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Absence of Cooperative Energy at the Heme in Liganded Hemoglobins†

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ABSTRACT: Using resonance Raman and infrared absorption spectroscopies, we show that there are no energetically significant structural changes at the heme upon the quaternary structure transition in six-coordinate hemoglobins. These observations are at variance with the presently accepted mechanism for cooperativity, which postulates severe strain in the T quaternary structure of liganded hemoglobin. By consideration of the present results, and studies on deoxyhemoglobins and photodissociated hemoglobins, a view of the distribution of the free energy of cooperativity emerges. In five-coordinate deoxyhemoglobins the iron–histidine bond is able to respond to the protein structure, thereby accounting for a wide variation (40 cm^{-1}) in its frequency. In contrast, when a sixth ligand is present and the iron is pulled into plane, the histidine–heme–ligand complex becomes structurally rigid, thereby preventing protein-induced changes at the heme. Instead, in liganded hemoglobin the changes in structure that occur at the subunit interface upon the quaternary structure transition are accommodated away from the heme by relatively weak bonds in the protein.

Despite many years of study, the molecular basis for cooperativity in hemoglobin is not understood (Rousseau & Ondrias, 1983). This results in part from the difficulty of studying all of the many possible interactions that could contribute to cooperativity. As a starting point, the quaternary structure dependent interactions at the binding site, i.e., the heme–protein–ligand interactions, must be elucidated. Only then can the amount of cooperative energy localized at the heme be evaluated and routes of information transfer within the tetramer be revealed. In order to study protein–heme interactions it is helpful to consider independently three separate regions of the heme pocket that may contribute to ligand stabilization and cooperative pathways: the proximal histidine–heme interaction; the interaction between the protein environment and the bound ligand; and direct interactions between the protein and the porphyrin. Additionally, in an assessment of the energetics of cooperativity, it is necessary to examine both liganded and deoxyhemoglobins (Ondrias et al., 1982). The effect at the heme of the quaternary structure transition in both deoxyhemoglobins (Ondrias et al., 1983; Nagai & Kitagawa, 1980; Nagai et al., 1980b) and methemoglobins (Henry, 1980; Nagai et al., 1980a; Scholler & Hoffman, 1979; Asher, 1981) has been extensively examined with resonance Raman scattering. Relatively few analogous studies of ferrous liganded hemoglobins have been reported (Nagai et al., 1980b; Tsubaki et al., 1982).

The importance of studying ferrous liganded hemoglobins becomes clear when the various mechanisms of cooperativity are considered. It is generally viewed that in the deoxy protein there is very little quaternary structure dependent strain at the heme but in liganded hemoglobin there is considerable strain (Baldwin & Chothia, 1979; Dickerson & Geis, 1983).

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In part, these arguments arise from the crystallographic results, which show a tilted orientation of the proximal histidine with respect to the heme plane in deoxyhemoglobin. It is proposed that the protein forces in the T structure give rise to this tilt. The forces are relaxed in the R structure, allowing the histidine to take on an upright (with respect to the heme) orientation. In deoxyhemoglobin in which the iron is out of plane, this tilt can be accommodated without excessive nonbonded repulsions. However, in six-coordinate hemoglobin in which the iron is pulled into plane (and consequently the histidine is closer to the heme plane), the tilted orientation of the histidine in the T structure is proposed to cause a severe nonbonded interaction between the histidine and the porphyrin. Thus, it has been predicted that the tilted orientation should result in strain in either the iron–histidine or the exogenous ligand–iron bond in the T structure of liganded hemoglobins.

Our present study concentrates on the properties of (carbon monoxy)hemoglobins. Carbon monoxide should be particularly sensitive to the stereochemistry on the distal side of the heme because its preferred linear bond to the iron atom should give rise to strong interactions between the CO and the distal histidine or Val E11 (Moffat et al., 1979). In contrast, ligands such as O_2 and NO, which bind preferentially in a bent configuration, would be expected to experience less stereochemical interference. Infrared absorption studies of the C–O stretching mode have indeed shown that it is sensitive to the environment of the ligand. Mutants lacking the distal histidine display significant splitting in the carbonyl carbon–oxygen stretching frequency (Caughey et al., 1978). For example, in HbM[1] Emory (β63His → Tyr) lines of equal intensity at 1950 and 1970 cm^{-1} were observed for this mode (Caughey et al., 1969). In Hb Zurich (β63His → Arg) lines at 1950.5 and 1958 cm^{-1} were detected (Choc & Caughey, 1981). The major line in native hemoglobin is at 1951 cm^{-1}, and in myoglobin it appears at 1944 cm^{-1} (Makinen et al., 1979). In several animal hemoglobins the principle line coincides with that of human hemoglobin at 1951 cm^{-1} but side bands of both lower and higher frequency are present in some (Caughey et al., 1978). It has been suggested that these effects are due in part to a direct interaction between the C–O and the distal histidine (Moffat et al., 1979; Caughey et al., 1969; Choc & Caughey, 1981; Satterlee et al., 1978). Whether or not such a direct interaction occurs, the sensitivity of the ligand to environment is clear. However, the physiological importance of these changes has not been determined since these variations do not correlate with affinity differences in any obvious way (Caughey et al., 1969).

Unlike deoxyhemoglobins and methemoglobins few fully liganded ferrous hemoglobins can be induced to undergo a quaternary structure transition. Liganded carp hemoglobin switches to the T structure upon the addition of IHP at low pH (Pennelly et al., 1975). Similarly, the liganded mutant hemoglobin Kansas (Asn α102 → Thr) may also be switched from the R to the T structure by the addition of organic phosphates (Shulman et al., 1975). These hemoglobins thereby allow the study of the variation in protein–heme interactions as a function of quaternary structure.

