Resonance Raman Spectroscopic Evidence That Carp Deoxyhemoglobin Remains in a T-like Quaternary Structure at High pH: Implications for Cooperativity†

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ABSTRACT: Resonance Raman spectroscopy shows the Fe-proximal imidazole stretching band to shift from 215 to 219 cm^{-1} between human deoxyhemoglobin (deoxy-Hb) and a Hb sample which is 75% oxygenated, demonstrating that the T–R quaternary structure switch can be monitored by resonance Raman spectroscopy in native Hb at equilibrium. For deoxy-Hb from carp, the band is at 215 cm^{-1} at pH 9 as well as pH 6, contrary to previous reports of an elevated frequency at high pH. The invariance of this frequency over a large affinity difference is in contrast to a recent report of continuously varying ν_{Fe-ImH} frequencies for human mutant deoxy-Hb's. The band shifts to 219 cm^{-1} for carp Hb at pH 9 when O_2 is bound to only 20% of the hemes. The spectra are consistent with a T–R switch upon binding $\sim$0.5 O_2 per Hb, on the average, although the number may be higher if the binding affinity is higher for α- than for β-chains. The 0.5 value, in conjunction with the weak cooperativity observed for carp Hb at pH 9, is incompatible with a value of the allosteric constant, $L = (T_0)/(R_0)$, large enough to prevent the ν_{Fe-ImH} band from shifting detectably at pH 9 in the absence of O_2. The possibility of functionally important intermediate structures is discussed.

The molecular mechanism of cooperative ligand binding to hemoglobin remains an important focus of research, with implications for protein allostery in general. A large body of equilibrium and kinetic data (Shulman et al., 1975) supports the idea that hemoglobin exists in two states (Monod et al., 1965), T (tense) and R (relaxed), which differ in ligand affinity, and that cooperativity is associated with a switch from the T to the R state at some point on the binding curve. These states can be associated with the different quaternary structures determined by X-ray crystallography for deoxyhemoglobin and ligated hemoglobin (Hb) (Perutz, 1978, 1980; Baldwin & Chothia, 1979). These structures provide the framework for analyses of cooperativity in molecular terms. Questions that remain to be answered include the degree of molecular flexibility within a quaternary structure, the pathway connecting ligation with protein conformation change, and the connection between structure and binding rates and energies.

In pursuing these questions, it is important to have spectroscopic probes of structure that can be applied to hemoglobin in its various functional states. A promising new probe has been developed through the finding that a vibrational mode corresponding to the stretching of the Fe-proximal imidazole (ImH) bond can be detected in the resonance Raman (RR) spectra of deoxyheme proteins (Kitagawa et al., 1979; Kincaid et al., 1979). This bond is the only covalent connection between heme and protein, and Nagai and Kitagawa (1980) have made the important observation that in human deoxy-Hb A, the stretching frequency is sensitive to quaternary structure in a chain-specific manner. Using valency hybrid Hb's and the effector molecule inositol hexaphosphate (IHP), they showed the α- and β-chain Fe–ImH frequencies to be 222 and 224 cm^{-1} for the R state but 207 and 220 cm^{-1} for the T state. These results were confirmed in a study of Co, Fe hybrids (Ondrias et al., 1982). The R–T frequency differences are substantial and imply changes in the Fe–imidazole bond strength resulting from the quaternary structure change. On the assumption that the bond strength scales directly with the stretching force constant, changes of 1.3 and 0.4 kcal/mol can be estimated for the α- and β-chains (Stein et al., 1980),[1] which are significant on the scale of the free energy of cooperativity, $\sim$3.4 kcal/mol per heme (Imai, 1973). The α- and β-chain Fe–ImH bands coalesce in native Hb to give a broad and asymmetric band, centered at 215 cm^{-1} for the deoxy T state, and a narrower band, centered at 222 cm^{-1} for the deoxy R state, as studied in chemically modified Hb's (Nagai et al., 1980), or via kinetic isolation (Stein et al., 1982; Scott & Friedman, 1984). In this study we demonstrate that the T–R frequency shift can be seen even at equilibrium for native Hb A.

