Proton Nuclear Magnetic Resonance Hyperfine Shifts as Indicators of Tertiary Structural Changes Accompanying the Bohr Effect in Monomeric Insect Hemoglobins†

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ABSTRACT: The proton nuclear magnetic resonance spectra of the cyano-met forms of three monomeric hemoglobins from the insect *Chironomus thummi thummi* have been recorded and analyzed. Two of these hemoglobins are known to exhibit significant alkaline Bohr effects. The patterns of the hyperfine-shifted heme resonances reveal that all these proteins exist in two forms, a major and a minor component. Although the two components are not interconvertible under mild conditions, their populations can be altered by incubation of the proteins from one to several days at pH >10 and 40 °C. The physical basis for the two forms of the proteins is suggested to be in a disorder either in the position of protoheme in the heme cavity or the position of histidine-E7 imidazole. Five prominent peaks which are observed in each of the three proteins and for both components of the two allosteric proteins are assigned to two methyls and the three protons of a single vinyl group on the protoheme prosthetic group, based on relative intensities and decoupling experiments. The pH dependence of these five peaks yields well-defined inflection points indicative of one-proton dissociation with p*K*s similar to those determined from the Bohr effect curves. Moreover, the relative magnitudes of the shift changes with pH for identical resonances in the three proteins closely parallel the relative amplitudes of the Bohr effect curves, indicating that we are monitoring directly the t ⇌ r transition. Although the perturbation on the heme due to the t ⇌ r transition appears to be focused on a vinyl group, direct electronic participation of this vinyl group in modulating the oxygen affinity at the heme iron can be discounted based on the observation of pH sensitive proton resonances and on oxygen-binding Bohr effect in the allosteric hemoglobins reconstituted with deuteroheme. We suggest that the pH sensitivity of the vinyl group resonances reflects changes in its rotational position due to steric interactions between the heme and globins, and hence may serve as a sensitive probe for change in tertiary structure near the heme cavity. Direct evidence for the interpretability of vinyl shifts in terms of rotational position is presented in the companion article (La Mar, G. N., Viscio, D. B., Gersonde, K., and Sick, H. (1978) *Biochemistry 17* (following paper in this issue)).

During the past decade, considerable effort has been devoted to investigating cooperativity and allosteric properties of vertebrate hemoglobins (see review articles by Antonini and Brunori, 1971; Braunitzer et al., 1974; Shulman et al., 1975). The present understanding of the molecular structural changes responsible for the altered oxygen affinity has resulted in large part from single crystal x-ray diffraction (Perutz, 1970, 1976) and solution proton NMR[1] spectroscopy (Davis et al., 1971; Lindstrom et al., 1972; Ogawa and Shulman, 1972; Mayer et al., 1973; Ogawa et al., 1974; Perutz et al., 1974; Morrow and Gurd, 1975). These two tools have addressed themselves to defining the details of the protein conformation and its effect on the electronic structure of the iron in the different states of the tetramer. Although the x-ray crystallographic studies have shown that the two affinity states, T, tense or low-affinity, and R, relaxed or high-affinity, differ primarily in quaternary structure, the details of how these effects of the T ⇌ R conversion are transmitted to the iron through the tertiary structure within the subunit remain yet to be completely understood.

One of the difficulties of applying proton NMR spectroscopy to this problem is that the complex nature of the tetrameric protein and its large size lead to relatively poorly resolved spectra with few resonances which can be assigned unambiguously. The use of paramagnetic forms of these tetrameric hemoglobins, low-spin met-cyano, high-spin met-aquo and deoxy, where the paramagnetism of the iron causes large hyperfine shifts for the protons of the prosthetic group, has improved resolution and expanded the information content of the spectra, but still has not provided direct information on the nature of the protein perturbation of the same heme iron (Davis et al., 1971; Ogawa et al., 1974).

There exist, however, two allosteric hemoglobins from the insect *Chironomus thummi thummi* (hemoglobin III and IV), which, although monomeric over the whole pH range, exhibit marked Bohr effects for O_2 and CO (Sick and Gersonde, 1969a, 1974; Gersonde et al., 1972, 1976). Thus these monomeric hemoglobins can undergo a pH-dependent tertiary structural change between a low-affinity (t) and high-affinity (r) state. This tertiary conformation change is controlled by the sixth ligand which is thermodynamically linked to one Bohr proton binding site (see Figure 13 in Gersonde et al., 1972). Another monomeric hemoglobin from *Chironomus thummi thummi*, hemoglobin I, shows no Bohr effect (Wollmer et al., 1972). These proteins may therefore serve as ideal models for elucidating the effect of structural changes within a subunit on ligand binding of the iron.

The pH dependence of the oxygen and carbon monoxide affinity for all three proteins has been determined and the pertinent data are reproduced in Table I. The feature of prime interest here is that the three hemoglobins have variable Bohr

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[1] Abbreviations used: NMR, nuclear magnetic resonance; ^{1}H NMR, proton magnetic resonance; DSS, sodium salt of 2,2-dimethyl-2-silapentane-5-sulfonate; $p_{1/2}(O_2,CO)$, half-saturation pressure for oxygen and carbon monoxide, respectively; Δ log $p_{1/2}(O_2,CO)$ represents $[\log p_{1/2}(O_2,CO)]_{max} - [\log p_{1/2}(O_2,CO)]_{min}$, respectively.

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TABLE I: Bohr Effect Parameters of Monomeric Hemoglobins from *Chironomus thummi thummi* at 25 °C.

		Hemoglobin I	Hemoglobin III	Hemoglobin IV
$p_{1/2}(O_2)_{max}$	[mmHg]	1.84[d]	1.60[e]	2.75[e]
$p_{1/2}(O_2)_{min}$	[mmHg]	1.84[d]	0.68[e]	0.45[e]
$\Delta \log p_{1/2}(O_2)$		0.0[d]	0.37[e]	0.79[e]
$-(\Delta \log p_{1/2}(O_2)/\Delta pH)_{pH7.2}$	[mol H^+/mol O_2]	0.0[d]	0.21[e]	0.42[e]
$n_{H^+,pH7.2}$[a]	[mol H^+/mol O_2]	nd[j]	0.20[f]	0.43[f]
$p_{1/2}(CO)_{max}$	[mmHg]	5.83×10^{-3}[g]	6.78×10^{-3}[g]	7.90×10^{-3}[g]
$p_{1/2}(CO)_{min}$	[mmHg]	5.83×10^{-3}[g]	3.08×10^{-3}[g]	3.38×10^{-3}[g]
$\Delta \log p_{1/2}(CO)$		0.0[g]	0.34[g]	0.37[g]
$\Delta pK_{HisG2(deoxy \rightarrow CO)}$[b]		nd	0.30[h]	0.35[g]
$-(\Delta \log p_{1/2}(CO)/\Delta pH)_{pH7.2}$	[mol H^+/mol CO]	0.0[g]	0.19[g]	0.21[g]
$n_{H^+,pH7.2}$[a]	[mol H^+/mol CO]	nd	0.04[f]	0.08[f]
$n_{H^+,pH7.1}$[c]	[mol H^+/mol CO]	nd	0.19[i]	0.31[i]
$M_{max} = p_{1/2}(O_2)/p_{1/2}(CO)$		300[g]	270[g]	350[g]
$M_{min} = p_{1/2}(O_2)/p_{1/2}(CO)$		300[g]	210[g]	135[g]