Vibrational spectroscopy is a powerful probe of protein–heme interactions since any changes in ground-state electronic properties and conformation directly influence the mode frequencies (Rousseau & Ondrias, 1983; Asher, 1981). The effects of the quaternary structure transition on the heme and axial ligand modes have been studied by infrared absorption spectroscopy and resonance Raman scattering (Rousseau & Ondrias, 1983). These include studies of deoxyhemoglobins (Ondrias et al., 1982; Nagai & Kitagawa, 1980), methemoglobins (Henry, 1980; Asher, 1981; Nagai et al., 1980a; Scholler & Hoffman, 1979), and ferrous liganded hemoglobins (Nagai et al., 1980b; Tsubaki et al., 1982; Ascoli et al., 1978; Kincaid et al., 1979; Onwubiko et al., 1982). Changes were detected in the iron–histidine stretching mode and in several porphyrin modes in deoxyhemoglobin (Ondrias et al., 1982; Nagai et al., 1980b; Nagai & Kitagawa, 1980; Shelnutt et al., 1979). In methemoglobins changes in porphyrin modes were also detected (Henry, 1980; Nagai et al., 1980a). In ferrous liganded hemoglobins no energetically significant quaternary structure dependent changes were detected (Tsubaki et al., 1982; Onwubiko et al., 1982). We report here an infrared absorption and resonance Raman scattering study of the quaternary structure dependence of the C–O and Fe–C stretching frequencies in HbK(CO) and carp Hb(CO). We also report Raman data from the porphyrin skeletal modes of these two hemoglobins. Protein structure dependent changes in the frequencies of the modes of the bound ligand were not detected. However, differences are evident in the porphyrin skeletal mode known to be sensitive to heme π-electron density.

Experimental Procedures

Resonance Raman spectra of the solution samples were obtained on a previously described Raman difference spectroscopy apparatus (Rousseau, 1981). The spectra of the liganded hemoglobins were obtained with 406.7-nm excitation (~100 mW) with a spectral slit width of about 2 cm^{-1}. Infrared spectra were obtained in a variable path length cell on a Beckman IR-12 spectrophotometer at 1-cm^{-1} resolution.

Carp Hb and Hb Kansas were isolated and purified according to the methods of Tan et al. (1972) and Boneventura & Riggs (1968), respectively. The T structure of the HbK(CO) in the presence of IHP was confirmed from its NMR spectrum (Ogawa et al., 1972). All samples were stored as the CO form under liquid nitrogen until ready for use. For the infrared measurements the heme concentration for carp Hb(CO) was 10 mM and for HbK(CO) it was 15 mM. The carp Hb(CO) and the HbK(CO) samples used for the Raman experiments were 100–250 and 500 μM, respectively. The carp Hb(CO) was buffered with a Bis-Tris solution at pH 5.8 (except for the pH studies) that was 0.1 M in chloride. The HbK(CO) was buffered with 0.02 M Bis-Tris, pH 7.0.

Results

The infrared absorption data for the C–O stretching frequency of HbK(CO) ± IHP are shown in Figure 1, and the corresponding data for carp Hb(CO) are shown in Figure 2. The atmospheric water vapor lines on both sides of the C–O mode were obtained by running the spectrometer in single-beam operation. They are included for calibration purposes. For HbK(CO) a slight shift to lower frequency (−0.8 cm^{-1}) is detected upon the addition of IHP. However, in carp Hb(CO) the addition of IHP causes a slight increase in frequency (0.7 cm^{-1}) and also approximately a 2-fold increase in the intensity of the line at ~1968 cm^{-1} relative to the line near 1952 cm^{-1}. A similar change in intensity was noted for the pH 5.8 samples when the chloride concentration was increased from 0.1 to 0.4 M. At higher pH (7.9) the chloride concen-

[1] Abbreviations: Hb, hemoglobin; HbM, hemoglobin M; HbA, hemoglobin A; Mb, myoglobin; CO, carbon monoxy; HbOp, opossum hemoglobin; HbK(CO), (carbon monoxy)hemoglobin Kansas; Lb(CO), (carbon monoxy)leghemoglobin; IHP, inositol hexaphosphate; TPP, tetraphenylporphyrin; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane.

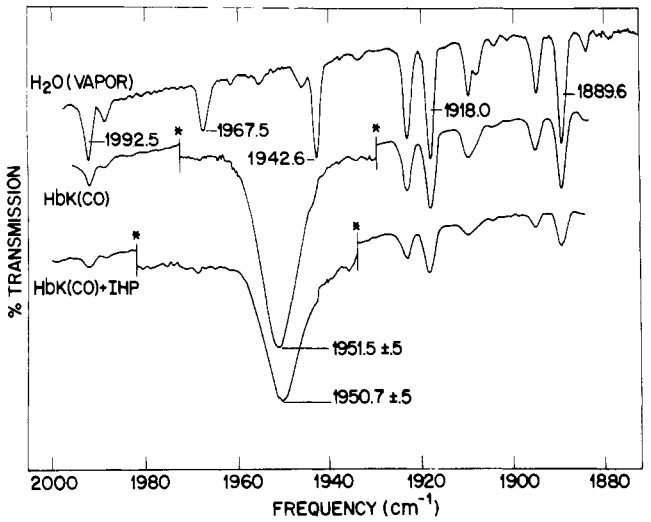

FIGURE 1: Infrared absorption spectra of the C–O stretching mode of (carbon monoxy)hemoglobin Kansas [HbK(CO)] with and without inositol hexaphosphate (IHP). The asterisks designate the frequency at which the instrument was converted from double-beam to single-beam operation so that water vapor lines could be used for calibration purposes.

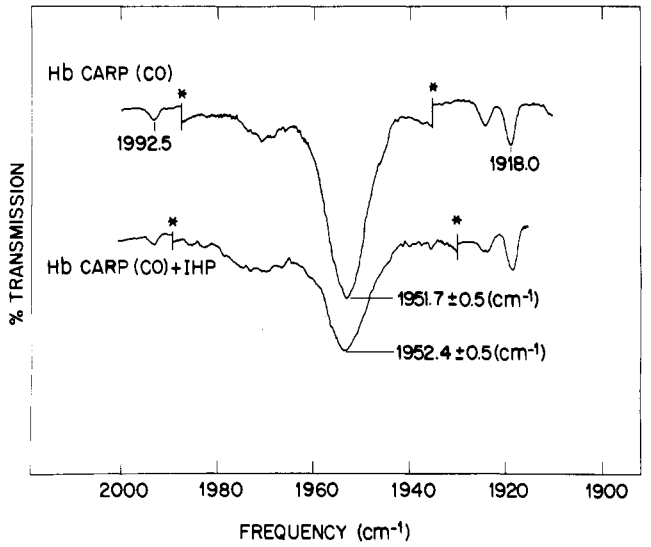

FIGURE 2: Infrared absorption spectra of the C–O stretching mode of carp (carbon monoxy)hemoglobin with and without IHP at pH 5.9. The asterisks designate a change from double- to single-beam operation so that the spectra could be calibrated with the water vapor lines.

tration had no significant effect on the relative intensity of these lines.

