Articles

Proton Nuclear Overhauser Effect Investigation of the Heme Pockets in Ligated Hemoglobin: Conformational Differences between Oxy and Carbonmonoxy Forms†

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ABSTRACT: Proton nuclear Overhauser effect (NOE) measurements have been used extensively to investigate the detailed conformations of peptides, proteins, and nucleic acids in the solution state. However, much of the published work has dealt with molecules of molecular weight less than 15000. It is generally thought that specific NOEs cannot be observed in larger molecules (due to spin diffusion), so that NOE is of little use in conformational studies of such systems. By use of truncated-driven NOE with an irradiation time of 100 ms, specific NOEs are observed in a protein of the size of human normal adult hemoglobin (Hb A, 65000 daltons). This technique has permitted us to assign several proton resonances arising from heme groups and from amino acid residues situated in the vicinity of the ligand binding site (such as E7 histidine and E11 valine) of the α and β chains of Hb A. In addition, two-dimensional $^1H\{^1H\}$ *J*-correlated spectroscopy (COSY) experiments as well as theoretical ring-current calculations have confirmed the spectral assignments obtained by the one-dimensional NOE experiments. These new results not only have permitted us to map the heme pockets and to investigate the conformational differences in the heme pockets between oxy and carbonmonoxy forms of Hb A but also have demonstrated that the technique of truncated-driven NOE can be used to investigate the detailed conformations of selected regions in larger macromolecules in a way heretofore thought not to be feasible.

In order to correlate the structure–function relationship in proteins, it is essential to supplement the structural information derived from X-ray crystallography by other techniques. Nuclear magnetic resonance (NMR)[1] spectroscopy is one of these techniques. ^1H NMR spectroscopy has been used extensively in our laboratory to investigate the structure–function relationship in hemoglobin (Hb). The heme iron atoms are the ligand binding sites of Hb. Thus, an understanding of the detailed conformations or environments of the heme pockets of the α and β chains of Hb A can provide insights into the molecular basis of the cooperative ligation process. The amount of information derived from an NMR measurement is directly proportional to our ability to make spectral assignments to specific amino acid residues in the Hb molecule. For a recent review on NMR studies of Hb, see Ho & Russu (1981).

Proton nuclear Overhauser effect (NOE), a technique of double resonance, has been recognized as a very powerful method to investigate the conformations of small biological macromolecules in solution. This technique is based on energy transfer between spins, and for a given pair of protons, the rate of energy transfer is a sensitive function of the interproton distance (Noggle & Schirmer, 1971; Bothner-By, 1979). The NMR relaxation parameter most important in an NOE experiment is the cross-relaxation rate between the irradiated proton and the observed proton. For a rigid rotor, the cross-relaxation rate between spins i and j is given by the equation (Solomon, 1955)

$$\sigma_{ij} = \frac{1}{10}\frac{\gamma^4\hbar^2}{r_{ij}^6}\left[\tau_c - \frac{6\tau_c}{1 + (2\omega\tau_c)^2}\right] \quad (1)$$

where r_{ij} is the interproton distance, τ_c is the correlation time that modulates the dipolar interaction between spins i and j, γ is the gyromagnetic ratio of a proton, $\hbar$ is the Planck constant divided by 2π, and ω is the Larmor frequency. Due to the sixth-power dependence of σ_{ij} on r, the NOE is a sensitive function of the interproton distance. Equation 1 also shows that for cases in which $\omega\tau_c \gg 1$ (such as macromolecules with $\tau_c > 10^{-9}$ s and $\omega > 10^9$ s^{-1}), σ_{ij} is directly proportional to τ_c. In such cases, cross relaxation can lead to nonspecific NOEs (Kalk & Berendsen, 1976). Hence, most of the published results in applying NOE techniques to macromolecules are limited to molecular weights of less than 10^4.

This limitation of NOE experiments can be overcome by using transient and truncated-driven NOE techniques. In these experiments, only the initial buildup rate is observed, and thus, selective ^1H–^1H NOEs can be detected even in larger macromolecules. Wüthrich and co-workers have applied truncated-driven NOE to investigate cytochromes *c* ($M_r \sim 12500$) [for a recent review, see Keller & Wüthrich (1981)]. Other proteins with similar molecular weight have been studied extensively by NOE techniques [for example, see Poulsen et al.

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[1] Abbreviations: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; COSY, two-dimensional $^1H\{^1H\}$ *J*-correlated spectroscopy; Hb A, human normal adult hemoglobin; HbCO, (carbonmonoxy)hemoglobin; HbO_2, oxyhemoglobin; metHb, methemoglobin; Mb, myoglobin; Lb, soybean leghemoglobin; DSS, 4,4-dimethyl-4-silapentane-1-sulfonate.

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(1980) and Mabbutt & Wright (1983)]. Stoesz et al. (1979) applied transient NOE to investigate superoxide dismutase (M_r 31 200). Redfield and Huang carried out a preliminary transient NOE investigation of Hb A in both deoxy and oxy forms and concluded that specific NOEs can be observed (Huang, 1979). However, they have not published their results.

In the present paper, we report several specific NOEs that can be observed in a protein molecule of the size of Hb A (M_r 65 000) by means of the truncated-driven NOE technique. These specific NOEs have permitted us to assign several proton resonances of the porphyrin ring and the proton resonances of two important amino acid residues located next to the ligand binding site of Hb A, namely, E7 histidine and E11 valine. Some of these resonance assignments are confirmed by theoretical ring-current calculations and two-dimensional COSY experiments. Using these resonance assignments, we are able to make a direct comparison of the conformation and dynamics of the heme pockets between oxy and carbonmonoxy forms of Hb A in solution.

Experimental Procedures

Materials. Hb A was prepared from normal human blood obtained from the local blood bank following the procedure of Drabkin (1946). Isolated α and β chains of Hb A in the CO form were prepared as described by Kilmartin et al. (1973, 1975). The oxygenated samples were prepared by standard procedures used in our laboratory (Lindstrom & Ho, 1972), where CO was replaced by O_2 in the presence of a bright light to facilitate the removal of CO by photodissociation at 4 °C. The samples were exchanged with D_2O (Bio-Rad) by repeated dilution with D_2O and subsequent concentration through an Amicon UM-20E membrane. All Hb samples used for NMR studies were in 0.1 M sodium phosphate and were 2–5 mM in heme. The pH values of the Hb solutions are given as the direct readings obtained from a Radiometer pH meter (Model 26) equipped with a standard combination electrode. Visible spectra of the Hb samples were taken with an Aminco DW-2 spectrophotometer before and after the NMR measurements, and the presence of methemoglobin (metHb) was checked at 630 nm. Only NMR spectra of samples that did not contain metHb were used in our analysis.

Methods. High-resolution ^1H NMR spectra were obtained on a Bruker WH 300-MHz spectrometer using a 16-bit digitizer. Each standard ^1H NMR spectrum is the average of 100–200 scans. The truncated-driven NOE difference spectra were obtained with the pulse sequence suggested by Keller & Wüthrich (1981), namely, $([t_1(\omega^{\text{on res}})\text{–obsd}–T_{ac}–T_d] - [t_1(\omega^{\text{off res}})\text{–obsd}–T_{ac}–T_d])_n$. A low-power radio-frequency pulse was applied to a given resonance for a period of time, t_1, followed immediately by the observation pulse (obsd). The aquisition time (T_{ac}) was 1 s. A delay time (T_d) of 2 s was applied before the next presaturation to allow for the recovery of magnetization. Eight scans with $\omega^{\text{on res}}$ and eight scans with $\omega^{\text{off res}}$ were taken each time (where on res means on resonance and off res means off resonance). The entire sequence of pulses was repeated 200–400 times (n) to achieve a satisfactory signal-to-noise ratio. The decoupler power (spectrometer settings 35–30L, equivalent to approximately 50– to 150 μW) was set lower than that required for complete saturation of the resonances in the isolated β chains and Hb samples in order to assure the selectivity of the irradiation. Duration of irradiation was typically 100–200 ms in order to observe only the initial buildup rate of NOE.

