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Proton Nuclear Magnetic Resonance Studies of Hemoglobins M Boston (α58E7 His → Tyr) and M Milwaukee (β67E11 Val → Glu): Spectral Assignments of Hyperfine-Shifted Proton Resonances and of Proximal Histidine (E7) NH Resonances to the α and β Chains of Normal Human Adult Hemoglobin†

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ABSTRACT: High-resolution proton nuclear magnetic resonance spectroscopy at 250 MHz has been used to investigate both the ferrous and ferric hyperfine-shifted resonances of naturally occurring valency hybrid hemoglobins, Hb M Boston (α58E7 His → Tyr) and Hb M Milwaukee (β67E11 Val → Glu), in order to make a definite assignment of these resonances to the α and β chains of normal human adult hemoglobin. In Hb M Boston ($\alpha_2^+\beta_2$), the iron atoms of the α chains are in the ferric state, while those in the β chains are in the ferrous state. On the other hand, the iron atoms of the β chains in Hb M Milwaukee ($\alpha_2\beta_2^+$) are in the ferric state, while those in the α chains are in the ferrous state. Due to the difference in the number of unpaired electrons, ferric and ferrous hyperfine-shifted proton resonances occur in different regions of the spectrum. The spectrum derived from the arithmetical sum of the ferrous hyperfine-shifted proton resonances of deoxy-Hb M Boston and Hb M Milwaukee in D_2O, which appear between 6 and 18 ppm downfield from residual HDO, is found to be essentially identical with that of normal human adult deoxyhemoglobin. Thus, the assignment of the ferrous hyperfine-shifted proton resonances in this spectral region to the α and β chains of normal human adult deoxyhemoglobin has been accomplished. By means of the spectral comparison of these three hemoglobins in the deoxy form, an assignment of the proximal histidine (E7) exchangeable NH resonances to the α and β chains of normal human adult deoxyhemoglobin has also been established. These resonances are found respectively at 58.5 and 71.0 ppm downfield from H_2O. Because of the similarity of the chemical shifts of ferrous hyperfine-shifted proton resonances, it is concluded that the ferrous heme environments of unligated Hb M Boston and Hb M Milwaukee are similar to those of normal human adult deoxyhemoglobin. On the other hand, the ferric hyperfine-shifted proton resonances of Hb M Boston and Hb M Milwaukee are found to be different from those of normal human adult methemoglobin. The results suggest that the detailed environments of the ferric hemes in these hemoglobins are different.

The proton nuclear magnetic resonance (NMR)[1] spectra of hemoglobin (Hb) in the deoxy and met forms show several characteristic resonances which are remote from the majority of diamagnetic resonances. They arise from the hyperfine interactions between the unpaired electrons of the iron atoms and the proton groups on the hemes and/or the nearby amino acid residues of the α and β chains. There are two types of hyperfine-shifted resonances, Fermi contact and pseudocontact shifted resonances. [For a recent discussion on hyperfine-shifted proton resonances in heme proteins, see Ho et al. (1978) and the references cited therein.] These resonances are sensitive to the conformation of the heme groups and to the electronic spin state of the iron atoms (Kurland et al., 1968; Lindstrom et al., 1972; Ho et al., 1973, 1978; Fung et al., 1976, 1977; Viggiano & Ho, 1979; Viggiano et al., 1979). A number

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[1] Abbreviations used: NMR, nuclear magnetic resonance; Hb, hemoglobin; Hb A, normal human adult hemoglobin; met-Hb, methemoglobin; Bis-Tris, [bis(2-hydroxyethyl)imino]tris(hydroxymethyl)-methane; pH*, the pH value in D_2O taken directly from the pH meter reading.

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of investigators have made use of these resonances for conformational studies of hemoglobins and for studies of ligand binding to hemoglobins (Davis et al., 1969, 1971; Lindstrom et al., 1971; Ho & Lindstrom, 1972; Ogawa & Shulman, 1972; Lindstrom & Ho, 1972; Breen et al., 1974; Huang & Redfield, 1976; Viggiano & Ho, 1979; Viggiano et al., 1979). However, a complete spectral assignment of hyperfine-shifted resonances can be classified into three levels. The first level is to assign the resonances to either the α or β subunits of Hb A. Ligand binding to the α or β chains of Hb A as well as conformational changes in a given subunit upon binding could be monitored by these resonances. A detailed understanding of structural change at the atomic level can be obtained from the second level of assignment, namely, the assignment of each resonance to an individual atom or group in the porphyrin ring or to the nearby amino acid residues in a given subunit. The third level of spectral assignment is to find out which resonances are due to the Fermi contact interaction and which are due to the pseudocontact interaction. The last spectral assignment would permit us to gain a deeper insight not only into the electronic structure of hemoglobin but also into the nature of conformational changes as manifested by changes of the hyperfine-shifted proton resonances as a function of experimental conditions. A complete spectral assignment at the last two levels is highly desirable but not yet feasible at the present stage. This study is undertaken, in part, to make a first-level assignment of ferrous hyperfine-shifted resonances by means of a spectral comparison among appropriate valency hybrid hemoglobins and Hb A.

