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Proton Nuclear Magnetic Resonance Study of the Rotational Position and Oscillatory Mobility of Vinyl Groups in Allosteric Monomeric Insect Hemoglobins†

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ABSTRACT: The changes in the heme hyperfine-shifted proton nuclear magnetic resonances arising from the pH-induced t ⇌ r transition in the monomeric met-cyanohemoglobins from the insect *Chironomus thummi thummi* are shown to be localized at one of the vinyl groups. This localized nature of the protein perturbations on the heme is taken as an indication that the structural change detected at the heme involves a pH-modulated change in the orientation of the vinyl group relative to the heme. Detailed analysis of the hyperfine-shifted peaks for the peripheral substituents of model heme compounds, the low-spin, biscyano ferric complexes of protoporphyrin IX and deuteroporphyrin IX, reveals that vinyl groups are sterically restricted from being coplanar with the heme and that the vinyl group yields a characteristic shift pattern which depends on its rotational position. A shift parameter is defined which provides an index of both the average rotational position and the oscillatory mobility of a vinyl group in any heme *b* containing protein. Application of this parameter to the insect hemoglobins reveals that a vinyl group in the low-pH, t, form of this protein is more coplanar with the heme and possesses less oscillatory mobility than in the high-pH, r, conformation of the protein. This is the first direct observation of any sort of strain in the t conformation of any O_2-binding heme protein and suggests that a "tense" description for the low-pH conformation is appropriate.

There exist two controversial views of the allosteric trigger mechanism at the heme iron. It has been proposed by Perutz (Perutz and Ten Eyck, 1971; Perutz, 1972) that the oxygen affinity in the T state is reduced by introducing tension, i.e., stretching in the proximal histidine–iron linkage, by which the high-spin character of the iron and its ionic radius increase, thereby inhibiting the iron from moving into the porphyrin plane as required for formation of a strong bond to the other axial ligand. When this tension is reduced in the R state, the iron can change to more low-spin character with a smaller ionic radius, thereby facilitating combination with the external ligand.

Although spin-state transition may have some influence on the triggering of allostery in hemoglobins, this hypothesis is undermined by the observation that cobalt hemoglobins, which do not exhibit a spin-state transition during ligation with oxygen, remain allosteric (Hsu et al., 1972; Dickinson and Chien, 1973). Based on the analysis of electron spin resonance data of the nitrosyl complexes of monomeric and tetrameric hemoglobins it has been proposed that the change of the ligand affinity arises primarily from the trans effect of the proximal imidazole of His-F8 (Trittelvitz et al., 1973, 1975; Overkamp et al., 1976; Twilfer and Gersonde, 1976). In contrast to the assumption of the spin-state hypothesis, these ESR[1] results

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[1] Abbreviations used: PP, protoporphyrin IX; DP, deuteroporphyrin IX; NMR, nuclear magnetic resonance; ppm, parts per million; ESR, electron spin resonance; DDS, 2,2-dimethyl-2-silapentane-5-sulfonate.

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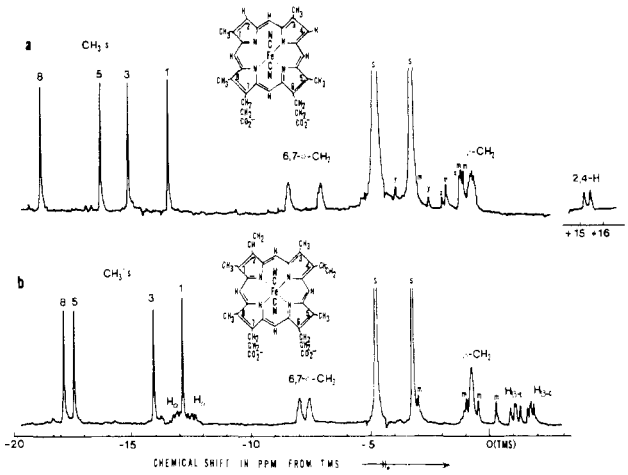

FIGURE 1: Proton NMR traces for (a) $DPFe(CN)_2^-$ and (b) $PPFe(CN)_2^-$ in CD_3OD (~2 mM) at 25 °C; s = solvent peaks, m = meso-H's, x = impurities, y = spinning side bands.

were interpreted as indicating that the low-affinity (tense) state is characterized by a relatively small imidazole–iron distance, thus weakening the σ bond of the ligand in the trans position. The high-affinity (relaxed) state, however, was suggested to possess a relatively large imidazole–iron distance, facilitating the formation of a σ bond of the ligand in trans position. Therefore the imidazole–iron bond behaves like a spring which is compressed by the constraints of the T structure and is relaxed in the R structure.