[a] Proton release upon transition from deoxy to ligated form, determined by pH measurements. [b] pK shift of the Bohr proton binding site, His-G2, upon the transition from deoxy to CO-ligated form, determined by NMR. [c] Proton release upon transition from deoxy to ligated form, determined by calorimetry. [d] Wollmer et al., 1972. [e] Gersonde et al., 1972. [f] Steffens et al., 1977. [g] Sick and Gersonde, 1974. [h] Sick et al., 1972. [i] Gersonde et al., 1976. [j] nd, not determined.

effects which can be correlated with changes with pH of the NMR spectra.

The most thorough characterization has been performed on hemoglobin III; its sequence has been determined (Buse et al., 1969; Braunitzer et al., 1974) and the single-crystal x-ray structures in the met-aquo, deoxy, and carbon monoxy forms have been reported (Huber et al., 1969, 1970).

The tertiary structure closely resembles that of sperm whale myoglobin or a subunit of tetrameric hemoglobin, although there are many substitutions even within the heme pocket (Huber et al., 1971). Only the proximal histidine-F8 and phenylalanine-CD1 are invariant; the histidine-E7, although present, is turned to the surface of the molecule in the met and CO-ligated form, interacting with the carboxyl group of a propionic acid. In the cyanide-ligated form, however, two positions for His-E7 are indicated by the difference Fourier map: one with an imidazole forming a salt bridge with a propionic acid at the surface, and another one with the His-E7 residue turned inward interacting with the CN^- bound at the sixth coordination site (W. Steigemann, personal communication).

The Bohr proton binding site has been suggested to be an imidazole based on the shift of the pK of this imidazole C-2 proton with the nature of the axial ligand (Sick et al., 1972; Trittelvitz et al., 1973; Sick and Gersonde, 1974). Although the Bohr effect cannot be determined directly from cyanide binding isotherms, the shift of the imidazole pK with cyanide suggested that this ligand is also linked to the Bohr proton (Sick et al., 1972; Sick and Gersonde, 1974). The sequence of hemoglobin IV is under present investigation and it appears that it is very similar to that of hemoglobin III (G. Braunitzer, personal communication); it has the same number of histidines (4) and hence presumably possesses the same allosteric proton binding site and possibly also lacks the distal histidine. In view of the great sensitivity of the hyperfine shifted heme resonances to protein tertiary structure in paramagnetic forms of hemoproteins and the indication that there is a coupling of the Bohr proton binding site and the coordination of cyanide in the met form of the protein, we will present and analyze here the hyperfine-shifted signals in the met-cyano forms. These forms yield extremely well-resolved proton NMR spectra in the region −30 to +5 ppm from the diamagnetic reference, the sodium salt of 2,2-dimethyl-2-silapentane-5-sulfonate, DSS, expanding greatly the possibilities for resolving and assigning the individual resonances to specific functional groups of the heme. In our companion manuscript (La Mar et al., 1978), we will present an interpretation of the effect of protein tertiary structural changes on the heme as reflected in the NMR spectra in terms of the rotational mobility of heme peripheral substituents.

Materials and Methods

Preparation of Hemoglobins. The ferric hemoglobin cyanide complexes will be designated metHb-I-CN, metHb-III-CN, metHb-IV-CN, where the 3+ oxidation state is designated by the met prefix, and the Roman numerals are reserved to reflect the labels of the individual monomeric hemoglobins as established previously. Similarly the ferrous oxygen complexes are designated as Hb-I-O_2, etc. The monomeric hemoglobins I, III, and IV from *Chironomus thummi thummi* were purified as described elsewhere (Sick et al., 1972). The separation of the monomeric hemoglobins was carried out by ion-exchange chromatography. The hemoglobin solutions were dialyzed against starting buffer (0.01 M Tris, pH 9.0) and then applied to a DEAE-Sephadex A50 column (6 × 60 cm) equilibrated with starting buffer. The individual hemoglobins were eluted with a linear gradient (starting buffer: 5 L of 0.01 M Tris, pH 9.0; mixing buffer: 5 L of 0.01 M Tris, pH 9.0, containing 0.15 M NaCl). The fractions were proven to be homogeneous by polyacrylamide gel (10%) disk electrophoresis at pH 9.5 (Mauer, 1968). The R_f values of hemoglobin I, III, and IV were 0.20, 0.47, and 0.60, respectively. The salt-free proteins were lyophilized and stored in the met form at −30 °C. Part of the protein was precipitated in concentrated ammonium sulfate and stored at +4 °C. No difference with regard to electrophoresis, O_2 binding, and paramagnetically shifted heme resonances was observed.

Reconstitution of Deuterohemoglobins. The preparation of globin and the reconstitution of hemoglobins with deuteroheme were carried out as described by Overkamp et al. (1976). The reconstituted protohemoglobins were identical with the respective native components as shown by polyacrylamide gel disc electrophoresis, O_2-binding properties, and NMR observables described in this paper. Protoheme IX was purified as described by Overkamp et al. (1976). Deuteroheme IX was prepared from protohemin IX according to Falk

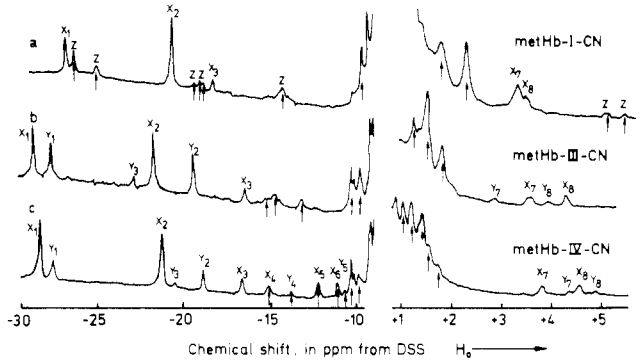


FIGURE 1: The 270-MHz heme proton resonance spectra of cyanide-ligated methemoglobin I (a), III (b), and IV (c) in 0.2 M $NaCl/D_2O$ at "pH" 7.42, 7.58, and 7.52, respectively, and at 25 °C. X component: x_1, x_2 = ring methyls; $x_3 = H_\alpha$; $x_7 = H_{\beta\text{-}t}$, $x_8 = H_{\beta\text{-}c}$ of one vinyl group; x_4, x_5, x_6 = single protons (x_4, x_5 = possibly α-CH_2). Y component: y_1, y_2 = ring methyls; $y_3 = H_\alpha$, $y_7 = H_{\beta\text{-}t}$, $y_8 = H_{\beta\text{-}t}$ of one vinyl group; y_4, y_5, y_6 = single protons. Arrows below peaks indicate that they shift with temperature.