The COSY experiments were performed as described by Wagner et al. (1981) and Wider et al. (1982) without the suppression of the water signal. The number of points sampled was 2048 over a sweep width of 4800 Hz in t_2 while 512 t_1 values were obtained. For each value of t_1, 192 free-induction decays were accumulated. Before Fourier transformation, the time domain data matrix was zero filled to 1024 × 1024 and multiplied in the t_1 and t_2 directions with a 0 phase-shifted sine bell function. The spectrum was further improved by symmetrization.

The ^1H chemical shifts are expressed as ppm with respect to the resonance of the methyl protons of a water-soluble standard, the sodium salt of 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), which is 4.73 ppm upfield from H_2O at 29 °C. The positive sign indicates that the observed resonance is downfield from that of DSS, and the negative sign indicates that the observed resonance is upfield from that of DSS.

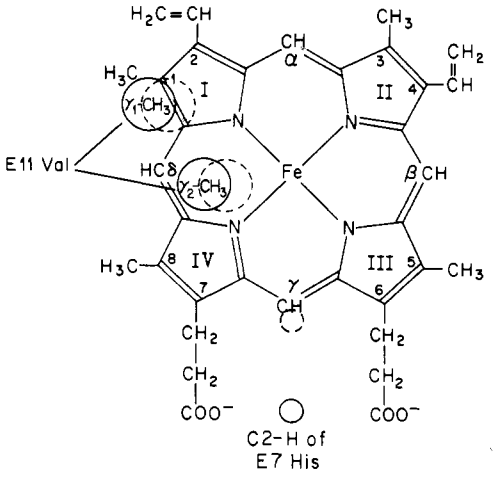

FIGURE 1: Structure of the heme group in hemoglobin and the positions of the γ_1- and γ_2-CH_3 groups of the E11 valyl residue and of the C2-H of the E7 histidyl residue in the CO and oxy form of α chains. The positions for the CO form (O) have been calculated from the X-ray coordinates for the α subunit of HbCO A (Baldwin, 1980), while the approximate positions for the oxy form (broken circles) have been calculated from our NMR data and theoretical ring-current calculations (see text).

Results

Spectral Assignments of the Heme Substituents and of the Distal Histidine and Distal Valine of the Heme Pockets of HbCO A by NOE. Figure 1 shows the heme structure and the positions of the two methyl groups of E11 valine (distal valine) and the position of the C2-H of E7 histidine (distal histidine) with respect to the porphyrin ring in both carbonmonoxy (O) and oxy (broken circle) forms. This figure is based on the X-ray coordinates of the α chain of HbCO A obtained from the Brookhaven National Laboratory Protein Data Bank and based on the structure of HbCO A determined by Baldwin (1980). The range of positions for the oxy form has been calculated from our NMR data and from ring-current calculations (see below).

The starting point of the present spectral assignments using the truncated-driven NOE is the resonance of the γ_2-methyl groups of E11 valyl residues in HbCO A. These resonances have previously been assigned by this laboratory by using mutant Hbs (Lindstrom et al., 1972).[2] Inspecting the structure and environment of the heme group as given in

[2] It should be pointed out that the previous assignment of the γ_1- and γ_2-CH_3 groups of E11 valyl residues in HbCO A by Lindstrom et al. (1972) has been reversed in our present work; namely, γ_2- and γ_1-CH_3 of E11 Val correspond to γ_1- and γ_2-CH_3 of E11 Val in our earlier paper. Also, in our present work, we reference the ^1H chemical shift to DSS whereas in the earlier paper HDO was used.

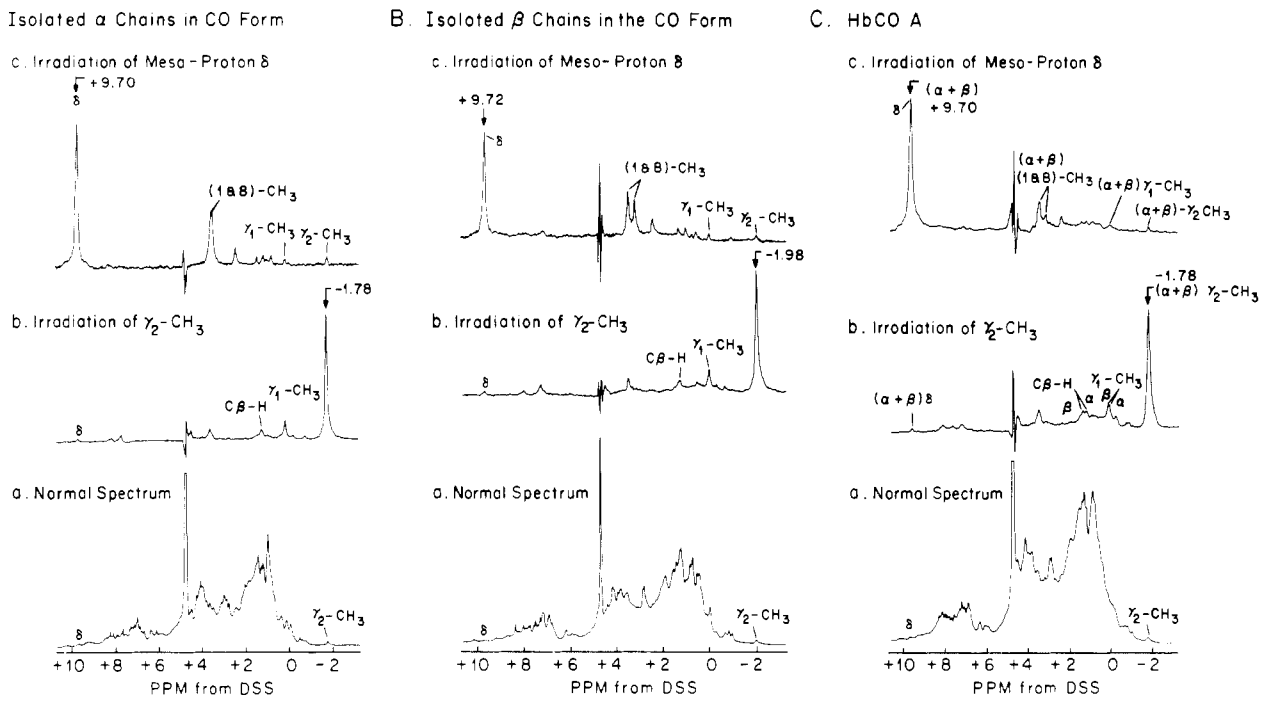

FIGURE 2: The 300-MHz ^1H NMR normal and NOE difference spectra of isolated α and β chains and of Hb A in the CO form in 0.1 M phosphate in D_2O at pH 7.3 and 29 °C: (A) isolated α chains; (B) isolated β chains; (C) HbCO A. The NOE difference spectra have been obtained with a preirradiation pulse of 100 ms at the positions indicated by the arrows. γ_1-CH_3, γ_2-CH_3, and Cβ-H are protons of E11 Val.

