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Protein–heme interaction in hemoglobin: Evidence from Raman difference spectroscopy

(electronic structure/vibrational modes/porphyrins/cooperativity)

J. A. SHELNUTT[ab], D. L. ROUSSEAU[ac], J. M. FRIEDMAN[a], AND S. R. SIMON[d]

[a]Bell Laboratories, Murray Hill, New Jersey 07974; and [d]State University of New York, Stony Brook, New York 11794

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ABSTRACT **Raman difference spectroscopy measurements on native and chemically modified human deoxyhemoglobins stabilized in either the R or the T quaternary structure revealed frequency differences in the oxidation state marker lines. The differences indicate that the R structure has an effective increase in the electron density of the antibonding π^* orbitals of the porphyrin rings. This increase is explained by a charge transfer interaction between donor orbitals and the π^* orbitals of the porphyrins. The relative amount of charge transferred, which is inferred from the Raman difference measurements, correlates with some but not all factors that influence the energetics of the quaternary structure equilibrium. In addition, the free energy of cooperativity for a variety of ligated proteins follows the same order as that of the degree of charge depletion of the π^* orbitals upon ligation as determined from the frequency of a Raman mode. The proposed electronic interaction between the protein and heme could result in energies large enough to provide a significant contribution to the energetics of hemoglobin cooperativity.**

The understanding of the molecular basis for cooperativity in the oxygenation of hemoglobin depends on being able to identify the origin of the difference, ΔG, in free energy of ligand binding between the low-affinity (T) and high-affinity (R) quaternary structures (1, 2). ΔG has been proposed by some to be localized at the heme (3) and by others to be distributed throughout the protein (4–6). We find evidence for a protein–heme interaction and we conclude that there is a contribution to ΔG, the free energy of cooperativity, which is detected at the porphyrin. This evidence, derived from Raman difference spectroscopy (RDS), suggests that ΔG contains an electronic stabilization energy resulting from a charge transfer interaction.

Due to resonance enhancement, the heme protein Raman spectra obtained with visible excitation frequencies contain only those vibrational modes associated with the porphyrin macrocycle. Consequently protein influences on the properties of the porphyrin ring may be studied by comparing the resonance Raman spectrum of molecules in which there are differences in the structure of the protein. In previous resonance Raman scattering studies of deoxyhemoglobins (7–11), which have attempted to locate bond strain and test cooperativity models, no differences in the Raman frequencies between the two quaternary structures were found. However, the lower limit for the detection of reliable frequency differences in hemoglobin has been about 1–2 cm^{-1}. Recently Shelnutt *et al.* (12) reported on a RDS technique in which they reliably detected frequency differences as small as 0.1 cm^{-1} in cytochromes *c* by simultaneously acquiring the Raman spectrum of two samples and by subsequent data processing on a minicomputer. We have now used this technique to compare native deoxyhemoglobin in the T structure and chemically modified deoxyhemoglobins stabilized in either the R or T structures.

MATERIALS AND METHODS

The modified human hemoglobins [NES-Hb A, des-Arg-Hb A, and NES-des-Arg-Hb A; NES is *S*-(*N*-ethylsuccinimido)-cysteinyl and Arg-141 is removed from the COOH terminus of the α chain] were prepared according to the procedure of Kilmartin and Hewitt (13). Inositol hexaphosphate (Ins-P_6) was added when appropriate at a concentration of 0.1 mM with Hb A and 0.4 mM with the derivatives. Stock solutions were stored at low temperature until ready for use, when small aliquots were diluted with 0.2 M sodium phosphate buffers (either pH 7 or pH 9) to obtain a heme concentration of between 200 and 400 μM. The deoxy samples were prepared by agitation in a nitrogen atmosphere prior to the addition of a slight excess of sodium dithionite. Methemoglobins were made by nitrite oxidation. Oxidation with ferricyanide was avoided because laser irradiation decomposed it, allowing the formation of methemoglobin cyanide even in samples that were chromatographed to remove the ferricyanide. While still in the nitrogen atmosphere the samples were transferred to the partitioned Raman scattering cell, which was then sealed. Spectra were obtained at room temperature, taking about 12 hr for completion. At the end of each run no change could be detected in either the Raman spectrum or the optical absorption spectrum of each sample. In those experiments in which the effects of a presumed quaternary conformation change were measured, this change was confirmed by measurement of the difference absorption spectrum.