(1964). A mixture of 400 mg of protohemin IX and 1.2 g of resorcinol was heated in a round-bottomed flask with air cooler at 150–160 °C for 45 min. After cooling, the dark-brown melt was washed with ether. The washing procedure was continued until the ether was colorless. The remaining solid was deuterohemin, which was crystallized several times for final purification as described by Fischer (1955). Five grams of deuterohemin was dissolved in 25 mL of pyridine. To this solution 40 mL of chloroform was added and after 15 min of shaking the solid material was filtered. The solution of deuterohemin was then added, with stirring, to a boiling mixture of 350 mL of glacial acetic acid, 5 mL of saturated sodium chloride solution, and 12 mL of concentrated HCl. After 15 h at room temperature, crystalline deuterohemin was isolated.

Preparation of Samples. For ^1H NMR experiments, 80 mg of lyophilized methemoglobin was dissolved in 0.5 mL of 0.2 M sodium chloride in D_2O (isotopic enrichment of 99.75%), to which 5 μL of a 10% sodium cyanide solution was added. Initially the pH was adjusted to pH 5 by addition of small amounts of 0.1 M DCl. After standing 10 min, any nondissolved materials were removed by centrifugation, and the desired pH value was adjusted by addition of 0.1 N NaOD. The pH was measured at 25 °C in the NMR tube with a glass electrode (Type 405-M3, a = 185 mm, Ingold, Frankfurt/Main) and a pH meter (Type PHM 63, Radiometer, Copenhagen). The accuracy of the pH measurement was ±0.02 unit. The pH values were not corrected for the isotope effect and are hence referred to as "pH" values.

Addition of DSS as internal marker had no effect on the proton NMR spectra of any of the proteins at "pH" $\geq$7. However, below "pH" 7, DSS significantly altered the hyperfine shift pattern, suggestive of a specific interaction between the marker and the heme cavity. *tert*-Butyl alcohol was found not to have any effect on the spectra at any pH employed and, hence, served as primary internal reference. Calibrations using both DSS and *tert*-butyl alcohol in the same sample indicate that the shift difference between the two references was independent of pH and temperature to $\leq$0.01 ppm. Hence, all shifts are given in ppm referenced to DSS and are accurate to $\leq$0.05 ppm.

For O_2-binding experiments, 30 mg of lyophilized methemoglobin was dissolved in 0.1 mL of 0.02 M sodium chloride. Aliquots of 20 μL of this hemoglobin solution were mixed with 20 μL of 0.4 M buffer containing 0.1 M sodium ascorbate as reductant. The pH was measured after oxygenation of hemoglobin with a glass electrode (Type 406-M3, a = 35 mm, Ingold, Frankfurt/Main).

Proton Magnetic Resonance Spectra. ^1H NMR spectra of the cyanide hemoglobins were recorded at 25 °C with a 270-MHz spectrometer (Type WH 270, Bruker-Physik, Karlsruhe) operating in the Fourier transform mode; 500 to 2000 transients were collected using a bandwidth of 7 kHz, 8K data points, and a 10-μs 90° pulse. Decoupling experiments were performed in the standard manner. Chemical shifts are reported in ppm, referenced against DSS. The temperature dependence of the paramagnetically shifted proton resonances was measured with a temperature device (Type B-ST-100/700, Bruker-Physik, Karlsruhe) calibrated with methanol (Van Geet, 1968).

O_2-Binding Experiments. The O_2-binding experiments were performed using the rapid diffusion technique at 25 °C (Sick and Gersonde, 1969b, 1972). The change of the absorbance caused by the oxygenation was measured at 436 nm in the case of protohemoglobin and at 424 nm in the case of deuterohemoglobin.

Calculation of Proton Dissociation Curves. Apparent pKs were determined by computer-fitting the pH-dependent chemical shifts to the Henderson–Hasselbalch equation. Good fits were obtained in all cases where the shift changes with pH were $\geq$0.3 ppm indicating that the dissociation of a single proton controls the change in environment responsible for the shifts.

Results and Discussion

The regions of the proton traces containing the hyperfine shifted resonances for CN^--ligated methemoglobins I, III, and IV at "pH" 7.5 are given in a, b, and c of Figure 1. The diamagnetic region, −8 to 0 ppm from DSS, provides relatively little useful information under the present circumstances. Inspection of the three traces reveals a definite similarity in the spread and the pattern of the resonances. However, such a cursory examination of the spectra also reveals immediate problems for the interpretation and assignment of the spectra.

Sample Heterogeneity and Protein "Disorder". Considerable experience with a variety of low-spin ferric hemoproteins (Wüthrich, 1970; Wüthrich et al., 1970; Keller et al., 1976; La Mar et al., 1977) as well as model compounds (La Mar et al., 1977; La Mar and Walker, 1977; La Mar et al., 1976; Satterlee and La Mar, 1976) has shown that the overwhelming majority of the low field hyperfine shifted resonances arise from the coordinated ligands, protoporphyrin IX and the proximal histidylimidazole. Prominent among these down-field peaks are heme methyls, of which two to three are generally resolved below −10 ppm. This leads us to the reasonable assumption that the most intense peaks below −10 ppm are heme methyls with intensities for three protons.

Thus for metHb-IV-CN (Figure 1c) peaks x_1 and x_2 can be confidently attributed to heme methyls. However, when the remaining peaks in Figure 1c are analyzed, it is found that, although peaks y_1, y_2, and x_3–x_8 have the correct intensity for single protons, at least five additional resonances, y_3–y_7, integrate for one-third of a proton. Conversely, if this last set of peaks is attributed to single protons, then x_1 and x_2 must each contain three methyl groups, although there are only a total of four methyls in a heme. A consistent interpretation of this proton trace can be arrived at only if we assume that there are present two forms of the protein giving rise to two separate sets of peaks. The major or X component of metHb-IV-CN gives rise to x_1, x_2 (methyls), and x_3–x_7 (single protons), while the

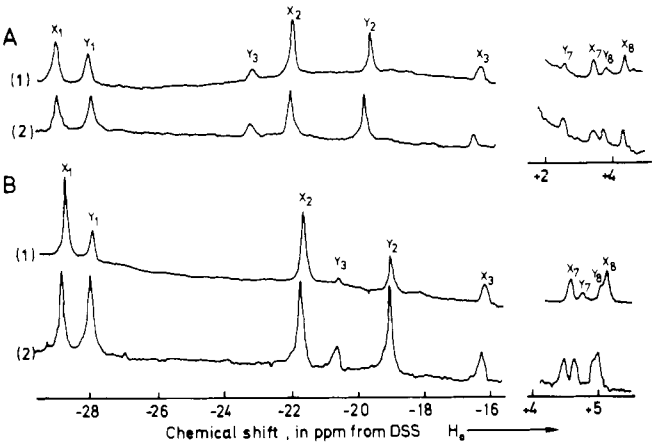


FIGURE 2: The 270-MHz heme proton resonance spectra of cyanide-ligated methemoglobins III (A) and IV (B) in 0.2 M $NaCl/D_2O$. (1) At "pH" 10.7 and 25 °C; (2) at "pH" 10.7 after 24 h of incubation at 40 °C.

