place at a later time. In low-temperature experiments the heme pocket relaxes as the temperature is increased.

to be completely general. The implication of the various models for cooperativity on them may now be considered.

For simplicity, the various models for cooperativity are taken to an extreme, i.e., we assume that the entire free energy of cooperativity is localized at the heme as one extreme and as the other we assume that no part of ΔG is stored as any interaction involving the heme, i.e., it is stored entirely in the protein and in protein interactions. We obtain the diagrams in Fig. 3. If all of the free energy of cooperativity is stored at the heme, depicted in the diagrams on the left in Fig. 3, then the bonds between the subunit interface and the heme pocket must be very rigid. (If they were soft, the cooperative energy could be stored in those bonds.) As such the tertiary environment around the heme is determined exclusively by the tetramer quaternary structure. This tertiary structure thereby becomes independent of the state of ligand binding. Under all cases then the tertiary structure present when the protein is in the T-quaternary structure may be designated as t, and similarly when the protein is in the R-quaternary structure the tertiary structure may be designated as r. From Eq. 7 this is the case in which $\Delta G = [H_{\ell}^{RT} - H_{d}^{RT}]$. Energetically there should be a large R-T difference in the deoxy preparations and and an even larger one in the R-T comparison between liganded proteins. This difference in the magnitude of the R-T difference for the two states of ligand binding could be expected from Fig. 3 by the difference between the interaction of the six-coordinate and the five-coordinate heme with the tertiary environment. Indeed, this is precisely a form of the "current wisdom" viewpoint of cooperativity (29), i.e., in the T structure of deoxyhemoglobin, the nonbonded interactions between the histidine and the heme are present but weak, whereas when the exogenous ligand is present and the iron moves into plane there develops a strong repulsive steric interaction between the histidine and heme. Data related to this model will be discussed below.

We now turn to the other extreme. In this limit the change in quaternary structure at the subunit interface is fully accommodated by bonds in the protein such that at the heme there remains no energetically significant quaternary structure-dependent differences. Thus, the environment around the heme is determined only by the state of ligand binding. Therefore, on the right side of Fig. 3 the tertiary environment for both quaternary structures of the deoxyhemoglobin is designated d. Similarly for the liganded heme the tertiary structure in this case, which is independent of quaternary structure, is designated as ℓ.

From Eq. 7, the energetics for the extreme case in which the cooperative energy is located away from the heme may be related to the structure diagrams. For this case $\Delta G = [P_{\ell}^{RT} - P_{d}^{RT}]$ so $[H_{\ell}^{RT} - H_{d}^{RT}] + B^{RT} = 0$. As will be discussed below it has been clearly found that $B^{RT} = 0$, and thus for the above condition to hold $[H_{\ell}^{RT} - H_{d}^{RT}] = 0$. Since this restriction only applies to the difference between H_{ℓ}^{RT} and H_{d}^{RT}, it places no restraints on the R-T difference within each state of ligand binding. Therefore, two extremes will be considered. First we assume that $H_{\ell}^{RT} = H_{d}^{RT} = 0$, i.e., there is no energetically detectable R-T difference within each state of ligand binding. This is in fact the case depicted on the right-hand side of Fig. 3. The tertiary structure around the heme in the deoxy hemoglobin, designated d, is the same in both the T- and R-quaternary structures; and in the liganded case, ℓ, it is also the same for both structures. Differences are only detected when comparing one of the photodissociated states [either T* or R*] to the corresponding deoxy molecule.

The other possibility that satisfies Eq. 7 for energy storage in the protein and not the heme is that the magnitude of each term is large and equal, i.e., $H_{\ell}^{RT} = H_{d}^{RT} \neq 0$. For this condition the R-T difference in both deoxy and liganded hemoglobin are large so a diagram more like that on the left of Fig. 3 is obtained, the only difference being in the energy balance. For protein energy storage $H_{\ell}^{RT} = H_{d}^{RT}$ and for localized energy at the heme $H_{\ell}^{RT} \neq H_{d}^{RT}$.