In the RDS technique the two samples to be compared are placed in a rotating cylindrical cell constructed with a partition along a diameter, so as to allow simultaneous and independent collection of data from each sample. At the completion of gathering data the difference spectrum is obtained by digitally subtracting one spectrum from the other. The difference spectrum of two closely spaced and equally intense Raman lines has the shape of the derivative of the line. The value of the frequency difference is obtained by comparing the intensity (peak to valley) in the difference spectrum to the intensity of the line in the Raman spectrum. Because the sensitivity of this method is limited by the signal-to-noise ratio, the data were extensively signal averaged. From model calculations it was determined that the spectral conditions used (2 cm^{-1} spectral slit width, 0.1 cm^{-1} frequency steps) resulted in less than a 2% error in the frequency differences.

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Abbreviations: RDS, Raman difference spectroscopy; NES, *S*-(*N*-ethylsuccinimido)cysteinyl; Ins-P_6, inositol hexaphosphate.

[b] On leave from: School of Physics, Georgia Institute of Technology, Atlanta, GA; present address: Sandia Laboratories, D5114, Albuquerque, NM 87185.

[c] To whom correspondence should be addressed.

In reporting our results we compare the Raman line frequencies of all the proteins to those of Hb A. However, in many experiments other combinations of proteins were examined to serve as a cross check on the accuracy of our measurements. In no case were frequency differences obtained that were outside our stated error limits. In addition, since the construction of the Raman difference apparatus was completed (March 1978) many controls have been run to ensure that artifactual differences do not occur. In the results reported here the error limits for frequency differences in isolated lines result from the noise limitation in the difference spectrum. When there are several closely spaced Raman lines in a spectrum, the accuracy is limited to our ability to separate out the relative contributions of each of the lines.

RESULTS

We have measured the Raman difference spectrum of several pairs of chemically modified deoxyhemoglobins, ranging from those proteins with normal stability of the deoxy quaternary structure (unmodified Hb A), through those with reduced stability and increased ligand affinity (des-Arg-Hb A at pH 7 and NES-des-Arg-Hb A plus Ins-P_6), to those in which the deoxy T structure does not form and ligand affinity is high (NES-des-Arg-Hb A at pH 7; des-Arg-Hb A at pH 9) (1). We have detected many spectral differences between these proteins: in some Raman lines there were intensity differences, in some there were line shape differences, and in some there were frequency differences. In this report we stress the results of the frequency differences that occur in the high-frequency regions of the spectrum (1300–1700 cm^{-1}).

The largest Raman frequency differences were observed between Hb A and NES-des-Arg-Hb A. The data comparing these two proteins are shown in Fig. 1 *lower*, in which the resonance Raman spectra of deoxy-Hb A and deoxy-NES-des-Arg-Hb A were obtained with 4579-Å excitation. In Fig. 1 *upper* is the RDS spectrum. The most prominent frequency differences occur in the porphyrin lines at 1357, 1471, 1545, 1567, and 1605 cm^{-1} (see Table 1). It was necessary to use other excitation frequencies to enhance the lines at 1545 and 1605 cm^{-1} in order to determine the frequency differences in these lines. In Fig. 1 the line at 1620 cm^{-1} and the shoulder on the high-frequency side of it show differences in relative intensity but no frequency differences. Changes in relative intensity and frequency differences as large as 1 cm^{-1} are observed in the low-frequency lines (below 800 cm^{-1}) but are difficult to interpret due to incomplete characterization of the origin of frequency differences in this region. The 674 cm^{-1} line shows the characteristic shape in the difference spectrum of a broadened but unshifted line. The vibrational mode known to be sensitive to the heme center-to-nitrogen (pyrrole) distance (14) is at 1556 cm^{-1} both in des-Arg-Hb A at pH 7 (T) and in des-Arg-Hb A at pH 8.6 (R). Due to overlapping lines our sensitivity was reduced for this line. Nevertheless, we were able to place a limit on the frequency difference of this line in the R and T structures of less than ±1 cm^{-1} by using the laser frequency and polarization that gave optimal enhancement. As may be seen in Table 1, in comparisons between Hb A and the modified proteins in which the deoxy structure was only partially destabilized, the lines that differed between Hb A and NES-des-Arg-Hb A also differed for the other proteins, but to a lesser extent. Frequency differences were not detected in any of the vibrational modes when Hb A at pH 7 and pH 9, Hb A and NES-Hb A, and Hb A and Hb A + Ins-P_6 were compared.

