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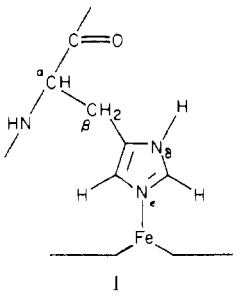
Proton Magnetic Resonance Investigation of the Influence of Quaternary Structure on Iron-Histidine Bonding in Deoxyhemoglobins†

Kiyoshi Nagai,‡ Gerd N. La Mar,* Thomas Jue, and H. Franklin Bunn

ABSTRACT: The proton nuclear magnetic resonance (NMR) spectra of the separated deoxy chains of normal adult hemoglobin (Hb A), the deoxy form of chemically modified hemoglobins, and Hb Kempsey [Asp-G1(99)β→Asn] have been investigated in H_2O solution with emphasis on the hyperfine-shifted proximal histidyl imidazole exchangeable $N_\delta H$ as indicators of the iron-histidine interaction. While the $N_\delta H$ hyperfine shift was found to be sensitive to chain origin and subunit assembly, the likelihood of change in both electronic effects and various steric strains precludes interpretation of the NMR data for different proteins at this time. However, upon introduction of conformational perturbations for any given protein, correlations between changes in NMR spectral parameters and altered function are interpretable in terms of changes in iron-imidazole bonding. For the individual chains, the α $N_\delta H$ shift, as well as the oxygen affinity, is insensitive to forming the *p*-(hydroxymercuri)benzoate (*p*MB) adduct, while the β-chain $N_\delta H$ shift decreases by 10 ppm and its oxygen affinity is reduced 6-fold by the same reaction. Since the *p*MB binding site is much closer to the proximal histidine of the β chain than the α chain, the β $N_\delta H$ upfield shift reflects some strain in the iron-histidine interaction. In the R quaternary structure, the deoxy subunits of NES-des-Arg(141α)-Hb, des-His(146β)-des-Arg(141α)-Hb, and des-Arg(141α)-Hb exhibit $N_\delta H$ resonances at approximately 77 ppm for both α and β subunits. The R → T conversion is accompanied by a 10-14-ppm decrease in the hyperfine shift of the α subunit; the β subunit is essentially unaffected. The decreased NH hyperfine shift is consistent with the introduction of some strain into the iron-histidine bond of the α subunit during the R → T transition. The observed $N_\delta H$ shift change for the α subunit is comparable to that observed upon binding *p*-(hydroxymercuri)benzoate to the isolated β chain and hence can account for only a small change in the oxygen affinity. In light of the resonance Raman data on $\nu(Fe-N_\epsilon)$, this study supports a dominant steric influence on iron-histidine bonding when Hb undergoes an R → T transition and argues against a major contribution of any electronic effect created by altered hydrogen bonding of $N_\delta H$ to a backbone carbonyl [Stein, P., Mitchell, M., & Spiro, T. G. (1980) J. Am. Chem. Soc. 102, 7795-7797]. In agreement also with the resonance Raman data [Nagai, K., Kitagawa, T., & Morimoto, H. (1980) J. Mol. Biol. 136, 271-289], the strain we observe is localized in the α subunit.

Central to the various hypotheses for the control of the iron reactivity in oxygen-binding hemoproteins in general, and hemoglobins in particular, is the state of the ubiquitous trans-proximal histidyl imidazole (I). Although the two



I

quaternary structures of hemoglobin (Hb)[1] (Monod et al., 1965; Perutz, 1970) have been characterized in some detail by X-ray crystallography, resolution is still insufficient to pinpoint the specific structural differences at the heme which characterize the contrasting O_2 affinities (Perutz, 1976; Baldwin & Chothia, 1979). A difference in steric effects has been postulated by Perutz, where the protein produces strain in the Fe-N_ϵ bonds in the low-affinity T structure which is relaxed in the high-affinity R structure (Perutz, 1979, 1980). Some support for weaker iron-imidazole bonding in the T rather than the R state has been provided by resonance Raman studies (Nagai et al., 1980; Nagai & Kitagawa, 1980). A prominent electronic influence on iron reactivity has been proposed on the basis of variable hydrogen bonding of the proximal histidyl imidazole $N_\delta H$ to a backbone carbonyl (Valentine et al., 1979) which would modulate the imidazole

† From the Department of Chemistry, University of California, Davis, California 95616 (K.N., G.N.L.M., and T.J.), and the Laboratory of the Howard Hughes Medical Institute, Peter Bent Brigham Hospital, Harvard Medical School, Boston, Massachusetts 02215 (H.F.B.). *Received May 11, 1981.* This work was supported by grants from the National Institutes of Health, HL-16087 (G.N.L.M.), and the Japanese Ministry of Education, 570105 (K.N.).