Hemoglobin from carp, which is believed to switch from T to R in the deoxy form upon raising the pH (Noble et al., 1970; Tan et al., 1972a,b), has appeared to fall nicely into this pattern. There have been two reports, one by Dalvit et al. (1982) and one from this laboratory (Walters et al., 1983), that the Fe–ImH frequency for deoxy carp Hb shifts by several cm^{-1} between low and high pH. We have found, however, that these results are incorrect and that the frequency upshift at high pH is associated with a small fraction of bound O_2. The increase in affinity at high pH makes it difficult to remove all of the bound ligand, but when this is accomplished, the

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[1] Nagai and Kitagawa (1980) gave much smaller values, based on a calculation of the energy required to displace a bond along a given potential curve. In our view, this procedure does not estimate the bond energy change, since the potential curve shifts between the two structures. It should be noted (Stein et al., 1980) that the changes are actually in the wrong direction to account for cooperativity, since a destabilization of the deoxy T state is implied by the lower Fe–imidazole bond strength; this should increase, not decrease, the ligand affinity, all other factors remaining equal. Presumably there are correlated changes in the ligated form which more than compensate this effect.

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Fe–ImH band is found to be identical with that observed at low pH. This behavior implies that, other indicators notwithstanding (McDonald et al., 1976; Dalvit et al., 1984), the protein remains in a T-like state at high pH.

This behavior, a T-state Fe–ImH frequency independent of affinity, is quite different from that reported recently by Matsukawa et al. (1985) for a series of mutant human hemoglobins of varying affinities. A continuous range of Fe–ImH frequencies, 215–224 cm^{-1}, was observed, the values correlating with the equilibrium constant for the binding of the first O_2 to the tetrameric molecules. Since the allosteric constant was determined from the binding curves to be large enough to assure that the deoxy tetramers remained in the T state, even for the high-affinity mutants, the authors developed a model involving a continuous range of T-state structures, with Fe–ImH frequencies directly related to the binding energy. Assuming that these data are not subject to the same binding artifact that led the earlier carp Hb studies astray, it must be concluded that carp Hb is different from human Hb mutants with respect to the plasticity of the T state.

Experimental Procedures

Human hemoglobin A was prepared according to Perutz (1968) and was stripped of phosphates on Bio-Rad AG-11 and AG-501-X8 columns. Hemoglobin from carp was prepared according to Tan et al. (1972a) by DEAE chromatography of hemolysate, the three components being eluted simultaneously with 0.05 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 8.5. It was stripped on a Bio-Rad AG-501-X8 column and stored at −20 °C as the CO adduct to prevent oxidation. HbCO was converted to HbO_2 by photolysis under a tungsten lamp in the presence of O_2, the 500–600-nm absorption changes being monitored. The deoxy form was then obtained by repeated flushing of HbO_2 with argon gas.

Raman spectra were obtained via backscattering, with 457.9-nm Ar^+ or 413.1-nm Kr^+ laser excitation; they were collected with a Spex 1401 monochromator equipped with a cooled photomultiplier and photon counting detection, under computer control (Walters et al., 1983). The Rayleigh background in the low-frequency region was subtracted by a computerized base-line correction program using a cubic polynomial fit to the background. The fraction of HbO_2 contained in partially oxygenated samples was determined from the relative intensities of the 1376- (HbO_2) and 1357-cm^{-1} (deoxy-Hb) Raman bands after making an appropriate absorption correction for the backscattering geometry used (Shriver & Dunn, 1974):

$$\frac{(HbO_2)}{(Hb)} = \frac{I_{1376}}{I_{1357}} \frac{I^0_{1357}}{I^0_{1376}} \frac{\epsilon_{l1} + \epsilon_{s1}}{\epsilon_{l2} + \epsilon_{s2}}$$

where I_{1376}/I_{1357} is the intensity ratio for the ν_4 RR bands of HbO_2 and Hb, at 1376 and 1357 cm^{-1} (Walters et al., 1983), I^0_{1376}/I^0_{1357} is the intensity ratio for pure (equimolar) samples, ϵ_{l1} and ϵ_{s1} are the HbO_2 molar absorptivities at the laser and scattering wavelengths, and ϵ_{l2} and ϵ_{s2} are the corresponding Hb absorptivities.