Whether the strain in the proximal histidine–iron bond arises from tension (Perutz and Ten Eyck, 1971; Perutz et al., 1976; Maxwell and Caughey, 1976) or compression (Trittelvitz et al., 1973, 1975; Overkamp et al., 1976; Twilfer and Gersonde, 1976), it can be controlled by the protein only if the porphyrin system is more or less fixed in the heme pocket, at least in the T structure. In order to prevent the porphyrin from moving to relieve this axial bond tension or compression, it is likely that the porphyrin is "clamped" into position more tightly by the surrounding amino acid side chains in the T than in the R state. The result of such tighter heme–protein contacts at the heme periphery may be expected to introduce some strain in the porphyrin substituents. The sizable molecular weight of tetrameric hemoglobins, unfortunately, causes severe difficulties in defining such small structural changes by either x-ray crystallography or NMR spectroscopy.

We have shown in the companion paper (La Mar et al., 1978) that the proton NMR spectra of the paramagnetic, ($S = \frac{1}{2}$), low-spin, met-cyano form of the two monomeric allosteric insect hemoglobins from the insect *Chironomus thummi thummi* exhibit a sensitivity to pH which can be correlated with the oxygen-binding Bohr effect (Sick and Gersonde, 1969, 1974; Gersonde et al., 1972, 1976; Sick et al., 1972; La Mar et al., 1978). This correlation indicated that the t (tense) $\rightleftharpoons$ r (relaxed) transition in these monomeric proteins can be induced in the cyanide-ligated form, and that the influence of the tertiary structural change accompanying this transition manifests itself at the heme primarily at one of the peripheral vinyl groups. Inasmuch as this degree of mobility may be sensitive to the "tightness" of the protein–heme contact, we will focus in this paper on a basis for interpreting the physical origin of the pH-induced change in the NMR shifts of the heme.

The ability to resolve the heme resonances in the met-cyano form of these proteins depends critically on the paramagnetism which induces sizable hyperfine shifts in the protons of the prosthetic group. Before formulating a picture of the pH-induced changes in the molecular/electronic structure of the protoporphyrin, we need to consider in somewhat more detail the physical origins of the paramagnetic shift (see Jesson, 1973). In general these hyperfine shifts $(\Delta H/H)_{hf}$ are the vector sum of contact and dipolar contributions, i.e.:

$$(\Delta H/H)_{hf} = (\Delta H/H)_{con} + (\Delta H/H)_{dip} \quad (1)$$

The contact shift reflects the nature of the delocalized spin density in the porphyrin π orbital, and, in the simplest case, is given by

$$(\Delta H/H)_{con} = \frac{-Ag\beta S(S+1)}{(\gamma/2\pi)3kT} \quad (2)$$

where A is the hyperfine coupling constant. In a case where the molecular structure of the prosthetic group remains intact, the contact shift varies inversely with the temperature (vide infra).

The dipolar shift, on the other hand, reflects a through-space interaction with the magnetically anisotropic spin on the iron and depends critically on the position of the proton in the magnetic symmetry axes of the iron. In the case of rhombic symmetry (Jesson, 1973), we have:

$$(\Delta H/H)_{dip} = -\frac{1}{3N}\{[\chi_z - \tfrac{1}{2}(\chi_x + \chi_y)](3\cos^2\theta - 1)r^{-3} - \tfrac{3}{2}(\chi_x - \chi_y)\sin^2\theta\cos 2\Omega r^{-3}\} \quad (3)$$

where χ_i are the components of the magnetic susceptibility tensor, θ is the angle between the proton–iron vector and the z axis, r is the length of this vector, and Ω is the angle between the projection of this vector on the xy plane and the x axis. The χ_i often obey the Curie law to a degree so that, in the absence of change in molecular structure, $(\Delta H/H)_{dip}$ should also vary as T^{-1}. Actually, in the case of the low-spin ferric systems of interest, it has been shown in model compounds that (La Mar and Walker, 1973), while the contact shift adheres reasonably well to the T^{-1} law, the dipolar shift has a slightly more complicated temperature dependence due to larger contributions from the second-order Zeeman effect (Horrocks and Greenberg, 1974).

The changes in $(\Delta H/H)_{hf}$ which accompany the t $\rightleftharpoons$ r conversion could effect either changes in anisotropy (χ_i in eq 3) and/or changes in electronic structure (A in eq 2). In the preceding paper we were able to assign unambiguously only a single vinyl group and two methyls. The vinyl groups can adopt different rotational positions with respect to the heme. Moreover, the coupling of the protons in these groups and the porphyrin π spin density depends on their rotational position (La Mar, 1973). In order to demonstrate that the contact shifts for peripheral substituents are sensitive indicators of the rotational positions, we will first investigate the NMR spectra of model compounds of the prosthetic group.

As subjects for our study of model compounds, we selected well-characterized low-spin biscyano complexes of ferric protoporphyrin IX, $PPFe(CN)_2^-$, and deuteroporphyrin IX, $DPFe(CN)_2^-$ (La Mar and Walker, 1973; Scheer and Katz, 1975). These complexes have had most of the signals assigned to specific side chains by a combination of deuteration and decoupling experiments. Their unusually narrow lines also permit resolution of all signals over a very wide temperature range (Wüthrich, et al., 1969, 1970; Viscio, 1977).