‡ Permanent address: The Second Department of Physiology, Nara Medical College, Kashihara 634, Japan.

[1] Abbreviations: NMR, nuclear magnetic resonance; Hb, hemoglobin; Hb A, normal adult hemoglobin; DSS, 4,4-dimethyl-4-silapentane-1-sulfonate; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)-methane; Tris, tris(hydroxymethyl)aminomethane; *p*MB, *p*-(hydroxymercuri)benzoate; IHP, inositol hexaphosphate; NES, 5-(*N*-ethylsuccinamido)cysteinyl; ppm, parts per million; EXAFS, extended X-ray absorption fine structure.

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basicity, as previously found in models (Stanford et al., 1980). More recent resonance Raman studies have shown that such H bonding is clearly detectable in $\nu(Fe-N_\epsilon)$ and could, in principle, serve as a control mechanism for O_2 affinity (Stein et al., 1980).

Proton NMR studies have provided a wealth of information on the solution structures of hemoglobins, and considerable effort has been devoted to elucidating the spectral features characteristic for the alternative quaternary states, as reviewed by Morrow & Gurd (1976). Since NMR spectral features can be compared only for proteins with the iron in the same oxidation/spin state (La Mar, 1979), emphasis has been placed on both the modified Hb's and the natural mutants which are capable of undergoing the T ⇌ R structural interconversion solely within the unligated deoxy state. Strong similarities in the hyperfine-shifted heme methyl region have been noted among the varous sets of T-state and R-state hemoglobins (Ogawa & Shulman, 1972; Perutz et al., 1974a; Morrow & Gurd, 1976; Fung & Ho, 1975), and an exchangeable resonance at 14 ppm (Ogawa et al., 1972; Mayer et al., 1973), assigned to the Tyr-42α H bond in the T structure (Fung & Ho, 1975), has been identified. However, no information has appeared to date on the difference in spectral parameters for the proximal histidines in the T and R states. A pair of resonances assigned to the $N_\delta H$'s for the nonequivalent chains had been located on the basis of work on model systems (La Mar et al., 1977) and the peaks recently assigned to individual subunits on the basis of both synthetic and natural (mutant) valency hybrids (La Mar, 1979; La Mar et al., 1980; Takahashi et al., 1980).

Since the hyperfine shifts for the axial imidazole in high-spin ferrous sytems reflect primarily spin transfer via $Fe-N_\epsilon$ σ bonding (Goff & La Mar, 1977; La Mar et al., 1977), the magnitude of the $N_\delta H$ hyperfine shift may provide information on the state of the histidine in the T and R structures. Moreover, comparison of the changes in $N_\delta H$ contact shifts and resonance Raman $\nu(Fe-N_\epsilon)$'s may permit some differentiation between purely steric and electronic effects because the alternative mechanisms influence the two spectral parameters in characteristically different ways (G. N. La Mar and J. S. de Ropp, unpublished experiments). We report here on the proton NMR study of these resonances in Hb A, its separated chains, the series of modified hemoglobins, and mutant Hb Kempsey for which the T ⇌ R transition can be induced by allosteric effectors solely within the deoxy state (Bunn et al., 1974; Kilmartin et al., 1975; Ogawa et al., 1974; Perutz et al., 1974 a,b). These properties, as characterized previously, are summarized in Table I.

Table I: Quaternary State of Modified and Mutant Hb's under Different pH and IHP Conditions

	pH 6.5 with IHP	pH 6.5 stripped	pH 9.0	ref
Hb A	T	T	T	*a, c*
des-His(146β)-Hb	T	T	T	*a*
NES-des-Arg(141α)-Hb	T	R	R	*a, b*
des-His(146β)-Arg(141α)-Hb	T	R	R	*a*
des-Arg(141α)-Hb	T	T	R	*a, b*
Kempsey Hb	T	R (?)	R	*c, d*

[a] Kilmartin et al. (1975). [b] Ogawa et al. (1974). [c] Perutz et al. (1974a,b). [d] Bunn et al. (1974).