Results and Discussion

ν_{Fe-ImH} and the Hb A T–R Equilibrium. Although the cooperative binding of O_2 to Hb means that most of the molecules are fully ligated or fully unligated, a significant number of partially ligated molecules can be obtained in the middle range of the binding curve. Since HbO_2 does not contribute significantly in the region of the deoxy-Hb ν_{Fe-ImH} band (Walters et al., 1983), we decided to search for evidence of the T–R frequency difference in human Hb A by examining the remnant ν_{Fe-ImH} band at high O_2 saturation. Figure 1 compares this band for stripped deoxy-Hb and a sample in which 75% of the heme groups are bound to O_2. A clear upshift is seen in the band maximum, from 215 to 219 cm^{-1}. According to the equilibrium binding data of Tyuma et al. (1983), the 25% of unligated heme groups are distributed as 9, 7.5, 4.5, and 5% in Hb molecules containing zero, one, two, and three bound O_2's, respectively. If, as is generally believed, the T → R switch occurs upon binding of ~2.5 O_2 molecules, on average (Shulman et al., 1975), then ~30% of the deoxyhemes are in molecules in the R state and the remainder are in the T state. This is consistent with the intermediate value observed for the peak frequency. When the effector molecules diphosphoglycerate (DPG) or inositol hexaphosphate (IHP) are present, the T → R switch shifts toward later stages of oxygenation (Imai, 1973). Consistent with this, we found the ν_{Fe-ImH} frequency shift at 75% oxygenation to be abolished by the addition of DPG or IHP.

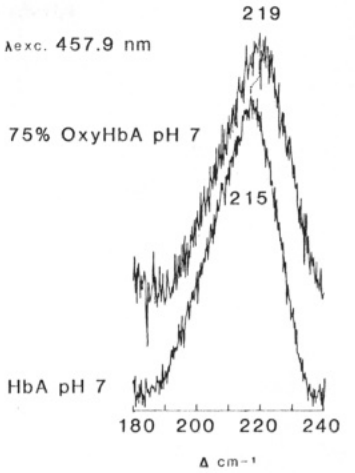

FIGURE 1: Resonance Raman spectra with 457.9-nm excitation in the 215-cm^{-1} region for human stripped deoxyhemoglobin, and for a sample which was oxygenated to a level of 75%. The latter spectrum was expanded vertically for visual comparison; oxy-Hb does not contribute significantly in this frequency region. The Rayleigh background for each spectrum was subtracted by a computerized base-line correction program using a cubic polynomial fit to the background.

Carp Deoxy-Hb Stays T-like at High pH. Hemoglobin from carp has attracted much interest because of the strong variation of its ligand binding properties with pH (Noble et al., 1970; Tan et al., 1972a,b; Tan & Noble, 1973; Pennelly et al., 1975; Chien & Mayo, 1980). The O_2 affinity is low in acidic solution but increases markedly as the pH is raised. The binding curve is cooperative at intermediate pH values but approaches being noncooperative at both low and high pH. Within the two-state model (Monod et al., 1965) this behavior is qualitatively accommodated by postulating a pH-dependent allosteric equilibrium constant, L, which is the ratio of unligated Hb in the T and R states. For a given ratio of intrinsic binding constants, $c = K_R/K_T$, low cooperativity is expected at both high and low values of L (Edelstein, 1971). For carp Hb, L clearly attains a high value at low pH, since ligated forms can be switched from R to T upon addition of IHP (Pennelly et al., 1975). This does not happen for Hb A, except for the NO adduct, in which the bond to the proximal imidazole is weakened via the NO trans effect, or for high-spin forms of metHb (Perutz, 1978).

It has generally been assumed that L decreases monotonically with increasing pH for carp Hb and that, at sufficiently high pH, the R state becomes predominant, even for the deoxy

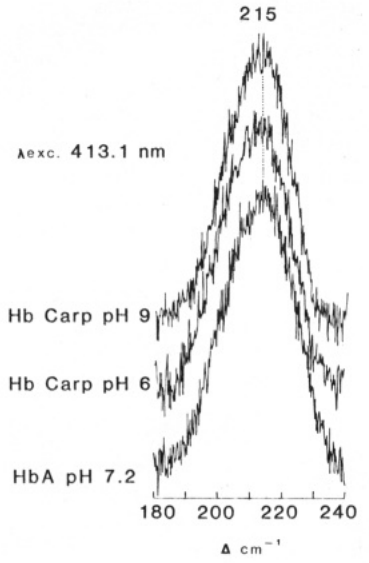

FIGURE 2: Comparison of the 215-cm^{-1} bands for deoxy-Hb A with deoxy-Hb from carp, at pH 6 (Bis-Tris) and pH 9 (Tris buffer). The spectra were obtained and processed as in Figure 1, except that excitation was at 413.1 nm.