Materials and Methods

Hemoglobins. The monomeric hemoglobins III and IV from *Chironomus thummi thummi* were purified and the recon-

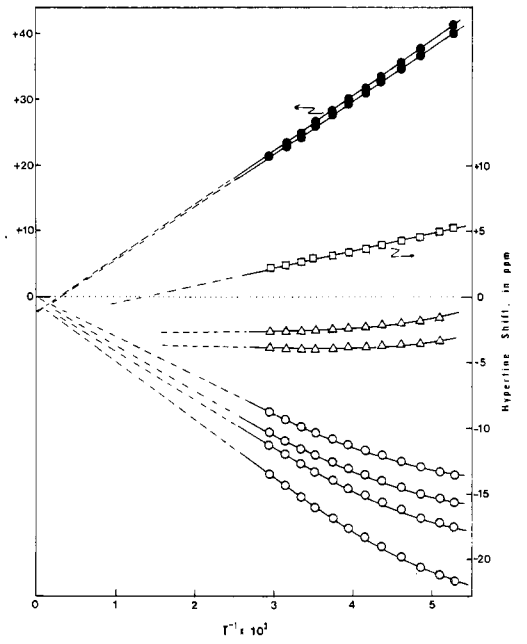

FIGURE 2: Curie plot of the pyrrole substituents of an ~2 mM CD_3OD solution of $DPFe(CN)_2^-$; (○) 1,3,5,8-CH_3; (●) 2,4-H; (△) 6,7-α-CH_2; (□) 6,7-β-CH_2.

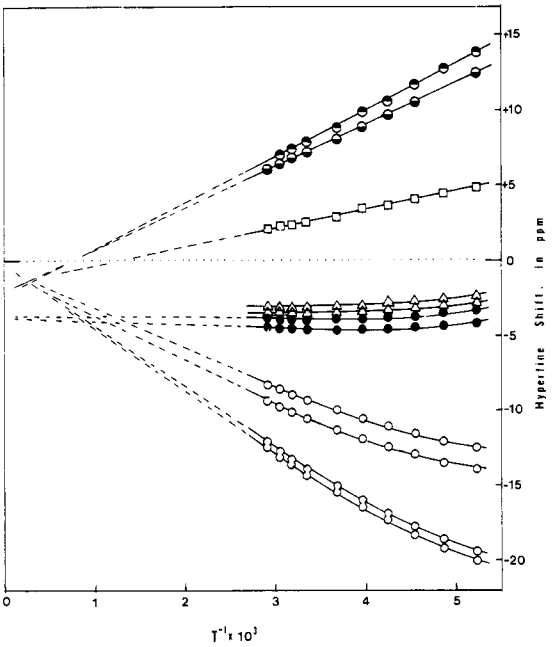

FIGURE 3: Curie plot of the pyrrole substituents of an ~2 mM CD_3OD solution of $PPFe(CN)_2^-$; (○) 1,3,5,8-CH_3; (●) 2,4-H_α; (◒) 2,4-H_β (trans); (◓) 2,4-H_β (cis); (△) 6,7-α-CH_2; (□) 6,7-β-CH_2.

stitution of deuterohemoglobins was carried out as described in La Mar et al. (1978). For preparation and nomenclature of hemoglobin derivatives, see companion paper (La Mar et al., 1978). All proton shifts relative to the internal calibrant, the sodium salt of 2,2-dimethyl-2-silapentane-5-sulfonate, DDS, were taken from La Mar et al. (1978), and converted to hyperfine shifts, by referencing to the position in diamagnetic iron(II) porphyrin complexes (Scheer and Katz, 1975).

Model Complexes. Protoporphyrin IX ferric chloride, PPFeCl, was obtained commercially (Sigma) and used without further purification. Deuteroporphyrin IX ferric chloride, DPFeCl, was made from the protoporphyrin complex in the standard manner (Falk, 1964). Methanol-d_4 solutions ~2 mM in complex and 8–10 mM in KCN were prepared which completely converted the complex to the low-spin biscyano species, $PPFe(CN)_2^-$ and $DPFe(CN)_2^-$. At higher concentrations, additional deviations from the Curie law are detectable due to dimerization (Viscio, 1977).

The proton NMR spectra for the model compounds were obtained on a JEOL PFT-100 Fourier transform spectrometer operating at 99.5 MHz. Spectra consisted of 500 to 5000 transients collected over a 5-kHz bandwidth using 8K data points and a 20-μs 90° pulse. Tetramethylsilane was used as internal calibrant. The hyperfine shifts for the functional groups of interest were obtained by referencing to the analogous diamagnetic iron(II) complexes (Scheer and Katz, 1975); downfield shifts are defined as negative, and all shifts are reported in parts per million, ppm.