The diagrams and the equations point the direction for clear cut experiments to distinguish between models for cooperativity by looking exclusively at the heme. For cooperative energy localized at the heme, R-T differences in the heme spectrum must necessarily be evident. For models with energy storage in the protein, differences needn't be detected at the heme but instead differences between the photodissociated metastable forms and the

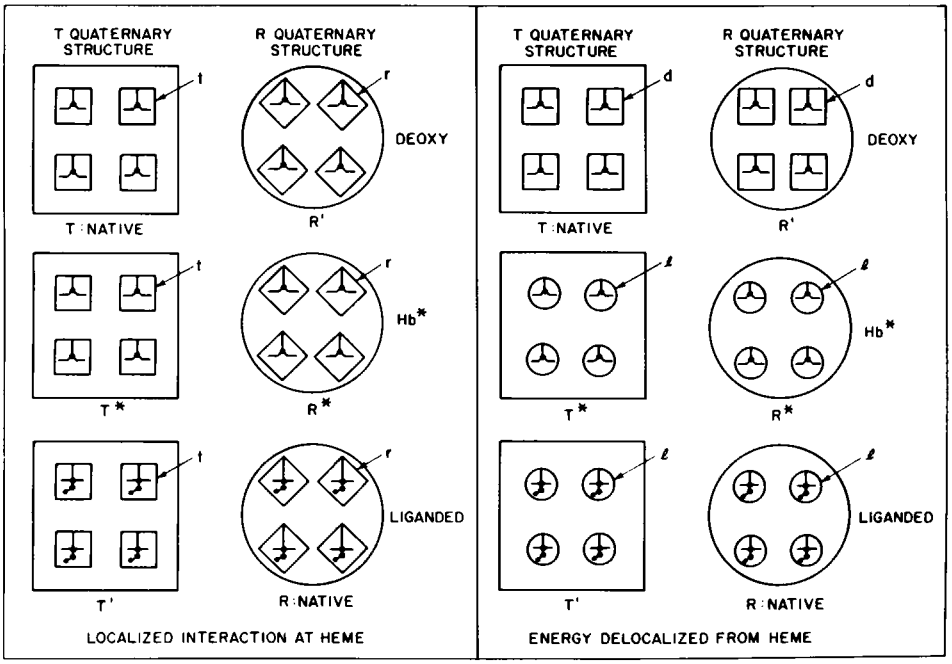

FIGURE 3 Special cases of the schematic representations of hemoglobin structures assuming, on the *left*, that the free energy of cooperativity is localized at the heme, and on the *right*, that no energy is stored in the heme. In the limit of localization at the heme the tertiary environment is determined by the quaternary structure and thereby is labeled (t) and (r) independent of the state of ligation. The square designates the t tertiary structure and the diamond designates the r tertiary structure. For the case on the *right* in which energy is not stored at the heme the quaternary structure changes at the subunit interface are fully relaxed in the protein. Thus, the tertiary environment at the heme depends only on whether the heme is deoxy (d) or liganded (ℓ). In this case we make the square represent the deoxy tertiary environment and the circle represent the liganded tertiary environment.

deoxy forms may be present. These latter differences are not present for the localized interaction at the heme. If such differences are found, they constitute positive evidence for the existence of cooperative energy in the protein part of the molecule. This contrasts sharply to arguments that have been made in the past in which distributed energy models were proposed owing to the absence of being able to detect any localized cooperative energy.

RESULTS AND DISCUSSION

Photodissociated hemoglobin may be studied by transient spectroscopy in which the metastable species is examined a short time (e.g., ~10 ns) after photodetachment or it may be studied with low-temperature spectroscopy in which the metastable form is held for long periods of time by freezing a liganded preparation to very low temperature (~10°K) before photoexcitation. It has been found that although some changes are induced by the freezing process, the differences between a deoxy form and the corresponding photodissociated form are the same in room temperature transient studies as they are in the cryogenic studies (29). For this discussion only the low-temperature results are presented.