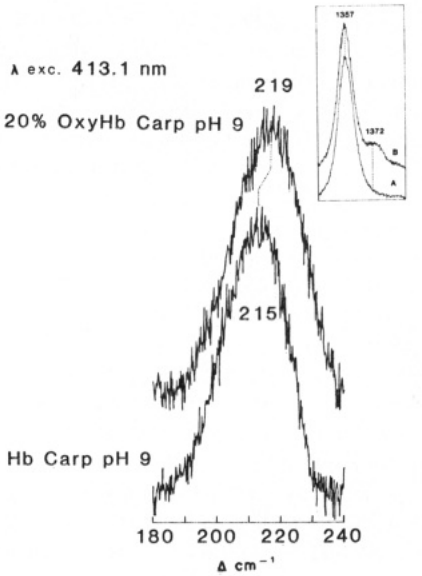

FIGURE 3: Comparison of the ~215-cm^{-1} RR band for carp deoxy-Hb at pH 9 and for a sample estimated to contain 20% HbO_2. The inset shows the ν_4 region of the RR spectrum for the same samples. The HbO_2 estimate was obtained from the relative intensity of the 1376-(HbO_2) and 1357-cm^{-1} (deoxy-Hb) bands (see Experimental Procedures).

form, thereby accounting for the loss in cooperativity. Figure 2 demonstrates, however, that the ν_{Fe-ImH} band of carp deoxyHb is the same at pH 6 and 9. Moreover, both samples show the same band center, 215 cm^{-1}, and width, as does deoxy-Hb A. This result differs from those reported previously (Dalvit et al., 1982; Walters et al., 1983), in which a pronounced upshift was seen at pH 9. Initially we obtained the same result but found it to be due to the presence of a small fraction of ligated Hb (see next section); when the photoconversion of HbCO to HbO_2 was carried out carefully, and the HbO_2 was flushed repeatedly with argon, the spectrum shown in Figure 2 was obtained reproducibly.

Although the RR spectra show no difference, we note that other spectroscopic indicators have shown differences. Thus, the optical absorption difference spectrum is similar to that observed for deoxy-Hb A between T and R states (McDonald et al., 1976). Also the NMR spectrum at low pH shows an exchangeable proton resonance at 9.1 ppm, similar to one seen in deoxy-Hb A and believed to be a marker for the T quaternary structure, which disappears at pH 8.5 (Dalvit et al., 1984). Certainly there must be some difference in the protein structure between low and high pH in order to account for the large affinity change, and the absorption and NMR spectra are presumably responding to these changes. In the case of NMR, dynamic factors may also be involved; for example, increased conformational flexibility at high pH might broaden and shift a resonance even in the absence of a change in the average structure. The RR result establishes, however, that whatever protein forces are responsible for the lowered ν_{Fe-ImH} frequency in T (or T-like), molecules are not relaxed at high pH for carp Hb.

A quite different result has been reported for mutant human Hb's by Matsukawa et al. (1985), who found a continuous range of ν_{Fe-ImH} peak frequencies, from 215 to 224 cm^{-1}, correlating with the binding constant for the first O_2 molecule, K_1. Since the tetrameric Hb's included in the study had allosteric constants, L, large enough to ensure that most of the molecules were in the T state, Matsukawa et al. inferred that the T state must accommodate a range of structural variation as reflected in the variable ν_{Fe-ImH} frequencies. Noting that ligated Hb's have shown no significant R–T differences in the Fe-exogenous ligand stretching frequency [the Fe–ImH RR band is unfortunately very weak in ligated Hb (Walters & Spiro, 1984)], they concluded that most of the binding free energy variation must reside in the unligated form and developed an equation based on this model, which fits the Raman frequency data:

