Proton Nuclear Magnetic Resonance Studies of Hemoglobin M Milwaukee and Their Implications Concerning the Mechanism of Cooperative Oxygenation of Hemoglobin†

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ABSTRACT: Hemoglobin M Milwaukee (β67E11 Val → Glu) is a naturally occurring valency hybrid containing two permanently oxidized hemes on the β chains. In this mutant, the two abnormal β chains cannot combine with ligands whereas the two α chains are normal and can combine with oxygen with a Hill coefficient varying from 1.1 to 1.3 [Udem et al. (1970), *J. Mol. Biol. 48*, 489]. High-resolution proton nuclear magnetic resonance spectroscopy at 250 MHz has been used to investigate the exchangeable, ring-current shifted, ferrous and ferric hyperfine shifted resonances of Hb M Milwaukee in the absence and presence of organic phosphates. The α-heme environment, as manifested by the ring-current shifted resonances in the liganded form as well as the ferrous hyperfine shifted resonances in unliganded form, and subunit interactions, as manifested by the exchangeable resonances, are similar in Hb M Milwaukee to those in normal adult human hemoglobin. Organic phosphates can partially or completely inhibit the structural transformation which normally accompanies the binding of oxygen or carbon monoxide to Hb M Milwaukee. Upon stepwise addition of oxygen to deoxy Hb M Milwaukee, the hyperfine shifted resonance spectra of ferric β chains show features which cannot be attributed to either fully deoxy or oxy species. However, the spectra for partially oxygenated Hb M Milwaukee can be described as an appropriately weighted average of the spectra of zero, singly, and doubly oxygenated species. The ferric hyperfine shifted resonance spectrum of the singly oxygenated intermediate has been calculated by a method employing least-squares analysis of the spectra of partially oxygenated Hb M Milwaukee at several values of oxygen saturation. The spectrum of this intermediate exhibits features which cannot be accounted for by a two-structure model. The present results are consistent with a sequential model for the oxygenation of this mutant hemoglobin. In view of the similarities between normal adult hemoglobin and Hb M Milwaukee, it is suggested that a two-state concerted allosteric model does not provide an adequate description of the structure-function relationship in normal adult hemoglobin.

Methemoglobinemia is a pathological condition arising from an abundance of non-oxygen-binding ferric or methemoglobin which is sufficient to impair oxygen transport and induces cyanosis. In many cases methemoglobinemia may be attributed to an inherited lack of methemoglobin reductase (Slosse and Wybauw, 1912; Hitzenberger, 1932; Dieckmann, 1932), but in some cases it is associated with the presence of one of several mutant hemoglobins M (Pisciotta et al., 1959). These mutants are characterized by amino acid substitutions in the heme pockets of either the α or β subunits, resulting in permanent oxidation of two of the four hemes in a tetrameric hemoglobin (Hb)[1] molecule under physiological conditions.

X-ray diffraction studies of deoxy Hb M Milwaukee (β67E11 Val → Glu) indicate that the βE11 glutamic acid side chain is positioned directly above the heme group, and that the γ-carboxylate oxygen atom of this residue occupies the sixth coordination position of the ferric β-heme iron (Perutz et al., 1972). No gross distortion in either the tertiary structure of the β subunits or the quaternary structure of the molecule as a whole is apparent at a resolution of 3.5 Å (Perutz et al., 1972). Although the oxygen-binding properties of half-oxidized Hb M Milwaukee differ from those of normal human adult hemoglobin (Hb A) (Hayashi et al., 1969; Udem et al., 1970), the ethyl isocyanide binding properties of fully reduced Hb M Milwaukee closely resemble those of Hb A (Nishikura et al., 1975). These findings, together with results to be reported and discussed in this paper, strongly suggest that, while ligand-binding equilibria are far simpler to analyze in Hb M Milwaukee than Hb A, the molecular mechanism of heme–heme interaction in Hb M Milwaukee does not differ qualitatively from that operating in normal hemoglobin. Thus, Hb M Milwaukee appears to provide a useful model system for the study of structure–function relationships, not only in this particular mutant, but also for hemoglobin generally.

By means of high-resolution proton nuclear magnetic resonance (NMR) spectroscopy, we have studied several features of the structure and structural changes accompanying the

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[1] Abbreviations used are: Hb, hemoglobin; Hb A, normal adult hemoglobin; HbO_2, oxyhemoglobin; HbCO, carbonmonoxyhemoglobin; NMR, nuclear magnetic resonance; Tris, tris(hydroxymethyl)aminomethane; Bistris, [bis(2-hydroxyethyl)imino]tris(hydroxymethyl)methane; P_2-glycerate, 2,3-diphosphoglycerate; Ins-P_6, inositol hexaphosphate; $Eu(DPM)_3$, tris(dipivalomethanato)europium(III); DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; p_{50}, the oxygen pressure in equilibrium with half-saturated hemoglobin; ACD, acid-citrate-dextrose.

oxygenation of Hb M Milwaukee. Several distinct low-field exchangeable proton resonances have been identified as arising from particular protons in intersubunit hydrogen bonds and have been used as spectroscopic probes of quaternary structure in various hemoglobins (Ho et al., 1975; Fung and Ho, 1975). By means of these resonances we have monitored the quaternary structure of Hb M Milwaukee under various experimental conditions. The hyperfine shifted and ring-current shifted proton resonances are sensitive probes for heme environments in the deoxy and oxy (or carbonmonoxy) form, respectively (Davis et al., 1971; Ogawa and Shulman, 1972; Lindstrom et al., 1972b; Ho et al., 1973). In deoxy Hb M Milwaukee, the α- and β-heme irons have different paramagnetic properties; the hyperfine shifted proton resonances of the high spin ferrous α-hemes (with spin 2 for ferrous iron) appear in a spectral region different from that of the high spin ferric β-hemes (with spin 5/2 for ferric iron) (Lindstrom et al., 1972a; Fung et al., 1976). Using these resonances it is possible to observe the structural alterations at the oxygen binding site of one type of subunits (β^+-hemes) which are associated with the binding of oxygen to the neighboring subunits (α-hemes). Therefore, the NMR studies of Hb M Milwaukee allow us to explore not only the structure of fully deoxygenated and fully oxygenated Hb M Milwaukee but also the changes at the β^+-hemes at intermediate stages of oxygen binding to the normal α-hemes. These measurements are regarded as a uniquely direct probe of the structural manifestation of the heme–heme interaction in Hb M Milwaukee.

In order to understand the molecular mechanism for the cooperative oxygenation of hemoglobin, it is essential to obtain information about the structures of partially heme-liganded intermediate species. It is in the detailed description or understanding of these intermediate species that various allosteric models of the heme–heme interaction in hemoglobin may be operationally distinguished. A major goal of the present investigation is to determine whether the spectral changes which are observed to take place in the vicinity of the β^+-hemes when oxygens are bound to the α chains of Hb M Milwaukee are consistent with the predictions of either the two-state concerted (for example, see Monod et al., 1965) or sequential (for example, see Koshland et al., 1966) class of models. Preliminary accounts of some aspects of our ^{1}H NMR studies of Hb M Milwaukee have been published (Lindstrom et al., 1972a; Fung et al., 1976).

Experimental Section

Materials. Whole blood containing Hb M Milwaukee was drawn from a heterozygous patient and treated with ACD (acid–citrate–dextrose) solution to prevent coagulation. The blood sample was shipped by air freight from Milwaukee to Pittsburgh in an insulated package containing chipped ice. The blood was first converted to the carbonmonoxy form by passing carbon monoxide gas over the sample. The red blood cells were washed repeatedly with 0.15 M NaCl (CO saturated) until the supernatant was clear and the cells were lysed with 2 volumes of CO-saturated distilled water. The ionic strength of the lysate was increased by the addition of 2.8 M phosphate at pH 6.8 until the phosphate concentration reached 0.1 M. The resulting stroma were removed by centrifugation at 17 000 rpm for 1 h at 4 °C.