Figure 3 shows the spectra of the 505–506-cm^{-1} line in both HbK(CO) and carp Hb(CO) upon the addition of IHP. This line has been assigned as the Fe–CO stretching mode by Tsubaki et al. (1982) and by Armstrong et al. (1982). At the top are the spectra of HbK(CO) ± IHP (heme concentration 500 μM). No difference could be detected in either the position or shape of the line at 505 cm^{-1} in HbK(CO) ± IHP, in accord with the results of Tsubaki et al. (1982). At a heme concentration of 500 μM the tetramer/dimer ratio is ~6 for HbK(CO), sufficiently high to ensure that it is the tetramer that is being studied (Edelstein, 1975). The spectra of carp Hb(CO) ± IHP are presented in the lower part of Figure 3. Again, there is no change in peak position of the line at 506 cm^{-1}. However, there is a change in shape resulting in more intensity on the low-frequency side at ~491 cm^{-1}.

In the high-frequency region containing the porphyrin skeletal modes, no significant changes in intensity or frequency

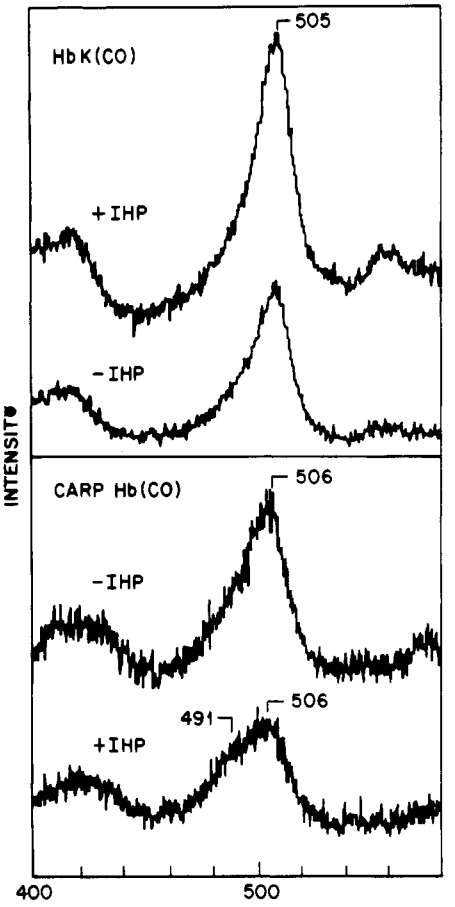

FIGURE 3: Resonance Raman spectra of the Fe–C stretching mode of (carbon monoxy)hemoglobin Kansas and carp (carbon monoxy)-hemoglobin with and without inositol hexaphosphate.

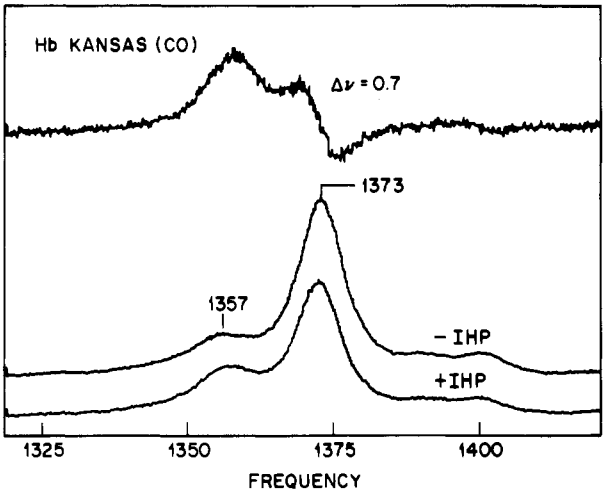

FIGURE 4: Resonance Raman spectra and difference spectrum of the electron density marker line in (carbon monoxy)hemoglobin Kansas with and without inositol hexaphosphate. The line in the IHP-containing samples is at lower frequency by 0.7 cm^{-1}. The line at 1357 cm^{-1} results from deoxyhemoglobin present at a higher concentration in the sample containing IHP.

were detected in any mode other than the electron density marker line at 1372–1373 cm^{-1}. For this line a 0.7-cm^{-1} and 0.8-cm^{-1} shift to lower frequency was detected upon the addition of IHP to HbK(CO) and carp Hb(CO), respectively (see Figures 4 and 5).

The effect of IHP on HbA(CO) was examined to determine if the effects detected in the Raman spectra resulted from tertiary structural changes due to the addition of IHP or from the quaternary structural transformation. No change was

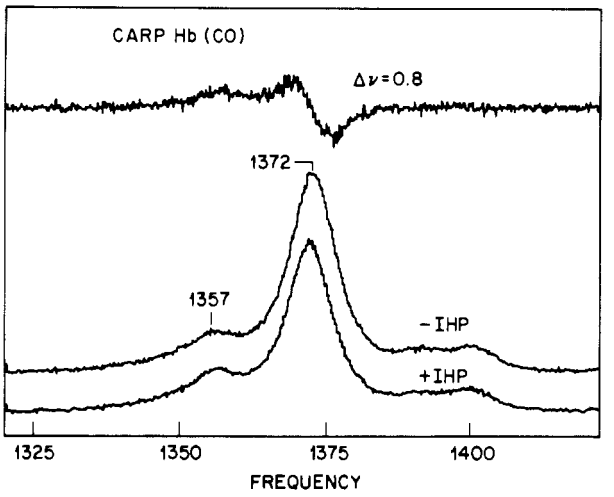

FIGURE 5: Resonance Raman spectra and difference spectrum of the electron density marker line in carp (carbon monoxy)hemoglobin with and without IHP. The line in the IHP-containing sample is 0.8 cm^{-1} lower than that without IHP. The line at 1357 cm^{-1} is from deoxyhemoglobin.

detected in the 507-cm^{-1} line in either the peak frequency or the shape. However, IHP induced an ~0.2-cm^{-1} shift to lower frequency in the 1372-cm^{-1} line.