Results and Discussion

Model Complexes. The proton NMR traces of $DPFe(CN)_2^-$ and $PPFe(CN)_2^-$ in CD_3OD are illustrated in a and b of Figure 1, respectively. Dilute solutions (2 mM) were used since at higher concentrations aggregation becomes a problem at very low temperatures (La Mar and Viscio, 1974). Analysis of the hyperfine shifts in methanol solutions has previously shown that the contact contribution dominates at all pyrrole positions; only the meso-H shifts exhibit primarily dipolar shifts (Frye and La Mar, 1976; La Mar et al., 1977). The temperature dependences, in the form of Curie plots (Jesson, 1973; La Mar and Walker, 1973), of the pyrrole substituent shifts for $DPFe(CN)_2^-$ and $PPFe(CN)_2^-$ are given in Figures 2 and 3, respectively. The 2,4-H shifts in $DPFe(CN)_2^-$, which are overwhelmingly contact in origin, reflect the carbon π spin density ρ_π, via the conventional equation (La Mar, 1973)

$$A_H = Q\rho_\pi \tag{4}$$

where Q is a negative constant. Since the ρ_π is independent of T, so is A, and hence $(\Delta H/H)_{con}$ should vary as T^{-1} (eq 2). As illustrated in Figure 2, the 2,4-H data points all fall on a straight line which exhibits only a very small nonzero intercept (ca. −1 ppm) at $T^{-1} = 0$, confirming that the contact shifts follow the Curie law reasonably well. The upfield meso-H shifts, (not shown in Figures 2 and 3), which are primarily dipolar (La Mar and Walker, 1973; La Mar et al., 1977), exhibit straight lines but with large negative intercepts at $T^{-1} = 0$. This confirms previous findings in model compounds that the contact contributions adhere to the Curie law much more closely than the dipolar contributions, with the latter term increasing faster than T^{-1} as the temperature is lowered.

The 1,3,5,8-CH_3 shifts for both complexes extrapolate to the origin in the high temperature limit, but exhibit a small curvature at lower temperatures. This curvature probably arises from the small *upfield* dipolar contribution (La Mar and Walker, 1973) to the net shift, which, as is the case of the meso H's, increases faster than T^{-1}.

The normal temperature dependence for 2,4-H, 1,3,5,8-CH_3's and meso H's, with shifts decreasing as the temperature is raised (Jesson, 1973; La Mar and Walker, 1973), is sharply contrasted by the behavior of the 6,7-α-CH_2's in both Figures 2 and 3, and the vinyl H_α's in Figure 3. This unusual behavior for these two groups, where the shift actually increases upon raising the temperature, can be explained by these groups being able to adopt a variety of rotational positions relative to the porphyrin plane with different A's (La Mar, 1973).

The hyperfine coupling of alkyl protons and its angular dependence is well understood based on extensive work with

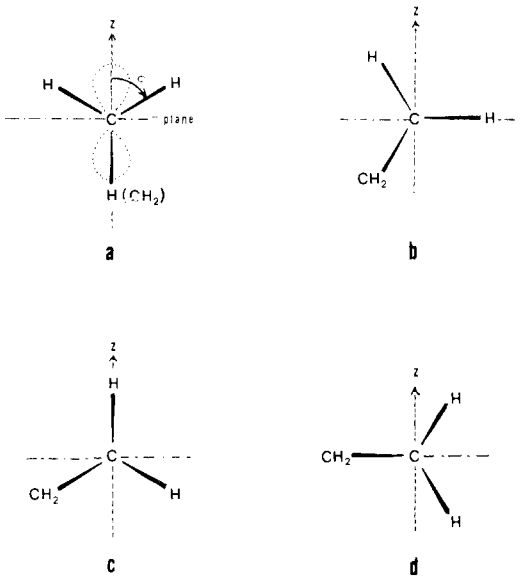

FIGURE 4: Possible orientations of methyl or methylene groups, looking along the C-C axis; ϕ is defined by the C-C-H plane and the aromatic carbon p_z axis. Orientation of the propionic acid side chain in the stablest configuration (a) and in rotomers (b-d) representing increasing energies due to steric interactions with neighboring groups.