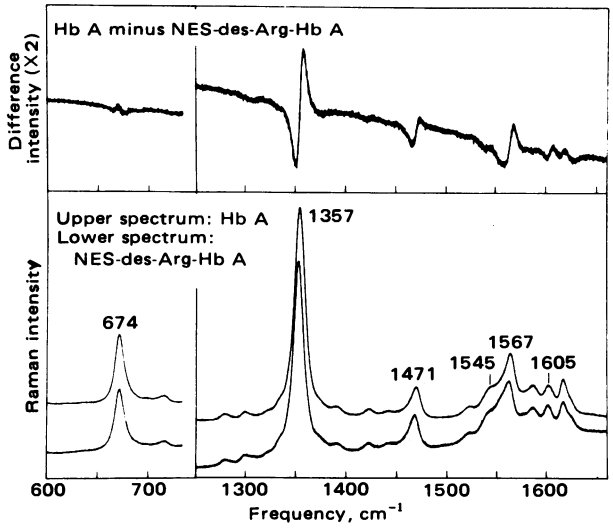

FIG. 1. Raman spectra (*Lower*) and Raman difference spectrum (*Upper*) of Hb A (T) and NES-des-Arg-Hb A (R). The scale of the difference spectrum is twice that of the Raman spectra. The spectrometer was advanced in 0.1-cm^{-1} increments for data collection and the signal was averaged by scanning the spectra 25 times. The scan rate was 0.5 sec per 0.1 cm^{-1}. The laser power at the sample was about 150 mW at 4579 Å. In this figure, which is a photograph of a plot of the digital data, the base lines were arbitrarily shifted for display purposes.

Hemoglobin consists of two α and two β chains. We have considered the possibility that our observed frequency differences occur in only one type of chain. The frequency differences for this chain would be twice as large as our measured differences for the tetramer, and there would be a linewidth difference in only those lines that show frequency differences. In this case the broadening of the 1357 cm^{-1} line is expected to be small but measurable, on the basis of a calculation of the sum of two displaced Lorentzians. Unfortunately, as may be seen in Fig. 1, the width of the 674 cm^{-1} line, in which no frequency shift is observed, increases on conversion to the R structure. The 1357 cm^{-1} line exhibits comparable broadening

Table 1. Raman frequency differences between Hb A and modified hemoglobins

Modified hemoglobin	Frequency difference (cm^{-1}) at Hb A line				
	1357 cm^{-1}	1471 cm^{-1}	1545 cm^{-1}	1567 cm^{-1}	1605 cm^{-1}
Des-Arg-Hb A (pH 7.0)	−0.3 (±0.1)	−0.5 (±0.5)	—[a]	−0.5 (±0.5)	−0.3 (+0.3, −0.8)
NES-des-Arg-Hb A + Ins-P_6	−0.5 (±0.1)	−0.9 (±0.5)	—[a]	−1.1 (±0.5)	−0.9 (±0.8)
Des-Arg-Hb A (pH 8.6)	−1.1 (±0.1)	−1.5 (±0.5)	—[a]	−1.7 (±0.5)	−1.0 (±0.8)
NES-des-Arg-Hb A (pH 7.0)	−1.3 (±0.1)	−1.8 (±0.5)	−1.5 (±1.0)	−2.2 (±0.5)	−1.5 (±0.8)

The signs of the frequency shifts are unambiguous, and the uncertainties in parentheses are derived from the signal-to-noise ratios.

[a] The frequencies of this line in the modified hemoglobins are lower than in Hb A, but for 4579-Å excitation the line is too weak for accurate determination of the frequency differences. In comparisons of des-Arg-Hb A at pH 7.0 and at pH 8.6 with 5287-Å excitation, the line was 1.0 ± 0.5 cm^{-1} lower at pH 8.6 than at pH 7.0.