Hb M Milwaukee was separated from the other hemoglobin components of the hemolysate, mainly Hb A, by column chromatography. A 4 × 20 cm demountable column of Bio-Rex 70 resins (Bio-Rad Laboratories) with 0.13 M sodium phosphate buffer at pH 6.4 (containing 38.07 g of NaH_2PO_4·H_2O and 17.31 g of Na_2HPO_4 in 4 L of distilled water) was used for the isolation procedure (Ranney et al., 1968). The column was equilibrated with the buffer until the pH and the conductivity (measured on a conductivity bridge Model RC16B2 of Beckman Instruments, Inc.) of the effluent matched that of the starting buffer. The hemolysate was dialyzed against the 0.13 M sodium phosphate buffer and 8–9 mL of 10 g % hemolysate was applied to the column. The column was developed at a maximum flow rate of ~10 mL/min at 4 °. A fast moving component (Hb A_2) came off in the void volume and the Hb A component was eluted over a period of 6 h. The Hb M Milwaukee component remained bound to the resins. When all the Hb A (about 60% of the hemolysate) had been eluted, the ionic strength of the buffer was increased by adding NaCl, so that the concentration of Na^+ reached 0.5 M, and the Hb M Milwaukee fraction was eluted in a fairly concentrated (~5%) solution. Early separations using the 0.19 M sodium phosphate buffer described by Hill et al. (1962) proved unsuccessful as Hb M Milwaukee would not bind to the Bio-Rex resins as required for separation. The purity of the hemoglobin solution was checked by electrophoresis on Gelman Sepraphore III cellulose polyacetate strips of the completely oxidized form of Hb M Milwaukee and was also monitored by NMR.

The NMR samples were prepared from the isolated Hb M Milwaukee fraction by first stripping the hemoglobin molecules of phosphates by chromatography on Sephadex G-25 column in 0.01 M Tris-HCl plus 0.1 M NaCl buffer at pH 7.5 (Berman et al., 1971). The sample was concentrated, exchanged with either D_2O or deionized H_2O by repetitive dilution, and then concentrated to the final concentration of ~15 g % by an ultrafiltration membrane (Amicon UM-20E). Stock solutions of [bis(2-hydroxyethyl)imino]tris(hydroxymethyl)- methane (Bistris; obtained from Aldrich), 2,3-diphosphoglycerate (P_2-glycerate; in the form of pentacyclohexylammonium salt, obtained from Calbiochem), inositol hexaphosphate (Ins-P_6; in the form of sodium salt obtained from Sigma), or potassium phosphate buffer, in D_2O or H_2O, were added to the hemoglobin solutions to obtain specific experimental conditions. For deoxy samples, the carbon monoxide was removed by flushing the hemoglobin solution with oxygen in an ice-water bath under a 150-W flood lamp and the oxygen was then removed by flushing with prepurified nitrogen. The deoxygenated solution was transferred directly to a sealed 5-mm NMR tube. An oxygen pressure of about 2 atm was required to obtain a fully oxygenated sample. Partially oxygenated samples were prepared by injecting appropriate amounts of the fully deoxygenated and fully oxygenated hemoglobin solutions into a sealed nitrogen-filled NMR sample tube. The fractional saturation of samples prepared in this manner was calculated as described in Appendix I. All samples were sealed inside nitrogen-gassed NMR tubes with rubber stoppers and epoxy (Hardman, Belleville, N.J.).

Hb M Milwaukee concentrations were determined spectrophotometrically on a Cary 14 spectrometer at 620 nm with a millimolar extinction coefficient (ϵ) of 4.4 for β^+-heme and at 540 nm with ϵ = 13.4 for α-heme (Antonini and Brunori, 1971). Hydrogen ion concentrations (pH) were determined on a Radiometer Model 26 pH meter equipped with a Beckman 39030 combination electrode. Deuterium ion concentrations (pD) were estimated by adding 0.4 pH unit to the pH meter readings for the D_2O solutions (Glasoe and Long, 1960).

The intensity "marker" used for calibrating the number of protons associated with a specific NMR resonance was pre-

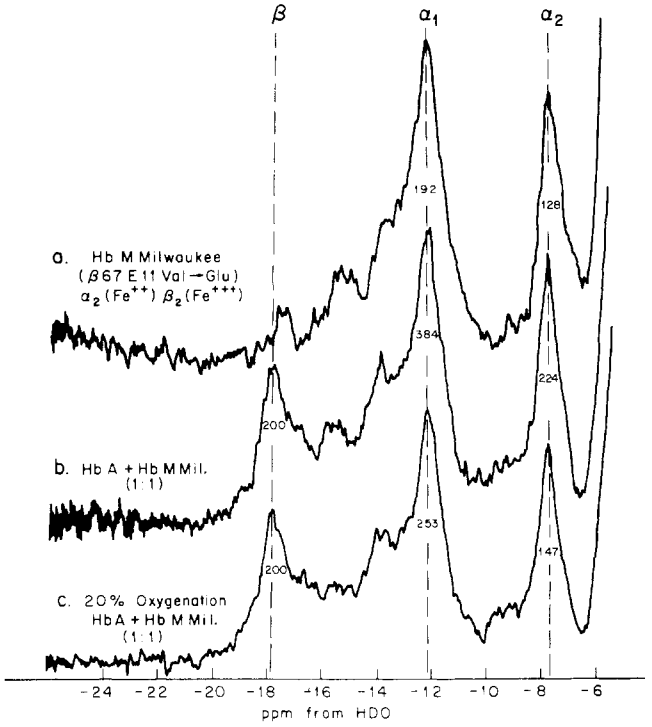

FIGURE 1: The 250-MHz ^1H NMR spectra of 15% Hb M Milwaukee and a mixture of Hb A and Hb M Milwaukee in D_2O and 0.1 M Bistris and 0.01 M Ins-P_6 at pD 7.0 and 30 °C. The number under each peak corresponds to the intensity of the resonance measured according to methods of Johnson and Ho (1974). Arbitrary units are chosen but scaled to allow the β resonance to have a value of 200.

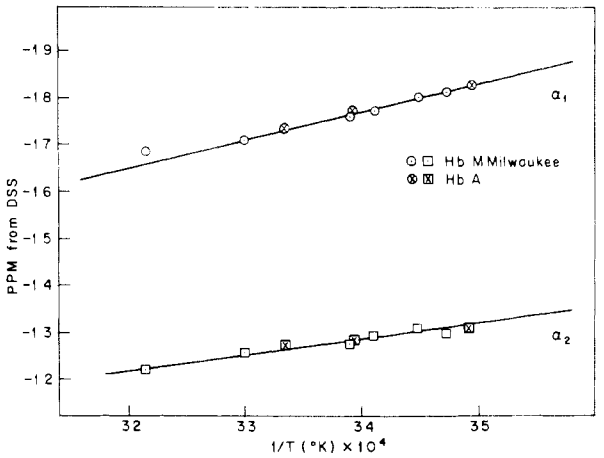

FIGURE 2: The temperature dependences of the paramagnetically hyperfine shifted proton resonances of deoxy Hb M Milwaukee and Hb A in D_2O and 0.1 M Bistris at pD 7.0.

pared by dissolving appropriate amounts of *tert*-butylcyclohexanol and shift reagent tris(dipivalomethanato)europium(III) [Eu(DPM)$_3$] (Merck) into deuterated chloroform (Demarco et al., 1970). The chemical shifts of the proton resonances of *tert*-butylcyclohexanol depend on the concentration ratio of the hexanol and the shift reagent in chloroform.

Methods. Standard 5-mm sample tubes (Wilmad) were used for all NMR measurements. For the proton calibration measurement, the "marker" was placed in a 1-mm inner NMR tube inserted coaxially into a 5-mm sample tube which contained the hemoglobin sample under investigation. The ^1H NMR spectra were obtained on the MPC-HF 250-MHz superconducting spectrometer interfaced with a Sigma-5 computer (Dadok et al., 1970). The signal-to-noise ratio was enhanced by the accumulation of multiple scans and by the NMR correlation technique (Dadok and Sprecher, 1974). For the deoxy hyperfine shifted and the exchangeable proton resonances, 500–1000 scans were needed; 100–200 scans were needed for the ferric hyperfine shifted and ring-current shifted resonances. Proton chemical shifts were referenced with respect to the residual water proton signal in the sample, which was 4.75 ppm downfield from the proton resonance of a standard, 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), at 30 °C, the ambient temperature of the probe. Chemical shifts downfield from the proton signal of water were assigned negative values and those upfield were assigned positive values. The uncertainties of the chemical shifts of the ferric hyperfine shifted proton resonances were ±1.0 ppm, of the ferrous (deoxy) hyperfine shifted and exchangeable proton resonances ±0.2 ppm, and of the ring-current shifted proton resonances ±0.05 ppm. It should be noted that the spectral baselines of the ferric hyperfine shifted resonances were matched throughout the entire study to obtain consistent and meaningful results. Due to the relatively low intensity and unusually large chemical shifts of the ferric hyperfine shifted proton resonances, the baseline was very sensitive to the spectrometer settings, such as the modulating frequency (50 kHz was used in this study), and spectral phase. When a different set of spectrometer settings was used, the envelope of these resonances as well as the baseline might appear quite differently. Thus, spectra presented in different figures may have slightly different baselines. However, the spectra shown within any one figure had comparable baselines. Comparison of peak intensity should only be made among spectra taken with identical spectrometer settings.