A group of mutant hemoglobins known as the M type is characterized by the presence of ferric atoms in either the α or β chains (Pisciotta et al., 1959). These naturally occurring valency hybrid hemoglobins contain both ferrous and ferric atoms, and only those subunits with ferrous atoms can combine with ligands under physiological conditions. Members of this group are Hb M Boston (α58E7 His $\rightarrow$ Tyr) and Hb M Milwaukee (β67E11 Val $\rightarrow$ Glu), samples of which are used in this study. According to the difference Fourier analyses of X-ray diffraction data, the tertiary structures of the normal ferrous heme pockets of these two mutants are essentially identical with the corresponding ones in Hb A, whereas there are considerable differences in the ferric heme environments of Hb M Milwaukee, Hb M Boston, and methemoglobin A (met-Hb A) (Perutz et al., 1972; Pulsinelli et al., 1973). It appears that a comparative ^{1}H NMR study of the two mutants would allow us to make a complete first-level spectral assignment of the hyperfine-shifted resonances due to the α and β chains of deoxy-Hb A and to gain insight into the detailed environments of the heme groups in these three hemoglobins.

In this communication, we report a 250-MHz ^{1}H NMR investigation of both ferrous and ferric hyperfine-shifted resonances, as well as the proximal histidine NH resonances of Hb A, Hb M Boston, and Hb M Milwaukee in the deoxy state. Some preliminary studies on Hb M Milwaukee have been undertaken previously by this laboratory (Lindstrom et al., 1972; Fung et al., 1976, 1977).

Experimental Section

Materials. Hb A was isolated from fresh whole blood samples obtained from the local blood bank according to the standard procedures of this laboratory (Lindstrom & Ho, 1972). Met-Hb A was obtained by oxidizing carbonmonoxyhemoglobin A with a fourfold molar excess of potassium ferricyanide per Hb tetramer. The met-Hb A solution was then passed through a column of Sephadex G-25 to remove ferri- and ferrocyanide. Hb M Milwaukee was purified according to Fung et al. (1977). Whole blood containing Hb M Boston was shipped to Pittsburgh by air freight from Osaka, Japan, in an insulated package containing chipped ice.[2] The isolation and purification procedures for Hb M Boston are the same as those used for Hb M Milwaukee (Fung et al., 1977). Sample preparation for NMR measurements followed the procedures of Lindstrom & Ho (1972). The concentrations of Hb M Milwaukee and Hb M Boston were determined spectrophotometrically by using an Aminco DW-2 spectrophotometer at 540 nm and using a millimolar extinction coefficient (ϵ) of 13.4 for the ferrous heme (Antonini & Brunori, 1971). For NMR measurements, appropriate amounts of a 1 M stock solution of [bis(2-hydroxyethyl)imino]tris(hydroxymethyl)methane (Bis-Tris, obtained from Aldrich) were added to the Hb preparations, giving 0.1 M solutions at pH* 6.6. pH* values were measured directly on a Beckman pH meter (Model 3500) equipped with a combination electrode (Model 39030) without correction for the deuterium isotope effect on the glass electrode.

Methods. ^{1}H NMR spectra were obtained on the MPC-HF 250-MHz spectrometer interfaced with a Sigma 5 computer. All the spectra shown were accumulated by the digital sweep mode with 6K data points and 9-bit word length and analyzed by the NMR correlation technique (Dadok & Sprecher, 1974). The ferrous hyperfine-shifted resonances were accumulated for $\sim$1000 scans at a sweep rate of 14 kHz/s from 1400 to 8458 Hz downfield from residual HDO with 20 kHz as the modulation frequency. A 10-Hz line broadening was applied to the spectral analysis so as to enhance the signal to noise ratio. For the ferric hyperfine-shifted resonances and proximal histidine NH resonances, the spectrometer was operated with 100-kHz modulation. The ferric hyperfine-shifted resonances produced reasonably good signal to noise ratios after 5000–10 000 scans of signal averaging with a 38 kHz/s sweep rate and 10 000–39 000-Hz sweep range. The spectra of the proximal histidine NH resonances were accumulated for $\sim$20 000 scans under the same spectrometer settings, but the receiver gain was attenuated by 6–9 dB. The base-line ramp was corrected by adding up an appropriate number of linear signals which were generated by a sawtooth oscillator for the analogue scan.

Proton chemical shifts are referenced to the residual water proton signal, which is 4.83 ppm downfield from 2,2-dimethyl-2-silapentane-5-sulfonate at 27 °C, the ambient probe temperature. Chemical shifts downfield from residual HDO or H_2O are presently assigned positive values[3] with an accuracy of $\pm$0.1 ppm for ferrous hyperfine-shifted resonances and $\pm$0.5 ppm for ferric hyperfine-shifted resonances and for proximal histidine NH resonances.

Results

Ferrous Hyperfine-Shifted Proton Resonances. Figure 1 shows the 250-MHz ^{1}H NMR spectra of 10% solutions of deoxyhemoglobins A, M Boston, and M Milwaukee in 0.1 M Bis-Tris buffer in D_2O at pH* 6.6 and 27 °C over the spectral region from 6 to 24 ppm downfield from residual HDO. Hb

[2] This blood was found in a Japanese family living in Osaka, Japan, and was, at first, designated as Hb M Osaka by Hayashi et al. (1964). The primary structure of Hb M Osaka was later found to be identical with that of Hb M Boston (Shimizu et al., 1965).