(about 4%). This general broadening of the Raman lines in NES-des-Arg-Hb A could be a result of loosening of the heme pocket in the R structure. Although we are unable to exclude the possibility that only one of the chains is displaying frequency differences with respect to Hb A, the absence of additional broadening in the 1357 cm^{-1} line suggests that the difference in frequency is similar in both chains.

DISCUSSION

The 1200–1700 cm^{-1} region we have investigated contains the Raman modes which have been shown (15) to be sensitive to changes in oxidation state through *cis* back donation to the π^* orbitals and to changes in the spin state of the iron.[e] The line at 1357 cm^{-1} is the most reliable marker of *cis* back donation because it appears to be relatively insensitive to spin state changes. On the other hand, the lines at 1471, 1545, and 1605 cm^{-1} are sensitive to spin state changes as well as to changes in oxidation state.[f] In addition, the line at 1556 cm^{-1} is sensitive to spin state in heme proteins but is insensitive to oxidation state. The latter Raman line is also known to correlate with the heme center-to-nitrogen (pyrrole) distance (14, 16).

We first consider the vibrational modes sensitive to the spin state and geometry about the iron. For the anomalously polarized mode at 1556 cm^{-1} we find that the difference in frequency between the T and R structure is less than ± 1 cm^{-1}. From the data of Spaulding *et al.* (14) the variation of the pyrrole nitrogen-to-center distance with the frequency of this mode is about 0.002 Å/cm^{-1}. We may conclude that any movement of the heme iron from the plane of the pyrrole nitrogen atoms, associated with the R–T transition in deoxyhemoglobin, is not caused by alterations in the core size. In a recent normal coordinate analysis the 674 cm^{-1} line was shown to be a breathing mode of the 16-membered macrocycle (17, 18) and thereby to have considerable pyrrole nitrogen-to-Fe stretching character.[g] In addition, in model Fe(II) complexes this metal-sensitive line changes frequency when the axial ligand is varied (19), but the frequency change does not correlate with the extent of back donation. We can place a limit on the frequency difference of this line between the R and T quaternary structures of less than 0.1 cm^{-1}. Furthermore, the absence of large frequency changes in any of the low-frequency modes that have been reported to involve iron motions (11, 17, 18, 20, [g]) is additional evidence that structure, spin, or strain changes are not occurring in the iron coordination sphere upon change in quaternary structure.

We have detected frequency differences of up to 2 cm^{-1} in all of those modes known to be sensitive to the electron density in the antibonding π^* orbitals (19). The relationship between the frequencies of these modes and changes in the π^* orbital electron density may be qualitatively understood in terms of back donation from the $e_g(d_\pi)$ metal orbitals to the π^* orbitals of the porphyrin macrocycle. The lowest unoccupied molecular orbital of the porphyrin macrocycle is the doubly degenerate $e_g(\pi^*)$ orbital 2–3 eV above the highest occupied π orbitals (21). Through back donation the metal $e_g(d_\pi)$ orbital mixes with the porphyrin π^* orbitals, and as a result charge is transferred from the metal orbital to the porphyrin π^* orbitals (22). Substantial electron density in the $e_g(\pi^*)$ orbital is supported by recent calculations showing significant back donation to this orbital in osmium porphyrin complexes [≈ 0.3 π electrons in Os porphine(pyridine)$_2$] (A. Antipas, J. W. Buchler, M. Gouterman, and P. D. Smith, personal communication). The Raman frequency differences observed upon change in oxidation state or upon variation of the π-acid strength of the axial ligand are consistent with changes in the porphryin π^* orbital density (19). Because the $e_g(\pi^*)$ orbital is antibonding with respect to the bonds that contribute to most of the Raman-active normal modes (23), increased charge density in this orbital weakens these force constants and thereby causes the observed lowering of the Raman frequencies of these modes.