Subsequent to photodissociation a deoxy heme is obtained so that the effects on the heme of the amino acid residues that form the heme pocket are seen by comparing the deoxy hemoglobin to the photodissociated form. In Fig. 4, the results of these experiments are shown. The experimental procedures used to obtain these data are described in reference 15. All data were obtained at 10°K with 441.6-nm excitation. The scattering intensity from each spectrum was similar but internal references were not used to compare intensities quantitatively. It was found that the relative intensity of the line at 366 cm^{-1} was higher for native deoxy hemoglobin than for any of the other preparations.

The top spectrum in Fig. 4 is of native deoxy hemoglobin. Below it is the spectrum of a photodissociated T-state hemoglobin. In this case the starting material was COHb Kansas plus IHP. There are several pronounced differences. A weak line at ~175 cm^{-1} increases in intensity and shifts to higher frequency by ~5 cm^{-1}. The Fe-His stretching mode at 235 cm^{-1} is stronger and shifted to high frequency in the photodissociated form. Similarly the line at 302 cm^{-1} is shifted to higher frequency. The line at 345 cm^{-1} appears to become a shoulder (at ~355 cm^{-1}) on the line at 366 cm^{-1}. Similar changes were recorded between the R-deoxy (NES-des-arg-HbA) and the R-photodissociated form. The starting material was CO native HbA in this case. Again in the photodissociated form the Fe-His stretching mode increased in frequency and intensity and the 301 cm^{-1} line shifted to 307 cm^{-1}, while the mode at 350 cm^{-1} appears to have split into a line at 327 cm^{-1} and a shoulder on the 366 cm^{-1} line.

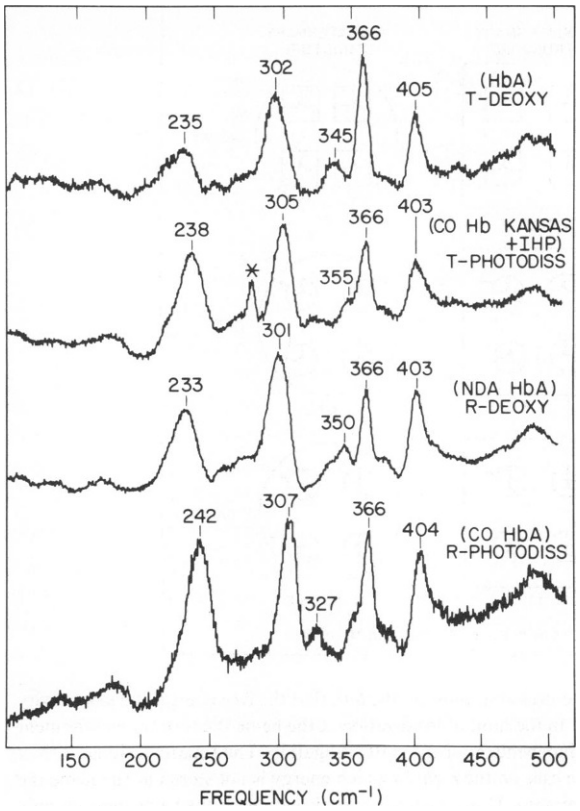

FIGURE 4 Resonance Raman spectra of deoxy and photodissociated hemoglobins obtained with 441.6-nm excitation at 10°K. The top spectrum is of native deoxy hemoglobin. Below it is the spectrum of photodissociated carbonmonoxy hemoglobin Kansas in the presence of IHP. In Figs. 1 and 3 this corresponds to T*. The third spectrum is of a chemically modified hemoglobin (NES-des-Arg) that is stabilized in the R structure. In Fig. 1 and 3 this corresponds to R'. The bottom spectrum is of photodissociated native carbonmonoxy hemoglobin. This corresponds to R* in Figs. 1 and 3. The line in the second spectrum marked with an asterick is a laser fluorescence line. The sloping background in the bottom spectrum results from a small amount of fluorescence in this particular sample.