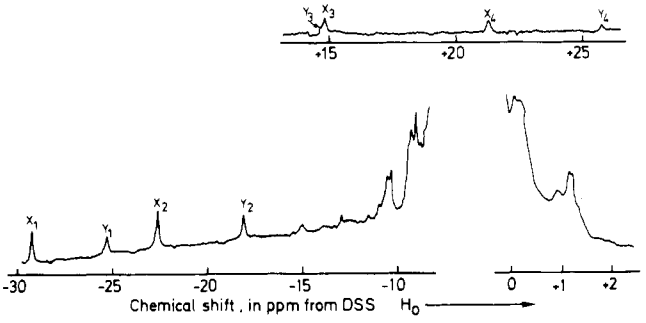


FIGURE 3: The 270-MHz heme proton resonance spectra of cyanide-ligated methemoglobin III, reconstituted with deuteroheme, in 0.2 M $NaCl/D_2O$ at "pH" 7.49 and 25 °C. X component; x_1, x_2 = ring methyls; x_3, x_4 = 2,4-pyrrole protons. Y component: y_1, y_2 = ring methyls; y_3, y_4 = 2,4-pyrrole protons.

minor or Y component yields y_1, y_2 (methyls), and y_3–y_7 (single protons) with the ratio of the two components $X/Y \sim 3$ (vide infra). Note when comparing intensities of the upfield and downfield portions of the traces in Figure 1, that, while the vertical expansions are identical, the horizontal expansion of the upfield portion is three times that of the downfield portion.

Inspection of the proton trace of metHb-III-CN in b of Figure 1 reveals a similar problem; x_1 and x_2 must be methyls of area 3, y_1 and y_2 have area for two protons; x_3, x_7, and x_8 are consistent with single protons, but then y_3, y_7, and y_8 must contain only two-thirds of a proton. This dilemma is again removed if we assume two components for which x_1 and x_2 are methyls and x_3, x_7, and x_8 single protons for the X component, while y_1 and y_2 are methyls and y_3, y_7, and y_8 single protons for component Y, with the ratio $X/Y \sim 3/2$. The existence of heterogeneity in this insect hemoglobin had also been suggested based on doubling of peaks found in the diamagnetic Hb-III-CO (Wüthrich et al., 1972).

Similarly, metHb-I-CN (Figure 1a) also exhibits peaks inconsistent with a single species. In fact, at least two minor components must also be present in addition to the major (X) component for which x_1 and x_2 are methyls and x_3, x_7, and x_8 are single protons (vide infra). However, this hemoglobin has not yet been isolated in a pure form and is known to contain minor components so that analysis of the remaining minor resonances has not proved possible to date.

The repeated purification by ion-exchange chromatography under slightly different conditions failed to alter the proton traces of the CN^--ligated forms of hemoglobins III and IV from those depicted in Figures 1b and 1c. Independent isolation of the protein from two different cultures of larvae also produced identical spectra. Furthermore, polyacrylamide gel disc electrophoresis showed neither a minor hemoglobin component nor a nonhemoglobin impurity. Therefore one may conclude that the two components in these two hemoglobins do not arise from any chemical heterogeneity which can be resolved by methods at our disposal.

Although the 3:2 ratio in metHb-III-CN is similar to the "genetic" heterogeneity in the form of neutral amino acid substitutions not far from the heme group, 39CD2 (Thr/Pro) and 57E6(Ile/Thr) (Buse et al., 1969), this origin can be discounted on the basis of the fact that the populations of X and Y components of both metHb-III-CN and metHb-IV-CN can be altered under severe conditions. Thus, while neither "pH" variations between 5.0 and 10.7 nor temperature variations between 0 and 45 °C for short durations produced detectable changes in the X/Y ratios, incubation of the proteins at high "pH" (>10) and high temperature (~40 °C) for one to several days equalized the two components for each of metHb-III-CN and metHb-IV-CN.

The portions of the proton trace of interest for metHb-III-CN and metHb-IV-CN at "pH" ~10.5 are reproduced in spectrum 1 of Figures 2A and 2B, respectively. In each of A and B of Figure 2 we compare the original trace (1) with that of the proteins after 24 h of incubation at 40 °C (trace 2). In each case it is obvious that there are changes in the relative intensities of the peaks which clearly separate them into two groups and provide the basis for the labeling of the X and Y resonances in Figures 1 and 2. Altering the populations of the two components appears to eliminate any sort of sample heterogeneity arising from amino acid substitutions.

Preparation of the apoprotein of hemoglobins III and IV and subsequent reconstitution with protoheme IX gave the native proteins with proton traces identical with those in b and c of Figure 1. Reconstitution of both apoproteins with deuteroheme IX gave deutero-metHb-III-CN and -metHb-IV-CN. The upfield and downfield portions of the proton trace for deutero-metHb-III-CN are illustrated in Figure 3. A similar trace is obtained for deutero-metHb-IV-CN (not shown). Again two sets of resonances, x_1, x_2 (methyls) and x_3, x_4 (single protons) for component X, and y_1, y_2 (methyls) and y_3, y_4 (single protons) for component Y are observed. However, for this prosthetic group, the component ratios are altered from that of the native proteins to $X/Y \sim 4$ for deutero-metHb-IV-CN and $X/Y \sim 2$ for deutero-metHb-III-CN.

Protein conformational differences due to cis, trans isomers of a proline peptide bond have been suggested as the origin of a similar double set of heme hyperfine resonances in ferricytochrome b_5 (Keller et al., 1976). However, on the basis of the refined x-ray data, cis–trans isomerization in hemoglobin III can be excluded (W. Steigemann, personal communication). Thus any conformational change responsible for the pair of spectra must involve major changes in the protein tertiary structure. One such possibility is suggested by recent x-ray crystallographic studies in metHb-III-CN (W. Steigemann, personal communication), which involves two structures, one of which has the distal histidine-E7 turned to the outside of the protein, and the other which has it turned into the heme cavity and hydrogen bonded to the coordinated cyanide.