Materials and Methods

Human Hb, des-Arg(141α)-Hb, NES-des-Arg(141α)-Hb, des-His(146β)-Hb, des-His(146β)-Arg(141α)-Hb, and the isolated α and β chains were prepared in the CO forms as previously reported (Kilmartin et al., 1975; Nagai & Kitagawa, 1980; Nagai et al., 1980). Hb Kempsey was purified in the CO form by ion-exchange chromatography on CM-52 (Whatman) and further on DE-52 (Whatman) as described by Bunn et al. (1974). All Hb solutions were concentrated in an ultrafiltration apparatus (Amicon) with a Dialfo membrane (PM-50), gel filtered against 1 mM Na_2HPO_4, and deionized by passage through a column of Amberlite AG-501-X8. Carbon monoxide was removed from Hb in a rotary evaporator under a stream of oxygen and strong illumination. Samples for NMR measurements were prepared in 85% H_2O/15% ^2H_2O in either 0.2 M NaCl or 0.1 M Bis-Tris–0.2 M Cl^-. The pH of the samples was adjusted with 2 M Tris by using a Beckman 3550 pH meter equipped with an Ingold microcombination electrode, and pH was expressed by direct reading of the pH meter. The Hb solutions were deoxygenated by repeated evacuation and flushing with N_2 gas under gentle shaking, and complete deoxygenation was achieved by addition of minimal amounts of sodium dithionite (Nakarai Chemicals, LTD; grade for amino acid analysis) solutions. The pH was determined before and after each run.

The NMR spectra were collected on Nicolet NT200 and NT360 FT spectrometers (200 and 360 MHz, respectively). Typical spectra required 2–6K scans, 4K data points, 20- or 40-kHz bandwidth, and a 7- or 10.5-μs 90° pulse, respectively. Notwithstanding field-dependent broadening effects (Vega & Fiat, 1976; Gueron, 1975; Johnson et al., 1977), the 360-MHz spectra displayed resolution comparable to the 200-MHz spectra. The H_2O resonance was reduced with a 60-ms presaturation pulse, and the signal to noise ratio improved with 50–100-Hz exponential apodization. Line-width measurements were made after subtracting apodization broadening. Presaturation did not affect the resonance intensity and pointed clearly to negligible saturation transfer which was further substantiated by the complementary Redfield experiments with and without H_2O saturation (Cutnell et al., 1981). All chemical shifts were referenced at 25 °C to the H_2O signal which in turn was calibrated against 4,4-dimethyl-4-silapentane-1-sulfonate (DSS). All spectra reported exhibited no detectable met-Hb, as indicated by the complete absence of characteristic peaks for the species.

Results

Figure 1 shows the low-field portion of the 200-MHz NMR spectra of Hb A, the α chain, α-*p*MB, the β chain, and β-*p*MB in the deoxy form at 25 °C. Deoxy-Hb A shows two exchangeable proton resonances at 63.6 and 76.7 ppm which have been assigned to the NH resonances of the proximal histidine in the α and β subunits, respectively (La Mar et al., 1977, 1980; Takahashi et al., 1980). The nonallosteric separated α and β chains show the NH resonances at 77.1 and 86.5 ppm, respectively. By attachment of *p*-(hydroxymercuri)benzoate (*p*MB) to the SH groups, the NMR spectrum of the α chain undergoes a drastic change in the region between 10 and 25 ppm, but the NH resonance remains essentially unshifted. However, the NH resonance of the β chain shifts upfield by 10 ppm. The differences in the NH line width for α and β chains probably arise because the former is primarily dimeric in solution while the latter form is tetrameric. The decreased line width of α-*p*MB relative to α chains is due to the former dissociating further into primarily a monomer (Antonini & Chiancone, 1977; Valdes & Ackers, 1978).

Deoxy-des-His(146β)-Hb has 5 times the oxygen affinity of a "T" structure and a reduced Bohr effect (Kilmartin et al., 1978). Its increased affinity has been attributed to a T-structure destabilization and therefore should be manifested