The changes detected in the comparisons reported here occur in both α and β hemes. This may be seen from the data directly in that the frequency shifts exceed the line widths, thus making it impossible that contributions from only one of the types of heme are changing frequency. We (Ondrias, M. R., D. L. Rousseau, M. Ikeda-Saito, and T. Yonetani, unpublished results) have confirmed that the changes occur in both types of heme; this was done by conducting analogous experiments in iron-cobalt hybrid preparations in which the separate subunits may be examined by appropriate selection of the laser excitation frequency (31). The fact that similar changes take place in each heme indicate that the heme response to the protein structural changes are the same, i.e., changes in the protein structure due to ligand release induce identical interactions in both hemes.

As evident from the spectra presented here and from spectra (not shown) obtained in the region of the skeletal modes of the porphyrin macrocycle (1,000–1,700 cm^{-1}), the photoproduct is a high spin five coordinate heme that is somewhat perturbed from its equilibrium structure. This interpretation is consistent with conclusions reached from transient absorption data in which it was found that the spin state transition precedes ligand detachment (32). Therefore any differences between the deoxy preparation and the photoproduct must result from molecular structural differences between the hemes and not from an unrelaxed electronic structure. In time-resolved studies and low-temperature studies on myoglobin (Argade, P., T. Scott, M. Ondrias, J. M. Friedman, D. L. Rousseau, manuscript in preparation), we do not detect the differences in the photoproduct that we report here for hemoglobin. Thus, the differences we detect must be protein induced and are specific to hemoglobin. We may consider two possible types of protein constraint. First, structural differences between the protein in deoxy hemoglobin and carbon monoxy hemoglobin could lead to a different long-range electrostatic field at the heme (33). However, such an effect would be expected to modify all the heme modes. Instead, we find that many modes do not change while others change dramatically. Thus, this mechanism does not appear to play a significant role in our data. The other possibility is that specific amino acids that form the heme pocket have positions that differ between deoxy hemoglobin and carbon monoxy hemoglobin. On generating the photoproduct, these amino acids must move to a new orientation before the fully relaxed deoxy spectrum of the heme is obtained. This model is consistent with the observation of only a few modes in the low-frequency region changing significantly, especially since one is dependent on the conformation of the proximal histidine (34) and others (see below) have been assigned as modes involving substituent groups (18, 22) and thereby would be expected to be subject to perturbations due to differences in amino acid residues that form the heme pocket. We are able to conclude that the differences we detect reflect tertiary structural differences in the heme pocket between the deoxy hemoglobins and the photoproducts.

It is challenging to speculate on the molecular origins of the differences we detect. First, it is necessary to consider which modes change frequency. In addition to the iron-histidine stretching mode, most prominent are the changes in frequency and intensity of the lines 302 and 345 cm^{-1}. From the most recent consideration of the normal modes (18, 21), these lines are in-plane modes involving peripheral substituents, the 302 line being assigned as a bending mode primarily involving the vinyl groups and the mode at 345 cm^{-1} being assigned as ν_8 a peripheral group stretching mode. This has been confirmed by its sensitivity to isotopic substitution of the peripheral methyl groups (21). Note that the mode at 366 cm^{-1}, which has been assigned as an out-of-plane peripheral substituent mode, displays no frequency difference upon photolysis. Thus, the data suggest that upon the removal of ligand the peripheral groups of the heme undergo an in-plane distortion. This conclusion is

fully consistent with type of changes that are observed in the comparison between the crystal structure of deoxy hemoglobin and CO hemoglobin (35). It was observed that upon going from deoxy to liganded hemoglobin, the hemes of both the α and β subunits shift significantly further into the heme pocket in a direction parallel to the heme plane. (For the α subunit the shift is 0.6 Å and for the β subunit it is 1.5 Å.) Thus, the protein forces restraining the full relaxation of the heme would be expected to cause an in-plane distortion to the peripheral groups.