Another possible origin for two sets of signals could be from "disorder" of the heme, where the two components differ by a 180° rotation of the heme group about its α–γ meso axis in

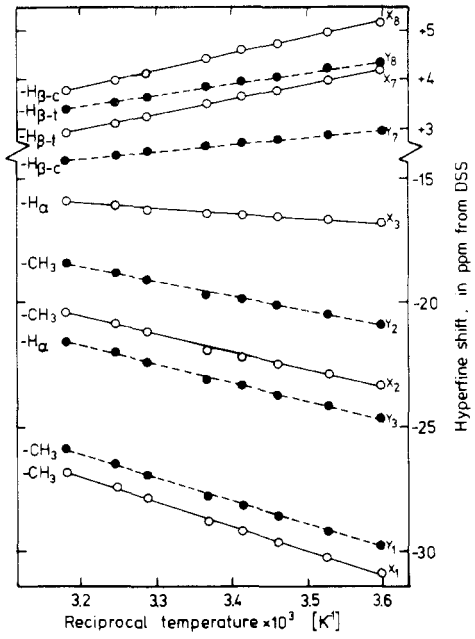


FIGURE 4: Dependence on the reciprocal of temperature of the heme proton resonance in cyanide-ligated methemoglobin III in 0.2 M NaCl/D_2O at "pH" 7.58. X component (O—O); Y component (●- - -●).

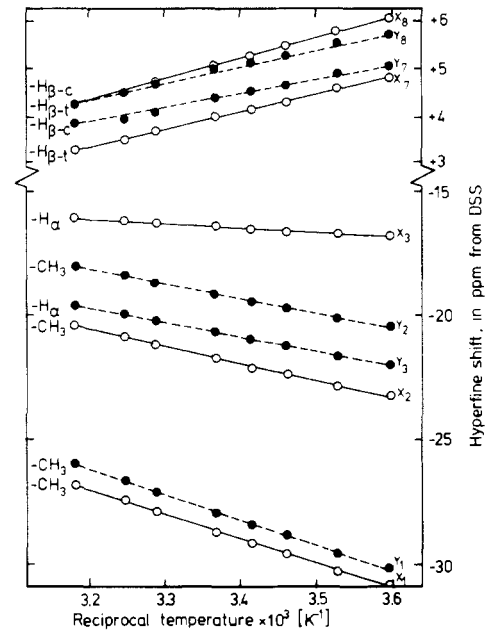


FIGURE 5: Dependence on the reciprocal of temperature of the heme proton resonances in cyanide-ligated methemoglobin IV in 0.2 M NaCl/D_2O at "pH" 7.52. X component (O—O); Y component (●- - -●).

the heme cavity. This will maintain identical environments for the propionic acid side chains but exchange the 1,3-methyls with the 2,4-vinyl environments. The different heme–protein contacts involving these methyls and vinyls could account for the slightly different hyperfine shifts for the two positions of the heme. This heme disorder, of course, could be related to the position of histidine-E7.

Further conclusions reached in this paper deal primarily with the major components in each protein and are therefore essentially independent of the physical origin of this heterogeneity.

Since incubation of the protein at low pH failed to alter the proton traces, the process appears to be facilitated by high pH. Thus the high pH form appears to provide more local mobility for the heme. Both the interpretation of the present NMR results (La Mar et al., 1978) and earlier considerations of the entropy of O_2 and CO binding (Gersonde et al., 1972, 1976) suggest that the high-pH form of the proteins is more flexible.

Assignment of Hyperfine Resonances. Although all downfield peaks below −10 ppm must experience paramagnetism, peaks above 0 ppm from DSS could originate from ring currents alone. The test for hyperfine shifted peaks is a temperature dependence where the hyperfine shift increases with decreasing temperature. The plots of observed shift from DSS vs. reciprocal temperature (Curie plot) for metHb-III-CN and metHb-IV-CN are given in Figures 4 and 5, respectively. The temperature dependences for peak x_1–x_5 for metHb-I-CN are similar to those in Figures 4 and 5. We plot in these graphs only the shifts for those peaks which can be unambiguously assigned to either one or the other component. Other peaks which also show a temperature dependence will be designated as such simply by a small arrow below the peak in Figure 1. Although we are confident of the assignment of x_1, x_2 (Figure 1) and y_1, y_2 as two heme methyls in the major and minor component of both metHb-III-CN and metHb-IV-CN on the basis of area, all other functional groups are expected to give rise to single proton resonances (each vinyl group→3 signals, each propionic α-CH_2→2 signals).

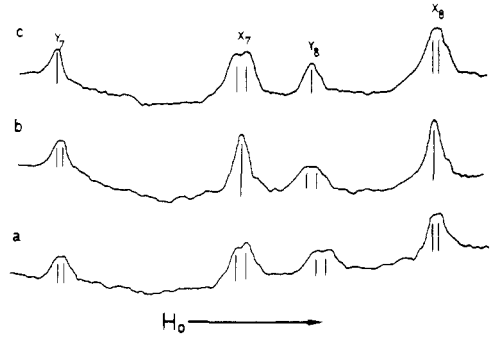
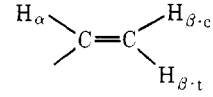


FIGURE 6: The 270-MHz heme proton resonance spectra of cyanide-ligated methemoglobin III in the upfield region. For conditions see legend of Figure 1b. No decoupling (a), irradiation of proton resonance x_3 (b), and y_3 (c), respectively.

The upfield pair of single proton resonance (x_7, x_8; y_7, y_8) is found in both components for each protein and provides an important clue as to their identity if the proton spectrum is particularly well-resolved (in metHb-I-CN there is apparently an overlap of X and Y single protons for the peak at +3.3 ppm). This upfield portion of the proton trace for metHb-III-CN is expanded under optimum resolution in a of Figure 6. One peak for each component, x_7 and y_8, clearly exhibits a doublet structure with a splitting of ~16 Hz. This splitting is much better resolved at 45 °C where peaks x_8 and y_7 also exhibit a flattening at the top suggestive of a doublet structure with J ~ 10 Hz. These multiplet patterns are expected (Wüthrich, 1970) for vinyl H_β, where J = 16 Hz corresponds to the trans proton $H_{\beta\text{-t}}$, and the 10-Hz splitting to the cis proton $H_{\beta\text{-c}}$, i.e.



This assignment of the upfield peaks of the H_β's in the X and Y components can be established by locating the vinyl H_α for each isomer and then irradiating it to remove the multiplet

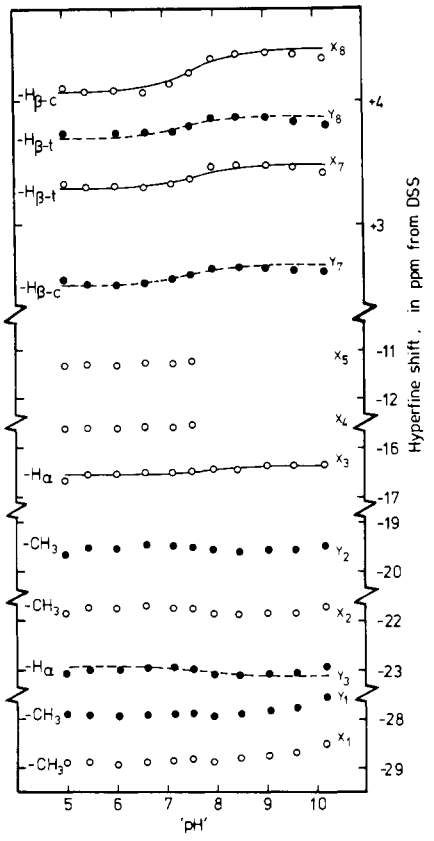


FIGURE 7: pH dependence of hyperfine shifted heme proton resonances in cyanide-ligated methemoglobin III in 0.2 M NaCl/D_2O at 25 °C. X component (O—O); Y component (●- - -●). Dissociation curves of x_3, x_7, x_8, and y_3, y_7, y_8 are calculated on the basis of a dissociation of one proton.