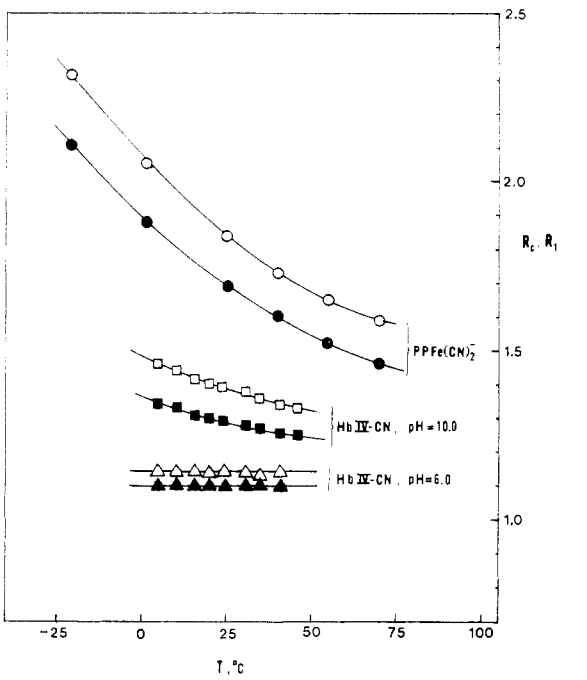

FIGURE 5: A plot of R_c and R_t for the vinyl group defined in eq 6 in the text, against temperature for the model compound, PPFe(CN)$_2^-$ (○ and ●), metHb-IV-CN at pH 10.0 in its r state (□ and ■), and metHb-IV-CN at pH 6.0 in its t state (△ and ▲). The open markers are for R_c, and the darkened markers are for R_t.

free radicals (La Mar, 1973). Much less is known about vinyl groups. Hence, we will first demonstrate that information on average rotational positions can be obtained from the alkyl groups and then extend our analysis to the vinyl groups.

Hyperfine Coupling in Alkyl Groups. The deviations from Curie law for 6,7-α-CH_2's are very large such that the hyperfine shift actually increases with lower temperature, indicating significant increase in A as the temperature is raised. The temperature dependence of α-CH_2 coupling constants, A_{CH_2}, has been recognized previously (La Mar, 1973) and can be explained on the basis of the hyperconjugative interaction between the α protons and the pyrrole carbon π spin density, and is given by the relation

$$A_{CH_{2,3}} = B_2 \cos^2 \theta \rho_\pi \qquad (5)$$

where B_2 is a positive constant and θ is the angle described in a of Figure 4.

For a rotating methyl group, $\langle \cos^2 \theta \rangle = \frac{1}{2}$ for any orientation, yielding $\langle A_{CH_3} \rangle = \frac{1}{2}B_2\rho_\pi$. The β-CH_2 group, however, imposes a sizable rotational barrier on the 6,7-α-CH_2's of the two propionic acid side chains. Molecular models indicate that the stablest rotamer is that depicted in a of Figure 4, for which $\langle A_{CH_2} \rangle = \frac{1}{4}B_2\rho_\pi$. At the lowest temperature, this is probably the only populated rotamer; this configuration is also most frequently found in crystal structures (Koenig, 1963; Little et al., 1975; Fermi, 1975; Seybert and Moffat, 1976). At higher temperatures, rotamers such as b with $\langle A_{CH_2} \rangle = \frac{3}{8}B_2\rho_\pi$, c with $\langle A_{CH_2} \rangle = \frac{5}{8}B_2\rho_\pi$, and d with $\langle A_{CH_2} \rangle = \frac{3}{4}B_2\rho_\pi$, depicted in Figure 4, become populated. These rotamers, a → d, reflect increasing degrees of oscillation and also increasing energy. Since the $\langle A_{CH_2} \rangle$ increases dramatically with the oscillation, it is clear that raising the temperature would increase the contact shift significantly, so that the shift actually increases rather than decreases with increasing temperature, as observed in both Figures 2 and 3.

In principle, the rotational barriers for the alkyl groups could be abstracted from the curvature in Figures 2 and 3. However, the lack of a quantitative separation of the hyperfine shift and the detailed temperature dependencies of the contact and dipolar components render the value of such an analysis doubtful.

Hyperfine Coupling in Vinyl Groups. The data in Figure 3 for the vinyl protons in PPFe(CN)$_2^-$ also exhibit an "abnormal" temperature dependence. For H_α, as for 6,7-α-CH_2, $\langle A_{H_\alpha} \rangle$ decreases dramatically with lower temperature. This is characterized best by an apparent large negative intercept at $T^{-1} = 0$, as well as by some curvature at low temperature.

The H_β's follow the Curie law more closely than the H_α, but even they exhibit a larger deviation (as percent of the observed shift at any temperature) than 2,4-H in DPFe(CN)$_2^-$, in the form of a negative intercept at $T^{-1} = 0$. This may suggest that $\langle A_{H_\beta} \rangle$ increases slightly upon lowering the temperature. Inspection of space filling molecular models again reveals that the vinyl groups are sterically restrained from being coplanar with the porphyrin π system; x-ray crystal structures in both model compounds (Koenig, 1963; Little et al., 1975), and proteins (Fermi, 1975; Seybert and Moffat, 1976) confirm the more stable out-of-plane configuration. Thus the stable rotational position for the vinyl group is that of a considerable out-of-plane configuration and may be expected to be the favored position at very low temperature. At high temperatures, thermal population of more coplanar rotamers may be expected, and the dramatic increase in $\langle A_{H_\alpha} \rangle$ and the slight decrease in $\langle A_{H_\beta} \rangle$ are most likely the manifestations of this change in average rotational positions.