$$\nu_{Fe-ImH} = 225.2 - 7.10(-\log K_1 + 0.488)^{1/2} \qquad (1)$$

On the basis of the O_2 binding constants measured by Chien and Mayo (1980), $K_1 = 0.0178$ and 1.17 at pH 5.92 and 9.06 (Bis-Tris buffer), we calculate from the above equation that ν_{Fe-ImH} should be 215 and 221 cm^{-1} at low and high pH. The latter value, which is the frequency expected for R-state Hb, agrees with the previous carp Hb measurement (Walters et al., 1983) at high pH, which we now find to be artifactual and due to incomplete deoxygenation. This artifact must be considered as a possible explanation for the trend observed by Matsukawa et al. (1985), since it would have the effect of raising the apparent ν_{Fe-ImH} frequency for high-affinity Hb's. (This possibility should also be considered in connection with the optical and NMR evidence discussed in the preceding paragraph.) If the trend is not artifactual, then it must be concluded that the T-state structure correlates with affinity for mutant human Hb's but is independent of affinity, at least as far as ν_{Fe-ImH} is concerned, for carp Hb. Possibly a range of different Hb's, as examined by Matsukawa et al. (1985), does show affinity tuning via ν_{Fe-ImH} variation, whereas a different mechanism is used to control affinity when a single protein (carp) responds to external conditions (pH).

Low-Saturation T–R Switch and the Two-State Model. Very little oxygenation is required to produce an appreciable increase in ν_{Fe-ImH} for carp deoxy-Hb at pH 9, as shown in Figure 3. The band in the upper spectrum, with a peak frequency of 219 cm^{-1}, was obtained on a sample that was determined, by the intensities of the ν_4 ["oxidation state marker" (Spiro, 1983)] porphyrin bands at 1357/1376 cm^{-1} (see Figure 3 inset and Experimental Procedures), to have 20% of its heme groups bound to O_2. Thus, a change in the deoxyheme structure occurs quite early in the oxygenation curve.

From the equilibrium binding data of Chien and Mayo (1980) for carp Hb at pH 9.06, we calculate that the deoxy-

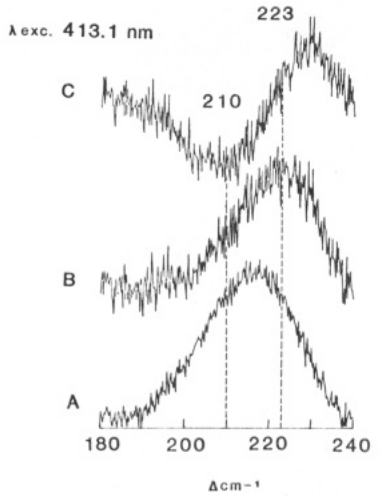

FIGURE 4: (A) Spectrum of the 219-cm^{-1} band for the 20% oxygenated carp Hb sample, as in Figure 3. (B) Spectrum A minus the spectrum of pure deoxy-Hb (Figure 3) after normalization for the deoxyheme content and multiplication by 0.35, the fraction of the deoxyhemes in molecules containing no O_2's. (C) Same subtraction as for (B) except the weighting factor was increased to 0.69, the fraction of deoxyhemes in molecules containing zero and one O_2.

hemes in the 20% HbO_2 sample are distributed according to the following percentages among molecules containing zero, one, two, and three O_2's: 35, 34, 7.5, and 1.2%. Figure 4 shows an attempt to deconvolute the contributions from these molecules to the 219-cm^{-1} band of the 20% HbO_2 sample, using computer subtraction. The two spectra shown in Figure 3 were scaled to the ratio 0.8, to account for the relative population of deoxyhemes, and the deoxy-Hb spectrum was then subtracted from the 20% HbO_2 spectrum by using a weighting factor of 0.35, the contribution expected for completely unligated Hb molecules. The result is curve B in Figure 4, which represents the aggregate spectrum from the deoxyheme groups of molecules containing one to three O_2's. It shows a significant upshift in the peak frequency, to 223 cm^{-1}, the value expected for deoxy-Hb in the R state. Appreciable intensity remains at 210 cm^{-1}, however, which must be associated with T-state molecules. Thus, some of the Hb molecules containing bound O_2 remain in the T state; most of these molecules contain one O_2.

Curve C shows the effect of increasing the scale factor to 0.69 in subtracting the deoxy-Hb spectrum, to allow for the deoxyhemes in Hb molecules containing one, as well as zero, O_2's. Clearly, this subtraction is excessive, since there is now a trough at $\sim$210 cm^{-1} and the frequency of the peak, 230 cm^{-1}, is higher than expected for R-state Hb. This result suggests that some of the Hb molecules with one O_2 are in the R state; i.e., the T $\rightarrow$ R switch occurs between zero and one bound O_2's. The form of the curve suggests roughly equal T and R populations for the monoligated Hb's. If this is correct, then the T $\rightarrow$ R switch occurs when the average number, i, of O_2's bound per Hb is $\sim$0.5.