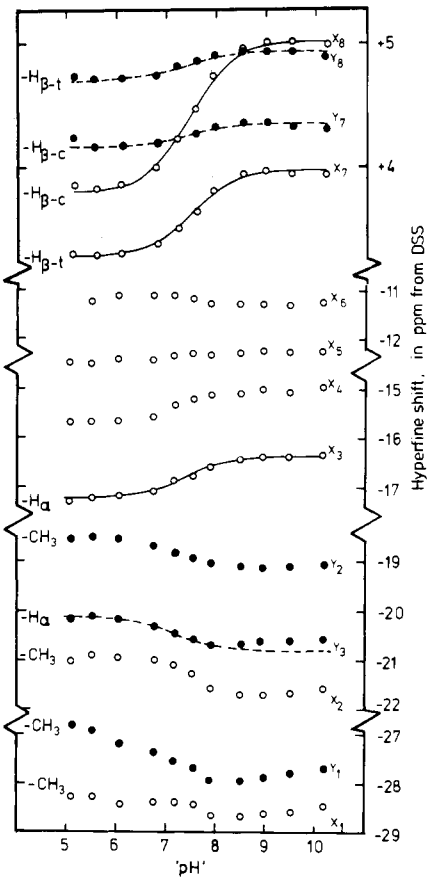


FIGURE 8: pH dependence of hyperfine shifted heme proton resonances in cyanide-ligated methemoglobin IV in 0.2 M NaCl/D_2O at 25 °C. X component (O—O); Y component (●- - -●). Dissociation curves of x_3, x_7, x_8, and y_3, y_7, y_8 are calculated on the basis of a dissociation of one proton.

structure on the H_β's. Collapse of the x_7, x_8 multiplets of metHb-III-CN was achieved only by irradiating x_3 (in Figures 1b and 2A), as illustrated in b of Figure 6. The doublet structure of x_7 is clearly eliminated and x_8 is simply narrowed. Hence x_3, x_7, and x_8 originate from the three protons of a single vinyl group. Similarly, irradiating y_3 collapses the multiplet structure for the minor component y_7, y_8, indicating that these three peaks comprise a single vinyl group in the Y component. The upfield multiplet structure for each component of metHb-IV-CN can also be removed by double irradiation (not shown) leading to the assignment of the vinyl groups for both components. In the case of metHb-I-CN, multiplet structure is not resolved and at least x_7 overlaps some peaks of the minor components. However, upon irradiating x_3, both x_7 and x_8 exhibit significant narrowing with the effect much larger in x_7 than x_8. Such narrowing of the single proton resonances upon double irradiation has also been used to assign the vinyl protons in cytochrome b_5 (Keller et al., 1976). Hence once again we have $x_7 = H_{\beta\text{-}t}$, $x_8 = H_{\beta\text{-}c}$, and $x_3 = H_\alpha$.

The relative areas of x_1, x_2, and the vinyl decoupling experiments therefore provide us with the assignment of two heme methyls and the three protons of a *single vinyl group* in each of the three proteins. In fact, the same five peaks are also assigned in the minor component of two of the proteins.

It should also be noted that the two single proton resonances x_3, x_4 (and y_3, y_4) in Figure 3 can be unambiguously assigned to the 2,4-pyrrole protons of deuteroporphyrin (La Mar and Walker, 1977). In view of the very similar peak positions of the two methyls and the vinyl group resonances in metHb-III-CN and metHb-IV-CN, it appears reasonable to conclude that we have assigned the same two heme methyls of the four possible methyls and the same heme vinyl of the two possible vinyls in each of the three proteins. Since the analogous methyl peaks of the minor component Y are upfield of the two methyls of the major component X in both metHb-III-CN and metHb-IV-CN, it is also reasonable to assume that the dominant component in each case corresponds to the same structural form of the protein (vide supra). These assignments of identical functional groups in the three proteins allow us to proceed with an analysis of the relative effects of pH on their hyperfine shifts.

The observation of large hyperfine shifts for only two methyl groups and a single vinyl group indicates that the electronic wave function containing the unpaired spin is highly asymmetric. The different amounts of spin density at the 2 and 4 positions (La Mar and Walker, 1977) are also illustrated by the sizable difference in the hyperfine shifts of the 2 and 4 pyrrole protons of deutero-metHb-III-CN (Figure 3).

Most of the other peaks which can be attributed to the X isomer cannot be assigned unambiguously at this time. For example, x_4, x_5 in metHb-IV-CN may arise from an α-CH_2 of a propionic acid side chain. These two peaks are present in both metHb-III-CN and metHb-IV-CN at low pH. However, for metHb-III-CN they decrease in intensity and disappear at pH >7.3 (Figure 7), suggesting a slow change in conformation of the side chain, possibly involved in formation of a salt bridge. Further studies on labeling of hemes should provide us additional assignments.

pH-Induced Tertiary Structural Changes. The sensitivity to pH of the five assigned, as well as some unassigned, peaks

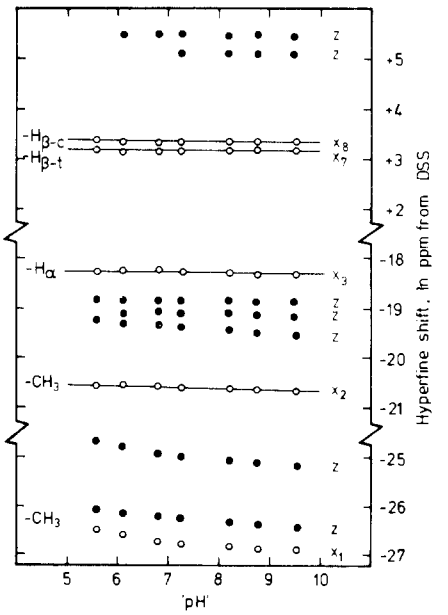


FIGURE 9: pH dependence of hyperfine shifted heme proton resonances of cyanide-ligated hemoglobin I in 0.2 M NaCl/D_2O at 25 °C. Major component, x (O—O); minor components, z (●- - -●).

for both components of the allosteric proteins metHb-III-CN and metHb-IV-CN are illustrated in Figures 7 and 8. The pH titration for the nonallosteric protein, metHb-I-CN, is given in Figure 9. These data in Figures 7–9 clearly reveal a degree of sensitivity to pH which qualitatively parallels the amplitudes of the O_2 Bohr effect curves. Most peak positions for metHb-I-CN are independent of pH and only one methyl exhibits a smooth decrease in shift with lower pH (see Figure 9). This smooth pH dependence, however, does not reflect an interconversion between two conformation states ($t \rightleftharpoons r$) which should be characterized by an inflection point near pH 7.5, but rather a non-ligand-linked local pH effect due to nonallosteric proton binding sites on the protein. Hence the NMR data appear to confirm a relatively pH-independent tertiary structure for hemoglobin I at 25 °C. The pH independent chemical shifts for the assigned peaks in metHb-I-CN at "pH" 7.3 and 25 °C are as follows: −20.59, −26.79 (methyls), +3.32 ($H_{\beta\text{-c}}$), +3.14 ($H_{\beta\text{-t}}$), −18.29 ($H_{\beta\text{-}\alpha}$) (shifts in ppm from DSS).