This temperature dependence of the change in the average rotational position for the vinyl group can be characterized better by the two ratios:

$$R_i = -(\Delta H/H)_{hf}^{H_{\beta-i}}/(\Delta H/H)_{hf}^{H_\alpha} \qquad (6)$$

one for $H_{\beta-c}$, R_c, and one for $H_{\beta-t}$, R_t. These ratios are plotted as a function of temperature in Figure 5. Thus, a more in-plane configuration is consistent with $R_{c,t} \sim 1$ (high temperatures, the right side of Figure 5), while increasing the extent of pop-

TABLE I: Hyperfine Shifts for High and Low pH Forms of Cyanide-Ligated Methemoglobins III and IV.[a]

Hemoglobin	Protons	Low pH form $\left(\frac{\Delta H}{H}\right)_{hf}$	Low pH form R_i[b]	High pH form $\left(\frac{\Delta H}{H}\right)_{hf}$	High pH form R_i[b]	δ[c]	Rel geometric factors[d]
metHb-III-CN	$H_{\beta-c}$	+10.2	+1.23	+10.5	+1.30	+0.3	+0.6
	$H_{\beta-t}$	+9.6	+1.16	+9.8	+1.21	+0.2	+0.8
	H_α	−8.3		−8.1		+0.2	+1.0
	CH_3	−18.3		−18.4		−0.2	+1.6
	CH_3	−25.4		−25.2		+0.1	+1.6
deutero-metHb-III-CN	2,4-H	+30.2 +23.6		+30.5 +23.8		+0.3 +0.2	+2.0 +2.0
	CH_3	−19.1		−19.1		0.0	+1.6
	CH_3	−25.6		−25.6		0.0	+1.6
metHb-IV-CN	$H_{\beta-c}$	+10.0	+1.12	+11.2	+1.37	+1.2	+0.6
	$H_{\beta-t}$	+9.6	+1.08	+10.3	+1.26	+0.7	+0.9
	H_α	−8.9		−8.1		+0.8	+1.1
	CH_3	−17.5		−18.4		−0.9	+1.6
	CH_3	−24.7		−25.1		−0.4	+1.6
deutero-metHb-IV-CN	2,4-H	+30.8 +23.6		+31.2 +23.6		+0.4 0.0	+2.0 +2.0
	CH_3	−19.0		−19.1		−0.1	+1.6
	CH_3	−25.5		−25.5		0.0	+1.6

[a] Shifts in ppm at 25 °C, in D_2O solution, referenced against the position in diamagnetic ferrous porphyrin complexes. [b] $R_i = R_c$ or R_t as defined in eq 6 in the text. [c] δ is the amplitude of the shift change in ppm; i.e., shift of high-pH form minus low-pH form. [d] Relative geometric factor $\sim r^{-3}$, since θ and Ω are essentially invariant with internal motions.

ulation of out-of-plane rotamers causes R to increase markedly (low temperatures, on the left side of Figure 5). It is interesting that the hyperfine shifts for a vinyl group attached to an aromatic π system in an unrelated complex of nickel(II) yield R_c, $R_t \sim 0.9$ for the in-plane vinyls (Eaton, 1973) which is consistent with our interpretation of the porphyrin data.

The change in contact shifts with vinyl rotational positions occurs because the unpaired spin density is in a filled π molecular orbital on the porphyrin (La Mar and Walker, 1973; Shulman et al., 1971). When the vinyl group is coplanar with the porphyrin plane, conjugation delocalizes the spin density into the vinyl π system, yielding shifts of comparable magnitude but opposite sign for H_α and H_β's (La Mar, 1973; Eaton, 1973). When the vinyl group is perpendicular, the two π systems are orthogonal, and spin transfer can occur only via the σ system or by polarization (La Mar, 1973). Similar changes in ring coupling constant with rotation have been predicted for the benzyl radical (Laidlaw and Adam, 1968).

The totally reasonable and consistent interpretation of the observed temperature dependencies of A_{H_α} and A_{H_β}'s, or their ratios R_c, R_t, suggests at least two valuable probes for the static and dynamic properties of the prosthetic group, either outside or within a protein environment. The values of the ratios, R_c, R_t, should be indices of the *average rotational position* of a vinyl group at a given temperature, with $R_{c,t} \sim 1$ indicating an in-plane configuration, and increasing R reflecting larger out-of-plane rotamer populations. The *rotational or oscillatory mobility* is manifested in two experimental observables; the H_α shift exhibits large deviations from Curie behavior such that the apparent intercept at $T^{-1} = 0$ is sizable and of the same sign as the observed shift, and the R_c, R_t values are temperature dependent, increasing from a value of $\sim$1 as the temperature is lowered. Both of these properties may be expected to yield useful information on the nature of the heme-protein contacts in hemoproteins.