Results

Ferrous Hyperfine Shifted Proton Resonances. In Figure 1a, the spectrum of 15 g % deoxy Hb M Milwaukee in 0.1 M Bistris buffer in D_2O at pD 7 and 30 °C in the presence of Ins-P_6 shows downfield resonances between −6 and −24 ppm from HDO. Instead of the three prominent hyperfine shifted proton resonances usually seen in deoxy Hb A at about −18, −12, and −8 ppm from HDO (Davis et al., 1971), there are only two major resonances at ca. −12 ppm (marked α_1 in the spectrum) and ca. −8 ppm (marked α_2). Other minor resonances of low intensity associated with the α chains include the ones at about −14, −15, and −17.5 ppm. The average line width at half-height of α_1 is ca. 300 Hz and of α_2 is ca. 200 Hz at a magnetic field corresponding to the proton resonance frequency at 250 MHz and at 30 °C. The line widths of these hyperfine shifted proton resonances are generally broader at lower temperatures. [For a recent discussion on the magnetic field and temperature dependence of the hyperfine shifted proton resonances of hemoglobin, refer to Johnson et al. (1977).] Six protons are assigned to the α_1 resonance. This assignment is based on an area calibration referenced with respect to the area of an OH proton signal of an intensity marker, *tert*-butylcyclohexanol, in the presence of Eu(DPM)$_3$. This sample is in an inner tube which is coaxially placed inside a regular NMR tube containg hemoglobin sample (see Experimental Section). The spectra of deoxygenated and partially oxygenated (20% oxygenation) samples of a 1:1 Hb M Milwaukee and Hb A mixture, in the presence of Ins-P_6, are shown in Figures 1b and 1c. Relative intensities of these resonances are measured and presented in Appendix IV.

We have also studied the spectra of deoxy Hb M Milwaukee

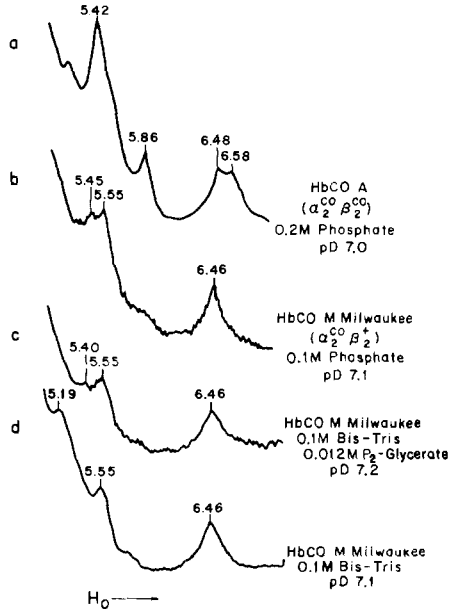

FIGURE 3: The 250-MHz ^{1}H NMR spectra of the ring-current shifted proton resonances of 15% carbomonoxy Hb M Milwaukee and in D_2O at 30 °C (units in ppm from HDO).

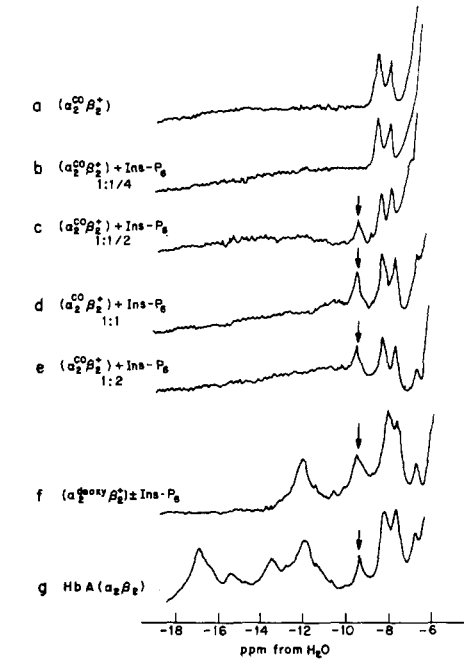

FIGURE 4: The 250-MHz ^{1}H NMR spectra of exchangeable proton resonances and deoxy hyperfine shifted proton resonances of 15% Hb M Milwaukee as a function of Ins-P_6 in H_2O and 0.1 M Bistris at pH 6.6 and 30 °C. The spectrum of Hb A is included for comparison.

at various temperatures. When the chemical shifts of the α_1 and α_2 resonances are plotted against the inverse of the temperatures at which these resonances are measured, a straight line is obtained (Figure 2), as expected in the case for paramagnetic hyperfine shifted resonances (McDonald and Phillips, 1967). A control study of deoxy Hb A reveals that the corresponding α resonances have the same temperature dependence as those of Hb M Milwaukee.

Ring-Current Shifted Proton Resonances. In diamagnetic proteins, some of the aliphatic proton signals are shifted away from their normal positions by the ring-current effects of aromatic systems (McDonald et al., 1969; Shulman et al., 1970; Wüthrich et al., 1972; Lindstrom et al., 1972b; Lindstrom and Ho, 1973; Ho et al., 1973). As shown in Figure 3, in the region between +5.0 and +7.0 ppm from HDO, the ring-current shifted proton resonances can be observed for carbonmonoxy Hb M Milwaukee at +5.45, +5.55, and +6.46 ppm in 0.1 M phosphate buffer at pD 7.1. This spectrum is very similar to the ring-current shifted spectrum of the isolated α chains from Hb A in the carbonmonoxy form (Lindstrom et al., 1972b). As can be seen in Figure 3, the removal of phosphate and the use of Bistris buffer cause a shift in the resonance at +5.45 to +5.19 ppm and the addition of 12 mM P_2-glycerate to the phosphate-free Bistris Hb M Miwaukee solution causes this line to shift back to +5.40 ppm from HDO.

Exchangeable Proton Resonances. Several exchangeable proton resonances of the hemoglobin molecule have been used as spectroscopic probes to monitor the quaternary structural transition during oxygenation. The two resonances at −9.4 and −6.4 ppm from H_2O are present in the deoxy quaternary structure but not in the oxy quaternary structure (Figure 4f; Fung and Ho, 1975). The −9.4 ppm resonance is associated with the OH proton in α42C7 Tyr, which is hydrogen bonded to the carboxy group of β99G1 Asp to form a characteristic $\alpha_1\beta_2$ intersubunit linkage in the deoxy form (Fung and Ho, 1975). We have observed the appearance of the −9.4 and −6.4 ppm resonances in fully deoxy Hb M Milwaukee samples (Figure 4f). These resonances disappear in fully oxy or carbonmonoxy sample (Figure 4a). However, some rather interesting results are obtained when the fully liganded sample is titrated with Ins-P_6. The deoxy spectroscopic probes at −9.4 and −6.4 ppm reappear in the spectra if a sufficient amount of Ins-P_6 is added (Figures 4c–e). The flat region beyond −10 ppm downfield indicates that the α chains are still fully liganded.

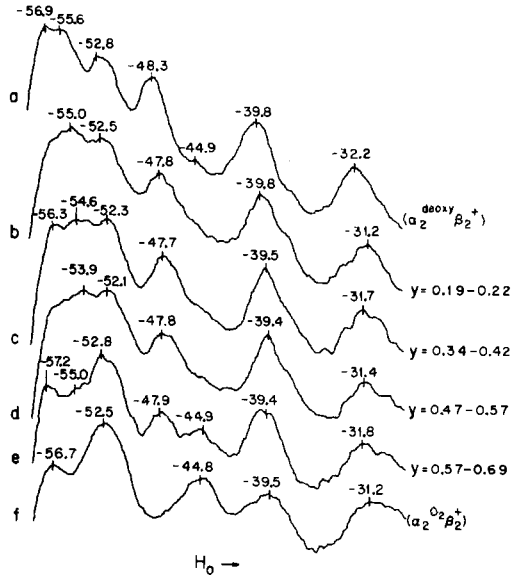

FIGURE 5: The 250-MHz ^{1}H NMR spectra of ferric hyperfine shifted proton resonances of 15% Hb M Milwaukee as a function of oxygenation in D_2O and 0.1 M Bistris at pD 7.0 and 30 °C (units in ppm from HDO). The range of fractional saturation is based on p_{50} = 5 Torr or p_{50} = 10 Torr. For details see Appendix I.