There is, however, an alternative explanation of curve C, namely, a higher O_2 affinity for the α-chains than for the β-chains. While the generation of curve B is unambiguous, provided that the fraction of unligated molecules is correctly estimated, further subtraction assumes that the deoxy-Hb spectrum is representative of all deoxyheme-containing molecules that remain in the T state. This assumption would be invalidated by selective binding to the α-chains, since this would specifically lower the RR intensity in the 210-cm^{-1} region, the frequency for β-deoxyhemes being 220 cm^{-1} even in the T state (Nagai & Kitagawa, 1980). Thus, it is theoretically possible for spectrum B to represent mostly T-state molecules if the O_2 is bound mainly to the α-chains. For this reason, the above estimate of $i = \sim 0.5$, must be considered a lower limit; it could be higher if O_2 binds selectively to the α-chains. The α- and β-chain affinities are equal for stripped human Hb A, but addition of DPG or IHP increases the α/β affinity ratio somewhat (Viggiano & Ho, 1979). To the best of our knowledge, such measurements have not been carried out for carp Hb.

Whatever the range of variation imposed on a protein by external conditions (pH, mutations, etc.) the two-state model requires that, under a given set of conditions, oxygenation of tetrameric protein must obey the two-parameter binding equation (Monod et al., 1965)

$$Y/(1 - Y) = \alpha[(1 + \alpha)^3 + Lc(1 + c\alpha)^3]/[(1 + \alpha)^3 + L(1 + c\alpha)^3] \quad (2)$$

where Y is the fractional O_2 saturation and α is a normalized partial pressure of O_2, $\alpha = K_R P_{O_2}$. It is possible to evaluate eq 2 numerically to obtain the Hill coefficient:

$$n_{max} = \text{d log } [Y/(1 - Y)]/\text{d log } \alpha|_{y=0.5} \quad (3)$$

According to Chien and Mayo (1980) $n_{max} = 1.21$ for carp Hb at pH 9.06, whereas for Hb A, $n_{max} = 2.51$ (Tyuma et al., 1973). The parameters c and L are connected by the relation (Hopfield et al., 1971) $i = -\log L/\log c$. Low values of n_{max} and i require that c and L be not very far from unity. For $n_{max} = 1.2$ and $i = 0.5$, we find that c must be 0.2, whence $L = 5^{0.5} = 2.24$. Such a low value of L, however, implies that a substantial fraction, 31%, of the unligated molecules is in the R state. This is incompatible with the invariance of the ν_{Fe-ImH} band at high pH (Figure 2). If 31% of the molecules had switched to the R state at pH 9, there should have been a spectral shift similar to that seen in the 75% oxygenated HbO_2 sample of Hb A (Figure 1). Thus, the spectroscopic and binding data are incompatible with the two-state model, assuming that the α- and β-chain affinities are the same. The incompatibility is avoided, however, if selective α-chain binding is assumed, since a higher value of i allows a higher value of L. For example, if $i = 3$, then $n_{max} = 1.25$ is obtained with $c = 0.25$ and $L = 64$.

The possible inadequacy of the two-state model bears on the question of the importance of intermediate structures. Spectroscopic evidence for such structures is accumulating. Thus, Viggiano and Ho (1979) observed that exchangeable proton resonances at 6.4 and 9.4 ppm in the NMR spectrum of Hb A, which arise from conformationally sensitive H bonds, decrease in a *nonconcerted* manner upon oxygenation, implying intermediate conformations for intermediate ligation states. Likewise Simolo et al. (1985) find an exchangeable proton resonance at 6.2 ppm, but not the one at 9.4 ppm, in a half-ligated Zn, Fe–Hb hybrid, implying that some, but not all, of the hydrogen bonds characteristic of the deoxy structure are broken. As noted above, carp Hb shows an exchangeable proton resonance at 9.1 ppm at low pH, which disappears at high pH, suggesting that the T-like structure ($\nu_{Fe-ImH} = 215$ cm^{-1}) loses a quaternary H bond at high pH. Thus, there is evidence for a range of variation within a structural class.

The question remains whether intermediate structures have major or minor influence on the mechanism of cooperativity. The present results, suggesting inconsistency in the two-state binding parameters for carp Hb at high pH, imply a major influence in this case. They are compatible with the possibility that molecules containing one O_2 attain an intermediate structure with an intermediate affinity, thereby lowering the cooperativity from that expected on the basis of the two-state

model. It will be possible to test this inference more rigorously when the relative affinities of the α- and β-chains are determined.