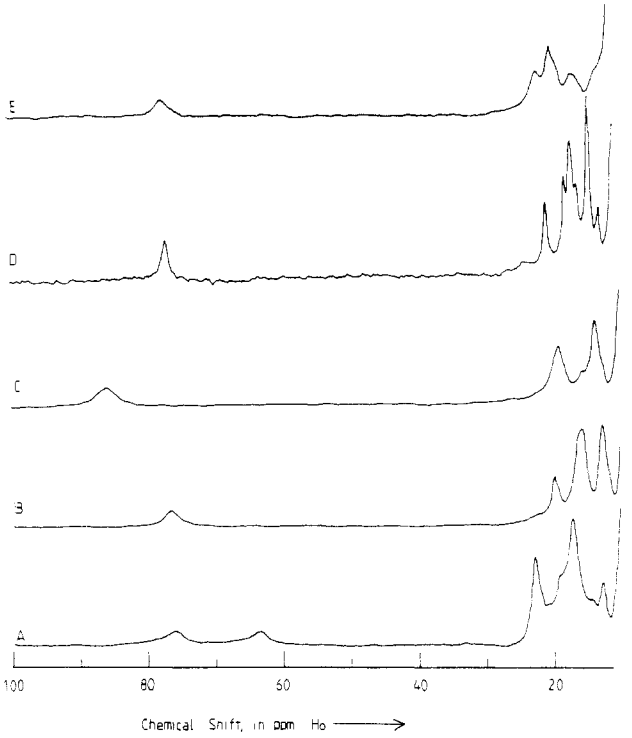

FIGURE 1: Downfield portions of the 200-MHz proton NMR spectra of Hb A (A), α chain (B), β chain (C), α-*p*MB (D), and β-*p*MB (E). All spectra were taken at 25 °C; all the samples were buffered with 0.1 M Bis-Tris and 0.2 M NaCl at pH 6.5 and referenced with respect to DSS.

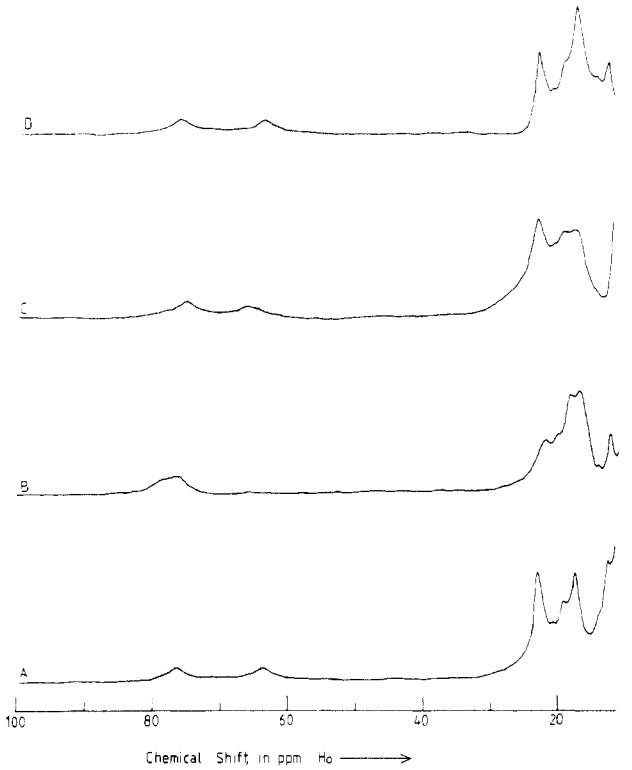

FIGURE 2: Downfield portions of the 200-MHz proton NMR spectra of des-His(146β)-Hb (A), NES-des-Arg(141α)-Hb (B), NES-des-Arg(141α)-Hb + IHP (C), and Hb A (D). All spectra were collected at 25 °C. The samples were buffered with 0.1 M Bis-Tris and 0.2 M NaCl at pH 6.5 and referenced with respect to DSS.

in a relaxing of any Fe–N_ϵ bonds in light of the strain model. However, the two proximal histidine $N_\delta H$ resonances (A in Figure 2) appear at 76.4 and 63.9 ppm at 25 °C, essentially at the same position as in deoxy-Hb A, 76.7 and 63.6 ppm.