Both metHb-III-CN and metHb-IV-CN exhibit pH dependences with the sensitivity in each protein greater in the major than in the minor component (see Table III). The resonances attributed to the three protons of *one* vinyl (x_3, x_7, x_8; y_3, y_7, y_8) and to the more pH-sensitive methyl group (x_2, y_2) change their position with pH following a one proton dissociation curve, with apparent p*K* values 7.4 ± 0.3 (see Table III). The correspondence of these p*K* values with the inflection point of the Bohr effect curves (Sick and Gersonde, 1969a, 1974; Gersonde et al., 1972), and with the intrinsic p*K* value of His-G2, which is assumed to be the Bohr proton binding site in hemoglobin III and possibly also in hemoglobin IV (Sick and Gersonde, 1974; Sick et al., 1972), allows the conclusion that the observed p*K* values reflect the allosteric transition between two protein conformations ($t \rightleftharpoons r + H^+$). Thus the pH-induced shift changes of the heme resonances again demonstrate that the equilibrium between the two tertiary structures is controlled only by a single proton binding site, i.e., by the Bohr proton binding site. All peaks which exhibit a pH dependence have such an inflection point, although fitting the points at very low and high pH to the Henderson–Hasselbalch equation was not always possible due to simultaneous smooth pH dependence of some shifts due to environmental effects similar to those observed for metHb-I-CN.

TABLE II: Correlation of O_2 Bohr Effect with pH-Dependent Chemical Shift Change in Cyanide-Ligated Methemoglobins I, III, and IV at 25 °C.[a]

Hemoglobin		Amplitude of the O_2 Bohr effect curve, $\Delta \log p_{1/2}(O_2)$	Amplitude of the vinyl proton chemical shift changes (ppm)		
			$H_{\beta\text{-c}}$	$H_{\beta\text{-t}}$	H_{α}
I		0	0.03	0.03	0.04
III	X	0.37	0.35	0.20	0.18
	Y		0.16	0.17	0.20
IV	X	0.79	1.25	0.72	0.89
	Y		0.22	0.24	0.67

[a] X = major component. Y = minor component.

If we restrict ourselves for the moment to the comparison of the major components, we find that, in each case in Figures 7 and 8, the lower field methyl (x_1) is less sensitive to pH than the other methyl (x_2), and that the largest shift change occurs for the vinyl $H_{\beta\text{-c}}$'s. Since we are looking at the same functional groups (two methyls and one vinyl) in each of the proteins, comparison of the pH sensitivity of the shifts is warranted and should monitor the magnitude of the perturbation effected on the heme by the $t \rightleftharpoons r$ transition. The magnitude of the perturbation is reflected by the magnitude of the shift change between the acid and base limiting values for the vinyl protons as shown in Figures 7 and 8. These amplitudes of the shift changes for the vinyl group for the three proteins are compared with the amplitudes of the O_2 Bohr effect in Table II. It is clear that there is a parallel between the magnitude of the Bohr effect and the pH sensitivity of the NMR shifts. The proton shifts for assigned signals in the high and low pH limiting pH regions and the apparent p*K* values as monitored by the heme resonances are listed in Table III.

If we now accept that the magnitudes of the NMR shift changes are in some way related to the amplitude of the structural change accompanying the $t \rightleftharpoons r$ transition, then a comparison of the NMR shift pH titrations in Figures 7 and 8 for the two components suggests the possibility that the Bohr effect may be considerably less important in the minor component for each protein. Since the relative amounts of the minor and major components differ for the two proteins, it suggests that the different Bohr effects measured experimentally reflect not only the intrinsic effect of the protein but also the distribution for the two components.

Inspection of Figures 7 and 8 or Table III reveals that the vinyl group shifts are more sensitive to pH than the methyls. Such a focus of the protein perturbation on the vinyl group suggests the possibility that the dramatic vinyl shift changes reflect a pH-induced change in the rotational position of the vinyl group relative to the heme. We will demonstrate in the companion paper (La Mar et al., 1978) that such an interpretation can indeed be advanced based on the analysis of the NMR spectra of some model compounds.

Bohr Effect in Monomeric Deuterohemoglobins. The possibility has been suggested that the rotational position of vinyl groups on the prosthetic group can be modulated by allosteric effectors in tetrameric hemoglobins (Sebyert et al., 1975, 1976; Sugita and Yoneyama, 1975) and that the change in heme-vinyl conjugation could alter the electronic structure of the iron so as to influence its oxygen affinity and allosteric response.

TABLE III: Chemical Shifts and Apparent pK Values of Cyanide-Ligated Methemoglobins III and IV at 25 °C.[a]

		MetHb-III-CN				MetHb-IV-CN			
Heme protons		Low pH	High pH	Shift change amplitude	pK	Low pH	High pH	Shift change amplitude	pK
$-H_{\beta\text{-c}}$	X	4.06	4.41	0.35	7.6 ± 0.2	3.78	5.03	1.25	7.4 ± 0.1
	Y	2.51	2.67	0.16	7.4 ± 0.2	4.15	4.37	0.22	7.4 ± 0.2
$-H_{\beta\text{-t}}$	X	3.29	3.49	0.20	7.6 ± 0.2	3.27	3.99	0.72	7.5 ± 0.1
	Y	3.70	3.87	0.17	7.4 ± 0.2	4.70	4.94	0.24	7.3 ± 0.2
$-H_{\alpha}$	X	−16.54	−16.36	0.18	7.7 ± 0.2	−17.25	−16.36	0.89	7.4 ± 0.2
	Y	−22.94	−23.14	0.20	7.7 ± 0.3	−20.13	−20.80	0.67	7.2 ± 0.3
$-CH_3$	X	−21.69	−21.94	0.25	nd	−20.91	−21.85	0.94	nd
	Y	−19.44	−19.64	0.20	nd	−18.51	−19.13	0.61	nd
$-CH_3$	X	−28.93	−28.74	nd	nd	−28.35	−28.79	0.44	nd
	Y	−27.96	−28.81	nd	nd	nd	nd		
x_4		−12.62	lb			−15.71	−15.01	0.70	nd
x_5		−11.32	lb			−12.48	−12.24	0.24	nd

[a] lb, line broadened. X, major component. nd, not determined. Y, minor component. Chemical shifts in ppm from DSS, and pK values were calculated by a least-squares fit on the basis of a dissociation of one Bohr proton linked to the tertiary structure change.