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Registry No. O_2, 7782-44-7; deoxy-Hb A, 9034-51-9; oxy-Hb A, 9062-91-3.

REFERENCES

Baldwin, J., & Chothia, C. (1979) *J. Mol. Biol. 129*, 175.
Chien, J. C. W., & Mayo, K. H. (1980) *J. Biol. Chem. 255*, 9790–9799.
Dalvit, C., Cerdonio, M., Fontana, A., Mariotto, G., Vitale, S., DeYoung, A., & Noble, R. W. (1982) *FEBS Lett. 140*, 303–306.
Dalvit, D., Muira, S., DeYoung, A., Noble, R. W., Cerdonio, M., & Ho, C. (1984) *Biochemistry 141*, 255–259.
Edelstein, S. J. (1971) *Nature (London) 230*, 224–227.
Hopfield, J. J., Shulman, R. G., & Ogawa, S. (1971) *J. Mol. Biol. 61*, 425–443.
Imai, K. (1973) *Biochemistry 12*, 798–808.
Kincaid, J., Stein, P., & Spiro, T. G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 549 (4156).
Kitagawa, T., Nagai, K., & Tsubaki, M. (1979) *FEBS Lett. 104*, 376.
Matsukawa, S., Mawatari, K., Yoneyama, Y., & Kitagawa, T. (1985) *J. Am. Chem. Soc. 107*, 1108–1113.
McDonald, M. J., Sawicki, C. A., & Gibson, Q. H. (1976) *Biophys. J. 16*, 40a.
Monod, J., Wyman, J., & Changeux, J.-P. (1965) *J. Mol. Biol. 12*, 88–118.
Nagai, K., & Kitagawa, T. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2033–2037.
Nagai, K., Kitagawa, T., & Morimoto, H. (1980) *J. Mol. Biol. 136*, 271–289.
Noble, R. W., Parkhurst, L. J., & Gibson, Q. H. (1970) *J. Biol. Chem. 245*, 6628–6633.
Ondrias, M. R., Rousseau, D. L., Kitagawa, T., Ikeda-Saito, M., Inubushi, T., & Yonetani, T. (1982) *J. Biol. Chem. 257*, 8766–8770.
Pennelly, R. R., Tan-Wilson, A. L., & Noble, R. W. (1975) *J. Biol. Chem. 250*, 7239–7244.
Perutz, M. F. (1968) *J. Cryst. Growth, 2*, 54–56.
Perutz, M. F. (1978) *Annu. Rev. Biochem. 48*, 327.
Perutz, M. F. (1980) *Proc. R. Soc. London, Ser. B 208*, 135.
Scott, T. W., & Friedman, J. M. (1984) *J. Am. Chem. Soc. 106*, 5677.
Shriver, D. F., & Dunn, J. B. R. (1974) *Appl. Spectr. 28*, 319–323.
Shulman, R. G., Hopfield, J. J., & Ogawa, S. (1975) *Q. Rev. Biophys. 8*, 325–420.
Simolo, K., Stucky, G., Chen, S., Bailey, M., Scholes, C., & McLendon, G. (1985) *J. Am. Chem. Soc. 107*, 2865–2872.
Spiro, T. G. (1983) in *Iron Porphyrins* (Lever, A. B. P., & Gray, H. B., Eds.) Part Two, Addison-Wesley, Reading, MA.
Stein, P., Mitchell, M., & Spiro, T. G. (1980) *J. Am. Chem. Soc. 102*, 7795–7797.
Stein, P., Terner, J., & Spiro, T. G. (1982) *J. Phys. Chem. 86*, 168–170.
Tan, A. L., & Noble, R. W. (1973) *J. Biol. Chem. 248*, 7412–7416.
Tan, A. L., DeYoung, A., & Noble, R. W. (1972a) *J. Biol. Chem. 247*, 2493–2498.
Tan, A. L., Noble, R. W., & Gibson, Q. H. (1972b) *J. Biol. Chem. 248*, 2880–2888.
Tyuma, I., Imai, K., & Shimizu, K. (1973) *Biochemistry 12*, 1491–1498.
Viggiano, G., & Ho, C. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3673–3677.
Walters, M. A., Scholler, D. M., Hoffman, B. M., & Spiro, T. G. (1983) *J. Raman Spectrosc. 14*, 162–165.