Figure 1, it is clear that the γ_2-CH_3 group of E11 Val is in close proximity to the meso proton δ and the heme methyl groups at positions 1 and 8 (1-CH_3 and 8-CH_3) and that the γ_1-CH_3 group of E11 Val is close to the methyl group at position 1, the meso proton δ, and the vinyl group at position 2. It has been suggested that the conformations of the heme pockets (as manifested by the ring current shifted proton resonances) of isolated α and β chains in the CO form are essentially identical with those in the intact HbCO A (Lindstrom et al., 1972). For both convenience and ease of spectral assignments, we start the discussion of our present NMR studies with the isolated α and β chains and then Hb A, all in the CO form. In the second part of this paper, we discuss the corresponding results for the isolated α and β chains and for Hb A in the oxy form.

(A) α Chains in the CO Form. (1) α62-E11 Valine. Figure 2Aa shows the 300-MHz ^1H NMR spectrum of isolated α chains of Hb A in the CO form. The upfield resonance at −1.78 ppm from DSS corresponds to the γ_2-CH_3 group of the α62-E11 valyl residue. The position of this resonance is shifted upfield by 2.71 ppm with respect to the diamagnetic position because of the ring-current effect produced by the π electrons of the porphyrin (Lindstrom et al., 1972). Figure 2Ab shows the truncated-driven NOE difference spectrum obtained upon preirradiation of the resonance at −1.78 ppm with a pulse of 100-ms duration. Only a few resonances appear in both aliphatic and aromatic resonance regions in the difference spectrum, indicating that the observed NOEs are extremely selective. The resonances that are present in Figure 2Ab must originate from the protons located very close to the γ_2-CH_3 of α62-E11 Val, and the resonance intensity reflects to a first approximation the proton–proton distance. The protons closest to the γ_2-CH_3 group of the E11 Val are the protons of the same amino acid residue, namely, γ_1-CH_3 and Cβ-H. From the relative intensities of these two resonances and from the COSY experiments (see below), we can assign the resonances at 0.13 and 1.21 ppm respectively to the γ_1-CH_3 and Cβ-H of E11 Val. The resonance at 9.70 ppm is tentatively assigned to the meso proton δ on the basis of Figure 1. This assignment is confirmed by the truncated-driven NOE difference spectrum of Figure 2Ac. On irradiating the resonance at 9.70 ppm, we observe very strong NOEs on two resonances at 3.50 and 3.57 ppm, which correspond to the ring-CH_3 groups 1 and 8 (1-CH_3 and 8-CH_3). Furthermore, as expected, we also observe NOEs on the γ_1- and γ_2-CH_3 groups of E11 Val.

Another method used to confirm our spectral assignment of E11 Val is the COSY technique. A typical COSY experiment can, in principle, provide a complete map of all $^1H\{^1H\}$ J connectivities, thus avoiding selectivity of irradiation in crowded spectral regions. The J connectivities between individual lines are manifested by pairs of cross peaks, which appear symmetrically with respect to the diagonal (Wagner et al., 1981; Wider et al., 1982). COSY spectra have been found to be very helpful in identifying the CH_3 groups of valyl and leucyl residues in proteins. Figure 3 shows the two-dimensional COSY contour plot in the region between 2 and −2.2 ppm with the connectivities for E11 Val. These results are in agreement with the one-dimensional NOE experiments discussed above. Thus, the present assignments for E11 Val can be considered definitive.

We have also used theoretical ring-current calculations to check our NMR assignment of the γ_1-CH_3 of E11 Val. Among the theories that have been developed to calculate the ring-current effects, we have chosen the method of Abraham et al. (1977), which has been shown to give the best agreement between the calculated ring-current shifts and the observed values in hemeproteins (Keller & Wüthrich, 1981). The coordinates for the hydrogen atoms of the γ_1-CH_3 of α62-E11 Val were generated from the X-ray coordinates of the corresponding carbon atoms (Baldwin, 1980). The distance of the center of the γ_1-CH_3 of α62-E11 Val from the heme plane was found to be 4.5 Å and from the heme center 6.1 Å. On the basis of these distances, the calculated ring-current shift for this methyl group was found to be 0.74 ppm. The measured ^1H chemical shift of this resonance is 0.13 ppm from DSS. Since the ^1H chemical shift of the CH_3 group of valyl

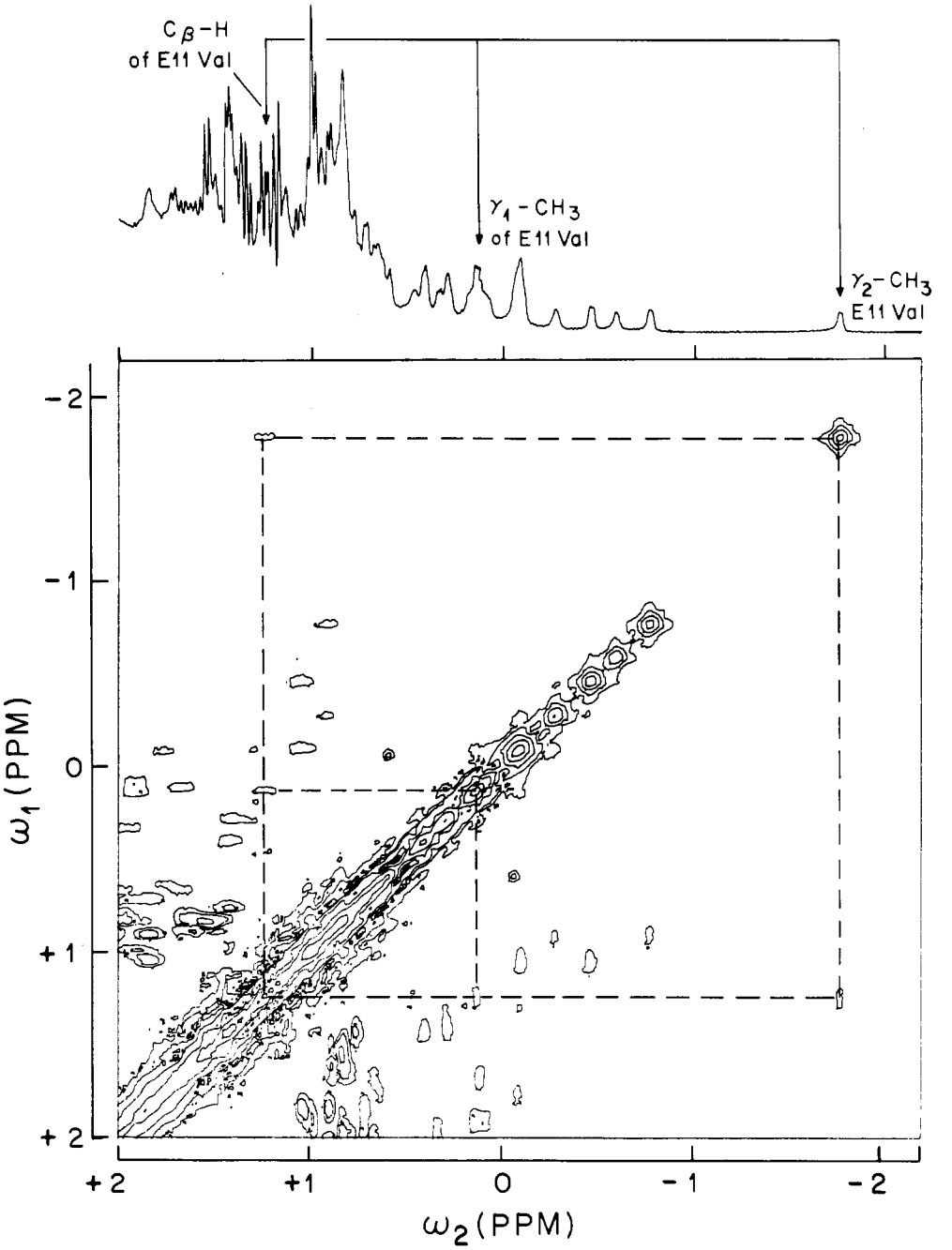

FIGURE 3: Spectral region from 2 to −2.2 ppm of the 300-MHz ^1H NMR spectrum (with resolution enhancement) and ^1H COSY spectrum of the isolated α chain of HbCO A in 0.1 M phosphate in D_2O at pH 5.3 and 29 °C.

residues in a random-coiled protein is 0.93 ppm from DSS (McDonald & Phillips, 1969), the corresponding ring-current shift on the γ_1-CH_3 of α62-E11 Val is 0.80 ppm. This value is in excellent agreement with the value predicted by the X-ray structure.