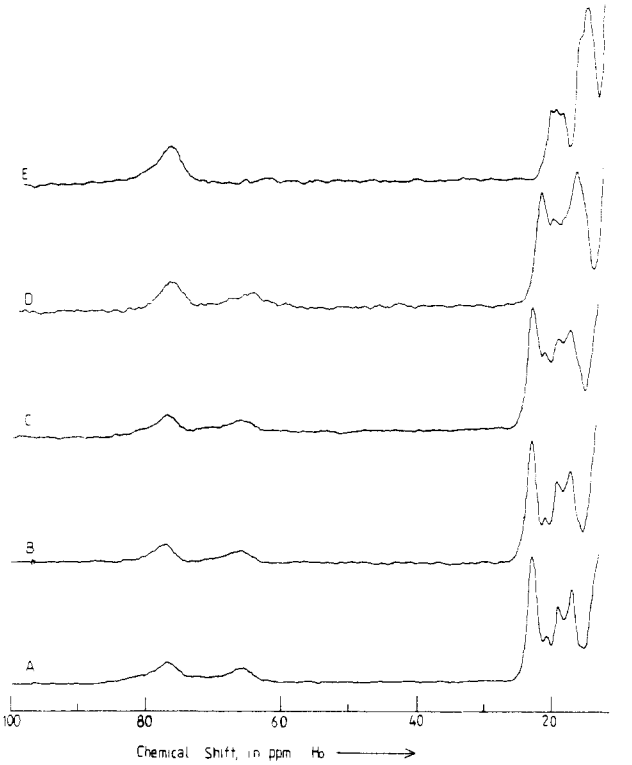

FIGURE 3: Downfield portions of the 360-MHz proton NMR spectra of des-His(146β)-Arg(141α)-Hb titrated with varying amounts (equiv) of IHP: 3 (A), 1 (B), 0.66 (C), 0.33 (D), and 0 (E). All spectra were taken at 25 °C. Samples were buffered at pH 6.5 with 0.1 M Bis-Tris and 0.2 M NaCl. Chemical shifts were referenced with respect to DSS.

Consequently, the removal of His-146β apparently does not influence the iron–histidine interaction. The NMR spectrum of the methyl region has been characterized by Fung & Ho (1975).

Stripped NES-des-Arg(141α)-Hb, which has a high O_2 affinity and no cooperativity (R structure), exhibits a composite exchangeable NH resonance indicative of peaks at 75.9 and 78.2 ppm (B in Figure 2). Hence, the two subunits exhibit very similar NH shifts in the R structure. Upon addition of IHP, it switches to the T structure (Table I). The NMR spectra reflect this in the upfield shift ($\sim$10 ppm) of the NH peak for one of the subunits (peaks at 65.5 and 74.8 ppm; see C in Figure 2). Thus, the NH shifts are now very similar to those of Hb A, and it appears reasonable to assume that it is the α-subunit NH peak which resonates upfield in both Hb A and NES-des-Arg(141α)-Hb. It has been noted (Ogawa et al., 1974) that the heme resonance region (10–25 ppm) of stripped NES-des-Arg(141α)-Hb is very different from that of Hb A, while in the presence of IHP it bears a strong resemblance to it.

Figure 3 shows the NMR spectra of deoxy-des-His-(146β)-des-Arg(141α)-Hb with different amounts of IHP. The stripped protein is in the R structure (Kilmartin et al., 1975), and the methyl region of the spectrum is very similar to that of deoxy-NES-des-Arg(141α)-Hb; the NH resonances of the α and β subunits are coincident at 77.8 ppm. With increasing amounts of IHP, the resonance at 77.8 ppm loses intensity, and a peak grows at 65.5 ppm. With 3 equiv of IHP per tetramer, the resonances at 65.5 and 76.4 ppm are almost equally intense and the methyl region of the spectrum becomes similar to that of deoxy-Hb A, which confirm that the molecule has been converted to the T structure.

The NMR spectra of deoxy-des-Arg(141α)-Hb at various pH values are illustrated in Figure 4. Consistent with its T

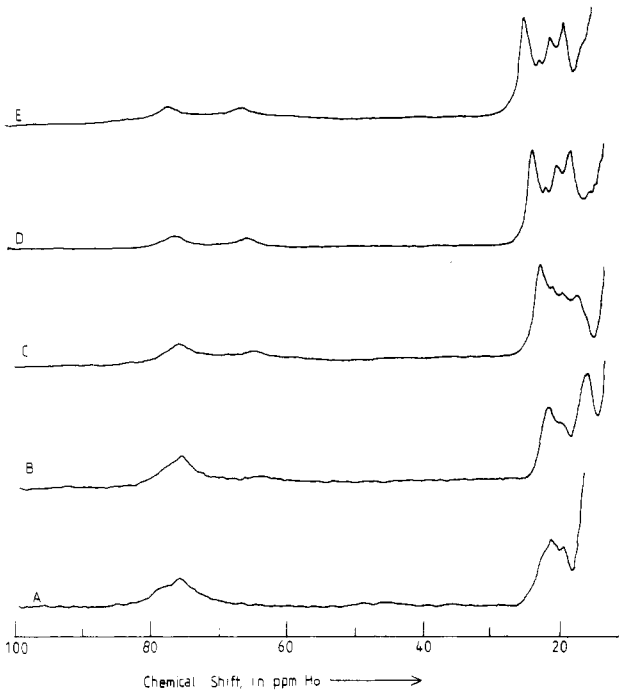

FIGURE 4: Downfield portions of the 360-MHz proton NMR spectra of des-Arg(141α)-Hb at various pHs: pH 9.0 (A), 8.4 (B), 7.6 (C), 6.9 (D), and 6.5 with IHP (E). All spectra were taken at 25 °C, and samples were buffered with 0.1 M Bis-Tris and 0.2 M NaCl. pH was adjusted with 2 M Tris. All chemical shifts were referenced with respect to DSS.