High chloride concentrations have been reported to influence the magnetic properties of carp Hb(CO) (Cerdonio et al., 1982). We measured the infrared absorption spectrum of carp Hb(CO) (heme concentration ~10 mM) and found that at high pH (7.9) the presence of 0.6 M Cl^- did not modify the spectra. However, at low pH (5.8) the increase of chloride from 0.1 to 0.4 M resulted in an increase in the intensity of the 1968-cm^{-1} component similar to that induced by the addition of IHP. In the Raman spectrum of carp Hb(CO) (heme concentration ~100 μM) the increase in the chloride concentration from 0.1 to 0.6 M (pH 5.6) had no effect on the frequency of the electron density marker line. However, the intensity of the 493-cm^{-1} shoulder in the Fe–CO stretching frequency region increased in the spectrum at high chloride concentration. The magnitude of the change was about one-third of that induced by the addition of IHP. It is also noteworthy that, in all comparisons between the presence and absence of IHP [including HbA(CO) ± IHP], the sample with IHP had a higher relative concentration of the deoxy species in the presence of our low-power laser probe beam than did the corresponding sample without IHP. This is indicative of either a reduction in CO affinity or an increase in photolysis efficiency. In contrast to this behavior, the ratio of the deoxy to liganded Hb in the chloride concentration comparison was the same.

Specific distal effects were probed by the examination of opossum hemoglobin (HbOp) in which the distal histidine residue in the α chain is replaced by a glutamine (Stenzel, 1974). It is evident that in HbOp(CO) the Fe–C mode is perturbed relative to HbA(CO) (Figure 6). It is broadened on the high-frequency side. This is resolved as a peak at 516 cm^{-1} in the difference spectrum. The electron density marker line, ν_4, is shifted to lower frequency from HbA(CO) by about 0.4 cm^{-1}. The data from all of these globins are summarized in Table I.

Discussion

Distal Interactions. The center frequency of the main infrared absorption band of the carbon–oxygen stretching mode (1951 cm^{-1}) in HbK(CO) slightly decreases on the addition of IHP and that of carp Hb(CO) slightly increases. (The line

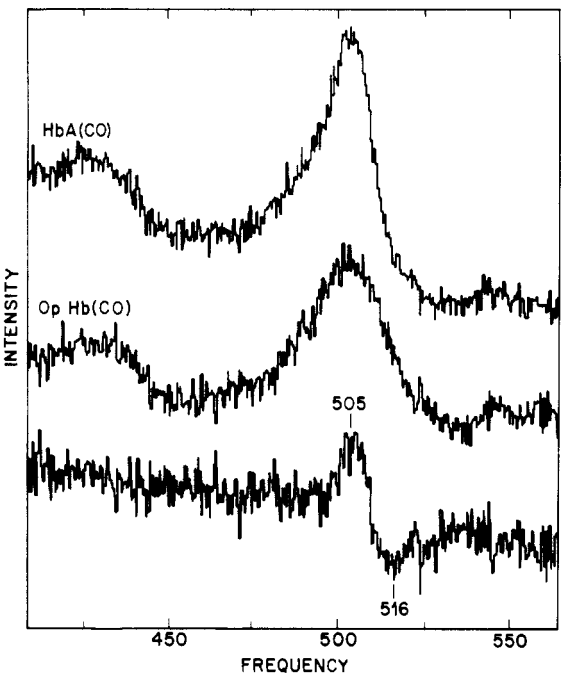

FIGURE 6: Resonance Raman spectra and difference spectrum (bottom) of human adult (carbon monoxy)hemoglobin and opossum (carbon monoxy)hemoglobin. The line from the opossum Hb has gained intensity at 516 cm^{-1} and lost intensity at 505 cm^{-1} in comparison to HbA(CO).

Table I: Representative Frequencies of Structurally Sensitive Vibrational Modes in Various Globins

globin	Fe–His	Fe–CO	π density	C–O
deoxy-HbA [T]	216		1357.0	
deoxy-Hb(A) [R] (des-Arg)	222		1355.5	
HbA(CO) [R]	?	505	1373.0	1951.5
Hb(CO) [R] (Kansas)	?	505	1373.0	1951.5
Hb(CO) [T] (Kansas)	?	505	1372.3	1950.7
Hb(CO) [R] (carp)	?	506	1373.0	1951.7
Hb(CO) [T] (carp)	?	506, 491	1372.8	1952.4, 1968
Hb(CO) (opossum)	?	505, 516	1372.6	1951, 1945
Lb(CO)	?	505	1375.1	1948.5
Mb(CO) (sperm whale)	?	510	1372.7	1944

at 1968 cm^{-1} will be discussed below.) These data are consistent with previous measurements on HbK(CO) by Kincaid et al. (1979), who detected a 0.7-cm^{-1} decrease in this mode on the addition of IHP, and also with measurements on Hb(trout IV)(CO) by Ascoli et al. (1978), who detected a 0.4-cm^{-1} increase in the frequency of the C–O mode upon generating a low-affinity structure by lowering the pH. [Prior workers (Kincaid et al., 1979) erroneously stated that the changes in the human and trout hemoglobins were in the same direction.] In contrast to the behavior of the C–O mode, it should be noted that the IHP-induced changes in the electron density marker line for HbK(CO) and carp Hb(CO) are in the *same* direction.

The position of the C–O stretching mode has been shown to be sensitive to bonding, steric, and dielectric properties of the distal environment as well as π back-donation from the porphyrin ring and the ligand at the trans position (Caughey et al., 1978). However, the small frequency shifts detected here correspond to energetic differences of less than 0.1 kcal/mol. Thus, the small changes we detect, which go in the opposite direction for the human and fish hemoglobins, do not appear to be related to the ligand binding affinity. In addition,

since the shift in the porphyrin electron density marker line is in the same direction for human and carp hemoglobins, the small shifts in the C–O stretching frequency cannot originate from the porphyrin macrocycle. Instead, it is likely that they represent small changes in the distal environments of the C–O ligands. In any event the opposite behavior of the C–O stretching mode for these two species under equivalent perturbations rules against energetic significance in the interactions between the bound ligand and the residues in the distal environment.

The absence of energetically important quaternary structure induced changes on the distal side of the heme is also indicated by the Fe–CO stretching frequency (505–506 cm^{-1}) observed in the Raman scattering data. No changes upon the addition of IHP were detected in this mode in HbK(CO). The peak frequency of the Fe–CO mode in carp Hb(CO) was also unshifted by the quaternary structure transformation. Assuming a harmonic bond potential, a frequency difference of less than 2 cm^{-1} corresponds to an energetic change of much less than 0.1 kcal. Similar conclusions have been drawn from the analysis of $HbK(O_2)$ with and without IHP (Nagai et al., 1980b) and from changes in Fe–ligand modes in methemoglobins (Rousseau & Ondrias, 1983; Henry, 1980; Asher, 1981).