(*2*) *Heme Substituents.* In the low-field end of the spectrum of isolated α chains of Hb A in the CO form in D_2O, there are four singlets (for example, see Figure 5A) that must originate, in analogy with myoglobin (Mb) and soybean leghemoglobin (Lg), both in the CO form (Bradbury et al., 1982; Mabbutt & Wright, 1983), from the four meso protons of the porphyrin.

Figure 4A shows the truncated-driven NOE difference spectra with saturation of these four resonances. Peak intensities in these spectra are related to the distance from the irradiated proton and can lead to specific assignments of the heme substituents. By irradiation of the resonance at 9.27 ppm (Figure 4Ad), strong NOEs are observed in a number of resonances: 2.50, 5.87, and 7.74 ppm. The resonance at 2.50 ppm occurs in the resonance region for the CH_3 group of the heme (Keller & Wüthrich, 1981; Mabbutt & Wright, 1983). The two resonances at 7.74 and 5.87 ppm are *J* connected (based on COSY experiments, results not shown). They correspond respectively to the methine proton and to the cis and/or trans methylene protons of one of the two vinyl groups of the heme. With preirradiation of the resonance at 9.43 ppm (Figure 4Ac), similar patterns are observed with a strong NOE at 2.48 ppm (a CH_3 group of the heme). The two resonances at 8.24 and 5.87 ppm in Figure 4Ac are also *J* connected (results not shown), and they correspond to the other vinyl group of the heme. On the basis of the structure shown in Figure 1, we can conclude that the two low-field resonances at 9.43 and 9.27 ppm must arise from the mseo protons α and β.

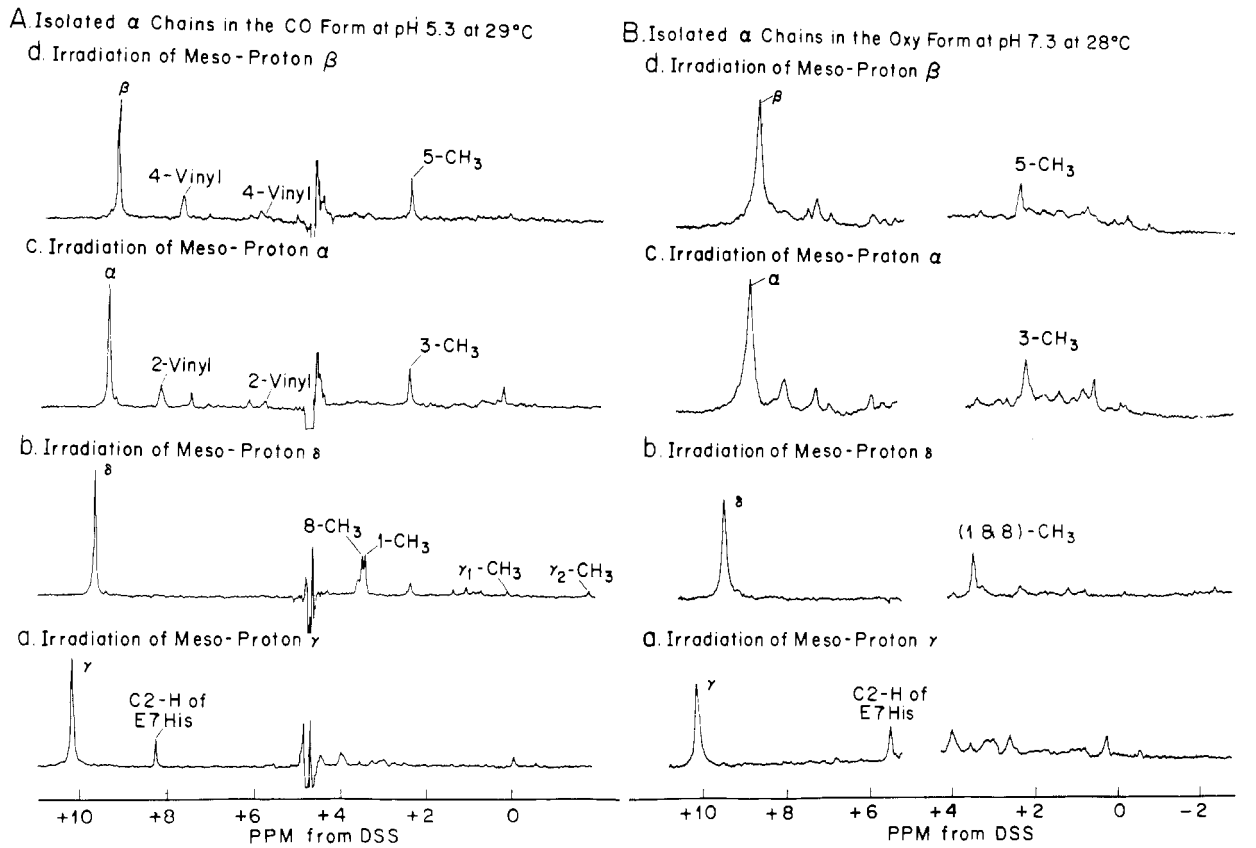

FIGURE 4: The 300-MHz ^1H NOE difference spectra of isolated α chains of Hb A in CO and oxy forms: (A) isolated α chains in the CO form in 0.1 M phosphate in D_2O at pH 5.3 and 29 °C; (B) isolated β chains in the oxy form in 0.1 M phosphate in D_2O at pH 7.3 and 28 °C. These NOE difference spectra have been obtained with a preirradiation pulse of 200 ms on each of the four meso proton resonances. γ_1-CH_3 and γ_2-CH_3 are protons of E11 Val.

Saturation of the meso proton δ at 9.70 ppm is shown in Figure 4Ab with the same results as those shown in Figure 2Ac. Irradiation of the resonance at 10.18 ppm gives no observable NOE on the four heme CH_3 groups (Figure 4Aa). This allows the assignment of this resonance to the meso proton γ. The next step is to assign the various meso protons and heme CH_3 groups to their specific positions in the porphyrin ring.

By irradiating the resonance at +5.87 ppm (observed in Figure 4Ac,d when the meso protons α and β were irradiated), NOEs on the two heme CH_3 groups at 3.50 and 2.48 ppm are observed (results not shown). The resonance at 5.87 ppm has been suggested as originating from the methylene protons of the two vinyl groups, while that at 3.50 ppm arises from either 1-CH_3 or 8-CH_3 and the resonance at 2.48 ppm arises from 3-CH_3 or 5-CH_3 of the heme. From Figure 1, we can conclude that the resonance at 3.50 ppm must arise from the 1-CH_3 group because this group is located close to the vinyl group at the 2-position and the resonance at 2.48 ppm from the 3-CH_3 group because this group is located on the same pyrrole as the vinyl group at the 4-position of the porphyrin ring. Table I summarizes the spectral assignments of heme substituents.