The $e_g(\pi^*)$ orbital is also involved in ligand bonding through mixing with the iron d_π orbitals. This is supported by substantial frequency differences between ligated and unligated hemoglobin in the Raman modes that are sensitive to the electron density in the π^* orbitals. These modes have roughly the same frequencies in oxyhemoglobin as they do in low-spin ferric heme proteins (24). Other studies suggest that the dioxygen ligand withdraws extensive charge density from the heme (25). It is apparent from the change in frequency of the back donation marker lines that the electron density of the porphyrin antibonding orbitals of the reduced heme are drained to about the same extent by oxygenation as they are by oxidation. *Because our experiments demonstrate that it is also these orbitals that are sensitive to the protein–heme interaction, they provide a link between oxygen bonding and quaternary structure of the protein.*

The observed change in electron density, which is due to a protein–heme interaction, may be interpreted in terms of a general charge transfer mechanism in which the charge in a porphyrin (acceptor) orbital is increased in the R structure by transfer from an electron donor. To see how a charge transfer interaction may contribute to cooperativity, we consider this interaction in greater detail by using a perturbation theory treatment of the mixing between donor and acceptor orbitals. In the R structure, in which we assume the near heme geometry is favorable for the charge transfer interaction to occur, the porphyrin orbital energies are raised and the donor orbital energies are lowered due to mixing of the orbitals. This mixing of the donor orbitals, which are nominally filled, and the porphyrin antibonding orbitals, which are only partially filled, results in increased electron density in the porphyrin orbitals. This interaction lowers the configuration energy of the charge transfer system, resulting in a stabilization of the R structure with respect to the T structure. In this model the stabilization energy is proportional to the amount of charge that is transferred. If this stabilization energy is functionally relevant, the electron density, which can be inferred from the RDS results, should correlate with factors that influence the instability of the T structure.

In Table 1 the deoxyhemoglobins that we have examined are ordered according to the degree of destabilization of the T structure (1). The corresponding Raman frequency differences are observed to increase with this order. Hence this ordering indicates that the π^* orbital electron densities deduced from the RDS data are associated with the degree of destabilization of the deoxy structure in these des-Arg-hemoglobins. However, the frequency differences do not quantitatively correlate with the degree of destabilization. Moreover, some hemoglobins, compared to Hb A at pH 7 (Hb A at pH 9, NES-Hb A + Ins-P_6) with significant affinity differences (1), do not show frequency differences in the RDS spectra. Chemical modifications of deoxyhemoglobin result in differences in the allosteric constant, L, and the ratio c ($c = K_R/K_T$, in which K is the dissociation

[e] By *cis* back donation we mean delocalization of charge from metal d_π orbitals to the porphyrin $e_g(\pi^*)$ orbitals.

[f] The mode at 1567 cm^{-1} in which we observe a difference has been seen only in hemoglobin, and its behavior upon changing the π^* electron density has not been determined.

[g] Nagai, K., Kitagawa, T. & Morimoto, H. (1978) Sixth International Biophysics Congress, Kyoto, Japan.

constant for ligand binding) (26). The electron density differences are detected in the heme and therefore are expected to reflect differences for the ratio c for these deoxyhemoglobins and the differences in the stabilization energies also affect L.

An additional consequence of the charge transfer interaction is *increased* stabilization of the R structure of the ligated hemoglobins relative to the R structure of the unligated hemoglobins. This would occur because the depletion of electron density in the antibonding orbitals, which takes place upon ligation and oxidation, favors increased charge transfer to the porphyrin in the ligated state and concomitantly increases the charge transfer stabilization energy. This increase in stabilization energy upon ligation contributes directly to ΔG, the free energy of cooperativity, and is proportional to the extent of charge depletion of the antibonding orbitals upon formation of the ligand bond. For different ligands the relative electron density in the antibonding orbitals may be inferred from the frequency of the marker line in the 1350–1380 cm^{-1} region. Thus the extent of charge depletion can be determined from the change in the marker line frequency upon ligation, and in the model this change for each ligand indicates the relative charge transfer contribution to ΔG. In Fig. 2 we have plotted the frequencies of this mode for several ligated proteins in order to determine the relative charge transfer stabilization for each. With the exception of HbNO, CoHb, and $CoHbO_2$, all of the data were obtained on our instrument. The absolute scale is accurate to within ± 0.5 cm^{-1} and the relative frequencies are accurate to within ± 0.2 cm^{-1}.