Ferric Hyperfine Shifted Proton Resonances. Both the deoxygenated and oxygenated samples of Hb M Milwaukee give NMR signals in the spectral region between −30 and −60 ppm downfield from HDO. These are the hyperfine shifted proton resonances of the β^+-hemes. As shown in Figure 5,

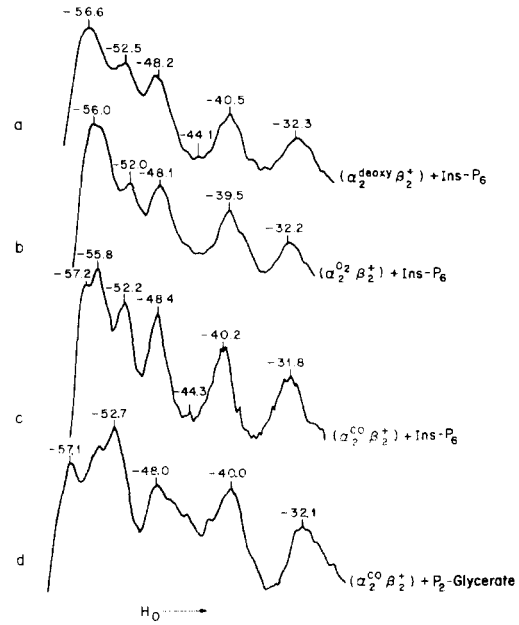

FIGURE 6: Effects of organic phosphates on the ferric hyperfine shifted proton resonances of 15% Hb M Milwaukee with and without ligation of O_2 or CO in D_2O and 0.1 M Bistris at pD 7.0 and 30 °C (units in ppm from HDO).

distinct resonances are observed at about −48, −53, and −55 to −57 ppm when the α chains are deoxygenated in 0.1 M Bistris buffer in D_2O at pD 7.0. When the α chains are fully saturated with oxygen or carbon monoxide, three major resonances at about −45, −52, and −57 ppm are present. Hereafter, we shall refer to spectra resembling that shown in Figure 5a as type a and spectra resembling that shown in Figure 5f as type f. Upon stepwise addition of oxygen to the α chains, the β^+-heme spectrum changes gradually from type a to type f, as shown in Figures 5b–e, with the ca. −48 ppm resonance being replaced by the ca. −45 ppm resonance and the ratio of the intensity of the resonance at −55 to −57 ppm to that of the resonance at −53 ppm decreases from greater than one to less than one. In this series of spectra it is important to note that, while the intensities of various peaks change significantly with changing oxygen saturation, their positions do not. This is a good indication that the chemical species which give rise to the various resonances interconvert slowly on the NMR time scale, i.e., the mean lifetime of a species is greater than ca. 0.4 ms (Pople et al., 1959).

Figure 6 shows the effects of organic phosphates upon the ferric hyperfine shifted resonance spectra of oxy and carbonmonoxy Hb M Milwaukee. Figure 6a shows that the addition of Ins-P_6 to deoxy Hb M Milwaukee has little effect on the spectrum. However, Figures 6b and c show that Ins-P_6 converts the type f spectrum of liganded Hb M Milwaukee to a type a spectrum. In order to assure that the α chains are still fully liganded when excess Ins-P_6 is added, the deoxy hyperfine shifted resonances are monitored simultaneously with no resonances being found in that region. The addition of excess P_2-glycerate to carbonmonoxy Hb M Milwaukee results in a hybrid spectrum; between −40 and −50 ppm it resembles type a, and between −50 and −60 ppm it resembles type f.

We have measured the spectra of fully oxidized Hb M Milwaukee (met Hb M Milwaukee) and met Hb A. In the absence of organic phosphate, met Hb M Milwaukee exhibits a type f spectrum over the frequency range between −30 and −60 ppm. In the presence of excess Ins-P_6, the spectrum is converted to type a (data not shown). The spectra of Hb M Milwaukee (types a and f) are compared with those of met Hb A under the same conditions at pD 7.0 over the same spectral region. We found that there is no simple correspondence between these spectra and that organic phosphate does not appear to have the same effect on met Hb A as it does on met Hb M Milwaukee.

Discussion

Resonance Assignments. In previous studies of the hyperfine shifted proton resonances in deoxy Hb A, we have suggested that the α- and β-hemes are nonequivalent and that the prominent resonance at ca. −18 ppm is a deoxy β-heme resonance while the ones at ca. −12 and ca. −8 ppm from HDO at room temperature are deoxy α-heme resonances (Davis et al., 1971; Lindstrom et al., 1972a; Ho et al., 1973). The NMR spectrum of deoxy Hb M Milwaukee shows that the major resonance at −18 ppm is missing (Figure 1), which is to be expected if the resonance at −18 ppm in Hb A is due to the deoxy β-heme. Therefore, this evidence confirms our previous assignment.

The ring-current shifted proton resonances in the spectrum of carbonmonoxy Hb M Milwaukee (Figure 3) are similar to those in the previously reported spectrum of isolated α chains of Hb A in the carbonmonoxy form (Lindstrom et al., 1972b). The similarity substantiates the assignment of the resonances at +5.19, +5.45, and +6.48 ppm from HDO to the α-heme pocket and those at +5.42, +5.86, and +6.58 ppm to the β-heme pocket.

Structure and Function of the α-Hemes. A nuclear resonance is characterized not only by its chemical shift but also by its line width which is governed by the relaxation processes associated with it. Therefore, the resonance can provide structural as well as dynamic information about a resonating species. We have demonstrated that the chemical shifts of the ring-current shifted resonances of the α-hemes in the presence and absence of organic phosphate in carbonmonoxy Hb M Milwaukee and of the temperature dependence of the deoxy hyperfine shifted resonances of the α-hemes in the Hb M Milwaukee are the same as the corresponding ones in the Hb A. Thus, genetic modification of the β subunit does not appear to have a significant effect upon the structure of the α-heme pocket in either the fully liganded or deoxygenated form. We have also observed that each individual resonance of the α-hemes in Hb M Milwaukee has essentially the same line width as the corresponding one in Hb A. Furthermore, we have examined the intensities (area measurements) of the deoxy hyperfine shifted proton resonances of α chains in a deoxy and a partially oxygenated mixture of Hb A and Hb M Milwaukee (Figure 1 and Appendix IV). All these results suggest that the α-hemes in Hb M Milwaukee are not only structurally but also functionally similar (if not identical) to those of Hb A. Recently, in a study of ethyl isocyanide equilibria, Nishikura et al. (1975) found that, in the fully reduced Hb M Milwaukee, the Hill coefficient of 2.0 is comparable to that of 2.4 for Hb A. They further reported that the Bohr effect and ligand affinity of reduced Hb M Milwaukee are also normal.

Quaternary Structure. The exchangeable proton resonances at −9.4 and −6.4 ppm from H_2O are associated with intersubunit interactions present in the deoxy quaternary structure and absent in the oxy quaternary structure. Their presence in the spectrum of deoxy Hb M Milwaukee and absence from the spectrum of stripped carbonmonoxy Hb M Milwaukee (Figures 4a and f) suggest that upon ligand binding Hb M Milwaukee undergoes a quaternary structure transition similar

to that undergone by Hb A. This result is consistent with the x-ray crystallographic and optical spectroscopic observations of Perutz et al. (1972), who noted that deoxy Hb M Milwaukee is isomorphous with deoxy Hb A and that met Hb M Milwaukee has a structure similar to that of met or oxy Hb A.

Structure of Methemoglobin M Milwaukee. The observation that the addition of a sufficient quantity of inositol hexaphosphate converts the type f ferric hyperfine shifted spectrum of met Hb M Milwaukee to a type a spectrum suggests that met Hb M Milwaukee has a structure resembling deoxy Hb M Milwaukee in the presence of Ins-P_6. The observation that Ins-P_6 does not have a similar effect upon the spectrum of met Hb A further suggests that the stability of the oxy-like structure of met Hb M Milwaukee, relative to its deoxy-like structure, is less than that of the oxy-like structure of met Hb A, relative to its deoxy-like structure.