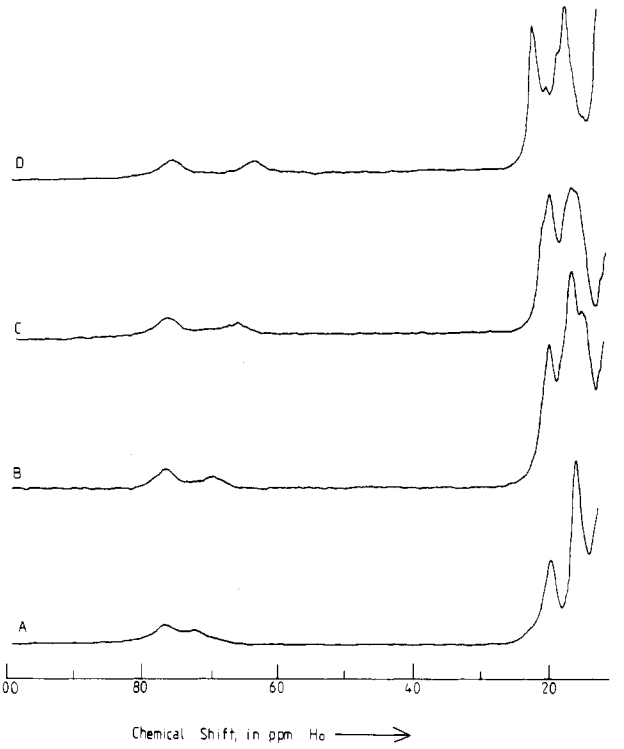

FIGURE 5: Downfield portions of the 360-MHz proton NMR spectra of Hb Kempsey at various pHs: pH 8.4 (A), 7.4 (B), 6.5 (C), and 6.5 with IHP (D). Spectra were obtained at 25 °C. Samples were buffered with 0.1 M Bis-Tris and 0.2 M NaCl. pH was adjusted with 2 M Tris. All chemical shifts were referenced to DSS.

structure at low pH (see Table I), two NH resonances are observed at 75.6 and 65.1 ppm (pH 6.9), and the methyl region of the spectrum is essentially identical with that of deoxy-Hb A. IHP imposes further constraint on the T structure but does not affect the positions of either the NH peaks or the methyl region. The peak at 75.6 ppm gains intensity with increasing pH, that at 65.1 ppm loses intensity, and the methyl region of the spectrum also undergoes drastic change, as described before (Perutz et al., 1974a; Fung & Ho, 1975). At pH 9.0, the NH peaks of the α and β subunits are coincident at approximately 78 ppm, in agreement with the conversion of the protein to the R structure at alkaline pH (Table I). It may be noted that as the pH is increased to 9 and the broad resonance at 75.6 ppm gains intensity at the expense of the broad peak at 65.1 ppm, a third, narrower exchangeable peak grows in at 76.3 ppm. It accounts for much less than a single proton and may suggest partial dissociation into dimers in order to account for the significantly decreased line width.

The NMR spectra of deoxy-Hb Kempsey are displayed in Figure 5. Stripped deoxy-Hb Kempsey is considered to be in the R structure (Table I), and the methyl region of the spectrum is somewhat similar to those of stripped des-His-(146β)-Arg(141α)-Hb (E in Figure 3) and NES-des-Arg-(141α)-Hb (B in Figure 2). However, in contrast to these R-structure deoxy-Hb's, deoxy-Hb Kempsey displays two NH resonances at 77.7 and 67.3 ppm at pH 6.5. The resonance at 77.7 ppm does not show any shift, but that at 67.3 ppm shifts further downfield with increasing pH. In the presence of IHP at pH 6.5, deoxy-Hb Kempsey exhibits two NH peaks at 77.1 and 64.6 ppm, which are very close to the positions in deoxy-Hb A, and the methyl region is the same as that of deoxy-Hb A, confirming that deoxy-Hb Kempsey is converted to the T structure.