(3) α58-E7 Histidine. In the NOE difference spectrum with preirradiation of the resonance of meso proton γ (Figure 4Aa), a strong NOE is observed at 8.24 ppm. Since this resonance is a singlet, it should originate from either a C2- or C4-H of a histidyl residue or from a C2-H of a tryptophan residue. The α chain of Hb A has only one tryptophan, at position 14 (A12) of the amino acid sequence (Dayhoff, 1972), and the C2-H of this tryptophan has been recently assigned to a singlet at 7.23 ppm from NOE experiments in H_2O (results not shown). Hence, the resonance at 8.24 ppm must originate from a C2-H or a C4-H of a histidyl residue that must be in the immediate vicinity of the heme group. There are three such histidyl residues in the α chain of Hb A (Dayhoff, 1972), α87-F8 His (proximal histidine), α58-E7 His (distal histidine), and α45-CD3 His. On the basis of geometrical and theoretical ring-current considerations, one would expect that the resonances for the C2- and C4-H of the proximal histidyl residue would be shifted upfield far away from the aromatic proton resonance region. The separation between the C2- of α45-CD3 His and the meso proton γ is ~7.7 Å (Baldwin, 1980), a distance too large to produce a significant NOE. We have estimated the distance between the C2-H of the distal histidyl residue and the meso proton γ of the heme to be only ~2.9 Å (see Figure 1). On the basis of all these considerations, we conclude that the resonance at 8.24 ppm is due to the C2-H of the distal histidine (α58-E7).

We have also calculated the ring-current shift for the C2-H of α58-E7 His by the method of Abraham et al. (1977). The C2-H of α58-E7 His is located at 2.7 Å from the heme plane and at a distance of 6.3 Å from the center of the porphyrin ring. The calculated ring-current shift for this proton resonance is 0.3 ppm downfield. The chemical shift for the C2-H resonance of a deprotonated histidine in a random-coiled protein is 7.75 ppm from DSS (Bundi & Wüthrich, 1979). The measured chemical shift of the C2-H of α58-E7 His at pH 7 is 8.14 ppm (Figure 5B). Hence, the measured ring-current shift for the C2-H of α58-E7 His of HbCO A is 0.39 ppm downfield, in good agreement with the theoretical ring-current shift for this proton resonance. Preliminary NMR results suggest a similar chemical shift for the C2- of E7 His of isolated β chains of Hb A in the CO form (results not

Table I: Spectral Assignments of Resonances due to Heme Substituents and Distal Valyl Residues of Isolated α and β Chains and Hb A in Carbonmonoxy and Oxy Forms in the Presence of 0.1 M Phosphate in D_2O at pH 7.2 and 29 °C

resonance	protein chemical shift (ppm)					
	α CO	β CO	HbCO	α O_2	β O_2	HbO_2
meso proton α	9.39			8.96		
	9.43[a]					
meso proton β	9.27			8.78		
meso proton γ	10.22			10.04		
	10.18[a]					
meso proton δ	9.70	9.72	9.70 (α and β)	9.43	9.70	9.49 (α and β)
1- and 8-CH_3	3.50	3.53	3.52	3.47	3.44	3.46
	3.57	3.26	3.17 (α and β)	3.47	3.29	3.19 (α and β)
3-CH_3	2.48			2.28		
5-CH_3	2.53			2.48		
	2.50[a]					
vinyl-2 α-CH	8.24					
vinyl-2 β-CH (trans and/or cis)	5.87					
vinyl-4 α-CH	7.74					
vinyl-4 β-CH (trans and/or cis)	5.87					
E11 Val γ_2-CH_3	−1.78	−1.98	−1.78 (α and β)	−2.38	−2.57	−2.38 (α and β)
E11 Val γ_1-CH_3	0.13	0.10	0.13 (α)	−0.11	−0.26	−0.11 (α and β)
			0.19 (β)			
E11 Val β-CH	1.21	1.36	1.22 (α)	0.99	1.04	
			1.45 (β)			
E7 His-58 C2-H	8.14			5.41		
	8.24[a]					

[a] Values obtained at pH 5.3.

shown).

Figure 5A shows the ^1H NMR spectrum of isolated α chains of HbCO A in 0.1 M phosphate at pH 5.4 and 29 °C over the spectral range from 10.5 ppm to 5.5 ppm. By following the chemical shift of the C2-H of α58-E7 His as a function of pH in D_2O at 29 °C, we have found that the total change in the chemical shift of this resonance is only about 0.1 ppm over the pH range from 4.8 to 8.2, with an apparent pK of 6.0 ± 0.1 (Figure 5B).

(B) β Chains in the CO Form. Figure 2Ba shows the 300-MHz ^1H NMR spectrum of isolated β chains of Hb A in the CO form. The resonances in Figure 2Ba are not as sharp as those for the isolated α chains shown in Figure 2Aa. This is because isolated β chains exist in solution as tetramers whereas isolated α chains exist as either monomers or dimers (Benesch & Benesch, 1964; Valdes & Ackers, 1977). The upfield resonance at −1.98 ppm has been assigned to the γ_2-CH_3 of β67-E11 Val (Lindstrom et al., 1972). The NOE difference spectrum with preirradiation on this resonance for 100 ms is shown in Figure 2Bb. The observed spectral features are very similar to those shown in Figure 2Ab. Following the same procedures and considerations used for the α chain, we can assign the resonances at 0.10 and 1.36 ppm respectively to the γ_1-CH_3 and Cβ-H of β67-E11 Val. The present assignment of the resonance at 0.10 ppm to the γ_1-CH_3 of β67-E11 Val is different from that previously suggested by Lindstrom et al. (1972), who assigned the resonance at −1.1 ppm to γ_1-CH_3 of β67-E11 Val. We observe only a very small NOE on the resonance at −1.1 ppm upon irradiating the resonance at −1.98 ppm, suggesting that this resonance must come from protons that are relatively far from the γ_2-CH_3 of β67-E11 Val. We believe that our present assignment of the γ_1-CH_3 of β67-E11 Val is more reliable than the previous work, and it is also confirmed by other studies.

The resonance at 9.72 ppm must be the meso proton δ of the β chain. By preirradiating this resonance, we have observed strong NOEs on two resonances at 3.26 and 3.53 ppm that can be attributed to the ring-methyl groups 1 and 8 of the heme (Figure 2Bc).

(C) HbCO A. Figure 2Ca shows the 300-MHz ^1H NMR spectrum of HbCO A. The resonance at −1.78 ppm corresponds to the γ_2-CH_3 groups of E11 Val from both the α and β chains (Lindstrom et al., 1972). Figure 2Cb shows the truncated-driven NOE difference spectrum with preirradiation at −1.78 ppm for 100 ms. Comparing the difference spectra of isolated α and β chains with that of HbCO A, we can assign several resonances to specific groups in the α and β subunits. Figure 2Cc shows the NOE difference spectrum of HbCO A with preirradiation on the resonance at 9.70 ppm, which must be analogous to the meso protons δ of the hemes of the isolated α and β chains. In this difference spectrum, we can observe

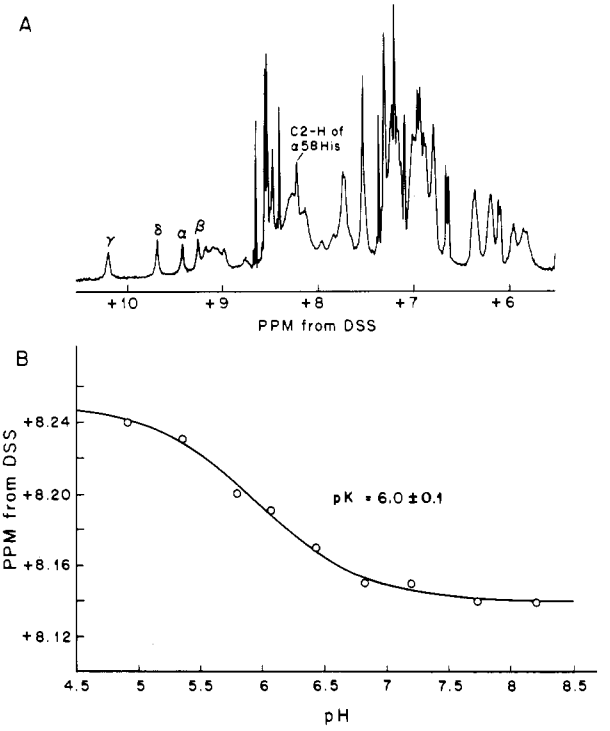

FIGURE 5: ^1H NMR investigation of isolated α chains of HbCO A: (A) 300-MHz ^1H NMR spectrum (with resolution enhancement) of the aromatic region of α chains of HbCO A in 0.1 M phosphate in D_2O at pH 5.3 and at 29 °C; (B) ^1H chemical shifts of the C2-H resonance of α58-E7 His (distal histidine) of isolated α chains of HbCO A as a function of pH in 0.1 M phosphate in D_2O and 29 °C.