The range of frequencies of the ligated proteins in Fig. 2 indicates substantial differences in the increase in the charge transfer stabilization energy for these ligands. To determine if the increase in the charge transfer stabilization energy is the same fraction of the free energy of cooperativity, ΔG, for each ligand, the values of ΔG are needed. Unfortunately very few have been determined, although rough estimates of ΔG may be made by examining the efficacy with which Ins-P_6 brings about the R-to-T quaternary structure change in the ligated state. To aid in ascertaining whether or not the increase in the charge transfer stabilization energy is the same fraction of ΔG for each ligand, we have placed a free energy scale for the ligated hemoglobins on the right side of the figure. If $\epsilon \Delta G$ is the fractional contribution of the increase in charge transfer stabilization energy to the ΔG for each ligand, and assuming the fraction ϵ is the same for all ligands, then the free energy scale should be linearly related to the charge transfer stabilization energy, which should also correlate with the frequencies of the Raman marker line. We have calibrated the free energy scale by using the free energy of cooperativity of binding O_2 to deoxyhemoglobin [$\Delta G = 3.6$ kcal/mol per heme (1)]. Using this scale, we placed the dashed lines on the figure to define the region in which 4 ΔG is approximately equal to the free energy stabilizing the T over the R structure in Hb A [8.5 kcal/mol of tetramer (1)] and hence defines the region of dynamic R/T equilibrium. The free energy scale we have constructed assumes a linear relationship between the Raman marker line and the stabilization energy. Calculations and corresponding experiments on model compounds are necessary to test this assumption.

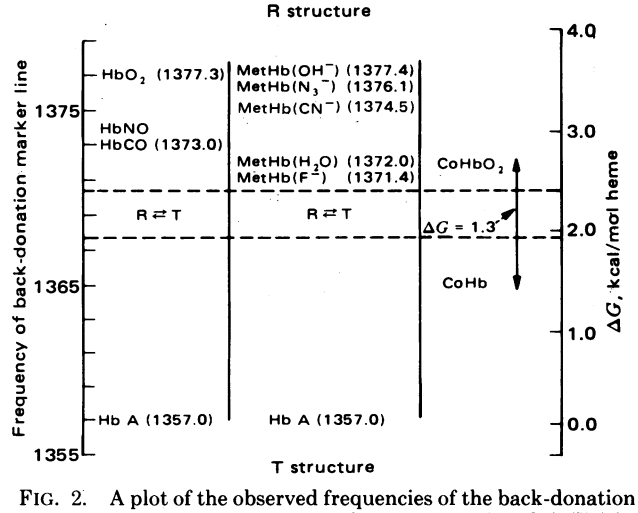

FIG. 2. A plot of the observed frequencies of the back-donation marker line for deoxyhemoglobin and various Fe(II) and Fe(III) liganded hemoglobins and cobalt-substituted hemoglobins. The difference in frequency between the unligated and ligated hemoglobin is a measure of the charge depletion of the π^* orbitals upon ligation, and the depletion is related to the contribution of the charge transfer stabilization energy to ΔG. A free energy scale for the ligated hemoglobins is shown on the right (1 kcal = 4.18 kJ). The significance of the scale is described in the text. All the frequencies listed in parentheses were obtained in the present study. The HbNO frequency was obtained from ref. 8 and those of the cobalt-substituted hemoglobins from ref. 27. The frequencies of the cobalt-substituted hemoglobins were scaled for the metal substitution effect (see text).