Discussion

The variation in the position of the $N_\delta H$ hyperfine shift in a variety of monomeric O_2-binding deoxyhemoproteins has shown it to be a sensitive indicator of the microenvironment of the proximal histidine (La Mar, 1979; La Mar et al., 1981). The present study confirms similar sensitivity for the analogous tetrameric species. The two prominent proposed mechanisms for modulating this iron–imidazole bonding are strain or tension, leading to lengthened $Fe–N_\epsilon$ bonds, and changes in the imidazole σ covalency via variable hydrogen-bond donation of $N_\delta H$ to protein-acceptor residues (Peisach, 1975; Stein et al., 1980; Valentine et al., 1979). The strain can take several forms such as simple bond stretching or tilting of the imidazole (Baldwin & Chothia, 1979). In either case, the increased strain will lead to reduced $Fe–N_\epsilon$ covalency and hence smaller $N_\delta H$ contact shifts (La Mar, 1973). Decreased $N_\delta H$ contact shifts, however, can also arise from an increase in proton donation of the axial imidazole (G. N. La Mar and J. S. de Ropp, unpublished experiments). Thus, it may not be surprising that the $N_\delta H$ contact shift varies with the nature of the protein chain and that there exists as yet no simple correlation between simple structural differences and shift changes among different proteins. As found in the present study, the α and β chains exhibit $N_\delta H$ shifts of 77.1 and 86.5 ppm, the latter of which is similar to deoxymyoglobin; all have very similar O_2 affinities and resonance Raman $\nu(Fe–N_\epsilon)$'s. Upon incorporation of the α and β chains into the R-state tetramer, the α $N_\delta H$ shift decreases only slightly (<2 ppm) while the β $N_\delta H$ shift decreases by nearly 10 ppm. The optical spectrum of Hb A in the R state has been shown to be simply the sum of those of the separate α and β chains (Perutz et al., 1974a; Brunori et al., 1968; Sugita, 1975; Olson & Gibson, 1972), and $\nu(Fe–N_\epsilon)$'s are indistinguishable for the separated chains and within experimental error (2 cm^{-1}) of the values in the tetramer (Nagai & Kitagawa, 1980). Hence, the difference in $N_\delta H$ shifts may reflect a combination of changes in bond distance, angles, and $N_\delta H$ hydrogen bonding whose effects clearly manifest themselves differentially in the NMR spectral

parameters but for which the resulting proximal histidine environments lead to similar O_2 affinities. Further interpretation of these shift changes is not possible at this time.

Perturbation of Separated Chains. The possibility for correlating $N_\delta H$ contact shift changes with structural changes can be expected to be improved by focusing on $N_\delta H$ shift variations induced by a perturbation or conformational change for a given protein, where a single factor may dominaate. When the α-*p*MB adduct is formed, the $N_\delta H$ shift, as well as the O_2 affinity (Antonini et al., 1965), is unaltered, indicating a negligible change in the proximal histidine environment. For the β chain, on the other hand, forming β-*p*MB decreases the $N_\delta H$ contact shift by 10 ppm, and this change is accompanied by 6-fold decrease in O_2 affinity. Since the *p*MB binding site is much closer to the proximal histidine in the β chain than in the α chain (Perutz et al., 1974b), introduction of some strain in the Fe–N_ϵ bond upon *p*MB adduct formation in the former chain is suggested.

T ⇌ R Conformational Change in Tetramers. A more direct correlation between $N_\delta H$ shifts and protein structure is apparent for the tetrameric Hb's. For each of the three chemically modified Hb's which exhibits the R → T switch, the $N_\delta H$ shifts for the two subunits are the same in the R structure, with that of one of the subunits decreasing by 10–12 ppm upon converting to the T structure. We conclude that it is the α-subunit NH peak which exhibits the upfield shift in each Hb and propose that these proximal histidine imidazole shifts directly reflect a characteristic tertiary structure at the proximal histidine in each subunit. The 10–12-ppm decrease in the hyperfine shift in the α subunit on converting from R → T deoxy-Hb can be interpreted in terms of some strain imposed on the Fe–N_ϵ bond in the α subunit,[2] with the result that less σ spin density is transferred to the imidazole (La Mar, 1973). In contrast to the negligible β-subunit $N_\delta H$ shift change, the variation in the α-subunit $N_\delta H$ shift during the R → T transition is significant and similar to that due to converting the β chain to β-*p*MB. This suggests that any tension introduced in the R → T transition can account for an O_2 affinity decrease of less than a factor of 10.