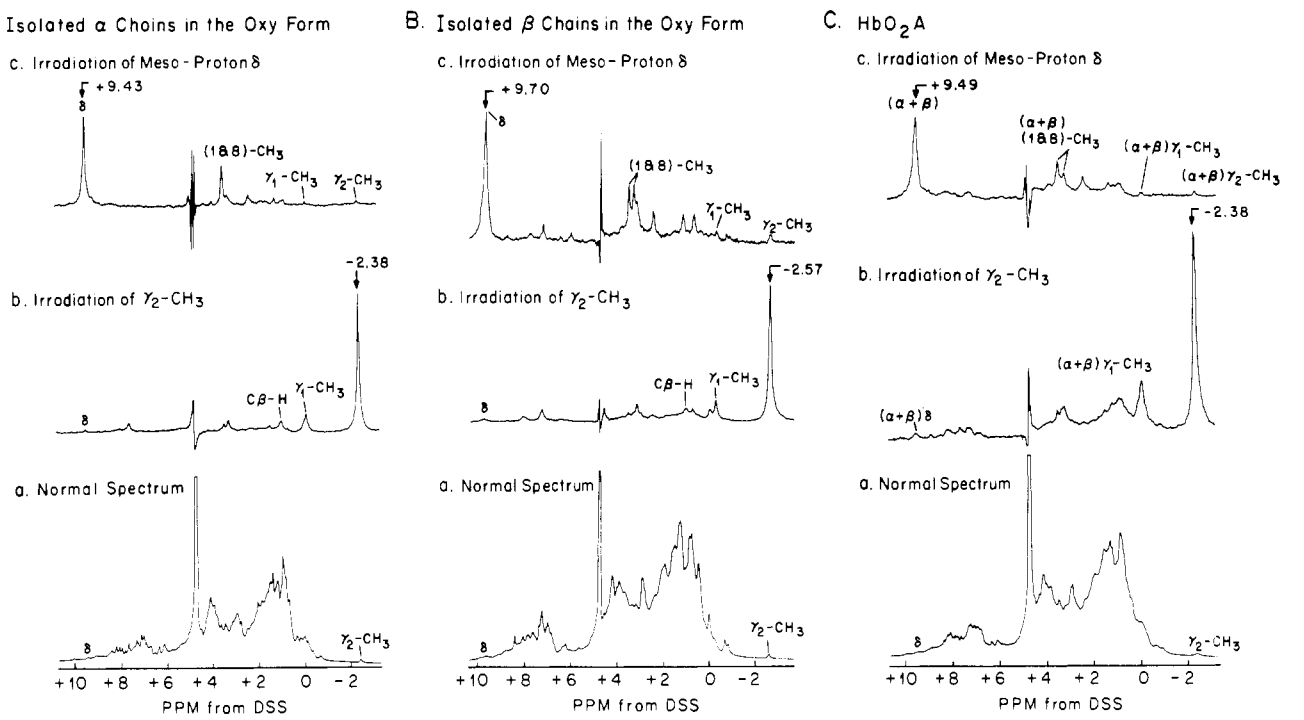

FIGURE 6: The 300-MHz ^1H NMR normal and NOE difference spectra of isolated α and β chains and of Hb A in the oxy form in 0.1 M phosphate in D_2O at pH 7.3 and 29 °C: (A) isolated α chains; (B) isolated β chains; (C) HbO_2 A. The NOE difference spectra have been obtained with a preirradiation pulse of 100 ms at the positions indicated by the arrows. γ_1-CH_3, γ_2-CH_3, and Cβ-H are protons of E11 Val.

the ring-methyl groups 1 and 8 of both α and β subunits of HbCO A and the γ_1- and γ_2-CH_3 groups of E11 Val (see Table I for spectral assignments).

Spectral Assignments of the Heme Substituents and of the Distal Histidine and Distal Valine of the Heme Pockets of HbO_2 A by NOE. We have carried out spectral assignments of the heme substituents and of the distal histidyl and valyl residues of the heme pockets of the isolated α and β chains and of Hb A, all in the oxy form, by using the same techniques that we have used in our spectral assignments for these three samples in the CO form. Due to the instability of HbO_2 A (tendency to form metHb and denaturation at low pH), we were not able to carry out our ^1H NMR investigation of isolated α and β chains and of Hb A in the oxy form as extensively as for their counterparts in the CO form.

(*A*) *α62-E11 Valine.* Figure 6Aa,Ba,Ca shows the 300-MHz ^1H NMR spectra of isolated α and β chains and of Hb A in the oxy form. The most upfield resonance has been assigned to the γ_2-CH_3 group of E11 Val (Lindstrom & Ho, 1973). Figure 6Ab,Bb,Cb shows the truncated-driven NOE difference spectra obtained upon preirradiation of this resonance. By using the same arguments as for the α and β chains in the CO forms, we can assign the resonances of the E11 valyl residue and the meso proton δ (see Table I). The assignment of the meso proton δ is confirmed by the truncated-driven NOE difference spectra of Figure 6Ac,Bc,Cc. It appears that the 1- and 8-CH_3 groups have the same chemical shift in the oxy form of the α chain but different chemical shifts in the oxy form of the β chain (see Table I for the spectral assignment).

(*B*) *Heme Substituents.* Figure 4B shows the truncated-driven NOE difference spectra obtained with irradiation of the low-field resonances that correspond to the meso protons of the heme analogous to those in the CO form (see Table I for the spectral assignments). Because we are not able to make specific assignments to the protons of the two vinyl groups, the tentative distinction between meso protons α and β is based on second-order NOE experiments carried out with irradiation

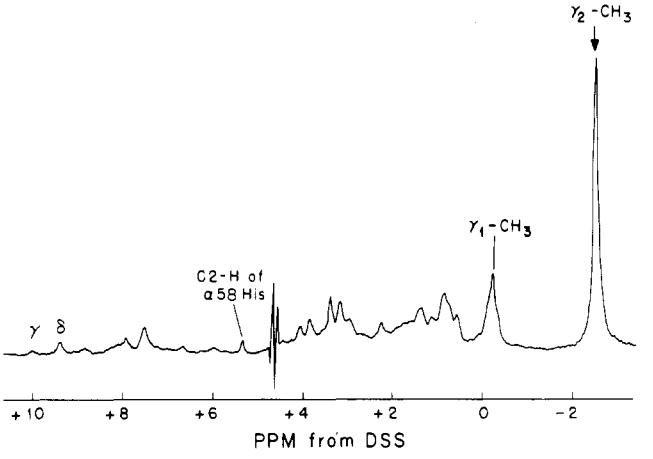

FIGURE 7: The 300-MHz ^1H NOE difference spectrum of isolated α chains of HbO_2 A in 0.1 M phosphate in D_2O at pH 7.3 and 28 °C. This spectrum has been obtained with a preirradiation pulse of 400 ms on the proton resonance of γ_2-CH_3 E11 Val.

of the meso-proton resonances (results not shown).