[3] In compliance with the recommendation for the presentation of NMR data for publication in chemical journals proposed by the International Union of Pure and Applied Chemistry (No. 38, August 1974), the chemical shift scale is defined as positive in the low-field (or high-frequency) direction. This change in the sign of the chemical shift scale should be noted when referring to earlier publications reported by this laboratory.

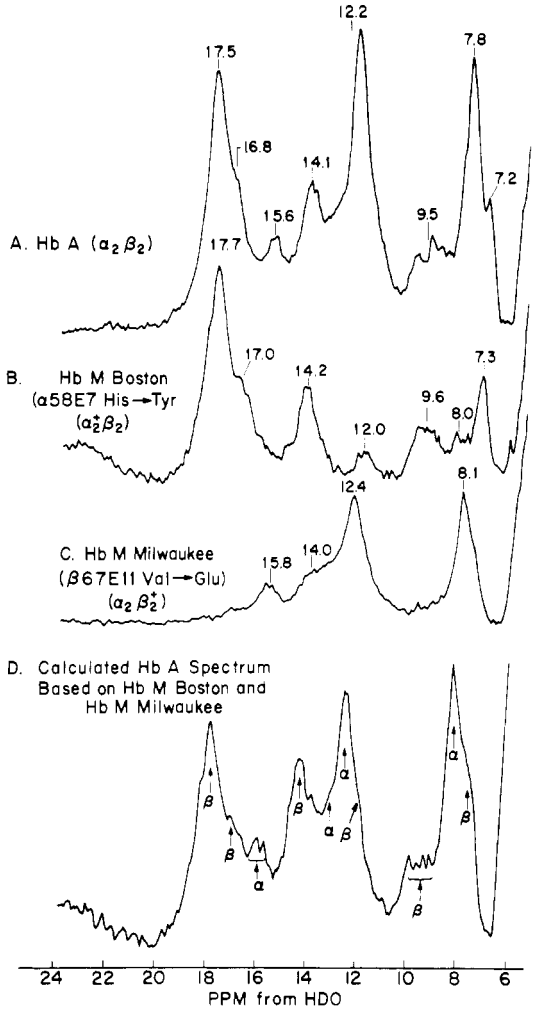

FIGURE 1: 250-MHz ferrous hyperfine-shifted proton resonance spectra of deoxyhemoglobins in 0.1 M Bis-Tris in D_2O at pH* 6.6 and 27 °C: (A) Hb A; (B) Hb M Boston; (C) Hb M Milwaukee; (D) calculated Hb A spectrum obtained by the spectral sum of Hb M Boston and Hb M Milwaukee.

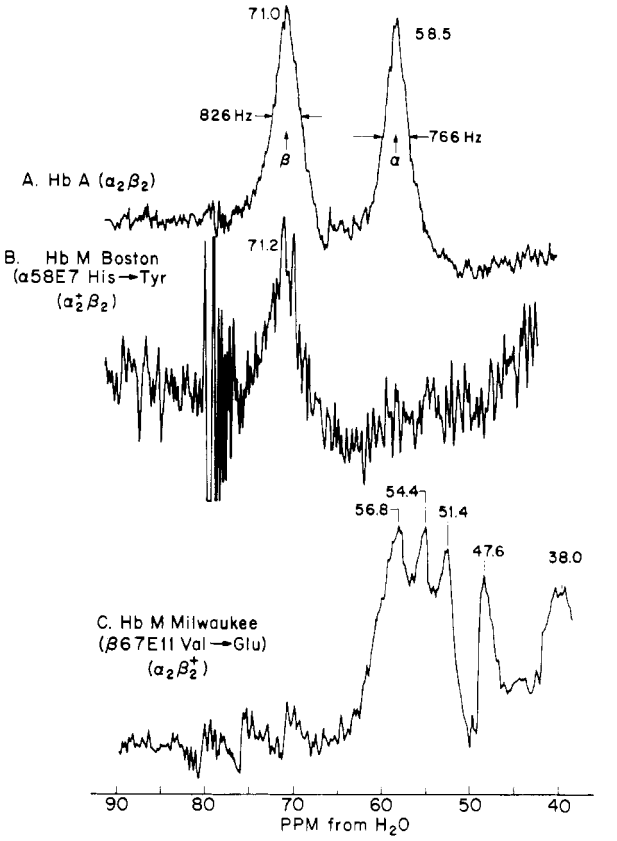

FIGURE 2: 250-MHz ^1H NMR spectra of deoxyhemoglobins in 0.1 M Bis-Tris buffer in H_2O at pH 6.7 and 27 °C over the spectral region from 40 to 90 ppm from H_2O: (A) Hb A; (B) Hb M Boston; (C) Hb M Milwaukee. The beat appearing at ~80 ppm in spectrum B is generated by the 20-kHz time-sharing operation of the spectrometer and is not a signal.