In the infrared spectra of carp Hb(CO) the addition of IHP leads to an increase in intensity of the line at 1968 cm^{-1} compared to the intensity of the line at 1951 cm^{-1}. A line at 1968 cm^{-1} was also reported in the infrared absorption spectrum of carp Hb(CO) + IHP by Onwubiko et al. (1982). By comparison with NMR data, they inferred that this indicated an IHP-induced change in the binding site structure of some of the β chains. The relationship between these structural changes affecting the binding sites of only a fraction of the β subunits and the IHP-induced quaternary structural change and ligand-binding affinity reduction could not be determined in the study by Onwubiko et al. (1982). The presence of a line at this frequency ($\sim$1968 cm^{-1}) has been reported in several other hemoglobins and myoglobins under a variety of conditions and has been attributed to a different (presumably more open) conformation of the distal side of the heme pocket (Choc & Caughey, 1981). It has been associated with changes in temperature and pH. Recently, Caughey et al. (1981) reported that in bovine (carbon monoxy)myoglobin the intensity of a line at this position is 5 times stronger at pH 5.2 than it is at pH 7.8. In addition, on increasing the temperature the relative intensity of the contribution at 1968 cm^{-1} increases. We also find that for carp Hb(CO) at low pH this line is relatively strong but at pH 7.8 it is absent. Furthermore, the addition of chloride increases the strength of this line at low pH but does not affect it at high pH. The absence of any increase in photodissociation by our laser beam in the high chloride samples implies that the presence of chloride does not affect the affinity of the liganded form (Cerdonio et al., 1983) since in cases in which the affinity does change (e.g., on addition of IHP), the equilibrium concentration of the deoxy form, due to photodissociation, also changes.

The position of the C–O stretching mode in CO heme proteins is governed by several factors, which may be classified as two general types of interactions—those that modify the electron density at the iron atom and those that affect the CO moiety directly. The direction of the shifts in the Fe–C stretching frequency and the C–O stretching frequency due to a structural change depends on the details of the particular interactions. However, one would expect that as the CO is bound more tightly, the Fe–C bond order would increase (resulting in an increase in the Fe–C stretching mode frequency) and the C–O bond order would decrease (lowering the C–O stretching mode frequency). Surprisingly, there are many cases in which a higher C–O frequency corresponds to a higher affinity. For example, in the binding of Zn^{2+} to Mb a prominent band at 1967 cm^{-1} develops in the infrared spectrum (Makinen et al., 1979) and the affinity for CO binding is *increased*.

Brown et al. (1983) have studied the effect of dehydration on the population of the conformer with the 1968-cm^{-1} C–O stretching mode in hemoglobin and myoglobin. From an extensive series of dehydration and denaturization experiments, they inferred that the conformer represented a partially unfolded heme pocket in which the distal interactions with the CO are reduced, thereby allowing it to assume a preferred linear orientation and a higher affinity. Equivalent observations have been made in strapped heme model compounds (Yu et al., 1983). These are hemes in which a molecular strap crosses the binding site hindering ligand attachment. It was found that those compounds with a high CO binding affinity had higher C–O stretching frequencies than did those with lower affinity (Yu et al., 1983). Concomitantly, lower Fe–C stretching frequencies are associated with the compounds with the higher C–O stretching frequencies (higher affinity). There is a linear negative correlation between these modes as might be expected since any steric or electronic perturbation should affect the electronic structure of the entire Fe–C–O moiety and thereby both frequencies. Thus, in the carp Hb(CO) we attribute the components with the high frequency of the C–O stretching mode and the lower frequency of the Fe–C stretching mode to the same conformer.

The addition of IHP at low pH to carp Hb(CO) gives a low-affinity hemoglobin. However, the additional conformer of the Fe–C–O moiety does not appear to be associated with this low affinity for several reasons. First, Onwubiko et al. (1982) reported that the additional conformer is associated with only the β subunits. If it is functionally significant, there should be a large subunit heterogeneity but no subunit heterogeneity in equilibrium binding has been detected (Tan et al., 1972). Second, the addition of Cl^- at low pH to carp Hb(CO) also gives an additional component to the Fe–C and C–O modes, but the presence of chloride does not affect the affinity of the liganded form (Cerdonio et al., 1983). Finally, a similar side band in the infrared spectrum (at 1968 cm^{-1}) in human (carbon monoxy)hemoglobin at low pH (4–5) has been reported by Onwubiko et al. (1982). However, under these conditions HbA(CO) does not have low affinity, but rather it takes on a higher affinity (Antonini et al., 1961). Therefore, there is no evidence to associate the conformers in the carp Hb(CO) having the anomalous stretching frequencies with low-affinity forms of the protein.

It is tempting to conclude on the basis of the above observations that the additional conformer seen in the presence of Cl^- or IHP at low pH originates from a steric interaction and is a high-affinity form. However, both changes in property on the proximal side such as the π acidity or the base tension and changes in the heme are known to affect the Fe–C–O grouping (Traylor, 1981). Consequently, the data do not allow a determination of the origin of the frequency shifts. Although the data are also suggestive that this minor component has high affinity, there is no evidence for a high-affinity component at equilibrium with the low-affinity T-structure form in the oxygen and carbon monoxide affinity measurements (Tan et al., 1972). We thus conclude that either the additional conformer is *not* in equilibrium with the remaining protein or if

it is then its affinity is controlled by other factors and is possibly similar to the affinity of the major conformer. The minor conformer does not appear to be a direct result of the quaternary structure transition.

The spectra of HbOp(CO) in which the Fe–CO stretching mode has a component at 517 cm^{-1} and the C–O stretching mode has a component at 1945 cm^{-1} demonstrate in this case as well that pronounced changes in the heme distal environment are reflected in the iron–exogenous ligand bond. However, there does not appear to be any obvious correlation between these values and ligand binding affinity. The affinity of Hb(Op) is lower than that of HbA whereas Mb, which also has an increased Fe–CO stretching frequency (510 cm^{-1}) and a decreased C–O stretching frequency (1944 cm^{-1}), has a high affinity. These data demonstrated that although the Fe–C and the C–O stretching frequencies are sensitive to tertiary structural effects in the heme pocket, they do not reflect ligand binding affinities in a simple way. Thus the ligand affinity must be regulated by some other mechanism.