Structure and Function of the β^+-Hemes. Since the oxidized β-hemes (β^+-hemes) in Hb M Milwaukee do not bind oxygen or carbon monoxide and provide hyperfine shifted proton resonances in a spectral region different from that of the normal deoxy α-hemes, we can, therefore, monitor the heme environment of the β^+ chains exclusively. We have found that when the α-hemes are free of ligand, the β^+ chains have a type a spectrum (Figure 5a). When the α-hemes are fully liganded in the absence of organic phosphate, the β^+ chains have a type f spectrum (Figure 5f). These two distinct spectra indicate that the environment of the ferric α-hemes is influenced by the oxygen saturation of the β-hemes. Although we have no direct experimental evidence that the structural changes occurring in the β^+ chains of Hb M Milwaukee correspond to those occurring in normal β chains of Hb A, our findings with respect to changes occurring in the α chains and in the intersubunit interactions upon oxygenation of Hb M Milwaukee suggest that this is indeed the case.

The optical spectroscopic data of Perutz et al. (1972) suggest that the allosteric effector, Ins-P_6, is capable of locking Hb M Milwaukee in the deoxy conformation, regardless of the ligation state of the α-hemes. We have used the ferric hyperfine shifted resonances to monitor the tertiary structure of the β^+-heme environment and the exchangeable proton resonances to monitor the intersubunit interactions. The results clearly indicate that the addition of a sufficient amount of Ins-P_6 will inhibit many of the structural changes that normally accompany the oxygenation of Hb M Milwaukee (Figure 6). Judging from the effect of excess P_2-glycerate upon the ferric hyperfine shifted resonance spectrum of the β^+-hemes (Figure 6d), it appears that when carbonmonoxy Hb M Milwaukee binds P_2-glycerate, the structure in the vicinity of the β^+-hemes in part resembles that of deoxy Hb M Milwaukee, and in part resembles that of oxy Hb M Milwaukee. A possible interpretation of this observation follows: Different parts of the ferric hyperfine shifted resonance spectrum reflect different sectors of the heme environment, which may in turn reflect interactions between a particular β^+ chain and different neighboring subunit chains. Thus, the binding of P_2-glycerate may constrain the interface between β^+ chains to remain in a deoxy-like conformation even when the α chains are liganded with oxygen or carbon monoxide, while the interface between α and β^+ subunits may be altered upon ligand binding of the α chains. In contrast, when the larger and more highly charged Ins-P_6 molecule is bound to oxy or carbonmonoxy Hb M Milwaukee, it may constrain both the β^+–β^+ interface and the α–β^+ interfaces to remain in a deoxy-like conformation, thus accounting for both the ferric hyperfine shifted and exchangeable proton resonance spectra of carbonmonoxy Hb M Milwaukee

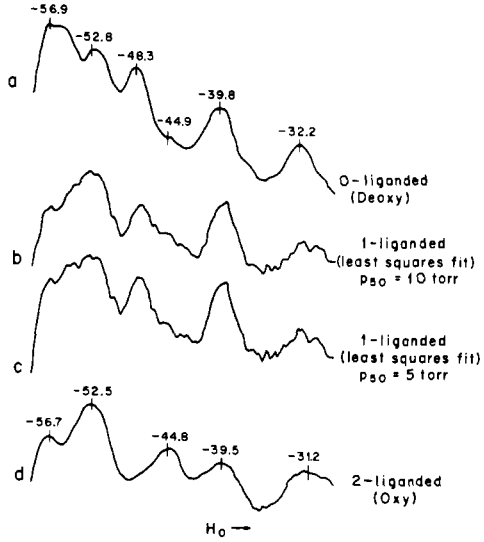

FIGURE 7: The 250-MHz ^{1}H ferric hyperfine shifted NMR spectra of deoxy and oxy Hb M Milwaukee (a and c, respectively), and calculated spectrum of "singly oxygenated intermediate" of Hb M Milwaukee (b). Curves b and c are calculated assuming $n = 1.0$. Test calculations for values of n up to and including 1.4 show no significant differences in shape of calculated curves. For details, see the text and Appendix III. The units are expressed as ppm from HDO.

plus Ins-P_6. According to this interpretation, the structure of carbonmonoxy Hb M Milwaukee plus P_2-glycerate may be classified neither as deoxy like nor oxy like.

Partially oxygenated solutions of Hb M Milwaukee contain an equilibrium mixture of zero, singly, and doubly oxygenated species. Since the hyperfine shifted proton resonance spectra of the β^+ chains in these solutions indicate that these species interconvert slowly (Results section), one may assume that, to a good approximation, each spectrum may be described as an appropriately weighted time-average of the spectra of the three-ligand species

$$a(x,y) = f_0(y)a_0(x) + f_1(y)a_1(x) + f_2(y)a_2(x) \quad (1)$$

where $a(x,y)$ is the observed amplitude of the spectrum at chemical shifts x and fractional saturation y, $f_i(y)$ is the fraction of Hb M Milwaukee to which i molecules of oxygen are bound at fractional saturation y, and $a_i(x)$ is the amplitude of the spectrum of the pure species binding i molecules of oxygen at chemical shift x.

Using the least-squares procedure described in Appendices II and III, we have calculated two possible ferric hyperfine shifted resonance spectra of the singly oxygenated intermediate corresponding to limiting low and high estimates of the oxygen pressure in equilibrium with half-saturated Hb M Milwaukee, p_{50}. These are shown in Figure 7 (curves b and c) together with the corresponding spectra of zero and doubly oxygenated species (curves a and d, respectively) for comparison.[2] Note that curves b and c are quite similar to each other and exhibit the same qualitative spectral features. Features common to

[2] The spectrum of singly oxygenated Hb M Milwaukee presented in a preliminary report [Figure 2b of Fung et al. (1976)] was calculated on the basis of a rough estimate of fractional saturation in partially oxygenated NMR samples. The spectra shown in curves b and c of Figure 7 of the present paper are calculated using more accurate estimates of fractional saturation in these samples (Appendix I). The qualitative features observed in Figure 2b of Fung et al. (1976) are also found in Figures 7b and 7c. In addition, the calculated spectra of partially oxygenated Hb M Milwaukee shown in Figure 9 are essentially identical with those shown earlier [Figure 1 of Fung et al. (1976)].

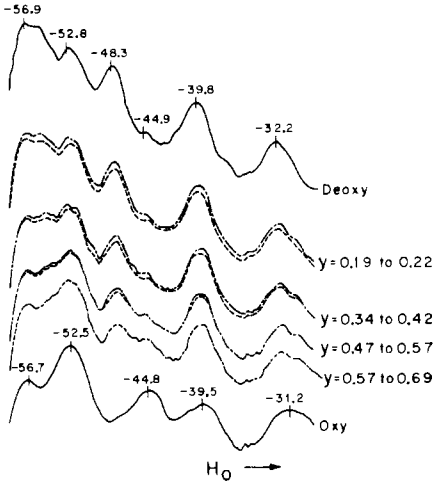

FIGURE 8: Ferric hyperfine shifted spectra of partially oxygenated Hb M Milwaukee calculated using eq 1 and spectra (- - - and - - -) curves are calculated with p_{50} = 5 and 10 Torr, respectively. For details, see Appendix II and the text. The units are expressed as ppm from HDO.