M Boston ($\alpha_2^+\beta_2$) has abnormal ferric α chains, while Hb M Milwaukee $(\alpha_2\beta_2)^+$ has abnormal ferric β chains. The resonances of the mutants shown in parts B and C of Figure 1 are derived from the ferrous β and α chains, respectively, except for a broad resonance appearing at ~24 ppm in the Hb M Boston spectrum. A comparison of the spectra of Hb M Boston and Hb M Milwaukee allows us to assign individual ferrous hyperfine-shifted proton resonances to the α and β chains of deoxy-Hb A. The ^1H NMR spectrum of deoxy-Hb A has three major resonances at 17.5, 12.2, and 8.0 ppm and several less intense or broader resonances. The three main peaks have been assigned to the protons from the β, α, and α chains, respectively (Davis et al., 1971; Lindstrom et al., 1972), and have been used for the study of ligand binding to the α and β chains of hemoglobins (Lindstrom et al., 1971; Ho et al., 1973; Lindstrom & Ho, 1972; Johnson & Ho, 1974; Wiechelman et al., 1974; Viggiano & Ho, 1979; Viggiano et al., 1979). It should be noted that the resonance at ~17 ppm consists of at least two components, both of which are derived from the β chains. A shoulder at 16.8 ppm and the sharper resonance at 17.5 ppm are not always clearly resolvable, such as at high pH and in the absence of organic phosphate (Viggiano et al., 1979; P. E. Johner and C. Ho, unpublished results). For the 12.2-ppm resonance, the β-chain contributions to the α-chain resonances are estimated to be less than 10%, and ~15% for the 8.0-ppm resonance. The calculated spectrum (Figure 1D) derived from Hb M Boston and Hb M Milwaukee agrees nicely with the experimental spectrum of deoxy-Hb A (Figure 1A). The spectra shown in Figures 1B and 1C, therefore, provide a direct measure of the intensities, line widths, and chemical shifts for individual contributions of each resonance to the spectrum of deoxy-Hb A. Thus, all of the observable resonances appearing in this region have now been completely assigned to the α and β chains of deoxy-Hb A.

Proximal Histidine NH Resonances. For deoxy-Hb A in H_2O, two resonances occur at 58.5 and 71.0 ppm downfield from H_2O (Figure 2A). The resonances disappear in the presence of D_2O (Figure 5D). La Mar et al. (1977) suggested that these two resonances arise from the exchangeable NH protons of the proximal histidines (E7). Our results have confirmed their suggestion, and we have further assigned these resonances to the α and β chains, respectively, as shown in Figure 2A. The basis of our assignment is a comparison of the spectra of Hb A, Hb M Milwaukee, and Hb M Boston in the deoxy form over the spectral region from 50 to 80 ppm downfield from H_2O. It is clear that the resonance at 71 ppm is missing in Hb M Milwaukee, suggesting that this resonance comes from the β chain. The resonance patterns from 31 to 56.8 ppm are essentially the same for Hb M Milwaukee in D_2O (Figure 3C) and in H_2O (Figure 2C). Thus, the resonances in this region must be due to the ferric hyperfine-shifted resonances. The resonance at 58.5 ppm for deoxy-Hb A in H_2O (Figure 2A), then, is due to the NH proton of the proximal histidine of the α chain. Hb M Boston has only one resonance at 71 ppm between 50 and 80 ppm, which confirms the assignment of the 71-ppm resonance as the NH proton

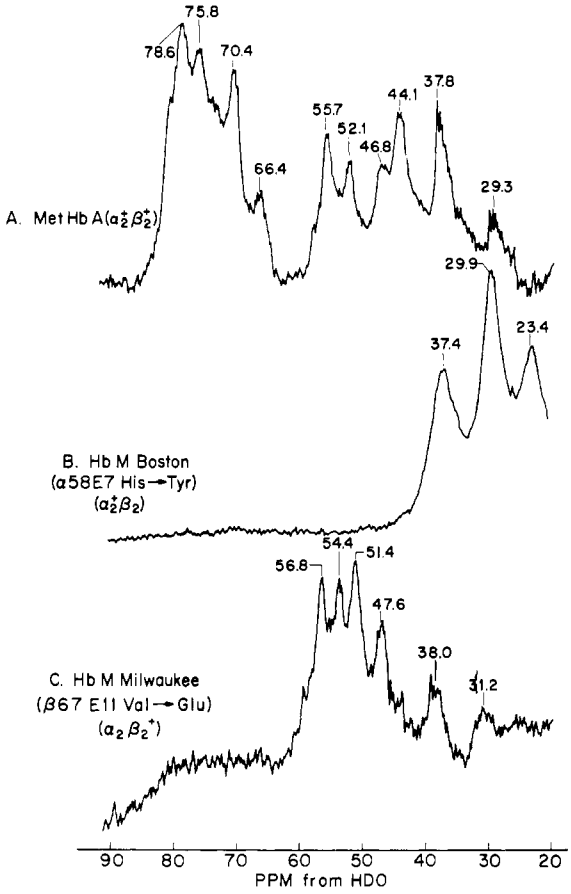

FIGURE 3: 250-MHz ferric hyperfine-shifted proton resonance spectra of methemoglobins in 0.1 M Bis-Tris buffer in D_2O at pH* 6.6 and 27 °C: (A) aquomet-Hb A; (B) Hb M Boston; (C) Hb M Milwaukee.