(*C*) *α58-E7 Histidine.* In the NOE difference spectrum with preirradiation of the resonance of meso proton γ (Figure 4Ba), a strong NOE is observed at 5.41 ppm. This resonance is a singlet as is its corresponding one in the CO form (Figure 4Aa). This resonance originates from the C2-H of α58-E7 His. The assignment of this resonance is assisted by the recent X-ray coordinates of HbO_2 A and by a truncated-driven NOE difference spectrum with a preirradiation of the resonance at −2.38 ppm (γ_2-CH_3 of E11 Val) (Figure 7). On the basis of the recent X-ray coordinates of HbO_2 A at 2.1-Å resolution (Shaanan, 1983), the distance between the C2-H of 85-E7 His and the meso proton γ is only 2.4 Å, and that between the C2-H of α58-E7 His and γ_2-CH_3 of α62-E11 Val is 4.3 Å. With irradiation of the γ_2-CH_3 of E11 Val (Figure 7) for 400 ms, NOEs are also observed at 10.04 and 5.41 ppm, resonances that correspond respectively to the meso proton γ and the C2-H of α58-E7 His. It should be mentioned that when a

Table II: Chemical Shift of the Meso Protons in Heme Proteins

resonance	α CO[a]	MbCO[b]	LbCO[b,c]	α O_2[a]	MbO_2[b]	LbO_2[b,c]
meso proton α	9.39	9.91	9.88	8.96	9.78	9.44
meso proton β	9.27	9.34	9.43	8.78	9.38	8.87
meso proton γ	10.22	10.14	10.15	10.04	9.89	10.02
meso proton δ	9.70	9.85	9.85	9.43	9.89	9.64

[a] Taken from this paper. [b] Taken from Bradbury et al. (1982). [c] Taken from Mabbutt & Wright (1983).

similar truncated-driven NOE experiment is carried out for the α chain in the CO form, we do not observe NOE on the C2-H of α58-E7 His (results not shown). This observation is consistent with the X-ray coordinates of HbCO A (Baldwin, 1980): the distance between the C2-H of α58-E7 His and the γ_2-CH_3 of α62-E11 Val is 6 Å. This distance is considerably larger than that in the oxy form. Thus, our present NMR results give an excellent indication that the conformation or location of the distal histidyl residue of the α chain is different between oxy and CO forms in the solution state.

Discussion

The significance of the present NMR results should be considered from the following three points of view: (i) selective NOEs in a protein of the size of Hb A; (ii) spectral assignments of the heme substituents and of the distal histidyl and valyl residues; (iii) conformational differences in the heme pockets between the oxy and carbonmonoxy forms of Hb A.

Selective NOEs in Hb A. The results presented in this paper have clearly demonstrated that selective NOEs can be observed in a protein of the size of Hb A when the truncated-driven NOE technique is used with a preirradiation pulse of 100 ms. In Hb A, the $\omega\tau_c$ term ($\sim$80) under our experimental conditions [using $\tau_c = 4.3 \times 10^{-8}$ (Johnson et al., 1977) and $\omega = 1.9 \times 10^9$ s^{-1}] is clearly much greater than 1. Hence, the proton relaxation is expected to be dominated by mutual cross relaxation. Indeed, in a typical steady-state NOE experiment on Hb, the magnetization is propagated through the entire molecule due to the adiabatic "flip-flop" transition, and no specific NOEs can be detected (results not shown). The specific NOEs observed suggest that the amino acid residues located on the distal side of the heme pocket are not so tightly packed. Hence, our NOE results suggest that there are only a few protons close to the group being irradiated and these protons are relatively isolated from the rest in the Hb molecule. In this "island" model as proposed by Bothner-By (1979), one would expect that cross relaxation between the protons of the island and the rest of the protons would be small compared to that between the protons of the island itself. Our suggestion that the residues on the distal side of the heme are less tightly packed and experienced a greater degree of internal motion is consistent with other, indirect experimental evidence. First, it has been known for a long time that alkyl isocyanides and other large compounds can bind to Hb A [Olson et al. (1982) and references cited therein]. More recently, a high-resolution ^1H NMR investigation has been carried out to investigate the effect of various alkyl groups of isocyanides on the resonances of E11 Val of Mb and of isolated α chains and β chains of Hb A and of Hb A (Mims et al., 1983). The fact that the heme cavity can accomodate such large compounds indicates the flexibility of the globin conformation on the distal side. Second, energy-minimization calculations carried out by Case & Karplus (1979) for Mb have suggested that E11 Val and E7 His are in broad potential-energy wells. Therefore, fluctuations of these residues would be expected.

Spectral Assignments of Heme Substituents and of Distal Histidyl and Valyl Residues. By means of truncated-driven NOE and COSY techniques as well as theoretical ring-current calculations, we have assigned the γ_1- and γ_2-CH_3 of E11 Val, the C2-H of E7 His, the great majority of the heme substituents of the isolated α chain (with the exception of the two propionic acid groups), and a large number of the heme substituents of the isolated β chain and of Hb A in both CO and oxy forms. As summarized in Table I, the chemical shifts of several heme substituents and of E11 Val protons in isolated α and β chains are essentially the same as their corresponding values in the tetrameric Hb A molecule (i.e., the differences in chemical shifts are less than 0.05 ppm between isolated α chains and the α chains of Hb A and are less than 0.2 ppm between isolated β chains and the β chains of Hb A). This suggests that there is very little rearrangement of the tertiary structures of the heme groups and their immediate environments of the α and β chains (on the distal side of the heme) when they assemble to form the tetrameric Hb A molecule in oxy and CO forms. An important consequence of this conclusion is that the structural and/or dynamic information derived from studies of isolated α and β chains in the CO or oxy form is relevant to their counterparts in HbCO A or HbO_2. As mentioned earlier, it is easier experimentally to obtain NMR data from isolated α and β chains (especially α chains) than from Hb A.

We also find it interesting to compare the heme environments and/or electronic structures of monomeric heme proteins with that of the isolated α chain of Hb A. Table II compares the chemical shifts of the four meso protons of the isolated α chains of Hb A to those in Mb and Lb in both CO and oxy forms. Table I shows that the resonances from the meso protons of the isolated α chains in the oxy form are shifted upfield with respect to those in the CO form; this shift is larger for the meso protons α and β and lesser for meso protons γ and δ. Similar results have also been observed in Lb (Mabbutt & Wright, 1983). On the other hand, there is a smaller difference in the chemical shifts of the meso protons of Mb in going from the CO to the oxy form (Bradbury et al., 1982). The observed differences in the chemical shifts of the meso protons of the isolated α chain and Lb may reflect a change in the conformation and/or electronic structure of the heme group in going from the CO to the oxy form. In isolated α chains, Mb, and Lb, the meso proton β is shifted the most upfield compared to the other three meso protons (Table II). A possible explanation of this upfield shift of the meso proton β could be due to its proximity to the phenylalanyl residue at CD1 in these three hemeproteins. Thus, the upfield shift of meso proton β could be ascribed, at least in part, to the ring-current effect produced by the aromatic ring of CD1 Phe. Similarly, the upfield shift for the meso proton α resonance in the α chain could be due to the ring-current shift induced by α98-G5 Phe, an amino acid residue situated on the proximal side of the heme close to the meso proton α. This suggestion is supported by the absence of a corresponding upfield shift for the meso proton α resonance in Mb, a molecule in which G5 Phe is replaced by a leucine. The chemical shift of the meso proton α of Lb suggests that the G5 Phe in Lb may be positioned either further away from the meso proton α or in

such a conformation that the ring-current effect on the meso proton α is small.