Decreases in ν(Fe–N_ϵ) (Nagai & Kitagawa, 1980; Kincaid et al., 1979) for both subunits during the R → T transition have been interpreted as originating in strains which are larger in the α than in the β subunits; this strain was concluded to make only a very small contribution to the overall heme–heme interaction energy (Nagai & Kitagawa, 1980). Resonance Raman studies with models, however, have shown that variable $N_\delta H$ hydrogen bonding can modulate ν(Fe–N_ϵ) and have implied that the same mechanism is operating in the deoxy T ⇌ R transition (Stein et al., 1980). Recent consideration of both NMR and resonance Raman data has shown that variations in these parameters originating primarily from changes in N_δ–H···X hydrogen bonding require that ν(Fe–N_ϵ) and the $N_\delta H$ contact shift change in opposite directions.[3] On the other hand, simple changes in iron–imidazole tension should yield changes in the same direction for both the $N_\delta H$ contact shift and ν(Fe–N_ϵ) (G. N. La Mar and J. S. de Ropp, unpublished experiments). Hence, the present NMR data provide some confirmation for the steric origin (Nagai & Kitagawa, 1980) of the change in ν(Fe–N_ϵ) accompanying the T → R *deoxy* transition and argue against important contributions from changes in the N_δ–H···X hydrogen bonding (Stein et al., 1980) in the unligated states.

R Structure of Hb Kempsey. This protein exhibits high O_2 affinity and essentially no cooperativity; however, about two-fifths of the alkaline Bohr effect is retained. Addition of IHP to Hb Kempsey results in a lowering of oxygen affinity and an increase in cooperativity (Bunn et al., 1974). These results, as well as the absorption and CD spectra, indicate that stripped deoxy-Hb Kempsey is in the R structure that is converted to the T structure on addition of IHP (Perutz et al., 1974a). The presence of a Bohr effect in the R structure, however, dictates that there can exist more than a single structure for the stripped protein. In contrast to the other characterized R-structure deoxy-Hb's studied here, stripped deoxy-Hb Kempsey exhibits a pair of resolved NH resonances, one at 78 ppm and the other at 67 ppm. This pattern of NH resonances is in fact intermediate between that characteristic of R and T states. While the peak at 78 ppm is consistent with the β-subunit peak in other R-state Hb's, the other peak (probably the α subunit) suggests an environment similar to that found in the T structure. However, the heme resonance region 10–25 ppm is quite different from that of known T-state deoxy-Hb's (Lindstrom et al., 1973; Davis et al., 1971). As the pH is raised, the upfield NH peak moves downfield, approaching the pH-independent peak at 77 ppm at high pH. This pH-dependent shift may reflect the conformational change responsible for the Bohr effect within the R structure. The possibility of an equilibrium between R and a modified T structure in stripped Hb Kempsey cannot be discounted at this time.

The present result, however, is consistent with the resonance Raman study of deoxy-Hb Kempsey (Kincaid et al., 1979) which showed that ν(Fe–N_ϵ) in deoxy-Hb Kempsey is unaffected by the addition of IHP and is the same as in deoxy-Hb A within experimental error (2 cm^{-1}). Eisenberger et al. (1976, 1978) found no difference in Fe–N_ϵ bond distance between deoxy-Hb's A and Kempsey using EXAFS. The present study, as well as that of Kincaid et al. (1979), however, suggests that deoxy-Hb Kempsey may not be a good model for R-structure deoxy-Hb and that the comparison of the EXAFS spectra of deoxy-Hb A with those of R state modified deoxy-Hb's may be more informative and pertinent.

Rate of T ⇌ R Interconversion. The previous observation of averaged heme signals has yielded only a lower limit to the interconversion rate (Ogawa et al., 1974). Both des-Hb-(146β)-Arg(141α)-Hb (with 0.5 equiv of IHP) and des-Arg-(141α)-Hb (at pH 7.56), which contain comparable amounts of T and R structures, exhibit line broadening due to exchange. For the latter protein, we obtain an exchange rate of $\sim 8 \times 10^2$ s^{-1} at 25 °C.

[2] It is interesting to note that in rabbit Hb the two proximal NH peaks appear at 76 and 62 ppm (Budd, 1973) and suggest that the α subunit experiences a greater tension than the human α subunit. The α heme environment difference between rabbit and human has been demonstrated in ^{13}CO NMR studies (Moon & Richards, 1974).

[3] A high-spin ferrous hemoprotein exhibiting a pH-modulated conformational change where large changes in the $N_\delta H$ shift originate primarily from changes in hydrogen bonding rather than steric strain is reduced horseradish peroxidase, where an increase in the $N_\delta H$ contact shift (G. N. La Mar and J. S. de Ropp, unpublished experiments) is clearly accompanied by a decrease in ν(Fe–N_ϵ) (Teroaka & Kitagawa, 1980).

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