Monomeric Hemoglobins. In Table I, we list the hyperfine shifts, referenced to the diamagnetic Fe(II) porphyrin (Scheer and Katz, 1975), for these five peaks in each of the acid and alkaline forms of metHb-III-CN and metHb-IV-CN. Also included in this table are the shift data for the four assigned peaks (the 2,4-pyrrole protons and two methyls) for the deuteroporphyrin-reconstituted proteins, deutero-metHb-III-CN and metHb-IV-CN, respectively. The δ represents the amplitude of the shift change on going from the low-pH to the high-pH form, with a positive sign indicating that the shift is further upfield in the alkaline form of the protein. It is clear from this table that the major effects are on the vinyl protons, which are all shifted upfield, while the methyls are affected much less and generally downfield. The 2,4-vinyl group as the focal point of the pH-induced perturbations of the heme hyperfine shifts is underscored by the observation of pH-sensitive shifts for one of the two pyrrole protons in deutero metHb-III-CN and -metHb-IV-CN, respectively, but essentially none for the methyl resonances.

Analysis of the Hyperfine Shifts. Before we attempt to interpret the vinyl shift changes in terms of rotational positions, we should establish whether the pH-induced hyperfine shift changes reflect changes primarily in the contact or the dipolar term in eq 1. The hyperfine shifts in these insect hemoglobins are very similar in pattern to those of met-myoglobin cyanides (Wüthrich et al., 1970) and their model compounds (La Mar and Walker, 1973; Viscio, 1977; Shulman et al., 1971), both of which have been shown to exhibit primarily contact shifts for the pyrrole substituents. The shift differences between the t and r conformations are unlikely to arise from changes in the dipolar shift, since the relative changes do not parallel the relative value of the axial geometric factor $(3\cos^2\theta - 1)r^{-3}$, also included in Table I. Both the axial and rhombic ($\sin^2\theta \cos 2\Omega r^{-3}$) geometric factors predict the largest shift change for H_α and the smallest for $H_{\beta-c}$, contrary to observation. Changes in the orientation of the in-plane magnetic axis are possible, but again would be expected to affect H_α the most, since Ω is very similar for all three vinyl protons. The dominance of changes in the contact shift during the t $\rightleftharpoons$ r transition is also underscored by the observation that in deutero-metHb-IV-CN, only the one pyrrole H exhibits a variable shift, indicating that

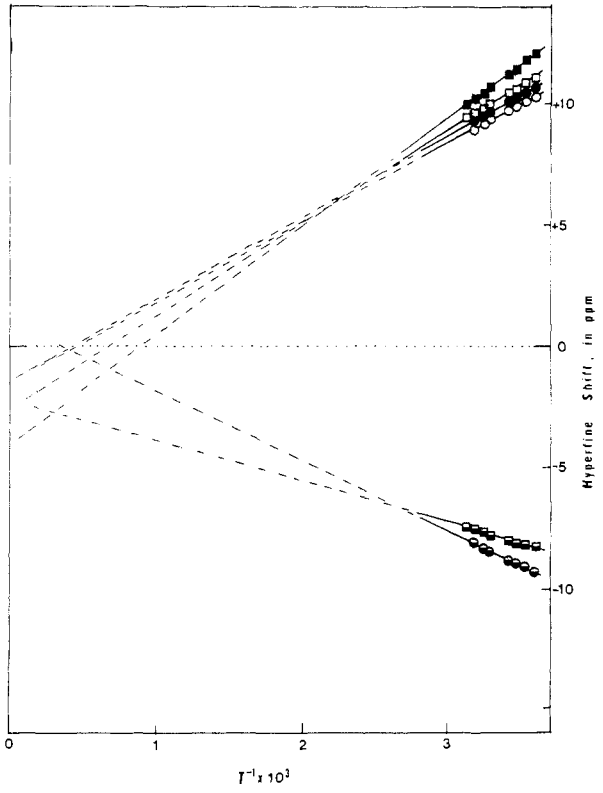

FIGURE 6: Curie plot for the three vinyl protons in metHb-IV-CN at pH 6 in its t state (circles) and at pH 10 in its r state (squares). H_α (■ and ◒); $H_{\beta-c}$ (■ and ●); and $H_{\beta-t}$ (□ and ○).

the perturbation is localized at the heme periphery and is not centralized at the metal ion. The qualitative conclusion that the shift changes are due primarily to changes in A in eq 2 allows us to make some comparisons between the protein data of the vinyl group and that from the $PPFe(CN)_2^-$ discussed above.

The Average Rotational Position. The R_c and R_t values in each of the metHb-III-CN and metHb-IV-CN differ between the t and r states such that $R(r) > R(t)$ for both the cis and trans positions. These data are illustrated in Table I and for metHb-IV-CN also in Figure 5 and lead us to conclude that the average rotational position of the vinyl group is more in-plane for the t than the r state in both proteins. Furthermore, the percentage increases in R_c and R_t in Figure 5 are larger in metHb-IV-CN (25% and 18%, respectively) than in metHb-III-CN (6% and 4%, respectively). Thus the change in the average rotational position for the vinyl group accompanying the t ⇌ r transition is larger in hemoglobin IV than in hemoglobin III in agreement with the observation of a larger Bohr effect in the former protein (Sick and Gersonde, 1969, 1974; Sick et al., 1972). Due to the fact that there may be differences in the relative importance of dipolar and contact contributions in the two proteins and in the model compounds, exact determinations of the average rotational positions in either the model compounds or the proteins and in either state of the protein are not possible at this time. Comparison of the average rotational position (i.e., R_c and R_t) in different proteins will require a more exact knowledge of the hyperfine shift origins. Such comparisons are deferred to the future. However, the relative changes in the rotational position within the protein appear unambiguous.