In the comparisons between deoxy and photodissociated hemoglobins for both the R- and the T-quaternary structures, as concluded above, we find differences in the Fe-His stretching mode and in the peripheral substituent sensitive modes. In deoxyhemoglobin (11) there are R-T differences in the Fe-His stretching mode and in the low-frequency porphyrin modes that are sensitive to the peripheral substituents. In liganded hemoglobin (14) there are no large R-T differences detected in the resonance Raman spectra. Thus, referring back to Fig. 3, it is evident that the data fit neither extreme for the models for sources of the free energy of cooperativity. Thus, ΔG cannot be stored in a single bond.

A popular view of the energetics of cooperativity is the model based on steric strain in the T-quaternary structure of liganded hemoglobin (29). In this model the proximal histidine is tilted in the T-quaternary structure and untilted in the R-quaternary structure. A postulate of the model is that the tilt in the T structure is unaffected by the presence or absence of a bound ligand in the six coordinate position. In the tilted configuration nonbonded repulsive interactions occur between the heme and the histidine. Upon binding a ligand to the heme the iron atom moves into plane from its out-of-plane deoxy position and the repulsive interactions become much greater than they were in the five coordinate case. Within the framework of the model this results in destabilization of either the Fe-His bond or the Fe-exogenous ligand bond.

The model described above localizes the free energy of cooperativity in the iron-histidine or iron-exogenous ligand bond. This model is clearly inconsistent with the resonance Raman data for several reasons. (*a*) There are no quaternary structure dependent changes in the iron-exogenous ligand bond (14). (*b*) It has been inferred (14) that there are also no differences in the iron-histidine bond because such changes should influence the iron-exogenous ligand bond. (*c*) The above model requires that the proximal histidine strain is significantly greater when the heme is six coordinated than when it is deoxygenated. However, from the Raman data cited above there is apparently less strain in the six-coordinate case. We conclude that the proximal strain concepts may account for correlations with physiological properties in deoxyhemoglobin but improperly place the free energy of cooperativity in iron axial bonds. In addition, in studies of the effect of IHP on methemoglobin the amount of energy localized at the iron atom observed by changes in the spin equilibrium is in the range of 0.1 to 1 kcal independent of whether or not the IHP induces a change in quaternary structure. (36; Henry, E. R., D. L. Rousseau, J. J. Hopfield, R. W. Noble, and S. R. Simon, manuscript submitted for publication). Thus, the data on methemoglobins also implies that a significant fraction of the 3.5 kcal of cooperative free energy is stored in bonds other than in the iron axial ligand bonds.

The data from photodissociated hemoglobin presented here directly address these questions. First a premise of the proximal strain model is that the tilt of the histidine is controlled exclusively by the quaternary structure (i.e., the tilt is the same independent of whether the heme is deoxy or six coordinate). If this were the case (and assuming that the Fe-His mode frequency reflects this tilt) (34), then the frequency of the Fe-His stretching mode should be identical in the deoxy species as in the corresponding photodissociated species. However, it is different in both the R and T structures. Note that in 10-ns transient data on photodissociated myoglobin, it is found that neither the Fe-His stretching mode nor any other mode differs from that of the deoxy preparation. (Argade, P., T. Scott, M. R. Ondrias, J. M. Friedman, and D. L. Rousseau, manuscript in prepration). Second, the observation of clear and significant differences between the photodissociated preparations and the deoxy samples, is positive confirmation that local tertiary structure is controlled by ligand binding and indicate energy storage away from the heme. Prior proposals that the free energy was not stored at the heme but in bonds of the protein have in general been based on the absence of finding any localized free energy although specific sites of cooperative energy storage at the $\alpha_1\beta_2$ subunit interface were recently proposed based on the examination of a large number of mutant hemoglobins (3). The data presented here, in the context of the models discussed, supply positive evidence that energy is stored away from the immediate heme tertiary environment. Because the resonance Raman technique is sensitive to only the heme we cannot determined if free energy is delocalized over many polypeptide bonds or is localized in but a few.