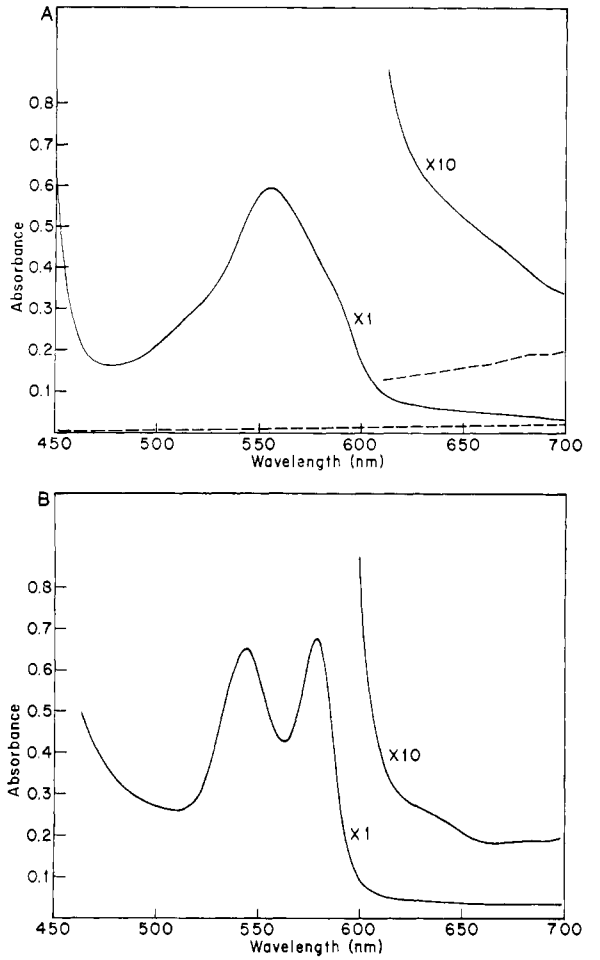

FIGURE 4: Optical spectra of Hb A: (A) deoxy-Hb A and (B) oxy-Hb A, both containing ~10% of met-Hb A. The broken line designates a base line.

of the proximal histidine of the β chain and the 58.5-ppm resonance as the corresponding one in the α chain. The line widths at half-height of these two resonances at 250 MHz are 826 Hz for the β chain and 766 Hz for the α chain, which are considerably broader than the line width of ~300 Hz at 100 MHz as reported by La Mar et al. (1977). The line width of these signals is magnetic field, temperature, and concentration dependent as expected for hyperfine-shifted resonances (Johnson et al., 1977).

Ferric Hyperfine-Shifted Proton Resonances of Hb M Boston and Hb M Milwaukee. ^{1}H NMR signals arising from the ferric α chains of Hb M Boston in D_2O are shown in Figure 3B. They occur quite close to the ferrous hyperfine-shifted proton resonance region. There are three main resonances whose chemical shifts are 37.4, 29.9, and 23.4 ppm downfield from HDO. The 23.4-ppm resonance shown in Figure 3B is identical with the broad resonance at the lowfield end of the spectrum shown in Figure 1B. Hb M Milwaukee shows six distinct resonances at 56.8, 54.4, 51.4, 47.6, 38.0, and 31.2 ppm from HDO as shown in Figure 3C [also see Fung et al. (1976, 1977)].

Ferric Hyperfine-Shifted Proton Resonances of Met-Hb A. In the course of our investigation of the proximal histidine resonances of Hb A, it was found that the contributions from met-Hb A severely interfere with the two NH resonances. Resonances due to met-Hb A show up prominently even when the deoxy-Hb A samples contain less than 10% of met-Hb A. To illustrate this effect, we took the ^{1}H NMR spectra of deoxy-Hb A in D_2O with various amounts of met-Hb A. Samples of deoxy-Hb A in D_2O were prepared according to the standard procedure used in this laboratory, and the met-Hb concentration was estimated by the conventional optical method (Antonini & Brunori, 1971) prior to NMR measurements. Figure 4 illustrates the optical spectra of deoxy-Hb A and oxy-Hb A containing ~10% met-Hb A. When the amount of met-Hb A becomes less than 10%, detection by the optical method demands meticulous care. ^{1}H NMR spectroscopy is, in this sense, a more sensitive method for detecting a small amount of met-Hb A (less than 10%) in deoxy-Hb A. Deoxy-Hb A samples contaminated with from 20 to 50% of met-Hb A were obtained by mixing a small amount of freshly prepared oxy-Hb A into the sealed deoxy-Hb A samples and allowing them to stand at room temperature for a few hours. The samples were then deoxygenated by passing N_2 gas through prior to taking a ^{1}H NMR measurement. Representative ^{1}H NMR spectra for deoxy-Hb A samples containing 10–50% met-Hb A are shown in Figure 5. Identical spectra are obtained even if the samples are prepared by mixing 100% met-Hb A with deoxy-Hb A. As shown in Figure 5, the relative intensities of the ferric hyperfine-shifted resonances vary with the amount of met-Hb A. However, a comparison between the spectra presented in Figure 5 and the spectrum of 100% met-Hb A (Figure 3A) shows that the ^{1}H NMR spectra at intermediate concentrations of met-Hb A are not simply the arithmetical sum of the spectra of met-Hb A and deoxy-Hb A. The meaning of these results requires further investigation.