Porphyrin Interactions. With the exception of the mode (ν_4) classified as the electron density marker line, no changes were detected as a function of quaternary structure in the high-frequency porphyrin skeletal modes. Frequency shifts in ν_4 on changing the quaternary structure also have been detected in deoxyhemoglobins (Ondrias et al., 1982; Shelnutt et al., 1979) and methemoglobins (Rousseau et al., 1980). In (carbon monoxy)hemoglobins and methemoglobins, ν_4 has lower frequency in the T state than in the R state whereas in deoxyhemoglobin the frequency is lower in the R state than in the T state.

An equivalent relationship has been observed in optical absorption studies (Perutz et al., 1976; Wang & Brinigar, 1979). The Soret band shifts slightly to the red on conversion from the R to the T structure in liganded ferrous hemoglobins (Giardina et al., 1975). In deoxyhemoglobins in the T structure the Soret band is blue shifted from its value in the R structure (Perutz et al., 1974). Thus the frequency shifts of the Soret absorption parallel the quaternary structure induced shifts of the ν_4 Raman line. This suggests that the data are consistent with the concept that the changes in ν_4 are a response to electronic structure changes rather than small conformational changes in the porphyrin resulting in small shifts in ν_4. It should be kept in mind that other parameters have been found to affect the Soret band (Cerdonio et al., 1983) but their effect on the Raman spectrum has not yet been explored.

The Raman data leave us with the same dilemma as that posed solely by the optical absorption data: Namely, "why does the R–T transition give the opposite frequency shift in deoxyhemoglobins as it does in liganded hemoglobins?" Perutz et al. (1976) suggested that in the deoxy protein further displacement of the iron from the heme plane causes the blue shift of the optical absorption and in the six-coordinate case a porphyrin distortion could raise the energy of the π levels in relationship to the π^* levels, thereby shifting the Soret transition to the red. However, the data presented here demonstrate that for the most part the porphyrin vibrational modes in the liganded complex are insensitive to the R–T transition. This argues against porphyrin distortion as the causative factor for the red shift of the Soret band.

Wang & Brinigar (1979) compared model heme absorption difference spectra to R–T difference spectra in hemoglobins in an attempt to interpret the optical absorption data. They found that the R–T differences in deoxyhemoglobin could be approximated by the difference spectrum of a heme complex with a less hindered minus a more hindered axial ligand. In contrast, they found that CO–protoheme complexes with more hindered proximal ligands best approximated spectra of R-state hemoglobin and myoglobins. Thus, spectra of complexes with a stronger minus a weaker axial ligand best approximated the T minus R optical absorption spectra in the liganded preparation, just the opposite of the behavior in deoxyhemoglobins. They proposed that in the T state of oxy- and (carbon monoxy)hemoglobins a steric interaction between the ligand and either the distal histidine (E7) or the Val E11 forces the Fe–CO and Fe–O_2 entities toward the proximal imidazole, thus relieving some of the proximal "strain" associated with the Fe displacement toward the heme plane upon ligand binding. If this distal steric interaction is in fact present in T-state liganded hemoglobins, it should give rise to changes in vibrational mode frequencies of either the Fe–exogenous ligand moles or the internal modes of the ligand. However, we do not detect such changes and can thereby rule out the proposal (Wang & Brinigar, 1979) of energetically significant distal interactions in T-state ferrous six-coordinate hemoglobins.

The Raman results reported here suggest a possible explanation for the different behavior between deoxyhemoglobins and liganded hemoglobins. In ferrous deoxyhemoglobins the uppermost π orbitals of the porphyrin are fully occupied and there is some electron density in the $e_g(\pi^*)$ antibonding orbitals (Antipas et al., 1980). Any interaction that increases the d_π to porphyrin π electron donation should increase the antiboding orbital density and thereby *lower* the frequency of the electron density marker line (ν_4). In ferrous six-coordinate hemoglobins, significant electron density has been withdrawn from the π orbitals of the porphyrin, possibly to the extent that the π^* orbitals are vacant and the π orbitals are not fully occupied. These different heme electronic structures of the five- and six-coordinate species might allow for different responses to the same perturbation. For example, an interaction that increases electron donation to the π system might, in deoxyhemoglobin, increase the π^* density (lowering the ν_4 frequency) and, in six-coordinate hemoglobins, increase only the π density (raising the ν_4 frequency). The effects of these putative electron density changes on the optical absorption bands are currently being explored.

A case in which the same perturbation gives the opposite effect in the ferrous and ferric states was recently reported in cytochrome *c* studies by Cartling (1983). When lysine is the sixth ligand (replacing methionine) in ferric cytochrome *c*, the electron density marker lines *increase* in frequency whereas in ferrous cytochrome *c* (generated in the frozen state by pulse radiolysis) they *decrease* in frequency when compared to the respective proteins with methionine as a sixth ligand. Thus changes in the opposite direction of the electron density marker line in five- and six-coordinate hemoglobin upon the quaternary structure transformation may be consistent with a single perturbation.

It is noteworthy that it is possible to modulate the porphyrin π density (as inferred from ν_4 frequency shifts) without affecting the strength of the Fe–CO bond. This is evident in the Hb(CO) and carp Hb(CO) quaternary structure comparisons ($\Delta\nu_4 \sim 0.8$ cm^{-1}) and most dramatically in the comparison of HbA(CO) and Lb(CO) ($\Delta\nu_4 \sim 2.0$ cm^{-1}), all of which have essentially the same value for the Fe–C mode (Rousseau et al., 1983). Similarly, there is no apparent correlation between the properties of the Fe–CO bonds of opossum Hb and the electron density marker line. Such uncoupled behavior probably results from the electronic properties of CO in which the Fe–CO bond is formed by a lone pair

donation to the Fe–d_{z^2} orbital resulting in σ character. This is in sharp contrast to the properties of deoxyhemoglobins in which there is a consistent inverse correlation between the ν_4 frequency and the Fe–His stretching mode frequency.