There is considerable controversy over the pK value of the distal histidyl residues of Hb A, isolated chains, Lb, and Mb. Published results on the pK value of this residue obtained by various physical–chemical techniques vary from 4.1 to 5.7 (Fuchsman & Appleby, 1979; Asher et al., 1981; Ikeda-Saito et al., 1977; Doster et al., 1982). In the present work, we have monitored the chemical shift of the C2-H of the distal histidyl residue (α58-E7 His) of the α chain in the CO form as a function of pH and have obtained an apparent pK value of 6.0 ± 0.1 for this residue (Figure 5B). The total change in the ^{1}H chemical shift of the C2-H of α58-E7 His over the pH range from 4.8 to 8.3 is only 0.1 ppm. This value is about 10% of the value normally observed for the C2-H of a histidyl residue in Hb A (Russu et al., 1982). Consequently, it is very likely that the ^{1}H NMR titration observed here for the C2-H of α58-E7 His of the α chain does not reflect the direct ionization property of this His residue and that it may result from a pH-induced conformational change in the heme pocket of the α chain. Further work is needed to elucidate the ionization property of the distal histidyl residue in heme proteins.

Conformational Differences in the Heme Pockets between Oxy and Carbonmonoxy Forms. Our present NOE experiments are focused on the detailed environments of the distal side of the heme pockets of isolated α and β chains and of Hb A in both oxy and CO forms. The atomic models of Hb A have clearly shown that the two distal residues, E7 His and E11 Val, are strategically located in the immediate vicinity of the ligand binding sites of both α and β chains (Perutz, 1970; Baldwin & Chothia, 1979; Baldwin, 1980; Shaanan, 1983; Fermi et al., 1984). It has been reported that there are considerable differences in the binding of CO and O_2 to Hb A [for example, see Moffat et al. (1979)]. Thus, the conformations of these two residues are thought to play an important role in regulating both the thermodynamics and dynamics of the ligation process of Hb A. The present assignments of the proton resonances of E11 Val in both isolated α and β chains of Hb A and of the C2-H of E7 His in isolated α chains of Hb A in CO and oxy forms allow us to use NMR spectroscopy to characterize the conformational differences of E7 His and E11 Val between CO and oxy forms in solution and to compare them to those derived from X-ray diffraction data in single crystals.

The C2-H of α58-E7 His of HbCO A is located at 2.7 Å from the heme plane and at a distance of 6.3 Å from the center of the heme plane (Baldwin, 1980). The C2-H of α58-E7 His of HbCO A experiences a ring-current shift of 0.39 ppm downfield. On the other hand, the observed ring-current shift for the C2-H of α58-E7 His in the oxy form of isolated α chains is 2.34 ppm upfield. This large upfield shift for the C2-H of the distal histidyl residue in the oxy form of the α chain may be explained by a movement of this histidyl residue toward the center of the heme. As a first approximation, let us assume that the distance between the C2-H of α58-E7 His and the heme plane in the oxy form remains the same as that in the CO form (namely, 2.7 Å). We can, then, use the ring-current equation developed by Abraham et al. (1977) to calculate an approximate position of the C2-H of α58-E7 His relative to the center of the heme plane for the oxy form. This calculated distance is ~4 Å, compared with the value of 6.3 Å found for the C2-H of α58-E7 His in the CO form as shown in Figure 1. The recent X-ray structure of HbO_2 A at 2.1-Å resolution (Shaanan, 1983) also indicates that the C2-H of α58-E7 His is closer to the center of the heme plane in HbO_2 A than in HbCO A (Baldwin, 1980), i.e., 4.7 Å vs. 6.3 Å. Thus, there is agreement between the NMR results obtained in the solution state and the X-ray diffraction results in the crystalline state.

The present NMR results (Figures 2 and 4) as well as those reported earlier by Lindstrom & Ho (1973) show that the γ_1- and γ_2-CH_3 of E11 Val of Hb A in the oxy form experience a larger ring-current shift than those in the CO form. On the basis of theoretical ring-current calculations and NOE results, we can conclude that in the oxy form the E11 Val is located closer to the normal of the heme with respect to the CO form. This movement of E11 Val toward the normal of the heme in the oxy form has to be less than 0.5 Å.

The present NMR results clearly show that there are conformational differences in the distal histidyl and valyl residues between the oxy and carbonmonoxy forms of Hb A in the solution state; namely, these two distal residues are being pushed further away from the heme plane in the CO form compared to the oxy form. This could be due to less steric hindrance shown by the bent ligand O_2 with respect to the linear ligand CO (Moffat et al., 1979). Our NMR data are consistent with those derived from X-ray crystallography. This implies that the conformations of the heme pockets of ligated Hb A are very comparable in solution and crystalline states. Additional research is urgently needed to correlate the functional differences between the oxy and carbonmonoxy forms to their differences in structure. NMR techniques offer an opportunity to gain some insights into this challenging problem.

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Registry No. HbCO A, 9072-24-6; HbO_2 A, 9062-91-3; heme, 14875-96-8; histidine, 71-00-1; valine, 72-18-4.

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NMR Relaxation of Protein and Water Protons in Diamagnetic Hemoglobin Solutions†

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ABSTRACT: We have measured T_1 and T_2 of protein and water protons in hemoglobin solutions using broad-line pulse techniques; selective excitation and detection methods enabled the intrinsic protein and water relaxation rates, as well as the spin-transfer rate between them, to be obtained at 5, 10, and 20 MHz. Water and protein T_1 data were also obtained at 100 and 200 MHz for hemoglobin in H_2O/D_2O mixtures by using commercial Fourier-transform instruments. The T_1 data conform to a simple model of two well-mixed spin systems with single intrinsic relaxation times and an average spin-transfer rate, with each phase recovering from a radio-frequency excitation with a biexponential time dependence. At low frequencies, protein T_1 and T_2 agree reasonably with a model of dipolar relaxation of an array of fixed protons tumbling in solution, explicitly calculating methyl and methylene relaxation and using a continuum approximation for the others. Differing values in H_2O and D_2O are mainly ascribed to solvent viscosity. For water-proton relaxation, T_1, T_2, and spin transfer were measured for H_2O and HDO, which enabled a separation of inter- and intramolecular contributions to relaxation. Despite such detail, few firm conclusions could be reached about hydration water. But it seems clear that few long-lived hydration sites are needed to explain T_1 and T_2, and the spin-transfer value mandates fewer than five sites with a lifetime longer than 10^{-8} s.

The hydration water of biological macromolecules has been studied with a variety of techniques, but its characterization remains vague and controversial. The time scales of the different methods vary widely, making comparisons difficult. Such questions have been thoroughly reviewed (Kuntz & Kauzmann, 1974). Packer (1977) has reviewed the role of water in more general heterogeneous systems, with emphasis on nuclear magnetic resonance (NMR)[1] work. NMR relaxation measurements provide a unique probe of the microscopic nature of hydration water, because the interactions responsible for relaxation are very short range. Studies of water relaxation

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[1] Abbreviations: NMR, nuclear magnetic resonance; FT, Fourier transform; MHz, megahertz; BPTI, basic pancreatic trypsin inhibitor; Hb, hemoglobin; M_r, molecular weight; NOE, nuclear Overhauser effect; CPMG, Carr–Purcell–Meiboom–Gill; FID, free induction decay; A/D, analog to digital; S/N, signal to noise; ZPC, zero-point crossing; WS, water suppression; rf, radio frequency; INV, inversion; TMV, tobacco mosaic virus; FET, field effect transistor.

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