We find that the ordering obtained from the Raman data roughly follows the corresponding ordering of ΔG established by considering the effect of addition of Ins-P_6. Those proteins with the highest Raman frequencies are well above the R/T equilibrium and are not switched from the R to the T structure upon the addition of Ins-P_6. Fluoromethemoglobin and aquomethemoglobin lie near the region of the dynamic R/T equilibrium and both are switched to the T structure upon addition of Ins-P_6 (28). The frequency difference between HbCO and HbO_2 may arise from different contributions of charge transfer to the stabilization of the R structure or from different values of ΔG associated with chain inequivalences. The position of CoHb in Fig. 2 shows that introducing Co instead of Fe into the heme decreases the stability of the T structure relative to that of FeHb. This partially destabilized T structure allows the R/T transition to occur between the second and third ligation steps (29). The frequency of the marker line for $CoHbO_2$ indicates a further stabilization of the R structure upon ligation, and the Raman frequency difference between deoxy- and oxy-CoHb of 7 cm^{-1} gives a value of 1.3 kcal/mol for this ΔG, in agreement with the value of 1.3 kcal/mol calculated by Woodruff *et al.* (27) from affinity measurements. In placing the CoHb data in Fig. 2 we have lowered the reported frequencies by 8 cm^{-1}, in accord with results of Kitagawa *et al.* (30), to reflect the metal substitution dependence of this line. (This does not affect the $\Delta G = 1.3$ kcal/mol determination.) The empirical relationship between ΔG and the change in frequency of the marker line on ligation for this variety of proteins indicates that the charge transfer stabilization energy is nearly the same fraction of ΔG for each ligand and is therefore roughly proportional to ΔG. However, it should be emphasized that this relationship provides no indication of the magnitude of the contribution of the charge transfer stabilization energy to ΔG.

There are several possible donor energy levels that could give rise to the increased orbital density that we have observed. For example, changes in orientation of the proximal histidine (31) or changes in its hydrogen bond conformation (25, 32, 33) could influence the histidine–iron bonding and hence the iron-to-porphyrin back donation. Alternatively, a charge transfer complex between the heme and a near heme aromatic residue may be formed such that electron density is transferred from filled orbitals of the aromatic residues to the partially filled

porphyrin antibonding orbitals. In addition, a change in van der Waals interaction between amino acid residues and the heme could cause redistribution of the charge among the porphyrin orbitals.

Evidence for a key role for the near heme aromatic residues in regulating the electron density in the π^* orbitals of the porphyrin comes from studies on the effects of species differences in these positions in cytochromes. In a recent RDS analysis of a series of cytochromes *c* (12), changes in the π^* electron density in the porphyrin macrocycle, comparable to those we have observed in hemoglobin, resulted from changes in the interaction between the porphyrin and the near heme aromatic residues. An extension of this interpretation to the present hemoglobin investigation suggests that upon the change in quaternary structure the heme–aromatic residue interaction changes, affecting the charge density of the antibonding orbitals. Comparison of x-ray crystallographic data on human deoxyhemoglobin (T structure) and horse aquomethemoglobin (R structure) confirms that the two aromatic residues in contact with the heme, phenylalanine CD1 and phenylalanine G5, change their orientation substantially with respect to the porphyrin ring in both α and β chains on going from the T to the R structure (34). The behavior of phenylalanine CD1 is most pronounced in this regard because in the R structure of both the α and β chains it occupies an equivalent position in van der Waals contact with the carbons of the porphyrin moiety. CD1 moves away from the heme in the T structure in both chains. In the mutant hemoglobins Hammersmith ($\alpha_2\beta_2^{42\text{Phe-Ser}}$) (35) and Bucuresti ($\alpha_2\beta_2^{42\text{Phe-Leu}}$) (36) substitution of this residue leads to loss of cooperativity in ligand binding and decreased O_2 affinity (37). The functional importance of this aromatic residue and its location make a direct charge transfer interaction an attractive possibility for the origin of the charge density change in the π^* orbitals. However, from the data currently available we cannot definitively distinguish between a direct electron-donating role of the near heme aromatic residues and a more complicated role in which they sterically regulate the formation of a charge transfer complex of the heme with an additional donor (37).

The energetics of the charge transfer interaction we propose depend on the amount of electron density that is transferred and on the energies of the donor and acceptor levels. Furthermore, the energetics that affect ΔG also depend on the absolute amount of charge that is drained from the antibonding orbitals upon ligation. By using values for these parameters that are not unreasonable we can calculate stabilization energies due to the charge transfer interaction; these energies could amount to a large fraction of ΔG. Although more experiments and calculations are needed to determine the contribution of this protein–heme interaction to the energetics of cooperativity, it is our belief that this interaction could account for a significant part of the enthalpic contribution to ΔG.

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