Discussion

Ferrous Hyperfine-Shifted Proton Resonances. X-ray difference Fourier analyses show that the normal subunits of

Table I: Proton Chemical Shifts of the Ferrous Hyperfine-Shifted Proton Resonances of Hb A ($\alpha_2\beta_2$), Hb M Boston ($\alpha_2^+\beta_2$), Hb M Milwaukee ($\alpha_2\beta_2^+$), Hb[α(Co)$_2\beta$(Fe)$_2$], and Hb[α(Fe)$_2\beta$(Co)$_2$] in the Deoxy Form at ~27 °C

nature of hemoglobin	chemical shift from HDO (ppm) for assignment									ref
	β	β	α	$\alpha + \beta$[b]	α	β	β	$\alpha(\beta)$[c]	β	
Hb A ($\alpha_2\beta_2$)	17.5	16.8	15.6	14.1	12.2		9.5	8.0	7.2	this work
Hb M Boston ($\alpha_2^+\beta_2$)	17.8	17.1		14.2		12.0	9.6	8.0	7.4	this work
Hb M Milwaukee ($\alpha_2\beta_2^+$)			15.8	14.0	12.4			8.1		this work
	β		β	β	$\alpha + \beta$		β	$\alpha + \beta$		
Hb[α(Co)$_2\beta$(Fe)$_2$]	17.9	16.7[a]		14.2		11.4	9.7		7.2	Ikeda-Saito et al. (1978)[4]
Hb[α(Fe)$_2\beta$(Co)$_2$]					12.2			7.7		Ikeda-Saito et al. (1978)[4]
Hb A ($\alpha_2\beta_2$)	18.4		15.7[d]	14.4	12.4		9.4	7.6		Ikeda-Saito et al. (1978)[4]

[a] 17.0-ppm resonance according to Figure 2 of Ikeda-Saito et al. (1978). [b] The assignment is presently being revised. [c] The α resonance is predominant. [d] This spectral assignment by Ikeda-Saito et al. (1978) is not consistent with this work. For details, see the text.

Hb M Milwaukee and Hb M Boston have conformations similar to the corresponding subunits in deoxy-Hb A (Perutz et al., 1972; Pulsinelli et al., 1973). Thus, a comparative ^{1}H NMR study of these two mutants should allow us to make an assignment of the ferrous hyperfine-shifted proton resonances due to α and β chains of deoxy-Hb A. A preliminary spectral assignment has been made by using Hb M Milwaukee (Lindstrom et al., 1972; Johnson & Ho, 1974; Fung et al., 1976, 1977). The present study has confirmed the earlier assignments by using Hb M Boston as a complementary chain mutant. As shown in Figure 1, the spectral sum of these two mutant hemoglobins over the region from 7 to 20 ppm from HDO is, indeed, in good agreement with that of Hb A. Our NMR data have also confirmed the X-ray results that the ferrous hemes of these two mutants have essentially the same conformations or environments as those of deoxy-Hb A. The two resonances at 17.5 and 16.8 ppm can be assigned solely to the β chains. We have also confirmed that the main peak at ~12 ppm arises from the α chains, with less than 10% coming from the β chains as shown in Figure 1B. The resonance at ~8.0 ppm has been reported to be an α-chain signal. Figure 1 shows that this assignment is essentially correct, though the contribution from the overlapped β-chain signal is probably slightly more than 15%. The resonance at 7.2 ppm overlaps considerably the 8.0-ppm signal, which makes it difficult to utilize the 8.0-ppm resonance for analysis of the data obtained by the partial oxygenation experiment. The β-chain contribution to the 12.0-ppm resonance could, however, be neglected as a first approximation. The resonance at 7.2 ppm is assigned to the β chain, in agreement with the data from the partial oxygenation experiments in the presence of inositol hexaphosphate reported by Viggiano et al. (1979). It is interesting to note that the intensity of the resonances at 16.8 and 7.2 ppm (both from the β chain) appears to be pH dependent (Viggiano et al., 1979; P. E. Johner and C. Ho, unpublished results). The complete characterization of these two resonances awaits further study, namely, the spectral assignment at the second and third levels.

Ikeda-Saito et al. (1978) investigated the hyperfine-shifted proton resonances at 360 MHz of iron–cobalt hybrid hemoglobins [α(Co)$_2\beta$(Fe)$_2$ and α(Fe)$_2\beta$(Co)$_2$] and those of deoxy forms of cobalt hemoglobin and iron hemoglobin, as well as their isolated chains. One of their aims was to assign these resonances to α and β chains of deoxy-Hb and cobalt Hb. They concluded that their peaks at 15.7, 12.4, and 7.6 ppm[4] represent contributions from β, $\alpha + \beta$, and $\alpha + \beta$ chains of Hb A, respectively. Our results clearly show that the 15.7-ppm resonance should be assigned to the α chain and that the 12.4- and 7.6-ppm resonances can be resolved into separate peaks with contributions from the α and β chains, in agreement with their conclusion. Caution should be exercised in using the iron–cobalt hybrid hemoglobins to make detailed spectral assignments in normal Hb A for the following reasons. First, the effect on substituting cobalt for iron in the structure–function relationship of the Hb molecule needs to be carefully assessed. The hyperfine-shifted proton resonances of cobalt deoxy-Hb do not appear to be the spectral sum of Hb[α-(Co)$_2\beta$(FeCO)$_2$] and Hb[α(FeCO)$_2\beta$(Co)$_2$] as shown in Figures 1 and 2 of Ikeda-Saito et al. (1978). Second, it appears in Figure 2 of Ikeda-Saito et al. (1978) that there are overlapping signals due to the contributions from the cobalt-