In the analysis presented here, we do not discuss the entropic contributions to the free energy of cooperativity although we recognize that there is considerable evidence pointing to the importance of such contributions (37). However, if entropic contributions played a substantial role in the heme interactions, changes in line widths with quaternary structure and temperature-dependent frequency shifts would be expected. Such line width differences are not detected (8) and temperature-dependent quaternary structure induced differences have not been reported. Thus, although entropic contributions in the protein can induce structural changes at the heme, it does not appear that large entropic contributions to the free energy of cooperativity are manifested at the heme. The present data do not allow the distribution of the enthalpic

and entropic contributions to the free energy of cooperativity in the protein to be made.

COOPERATIVITY

On the basis of the large body of resonance Raman data, a picture emerges of a mechanism for hemoglobin cooperativity and the effects of the protein structural changes at the heme. Changes at the subunit interface due to the quaternary structural change or to tertiary changes within a given quaternary state induce changes throughout the protein. The weakest bonds, wherever they may be, store enthalpic changes. In deoxyhemoglobin the changes at the interface cause widespread structural changes in the protein that propagate all the way down to bonds associated with the heme thereby giving it sensitivity to the protein structure and hence to K_1, the equilibrium constant for binding the first ligand. Based on the change in the Fe-His bond only, the amount of energy at the heme associated with the quaternary structure change is ~1 kcal. A lesser amount of energy may also be associated with changes in some of the other bonds. In the liganded case the structure at the heme tightens up so that the change in quaternary structure is accommodated in bonds of the surrounding polypeptide. No significant R-T structural changes are detected in the heme in this case. Furthermore, the iron-ligand bond energies and the internal bond energies of the bound ligands have no quaternary structure dependence. (14; Henry, E. R., D. L. Rousseau, J. J. Hopfield, R. W. Noble, and S. R. Simon, manuscript submitted for publication). Hence, the exogeneous ligand is also insensitive to quaternary structural changes. Therefore, no single bond or set of bonds may be viewed as storing the free energy. Instead, it is distributed differently in deoxyhemoglobin than it is in liganded hemoglobin. Energetically of course, it makes no difference where ΔG is found. All that is necessary is that Eq. 7 is satisfied.

The data reported here illustrate that the frequencies of the deoxy heme are different for deoxyhemoglobin than for photodissociated hemoglobin. These differences are induced by differences between the tertiary structure of deoxy hemoglobin and that of liganded hemoglobin. In other words, the optimum protein conformation surrounding a deoxy heme is not the same as that surrounding a six coordinate heme. This is in sharp contrast to the Mb case in which in 10-ns transient experiments the photodissociated and the deoxy spectra are identical (Argade, P., T. Scott, M. R. Ondrias, J. M. Friedman, D. L. Rousseau, manuscript in preparation). The heme pocket differences for the two states of ligand binding in hemoglobin are apparently widespread since the vibrational modes sensitive to peripheral substituents as well as the Fe-His stretching mode differ in the deoxy-photodissociated heme comparison. Evidently the protein has tailored the heme pocket to be very specific to the presence or absence of bound ligand. Furthermore, the data show that when a ligand is bound (or released) the orientation of amino acid residues near the hemes of both the α and β subunits must change. The absence of equivalent changes in myoglobin illustrate that such changes are not a general property of oxygen carrying and storage heme proteins and may thereby be a critical difference between cooperative and noncooperative proteins. Thus, in hemoglobin immediately following binding, or release, of ligand, since the heme cannot be accommodated by the same tertiary structure, a strain is generated. This strain causes an amino acid readjustment in the heme pocket that, in turn, may drive a more global conformational change in the protein and thereby trigger the cooperative transition in the hemoglobin tetramer.

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