Oscillatory Mobility. Since the vinyl group is constrained to be more in-plane in the t state for both proteins than either in the r state or in the model compound, it may be anticipated that this local "strain" also inhibits its usual oscillatory mobility.

The Curie plots for the three vinyl protons of metHb-IV-CN in the t (pH 6.0) and r (pH 10.0) states are illustrated in Figure 6. It is clearly observed here that H_α for the t state (pH 6.0) does not deviate significantly from the Curie law, as indicated by near-zero intercepts at $T^{-1} = 0$. Hence, in this state the vinyl group appears to be essentially rotationally immobile, and A_{H_α} is independent of the temperature in the range 5 to 45 °C. On the other hand, in the r state (pH 10.0), H_α exhibits the decreases in A with decreasing temperature and the resulting large nonzero intercept at $T^{-1} \rightarrow 0$, as observed in the model compounds, indicating a rotationally mobile vinyl group. Most importantly, however, we show that the extent of deviations from Curie law for the protein differs characteristically in the high and low pH regions, making the confirmatory comparison with the model compounds interesting but not necessary for our interpretation. In Figure 5 we also included the temperature dependence of R_c and R_t in the t and r states for metHb-IV-CN. Again, the data confirm our interpretation, in that the immobile vinyl in the t state exhibits a constant R_c and R_t, while these ratios increase on lowering the temperature in the r state, as observed in $PPFe(CN)_2^-$.

Conclusions

The characteristically different vinyl shift patterns and their temperature dependencies in the high and low pH regions for these monomeric insect met-hemoglobin cyanides lead us to the conclusion that the average vinyl rotational position in the tense or t form is more in-plane and has less oscillatory mobility than in the relaxed or r state. Since the more in-plane, restricted vinyl group represents a local strain at the heme periphery, a tense description for the heme pocket in the t or low pH state appears appropriate.

Our qualitative results clearly demonstrate that the hyperfine shift patterns of vinyl groups provide us with a novel new probe of heme–protein interactions in allosteric proteins and suggest that the location and assignment of such vinyl resonances in the tetrameric hemoglobins, however difficult, should be undertaken.

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Ligand Binding to the Adenine Analogue Binding Protein of the Rabbit Erythrocyte†

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ABSTRACT: Adenine analogue binding protein of rabbit erythrocytes reversibly binds [^{3}H]adenosine with a K_D of 5.3 $\times 10^{-9}$ M, an association rate constant of 1.4×10^{-12} M^{-1} min^{-1} and a dissociation rate constant of 7.5×10^{-3} min^{-1}, as estimated by a nonlinear curve-fitting program applied to data on the time course of the binding reaction. Independent estimates of K_D by Scatchard plots and of the dissociation rate constant by dilution or adsorption of free [^{3}H]adenosine on charcoal or by the addition of excess adenosine agreed closely with the estimates from the curve-fitting program. Inhibition of [^{3}H]adenosine binding by a series of 77 adenosine analogues was used to define the factors determining the binding affinity of this nucleoside. These are: (1) the size and aromaticity of the purine base; (2) a glycosylic torsion angle of approximately $-120°$; (3) the ribo configuration of the 2′- and 3′-hydroxyls and also the 5′-hydroxyl. Bulky substituents in the region of C-2′ and to a lesser extent in the region of C-3′ reduce binding affinity.

In the course of studies of the cAMP[1]-dependent protein kinases of rabbit erythrocytes, Yuh and Tao (1974) purified two proteins, both of which bound cAMP and adenosine but neither of which had a regulatory effect on the catalytic subunits of rabbit erythrocyte protein kinases I, IIa, or IIb. Detailed studies on the more abundant of the two proteins showed that adenosine in equimolar concentrations inhibited cAMP binding but not vice versa. Because the binding affinity (K_m) of adenosine was similar to that of cAMP, 10^{-7} and 3×10^{-7} M, respectively, but the binding capacity for adenosine was at least three times greater than for cAMP, they concluded that the protein possesses two types of binding sites. The more abundant site accommodates only adenosine, the other either adenosine or cAMP.

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[1] Abbreviations used: AABP, adenine analogue binding protein; BSA, bovine serum albumin; cAMP, cyclic adenosine 3′,5′-monophosphate; Hepes, *N*-2-hydroxyethylpiperazino-*N*′-2-ethanesulfonic acid; SEM, standard error of the mean; Tris, tris(hydroxymethyl)aminomethane; PEI, polyethylenimine.

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