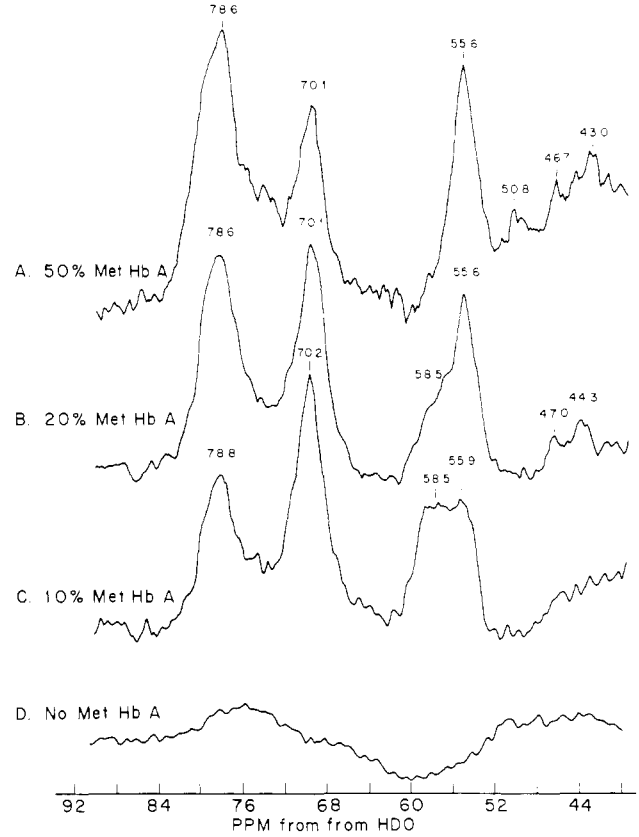

FIGURE 5: 250-MHz ^{1}H NMR spectra of deoxy-Hb A in D_2O containing (A) 50, (B) 20, (C) 10, and (D) 0% of met-Hb A. The total concentration is ~1.8 mM as a Hb tetramer. The amounts of met-Hb A are estimated by the optical method.

[4] We have adjusted the values of Ikeda-Saito et al. (1978) to our values which are referenced to the proton signal of residual HDO in the sample by subtracting 4.9 ppm from their results.

and iron-containing subunits in these hybrid hemoglobins. Table I gives a comparison of the hyperfine-shifted proton resonances between our results and those reported by Ikeda-Saito et al. (1978).

Proximal Histidine NH Resonances. Recently, La Mar et al. (1977) have attempted to assign the proximal histidine NH resonances of deoxy-Hb A and myoglobin based on a study of model compounds. Two relevant resonances were found for Hb, but only one was found for myoglobin. They suggested that the two resonances in deoxy-Hb A must originate from the nonequivalence of the α and β chains, but they could not make a definite spectral assignment. Hb M Boston and Hb M Milwaukee are well suited to test their suggestion. A series of control experiments show that the proximal histidine NH resonances are apt to overlap with the ferric hyperfine-shifted resonances when deoxy-Hb A is contaminated with small amounts of met-Hb A as shown in Figure 5. In this case, the intensity of the 71.0-ppm resonance always appears higher than that of the 58.5-ppm resonance, and another resonance is observed around 50–56 ppm. The relative intensities vary with the amount of met-Hb content, but even when present in small amounts the met-Hb substantially disturbs the spectra. Samples prepared lacking trace amounts of met-Hb A show a pair of signals of exactly equal intensity. It should be mentioned that the line shape and the spectral intensities of these two resonances as reported by La Mar et al. (1977) suggest that the deoxy-Hb A sample used by them contained a substantial amount of met-Hb A.

Ferric Hyperfine-Shifted Proton Resonances. High-spin ferric hyperfine-shifted proton resonances of met-Hb and model compounds are well-known to be affected by a number of factors, such as pH, ligand, temperature, solvent, etc. (Kurland et al., 1971; Morishima et al., 1978a,b). We have been unsuccessful in making spectral assignments of ferric hyperfine-shifted proton resonances to the α and β chains of met-Hb A using the M-type hemoglobins. The difficulty is probably due to a difference in the detailed structure and/or conformation around the heme region in the met-Hb derivatives. Morishima et al. (1978a,b) have pointed out that Hb M Milwaukee has a glutamic acid at β67 which ligates to the ferric atom in place of H_2O as the sixth ligand and that is why the spectrum of metmyoglobin in the presence of formate ion is similar to that of Hb M Milwaukee. The same explanation would apply if the mode of binding was the same and the conformations were also similar to each other. Hb M Boston has a tyrosine residue at the distal position α58(E7) instead of a histidine (Shimizu et al., 1965). According to the X-ray data, the distal tyrosine which is ligated to the ferric atom is, in fact, pulled to the distal side, which causes a breaking of the bond linking the iron atom to the proximal histidine (Pulsinelli et al., 1973). We believe that this is the first case in which a ^{1}H NMR spectrum has been observed for a pentacoordinated ferrihemoglobin molecule. The spectrum of Hb M Boston should, as a consequence, be similar to metmyoglobin ligated to phenol in a pentacoordinated form. Though we have no data to test this idea, it is interesting to note that at high pH, metmyoglobin has a spectral pattern similar to that of Hb M Boston (Morishima et al., 1978b). Metmyoglobin is ligated, in this case, very tightly to a hydroxide ion. Therefore, it may be said that the ferric atom of metmyoglobin binds to the hydroxide ion more strongly than the ferric atom of Hb M Milwaukee binds to glutamic acid. The fact that Hb M Boston is stabilized in the deoxy quaternary state even after oxygenation of the β chains is consistent with this hypothesis (S. Takahashi and C. Ho, unpublished result). Thus, a spectral comparison of Hb M Boston and Hb M Saskatoon (β63E7 His → Tyr) or Hb M Iwate (α87F8 His → Tyr) and Hb M Hyde Park (β92F8 His → Tyr) should provide a further test of this hypothesis.