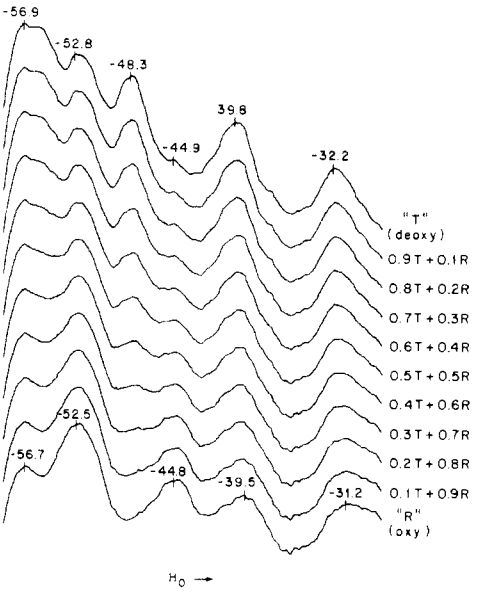

FIGURE 9: A family of possible spectra of partially oxygenated Hb M Milwaukee calculated according to a "two heme-state" model. For details, see the text. The units are expressed as ppm from HDO.

both calculated curves are therefore taken to be characteristic of the spectrum of singly oxygenated Hb M Milwaukee independent of the precise value of p_{50}. We do not attribute significance to the irregular fine structure of curves b and c, which probably reflects an accumulation of experimental errors in the four spectra (Figure 5b-e) from which they were extracted. In Figure 8 are shown spectra of partially oxygenated Hb M Milwaukee which are calculated from eq 1 with the appropriate set of f_i, and the spectra of zero, singly, and doubly oxygenated Hb M Milwaukee. The calculated spectra obtained using curve b of Figure 7 (based on p_{50} = 10 Torr) for the spectrum of singly oxygenated Hb M Milwaukee are essentially superimposable upon those obtained using curve c of Figure 7 (based on p_{50} = 5 Torr) for the same spectrum. The calculated spectra shown in Figure 8 agree with the observed spectra shown in Figure 5 to well within the uncertainty introduced by experimental errors and a lack of precision in the input values of y and the Hill coefficient, n. As far as is known to the authors, the calculated ferric hyperfine shifted spectra of singly oxygenated Hb M Milwaukee shown in Figure 7 represent the first reported measured property (albeit measured indirectly) of any equilibrium intermediate heme-ligand species of hemoglobin. Functional interpretations of structural data, including the NMR results presented in this paper, are subject to certain limitations which have been discussed by Fung et al. (1976). With these limitations in mind, the structural information in Figure 7 may be discussed in the context of various allosteric models for hemoglobin.

There exists a class of allosteric models in which the heme (ligand binding site) is postulated to exist instantaneously in one of two states or conformations which are characterized by either a high or low affinity for heme ligand. According to these models, all of the hemes in deoxyhemoglobin exist predominantly in the low affinity conformation. When one or more of the hemes bind ligand, the equilibrium between low and high affinity conformations of the remaining unliganded binding sites shifts toward the high affinity conformation, and the equilibrium average affinity of these sites for heme-ligand increases. This class of models includes not only the "two-state" model in which the quaternary structure of the tetrameric hemoglobin molecule determines the tertiary structure and ligand affinity of each subunit [as typified by the model of Monod et al. (1965)], but also several models in which the tertiary structures of subunits may vary within a given quaternary structure (Perutz, 1970; Shulman et al., 1972; Szabo and Karplus, 1972; Herzfeld and Stanley, 1974). Such models will be termed "two heme-state" models to indicate the number of allowed states of an unliganded heme. Another approach to the description of the cooperative oxygenation of hemoglobin is typified by the sequential model of Koshland et al. (1966). According to a sequential description, the ligation of a subunit alters intersubunit interactions in such a manner as to destabilize the conformation of a neighboring unliganded subunit relative to the conformation which the subunit adopts upon ligation. In this manner the ligation of one subunit directly increases the oxygen affinity of the neighbors.

The ferric hyperfine shifted spectrum of Hb M Milwaukee is a sensitive probe of the conformation of the β^+-hemes and their immediate environments. If the two-heme-conformation postulate is correct, then it follows that the ferric hyperfine shifted spectrum of the β^+-hemes in singly oxygenated Hb M Milwaukee should be some equilibrium average of the corresponding spectra in fully deoxygenated (zero oxygenated) and fully oxygenated (doubly oxygenated) Hb M Milwaukee. In Figure 9 is plotted a family of possible spectra generated by averaging the observed spectra of deoxy and oxy Hb M Milwaukee in various proportions. It is apparent upon inspection of Figure 9 that the ferric hyperfine shifted spectrum of singly oxygenated Hb M Milwaukee (Figure 7b or 7c) cannot be obtained as an average of the corresponding spectra of fully deoxygenated)figure 7a) and fully oxygenated (Figure 7d) Hb M Milwaukee. Because the singly oxygenated species does not have the twofold symmetry of the zero and doubly oxygenated species, the data do not exclude the possibility that one of the two β^+-hemes exists in a deoxy-like conformation. However, it seems clear that the conformation of at least one of the two β^+-hemes in singly oxygenated Hb M Milwaukee cannot be described as an equilibrium average of the deoxy-like and oxy-like conformations (Figure 10). The data thus indicate that, during the course of oxygenation, unliganded binding sites may adopt intermediate as well as deoxy-like and oxy-like conformations. It follows that the postulate of two heme affinity states lacks structural justification, and that "two

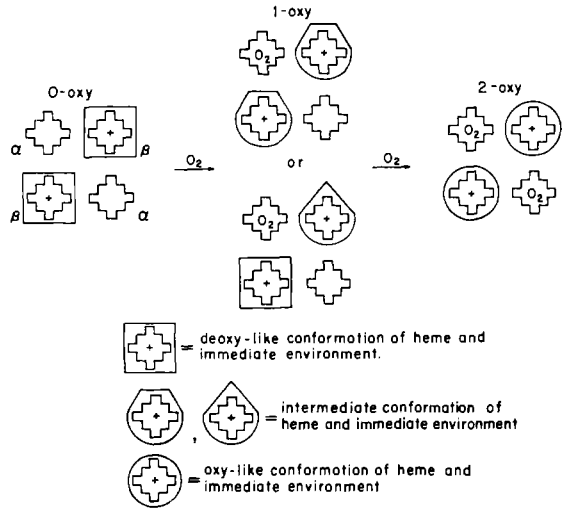

FIGURE 10: Schematic diagram depicting sequential changes in the conformation of β^+-hemes in Hb M Milwaukee upon oxygenation.

heme-state" models provide an oversimplified representation of the structure–function relationships in Hb M Milwaukee. Since the NMR results presented here (together with other data cited in the introductory section) indicate that the structure–function relationships in Hb M Milwaukee are qualitatively similar to, but much simpler than, the corresponding relations in normal hemoglobin, we consider it highly improbable that "two heme-state" models could provide an adequate conceptual basis for the understanding of structure–function relationships in the more complex system of normal adult hemoglobin.

Acknowledgment

We thank Dr. Max F. Perutz and Dr. Martin Karplus for several stimulating discussions during the course of this work.

Appendix I: Calculation of Fractional Saturation of Mixtures of Deoxy and Oxy Hb M Milwaukee

A 15 g % solution of Hb M Milwaukee is brought to equilibrium at 30 °C with O_2 at pressure of 1500 Torr. The hemoglobin is essentially completely saturated with oxygen ($y \approx 1.0$). Thus the number of moles of oxygen in volume V of solution is equal to the number of moles of ferrous hemes plus that amount of unbound oxygen which is dissolved in solvent (calculated using Henry's law). Expressing V in units of liters we obtain

$$\text{moles of oxygen bound to Hb M Milwaukee} = 4.62 \times 10^{-3} V$$

$$\text{moles of oxygen dissolved} = 2.12 \times 10^{-3} V \qquad \text{(I.1)}$$

A volume X_2V of this solution is mixed at 30 °C with a volume $(1 - X_2)V$ of a 15% solution of completely deoxygenated Hb M Milwaukee in a nitrogen-filled NMR tube of volume $5V$. The oxygen present in the mixture then equilibrates with the gaseous volume, coming to a final partial pressure p of oxygen. The number of moles of oxygen in volume V of Hb M Milwaukee solution is equal to the number of moles of ferrous heme times the fractional saturation $y(p)$. The number of moles of unbound oxygen dissolved in solution is calculated from Henry's law for oxygen partial pressure p. The number of moles of gaseous oxygen in volume $4V$ is calculated from the ideal gas law for oxygen partial pressure p.

$$\text{moles of oxygen bound to Hb M Milwaukee} = 4.62 \times 10^{-3} y(p) V$$

$$\text{moles of oxygen dissolved} = 1.41 \times 10^{-6} pV$$

$$\text{moles of gaseous oxygen} = 2.12 \times 10^{-4} pV \qquad \text{(I.2)}$$

where p is given in units of Torr. Since the total amount of oxygen is conserved in the mixing process, we may write (canceling V)

$$(4.62 \times 10^{-3} + 2.12 \times 10^{-3})X_2 = 4.62 \times 10^{-3} y(p) + p[1.41 \times 10^{-6} + 2.12 \times 10^{-4}] \qquad \text{(I.3)}$$

$y(p)$ may be expressed as a function of p, eq I.3 solved for p as a function of X_2, and y evaluated for the equilibrium value of p. Alternatively, the function $y(p)$ may be inverted to yield $p(y)$, and eq I.3 solved directly for y. We have used for this purpose the Hill equation

$$y = \frac{(p/p_{50})^n}{1 + (p/p_{50})^n} \qquad \text{(I.4)}$$

which may be inverted to yield

$$\log p = \log p_{50} + \frac{1}{n} \log \left(\frac{y}{1 - y}\right) \qquad \text{(I.5)}$$

It is found that for $1.0 \leq n \leq 1.3$ the final results are not strongly dependent upon the value of n. Thus without loss of generality, we may write

$$p \cong p_{50} \left(\frac{y}{1 - y}\right) \qquad \text{(I.6)}$$

Substituting eq I.6 into eq I.3, an equation quadratic in y is obtained which may be solved analytically as a function of X_2 and p_{50}.