TABLE IV: Chemical Shifts and Apparent pK Values of Cyanide-Ligated Deuteromethemoglobins III and IV at 25 °C.[a]

		MetHb-III-CN				MetHb-IV-CN			
Heme protons		Low pH	High pH	Shift change amplitude	pK	Low pH	High pH	Shift change amplitude	pK
Pyrrole H	X	21.40	21.81	0.41	7.7 ± 0.2	22.11	22.50	0.39	nd
	Y	26.15	26.15	0.00		25.70	26.04	0.34	nd
Pyrrole H	X	14.91	15.11	0.20	7.8 ± 0.3	14.90	14.90	0.00	
	Y	14.75	15.08	0.33	7.3 ± 0.3	14.66	15.50	0.84	7.3 ± 0.1
$-CH_3$	X	−22.65	−22.65	0.00		−22.45	−22.50	0.05	
	Y	−18.10	−18.10	0.00		−17.43	−18.30	0.87	7.4 ± 0.1
$-CH_3$	X	−29.20	−29.20	0.00		−29.00	−29.00	0.00	
	Y	−25.20	−25.20	0.00		−23.87	−25.46	1.59	7.1 ± 0.3

[a] X, major component. Y, minor component. Chemical shifts in ppm from DSS and pK values were calculated by a least-squares fit on the basis of a dissociation of one Bohr proton linked to the tertiary structure change. nd, not determined because of data scatter.

In the case of the monomeric insect hemoglobin III, it has been reported that deuterohemoglobin III, in contrast to the native form, has no Bohr effect (Amiconi et al., 1972). However, if we inspect the pH titration of the hyperfine shifted resonances in the spectrum of deutero-metHb-III-CN in Figure 10 (similarly for deutero-metHb-IV-CN) we find that it exhibits well-defined pH sensitivities, indicating that the t ⇌ r structural interconversion still takes place. The shifts, relative to DSS, in these reconstituted hemoglobins are compiled in Table IV.

In view of the possibility that rapid autoxidation interfered with the previously reported oxygen affinity studies of the reconstituted insect deutero-hemoglobin III, we have repeated these measurements using the improved experimental methods described in Materials and Methods. In Figure 11 we present the pH effect on $p_{1/2}(O_2)$ of deuterohemoglobins III and IV, which clearly shows that the proteins with the modified heme exhibit essentially the same amplitude of the Bohr effect curve as in the native proteins, although the O_2 affinity is increased. This leads us to conclude that electronic participation of the vinyl group in the Bohr effect is unimportant, although its hyperfine shifts are sensitive indicators of the t ⇌ r transition which modulates the oxygen affinity via some other manner, possible via the trans effect of the proximally bound imidazole (Overkamp et al., 1976).

Conclusions

The present study shows the proton NMR can be utilized to obtain valuable information on protein heterogeneity and the effect of pH-induced protein tertiary structural changes on the molecular and electronic structure of the heme. The types of information obtained in this study indicate that these allosteric monomeric hemoglobins may serve as models for understanding the intrasubunit structural changes which give rise to the Bohr effect in the more complicated tetrameric hemoglobins.

Some of the more specific conclusions which we have reached are listed below.

1. Both allosteric monomeric insect hemoglobins exhibit heterogeneity as detected by proton NMR hyperfine shifts, which is tentatively suggested to arise from either a "disorder" of the heme with respect to a 180° rotation about its α-γ meso axis within the heme pocket or a "disorder" of the distal histidine-E7 position.

2. Two heme methyls and the three protons of a single vinyl group exhibit the largest hyperfine shifts which reflect a high

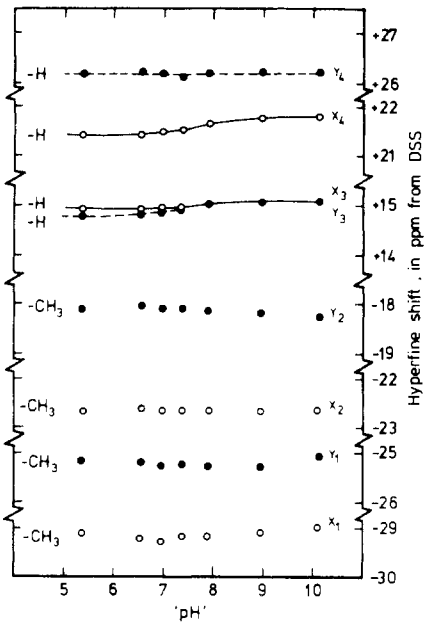


FIGURE 10: pH dependence of heme proton resonances in cyanide-ligated deuteromethemoglobin III in 0.2 M NaCl/D_2O at 25 °C. X component (O—O); Y component (●- - -●). Dissociation curves of x_3, x_4, and y_3 are calculated on the basis of a dissociation of one proton.

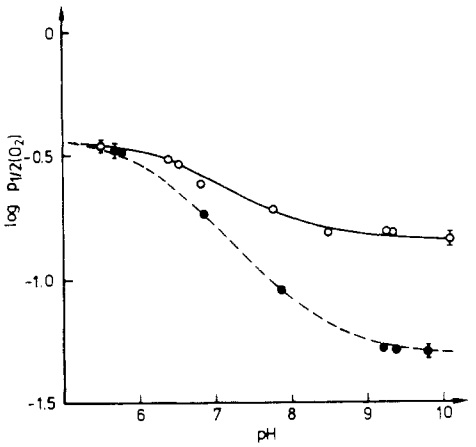


FIGURE 11: Oxygen Bohr effect of deuterohemoglobin III (O—O) and IV (●- - -●) at 25 °C.

degree of in-plane asymmetry in the electronic structure of the heme.

3. The hyperfine shifts of the major components for these proteins are pH dependent, with well-defined inflection points indicative of a labile equilibrium between two forms differing in the tertiary structure of the protein.

4. The pH-dependent vinyl group hyperfine-shift changes parallel the amplitudes of the oxygen Bohr effect curves, indicating that we are monitoring the t ⇌ r transition responsible for the Bohr effect.

5. The vinyl groups exhibit the largest pH effects and appear to be the focal point of the pH-induced perturbation of the heme, suggesting the possibility that the t ⇌ r transition alters the conformation of the vinyl group relative to the heme plane.

6. A direct electronic effect of the vinyl group as a source of the Bohr effect is eliminated by demonstrating that pH sensitivity of the hyperfine shifts is maintained to some degree in the deuteroporphyrin substituted proteins. The oxygen Bohr effects are also found to be essentially unaltered upon removing the vinyl groups, although the O_2 affinity increases by a factor of ten in deuterohemoglobins.

Acknowledgments

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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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