The present results suggest that the ferric hyperfine-shifted proton resonances are very sensitive to the electronic state of the ferric atom, coordination state, nature of ligands, stereochemical difference of the heme environment, etc. The complexity of the ferric hyperfine-shifted resonances reflects how valuable these resonances may be for a conformational study of hemoglobin.

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Conformation of Two Homologous Neurotoxins. Fluorescence and Circular Dichroism Studies†

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ABSTRACT: Two homologous short neurotoxins isolated from snake venoms (*Laticauda semifasciata* erabutoxin b and *Naja nigricollis* toxin α) have been studied by means of fluorescence spectroscopy and in aqueous solution at various pH values. In parallel experiments, the stability of toxin conformations was analyzed on the basis of ultraviolet circular dichroism. Total luminescence spectra (77 K) were recorded for both toxins in neutral and alkaline solutions. The data obtained indicate that, at neutral pH, the fluorescence emission is only due to the single and invariant tryptophan (29). From a comparative study with erabutoxin a, which differs from erabutoxin b by a single residue, it is unambiguously shown that the protonation of His-26 of erabutoxin b is responsible for a decrease of Trp-29 fluorescence. Also, on the basis of available X-ray data it is proposed that the protonation or deprotonation of the following titrable groups is responsible for an alteration of Trp-29 fluorescence. These are Asp-31 ($pK \simeq 4$) and Lys-27 ($pK = 9.6$) for both toxins and Lys-26 ($pK \simeq 9.6$) for toxin α. No tyrosinate emission can be observed at neutral pH and 77 K. Excitation spectra of toxin α revealed that 50% of the light absorbed by Tyr-25 in water is transferred to Trp-29. From the energy transfer measurements, the distance separating these two aromatic chromophores in the native toxin was estimated to be 13 Å. A similar experiment was made for toxin α dissolved in trifluoroethanol. The data indicate that the distance separating the two aromatic side chains does not depend greatly on the nature of the solvent.

Elapidae and *Hydrophiidae* venoms possess neurotoxins which block the nicotinic acetylcholine receptor specifically and with a high affinity (Lee, 1972; Bourgeois et al., 1972). These compounds are single chain polypeptides of either 60–62 amino acids and 4 disulfide bonds (short neurotoxins) or 66–74 amino acids and 5 disulfide bonds (long neurotoxins) (Maeda & Tamiya, 1974). A chart alignment of 52 neurotoxin sequences shows that 22 residues or groups remain invariant (Menez et al., 1978).

On the basis of circular dichroism analysis and secondary structure predictions, it has been shown that short neurotoxins share a homologous conformation (Hseu et al., 1977; Dufton & Hider, 1977; Menez et al., 1978), essentially organized into β-pleated sheets and resembling the X-ray structure solved from erabutoxin b crystals (Tsernoglou & Petsko, 1976; Low et al., 1976). This structure reveals that a short toxin molecule largely consists of three β-sheet loops which are joined side by side. It also shows that most of the "functionally" invariant residues (or groups) are concentrated on the central loop or in its spatial proximity. This loop seems therefore of importance for the neurotoxic activity of the whole molecule and it is noteworthy that the sole invariant aromatic amino acids, Tyr-25 and Trp-29, are precisely located in the core of this region. Taking advantage of the fact that most of the short neurotoxins have no additional tyrosine or tryptophan residues, we used Tyr-25 and Trp-29 as fluorescent probes to investigate local and/or environmental perturbations occurring in the toxin molecules. Erabutoxin b[1] from *Laticauda semifasciata* and toxin α from *Naja nigricollis* were used for this work.

In the present study (i) ionizable groups responsible for changes of Trp-29 fluorescence emission are tentatively assigned, (ii) an average distance between Tyr-25 and Trp-29 is estimated from the determination of excitation energy transfer efficiency, (iii) conformational modifications induced by pH changes are investigated by circular dichroism (CD), and (iv) total luminescence spectra at 77 K are examined for both toxins.

Materials and Methods

Toxin α from *N. nigricollis* was prepared as described (Fryklund & Eaker, 1975). Erabutoxins a and b from *L. semifasciata* were kindly supplied by Professor N. Tamiya (Sendai, Japan). *N*-Ac-Trp-NH_2 and the dipeptide L-Tyr-L-Trp were purchased from Sigma Chemical Co. Trifluoroethanol was from Merck and was used without further purification. Methanol was from Sigma.

Fluorescence and energy transfer experiments were performed at 8 °C with dilute protein solutions [$(2–5) \times 10^{-6}$ or $(2–5) \times 10^{-5}$ M] by using a FICA 55000 differential absolute spectrofluorometer. The appropriate buffer was placed in the reference compartment. Excitation and emission slits were adjusted to 7.5 and 2.5 nm, respectively, for emission spectra

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[1] Abbreviations used: toxin α, toxin α from *Naja nigricollis*; erabutoxins a and b, erabutoxins a and b from *Laticauda semifasciata*; TFE, trifluoroethanol.

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