Unfortunately, the value of p_{50} under the conditions chosen for our NMR experiments has not been measured. However, we may estimate this value to within a factor of 2 as follows. The p_{50} of phosphate-stripped Hb M Milwaukee in 0.1 M NaCl at pH 7.0 was reported to be 3.25 Torr at 10 °C by Udem et al. (1970). Extrapolating the results of Hayashi et al. (1969), we estimate that the p_{50} of Hb M Milwaukee is roughly doubled upon increasing the temperature from 10 to 30 °C. Replacement of H_2O by D_2O would not be expected to have a large effect upon p_{50} (Tomita and Riggs, 1970). Solutions (0.1 M) of Bistris and chloride ion are of comparable ionic strength. Thus the p_{50} of Hb M Milwaukee under the conditions of our NMR experiments (i.e., 0.1 M Bistris at pD 7 and 30 °C) is probably between 5 and 10 Torr. The values of y calculated for each of the partially oxygenated samples are given in Table I below for the limiting estimates of p_{50}.

Appendix II: Procedure for Extracting the Ferric Hyperfine Shifted Proton NMR Spectra of Singly Oxygenated Hb M Milwaukee

The ferric hyperfine shifted proton NMR spectrum of Hb M Milwaukee at each level of oxygen saturation was digitized to yield a table of $370(x,a)$ pairs (x = chemical shift, a =

TABLE I: Calculated Values of Fractional Saturation.

X_2	p_{50} = 5 Torr	p_{50} = 10 Torr
0.2	0.22	0.19
0.4	0.42	0.34
0.6	0.57	0.47
0.8	0.69	0.57

spectral amplitude). It may readily be shown that the procedure described below is unaffected by baseline differences between different spectra so long as the scale of relative amplitudes *within* a given spectrum is the same for all spectra. The digitized raw data were scaled in accordance with this requirement.

In the text, evidence is presented to show that to a good approximation, the ferric hyperfine shifted spectra of partially oxygenated Hb M Milwaukee may be quantitatively described by eq 1. The fraction of Hb M Milwaukee to which i molecules of oxygen are bound at fractional saturation y, $f_i(y)$, is calculated as a function of y and the Hill coefficient, n, as described in Appendix III.

If the values of n and y, and, hence, the f_i were known precisely, then the optimal procedure for extracting the spectral component corresponding to the singly oxygenated hemoglobin, $a_1(x)$, would be to simultaneously vary $a_0(x)$, $a_1(x)$, and $a_2(x)$ in order to achieve a least-squares fit of eq 1 to all six ferric hyperfine shifted spectra (curves a–f of Figure 5). However, because n and y, and, hence, the f_i are not precisely known for the four spectra taken at intermediate saturation levels, there is more uncertainty in the composition of the hemoglobin for these spectra than for the spectra of fully deoxygenated and fully oxygenated Hb M Milwaukee. Thus we set $a_0(x) = a(x,0)$, and $a_2(x) = a(x,1.0)$. The value of $a_1(x)$ which provides the best fit of text eq 1 to the four intermediate spectra (y = 0.22, 0.42, 0.57, and 0.69 for p_{50} = 5 Torr or y = 0.19, 0.34, 0.47, and 0.57 for p_{50} = 10 Torr) in the least-squares sense is then

$$a_1(x) = \frac{\left[\sum_y a(x,y)w(y)f_1(y) - a_0(x)\sum_y w(y)f_0(y)f_1(y) - a_2(x)\sum_y w(y)f_2(y)f_1(y)\right]}{\sum_y w(y)f_1(y)^2} \quad \text{(II.1)}$$

where $w(y)$ is the statistical weight assigned to the data for saturation value y, and Σ_y indicates a sum over the y values (0.22, 0.42, 0.57, and 0.69 for p_{50} = 5 Torr or 0.19, 0.34, 0.47, and 0.57 for p_{50} = 10 Torr); for example at p_{50} = 5 Torr

$$\sum_y a(x,y)w(y)f_1(y) = a(x,0.22)w(0.22)f_1(0.22) + a(x,0.42)w(0.42)f_1(0.42) + a(x,0.57)w(0.57)f_1(0.57) + a(x,0.69)w(0.69)f_1(0.69)$$

Appendix III: The Calculation of f_i as a Function of y and n

Let the oxygenation of Hb M Milwaukee by represented by the following Adair scheme:

$$\mathrm{Hb} + \mathrm{O_2} \overset{2K_1}{\rightleftharpoons} \mathrm{Hb(O_2)_1}$$

$$\mathrm{Hb(O_2)_1} + \mathrm{O_2} \overset{K_2/2}{\rightleftharpoons} \mathrm{Hb(O_2)_2}$$

We may arbitrarily set $K_1 = \alpha K_2$. Then

$$[\mathrm{Hb(O_2)_1}] = [\mathrm{Hb}]2\alpha K_2[\mathrm{O_2}] = [\mathrm{Hb}]2\alpha c \quad \text{(III.1)}$$

and

$$[\mathrm{Hb(O_2)_2}] = [\mathrm{Hb}]\alpha(K_2[\mathrm{O_2}])^2 = [\mathrm{Hb}]\alpha c^2 \quad \text{(III.2)}$$

TABLE II: Fraction of Hb M Milwaukee Existing as Zero, Singly, and Doubly Oxygenated Species, Calculated for Various Values of Hill Coefficient n and Fractional Saturation y.

n	y	f_0	f_1	f_2
1.0	0.2	0.640	0.320	0.040
	0.4	0.360	0.480	0.160
	0.6	0.160	0.480	0.360
	0.8	0.040	0.320	0.640
1.2	0.2	0.663	0.273	0.063
	0.4	0.407	0.387	0.207
	0.6	0.207	0.387	0.407
	0.8	0.063	0.273	0.663
1.4	0.2	0.692	0.216	0.092
	0.4	0.454	0.291	0.254
	0.6	0.254	0.291	0.454
	0.8	0.092	0.216	0.692

where $c \equiv K_2[\mathrm{O_2}]$. Let $[(\mathrm{Hb})] \equiv [\mathrm{Hb}] + [\mathrm{Hb(O_2)_1}] + [\mathrm{Hb(O_2)_2}]$. Then the fractions of zero, singly, and doubly oxygenated Hb M Milwaukee are given respectively by

$$f_0 = \frac{[\mathrm{Hb}]}{[(\mathrm{Hb})]} = \frac{1}{1 + 2\alpha c + \alpha c^2} \quad \text{(III.3)}$$

$$f_1 = \frac{[\mathrm{Hb(O_2)_1}]}{[(\mathrm{Hb})]} = \frac{2\alpha c}{1 + 2\alpha c + \alpha c^2} \quad \text{(III.4)}$$

$$f_2 = \frac{[\mathrm{Hb(O_2)_2}]}{[(\mathrm{Hb})]} = \frac{\alpha c^2}{1 + 2\alpha c + \alpha c^2} \quad \text{(III.5)}$$

The fractional oxygen saturation is given by

$$y = \frac{\alpha c + \alpha c^2}{1 + 2\alpha c + \alpha c^2} \quad \text{(III.6)}$$

The value of c at half saturation (c_{50}) is obtained by solving eq II.6 for c when y = 0.5

$$c_{50} = \alpha^{-1/2} \quad \text{(III.7)}$$

The value of the Hill coefficient n is given by

$$n \equiv \left.\frac{\mathrm{d}\log\left(\frac{y}{1-y}\right)}{\mathrm{d}\log[\mathrm{O_2}]}\right|_{y=0.5} = \left.\frac{\mathrm{d}\log\left(\frac{y}{1-y}\right)}{\mathrm{d}\log c}\right|_{c=c_{50}} = \frac{2}{\alpha^{1/2}+1} \quad \text{(III.8)}$$

Therefore

$$\alpha = \left(\frac{2}{n} - 1\right)^2 \quad \text{(III.9)}$$

The value of c for particular values of y and α may be obtained by inserting those values into eq III.6 and solving the resulting quadratic equation for c. Thus application of eq III.9, III.6, III.3, III.4, and III.5 in sequence yields the values of the f_i as functions of y and n. Values of f_i calculated for various values of n and y are presented in Table II.