Proximal Interactions. The iron–histidine stretching vibrational mode has been assigned in deoxyhemoglobin and deoxymyoglobin. In ferrous six-coordinate oxymyoglobin Walters et al. (1982) recently proposed that a weak shoulder at 272 cm^{-1} is the iron–histidine mode. However, the evidence was weak and an assignment of this mode in hemoglobin was precluded by the complexity of the spectrum of hemoglobin in the low-frequency region. It is not surprising that a strong Fe–His mode is apparently absent in six-coordinate heme proteins since in five-coordinate hemes the Fe–His stretching mode is enhanced by charge transfer or iron d–d transitions rather than the porphyrin π–π^* transitions, which enhance the porphyrin skeletal modes (Ondrias et al., 1983). The electronic reorganization that occurs on six-coordination would not be expected to leave the transition responsible for the enhancement of the Fe–His mode unaffected. Therefore, conclusions about proximal interactions may be drawn only from inference by examination of other modes.

In model compounds, several cases have been reported in which there are detectable differences in the properties of the exogenous ligand when properties of the ligand in the fifth-coordinate position are changed. First, the N–O stretching frequency on the distal side of the porphyrin was found to vary with the iron–nitrogen distance on the proximal side when the axial ligand and crystal structure of six-coordinate nitrosylpyridine–TPP complexes were changed (Scheidt et al., 1977). The sensitivity was found to be about 10 cm^{-1}/0.1 Å. It should be noted that in (NO)HbA + IHP when the iron–histidine bond in the α chains is broken, the N–O stretching frequency in the β chains is unaffected. Second, in comparisons between porphyrins with sterically hindered and unhindered substituted imidazole axial ligands, Kerr et al. (1983) detected a 7-cm^{-1} frequency shift in the Fe–CO stretching mode, which presumably results from increased proximal strain. Third, changing the basicity of a series of substituted pyridine axial ligands trans to CO in deuteroporphyrin complexes results in a 17-cm^{-1} change in the C–O stretching frequency (Alben & Caughey, 1968). In view of these results the observations on hemoglobins suggest that either the heme–histidine–ligand complex in hemoglobin uniquely lacks any trans interactions or there is no significant strain in the proximal histidine bond in the six-coordinate case.

In deoxyhemoglobins an inverse correlation was reported recently between the Fe–His stretching mode and ν_4, the π-electron density marker line (Ondrias et al., 1982). This correlation was found to extend over a wide variety of hemoglobins. Since an electronic reorganization accompanies the transition to six-coordinate hemoglobin, the same correlation would not be expected to exist in the (carbon monoxy)-hemoglobins. Therefore, conclusions regarding the degree of proximal strain cannot be drawn from the changes seen in ν_4.

Although from the data presented here and elsewhere we can infer that there is no significant difference in the Fe–His mode in six-coordinate hemoglobins, the transient deoxy species measured 10 ns after photolysis of CO exhibits a characteristic quaternary structure difference in the Fe–His mode (Freidman et al., 1982). The time scale in these measurements (10 ns) is much shorter than that of the quaternary rearrangement (hundreds of microseconds) and thus allows for an assessment of quaternary structural forces. The transient results imply the presence of differences in the six-coordinate case since on a 10-ns time scale the protein quaternary structure about the transient heme remains that of the liganded speceis. However, these differences in the Fe–His mode, which would be expected to be present in the six-coordinate case as well, do not induce changes in any of the modes we have examined in liganded hemoglobins. Thus, we must presume that any strain in the Fe–His bond does not exert any energetic influence at the heme in the ferrous six-coordinate case. The implications of the differences between the transient and the six-coordinate results will be discussed below.

Free Energy of Cooperativity. The free energy of cooperativity, ΔG, is defined as the difference for binding oxygen (or some other ligand) in the R state and the T state. Thus if E represents the total energy of a system and L and D represent liganded and deoxy molecules, respectively, ΔG may be written (Rousseau & Ondrias, 1983; Ondrias et al., 1982) as

$$\begin{aligned}\Delta G &= (E_D^T - E_L^T) - (E_D^R - E_L^R) \\ &= \Delta E^T - \Delta E^R \end{aligned} \quad (1)$$

and rearranged to

$$\begin{aligned}\Delta G &= (E_L^R - E_L^T) - (E_D^R - E_D^T) \\ &= \Delta E_L - \Delta E_D \end{aligned} \quad (2)$$

If ΔG is confined to a single region of the protein (e.g., the heme), then the free energy of cooperativity is given by the difference between the R–T difference in the liganded state and that in the deoxy state. It should be noted that if, for a given interaction, the R–T differences are the same for the liganded and deoxy states (i.e., $\Delta E_L = \Delta E_D$), then that interaction makes no contribution to ΔG.

It has been postulated that there is a heme–protein interaction in which the T structure is destabilized such that part of ΔG is stored in the iron–histidine bond. Furthermore, it has been predicted that most of the strain will be present in the liganded rather than the deoxy protein (Baldwin & Chothia, 1979; Dickerson & Geis, 1983). The argument proceeds as follows: For deoxyhemoglobin the iron is out of plane. In the T structure the histidine is tilted with respect to the heme plane, giving rise to a repulsive nonbonded interaction between the histidine and the heme. Upon ligand binding the iron atom is pulled into plane, owing to the nonbonded interactions of the exogenous ligand that serve as a counterforce to the nonbonded interactions of the histidine. This movement into plane serves to increase the strain energy of the iron–histidine bond in both the T and R structures. However, in the T structure the histidine maintains the same tilt in the liganded protein as it had in the deoxy state, resulting in an increased destabilization of the iron–histidine bond as compared to the R structure. Thus in the six-coordinate case the strain in the T structure with respect to the R structure must be increased compared to the five-coordinate case. It should also be noted for this case that because of eq 2 the increased destabilization of the T structure in the six-coordinate hemoglobin is a necessary condition for localization of ΔG in this bond (i.e., $|\Delta E_L| > |\Delta E_D|$).

The general features of the arguments presented above are not limited to the Fe–His bond. In any models for cooperativity for which a local interaction in the T structure is destabilized, the destabilization must be greater in the liganded case (Rousseau & Ondrias, 1983; Ondrias et al., 1982). Under these conditions, if the strain is localized at the heme, it should be detectable in either the iron–histidine bond, in the exogenous ligand–iron bond, or in the bonds of the porphyrin macrocycle. However, the data reported here show that there is no quaternary structure dependence in the exogenous ligand–iron

bond, that the changes in the heme modes are smaller than those detected in deoxyhemoglobins, and by inference that the strain in the iron–histidine bond is small. All of the current models in which cooperative energy is localized at the heme propose to have larger T-structure strain in the liganded heme than in the unliganded heme. The studies reported here have failed to locate such tension.

The comparison between the transient Raman results (Freidman et al., 1982) and the liganded hemoglobin results poses a dilemma. The transients show a change in the Fe–His bond with quaternary structure, but by inference from the six-coordinate studies there is no large change in the Fe–His mode. We explain this apparent dilemma as follows: In the fully liganded six-coordinate hemoglobin the histidine is rigidly held in place by the counterforces of the nonbonded interactions between the histidine and the porphyrin and the exogenous ligand and the porphyrin. These forces are very strong and thereby determine the stereochemistry of the histidine–porphyrin–ligand complex independent of the quaternary structure. The quaternary structural strain from the subunit interface is accommodated by weaker bonds in the pathway between the interface and the heme (Hopfield, 1973; Perutz, 1982). The energetics of a mixture of strong and weak bonds in series may be understood from elementary considerations. If we assume that a constant force is exerted on a strong bond, s, in series with a weak bond, w, with force constants k_s and k_w ($k_s \gg k_w$), then the resulting motion will be x_s and x_w, where $x_w \gg x_s$ (i.e., $F = k_s x_s = k_w x_w$). The work done at each bond, hence the amount of stored energy, is $(1/2)k_s x_s^2$ and $(1/2)k_w x_w^2$ so $\text{work}_w/\text{work}_s$ is equal to the ratio of the force constants (k_s/k_w). More energy is therefore stored in the weak bond since k_s is greater than k_w. In the six-coordinate case the weakest bonds do not appear to be localized at the heme. Thus, the free energy of cooperativity resides both in enthalpic changes at the interface and in weak bonds within the protein, as well as in entropic changes in the protein which would not be detected in our resonance Raman measurements.

Upon breaking the iron–ligand bond the iron moves out of plane to alleviate the nonbonded forces. In the resulting five-coordinate out-of-plane configuration the histidine tilt becomes very structure sensitive. Indeed, in hemoglobin the frequency of the iron–histidine mode varies from ~200 to ~245 cm^{-1} under various conditions (Ondrias et al., 1982; Ondrias et al., 1983). Therefore, upon dissociating the ligand some of the strain in the pathway between the subunit interface and the heme is realized in the iron–histidine bond, thereby accounting for its detectability in the transient spectra.

Conclusions

The experiments reported here address the basic questions of the mechanisms of cooperativity. Those mechanisms that invoke a T-structure destabilization and localize the free energy of cooperativity on the heme require a larger energetic difference for that interaction between the quaternary structures in the liganded protein than in the deoxy protein. However, the data show that no energy is stored in the distal interactions, and we infer that the energy stored in the proximal histidine–iron complex is small. For liganded hemoglobin the quaternary structural changes resulting from the subunit rearrangement appear to be largely accommodated in the protein bonds before reaching the heme environment. The state of ligand binding, not the quaternary structure, is the dominant determining factor in the tertiary environment around the heme. Thus, we conclude that no large fraction of the free energy cooperativity is stored at the liganded heme. The proximal strain model, which places a significant fraction of the free energy of cooperativity at the iron–histidine bond, due to nonbonded interactions between the histidine and the heme, is therefore at variance with these data. Instead, the data reported here are consistent with a model in which the free energy of cooperativity is either distributed in several weak bonds or localized in bonds that are not part of the primary tertiary environment of the heme.

On the other hand, there are some systematic quaternary structure dependent changes that are detected at the heme. Just as in deoxyhemoglobins (Ondrias et al., 1982) and methemoglobins, (Rousseau et al., 1980), the porphyrin π-electron density sensitive line (ν_4) is sensitive to quaternary structure in the (carbon monoxy)hemoglobins, and changes in this mode are interpreted as resulting from an electron density change. The amount of cooperative energy resulting from this change can not be readily assessed. Whether this change in electron density results from a direct porphyrin–protein interaction or is a consequence of back-donation from the iron due to a change in the iron–histidine bond also awaits further experimental and theoretical studies to resolve.

A comparison of the quaternary structure dependent mode changes detected in deoxyhemoglobins with those detected in liganded hemoglobins leads to some further observations on the apportioning of the cooperative energy. The quaternary structure dependent changes at the heme in deoxyhemoglobins are more widespread and larger than those detected in liganded hemoglobins. Thus the deoxyheme is apparently more flexible in its response to the changing tertiary environment induced by the quaternary structural change. In liganded hemoglobins the bonds and interactions associated with the heme are "tighter" and thus do not respond as much to changing the quaternary structure. Instead, the structural changes that occur at the interface upon the quaternary structure transition strain relatively weak chemical bonds and interactions (e.g., van der Waals interactions) in the protein rather than the stronger bonds involving the heme.

It is interesting to consider the quaternary structure dependence in methemoglobins in relation to the data presented here as well. Again, no changes in the iron–ligand modes have been detected as the quaternary structure is changed (Henry, 1980; E. R. Henry, D. L. Rousseau, J. J. Hopfield, R. W. Noble, and S. R. Simon, unpublished results). However, distinct changes in spin equilibrium were detected for several methemoglobins (Messana et al., 1978). For those ligands that normally confer a spin equilibrium to the heme iron, the subtle changes in the heme pocket upon the R–T transition cause a shift to higher spin. Messana et al. (1978) concluded, for example, that in carp Hb(N_3^-) the fraction of high-spin hemes changed from ~10 to 50% upon the addition of IHP. This corresponds to a free energy change of about 1 kcal. A recent analysis of methemoglobin data by E. R. Henry, D. L. Rousseau, J. J. Hopfield, R. W. Noble, and S. R. Simon (unpublished results) has shown that the contribution of ΔG that is found at the heme in human hemoglobins is 0.3 kcal/heme and in carp hemoglobin it is 0.6 kcal/heme. For the ferric case, therefore, the weakest degree of freedom at the heme is the equilibrium between the two spin states. Within each spin state the structural changes detected at the heme are very small (Henry, 1980; E. R. Henry, D. L. Rousseau, J. J. Hopfield, R. W. Noble, and S. R. Simon, unpublished results).

From a now large body of data it does not appear that a clear separation between models for cooperativity that cite localized interactions at the heme vs. those that place the cooperative energy in the protein can be made. Instead, some

fraction of the energy (an amount difficult to determine quantitatively) may be stored at the heme in a few interactions, and the remainder is in the protein.

Registry No. HbK(CO), 39320-10-0; IHP, 83-86-3; HbA(CO), 9072-24-6; Cl, 16887-00-6; heme, 14875-96-8.

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