Appendix IV: Check of Experimental Technique and Analytical Assumptions

Inositol hexaphosphate containing solutions of Hb A and Hb M Milwaukee at nominal oxygen saturations of 0.2 were prepared by mixing appropriate amounts of fully deoxygenated

TABLE III: Experimentally Measured Intensity Ratio of the α-Heme Resonance at -12 ppm to That of the β-Heme "Marker" Resonance at -18 ppm.

	Intensity Ratio, IR	
y	$IR_A(y_A)$ Hb A[a]	$IR(y)$ Hb A + Hb M Milwaukee (1:1)
0.0 (deoxy)	0.96	1.92
0.2 (nominal)	0.62	1.27

[a] Johnson and Ho, 1974.

and oxygenated solutions as described in the Experimental Section of the text. Appropriate volumes of each of these solutions were in turn mixed to yield a solution containing equal amounts of Hb A and Hb M Milwaukee at a nominal average oxygen saturation of 0.2. (In the absence of oxygen equilibrium data for either of these hemoglobins under the experimental conditions employed here, it is difficult to quantitatively estimate the fractional oxygen saturations of either species individually.)

The data of Johnson and Ho (1974) indicate that, within experimental error, oxygen binds almost exclusively to the α-hemes of normal hemoglobin up to a total saturation of $\sim$0.3 in the presence of excess Ins-P_6. Thus under these conditions the intensity of the β-chain deoxy hyperfine shifted resonance at -18 ppm will be essentially independent of oxygen saturation below $y = 0.3$ and may, therefore, be used as an intensity marker in partially oxygenated solutions of Hb A and mixtures of Hb A and Hb M Milwaukee.

The ratio of the experimentally measured intensity of the α-heme deoxy hyperfine shifted resonance at -12 ppm to that of the β-heme "marker" resonance at -18 ppm, denoted by IR, is reported in Table III (see Figures 1b and 1c). The intensity ratio at average saturation y is assumed to be a sum of contributions from α chains of hemoglobins A and M Milwaukee at their respective saturations, y_A and y_M:

$$IR(y) = IR_A(y_A) + IR_M(y_M) \quad \text{(IV.1)}$$

$$IR_A(y_A) = (1 - y_A^{\alpha})IR_A(0) = (1 - 2y_A)IR_A(0) \quad \text{(IV.2)}$$

$$IR_M(y_M) = (1 - y_M^{\alpha})IR_M(0) = (1 - y_M)IR_M(0) \quad \text{(IV.3)}$$

where $IR(y)$ is the total intensity ratio at average saturation y, $IR_A(y_A)$ is the contribution from Hb A, $IR_M(y_M)$ is the contribution from Hb M Milwaukee, and y_A^{α} and y_M^{α} are the fractional saturations of α-hemes in hemoglobins A and M Milwaukee, respectively.

Comparing the results for fully deoxygenated Hb A and fully deoxygenated Hb A + Hb M Milwaukee in Table III, we find that $IR_M(0) = 0.96$. The identity (to within experimental error) of these two intensity contributions is an indicator that the conformations of the α-chain binding sites in deoxy Hb A and deoxy Hb M Milwaukee are quite similar. By substituting this result into eq IV.2 and IV.3 and combining them with eq IV.1, we obtain

$$IR(y) = 0.96(2 - 2y_A - y_M) \quad \text{(IV.4)}$$

Since IR(0.2) is reported to be 1.27 (from Table III), eq IV.4 further reduces to

$$2y_A + y_M = 0.68 \quad \text{(IV.5)}$$

Fractional oxygen saturation is defined as the average number of bound oxygen molecules per binding site. Since there are four oxygen binding sites for each molecule of Hb A and two for each molecule of Hb M Milwaukee, the average fractional oxygen saturation of a 1:1 mixture of Hb A and Hb M Milwaukee is given as a function of the saturation of each species by

$$y = (4y_A + 2y_M)/6 = (2y_A + y_M)/3 \quad \text{(IV.6)}$$

Although the NMR results do not permit us to estimate either y_A or y_M individually, eq IV.5 does provide the value of $2y_A + y_M$, so the average oxygen saturation of the 1:1 mixture may be calculated from eq IV.6 to be 0.22. The reasonably good agreement of this value with the nominal value of 0.2 (within experimental error) establishes the self-consistency of the various assumptions made here, verifies the functional integrity of the hemoglobin solutions used as NMR samples, and validates the mixing technique as a method for obtaining NMR samples of hemoglobin whose fractional oxygen saturation is known to within a few percent.

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A Proton Magnetic Resonance Study of the Conformation of Methionine-Enkephalin as a Function of pH†

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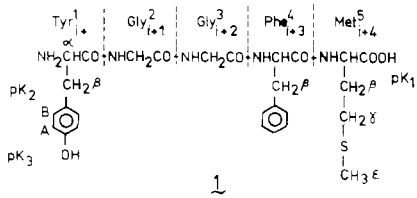
ABSTRACT: It is found that methionine-enkephalin has at least two different conformations in aqueous solution, one at low and one at high pH. From inspection of titration curves and coupling constant values, it seems reasonable to conclude that these conformations are characterized by a folding so as to bring the two ends of the molecule in close proximity. This behavior parallels that found recently in $(CD_3)_2SO$ as the solvent. It follows that the Phe-Met region of the molecule constitutes a relatively rigid region, but that the chain possesses flexibility around the first Gly residue. Possible implications of this behavior with respect to the receptor site are discussed.

It has been reported (Hughes et al., 1975) that two pentapeptides (enkephalins) act as endogenous agonists of opiates in brain. A recent report (Bradbury et al., 1976a) on binding of peptide fragments of lipotropin, which includes the Met-enkephalin (Met-E, **1**),[1] to crude opiate receptor preparations



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has further led to the suggestion that these peptides are the natural substrate for opiate receptor. As a result of the general belief that the conformation of a hormone may be related to its biological activity (Bumpus et al., 1961) and the known activity of opiates, attempts have been made to predict the conformation of Met-E and relate it to the structure and conformation of opiates (Bradbury et al., 1976b; Horn and Rodgers, 1976). The conformation of Met-E proposed by Bradbury et al. (1976b) on the basis of statistical studies (Chou and Fasman, 1974a,b) or theoretical calculations (Lewis et al., 1971) is characterized by a β turn involving the residues Tyr-Gly-Gly-Phe and a hydrogen bond between the CO of Tyr and the NH of Phe. It should be pointed out here that the theoretical calculations used in the above mentioned proposal are based on the x-ray data of proteins and enzymes and thus do not involve solvent effects. Therefore the conformation of Met-E in solution can differ from the one proposed above. However, it will be interesting to see if the conformation determined by x-ray is similar to the one predicted by these calculations or not. Horn and Rodgers (1976) have tried to predict the conformation of the tyrosine portion of enkephalins at the receptor. It is argued that, as opiates are fairly rigid molecules, the conformation obtained by x-ray data can be reasonably assumed to be similar to the one at the receptor and thus a structural and conformational correlation between the opiates and the enkephalins can reflect the conformation of the latter at the receptor. Recently we have advanced evidence (Roques et al., 1976a,b) for a β_I-bend conformation (Lewis et al., 1973) of Met-E, involving the sequence Gly-Gly-Phe-Met and a

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[1] Abbreviations used: Met-E, methionine-enkephalin; (Tyr-Gly-Gly-Phe-Met); $(CD_3)_2SO$, dimethyl-d_6 sulfoxide; NMR, nuclear magnetic resonance; Unc, uncorrected. The nomenclature used in this paper follows the rules suggested by the IUPAC-IUB Commission on Biochemical Nomenclature ((1972), *Biochemistry 11,* 1726). All optically active amino acids are in the L configuration.