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^{1}H NMR Study of Dynamics and Thermodynamics of Heme Rotational Disorder in Native and Reconstituted Hemoglobin A†

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ABSTRACT: The reaction of heme and apoprotein has been studied in detail by ^{1}H NMR spectroscopy in order to elucidate the conditions for reconstitution of hemoglobin (Hb) to yield the native protein. The initially formed holoprotein exists as a mixture of isomers with individual subunits possessing the two heme orientations differing by a 180° rotation about the α,γ-meso axis [La Mar, G. N., Yamamoto, Y., Jue, T., Smith, K. M., & Pandey, R. K. (1985) *Biochemistry 24*, 3826–3831]. We characterize in detail herein the rates and mechanism of heme reorientation and show that the rates differ dramatically for met-aquo and met-azido derivatives and are highly pH dependent in both subunits in a fashion that allows selective equilibration in either subunit. Nonequilibrium mixtures of such isomers can be kinetically trapped in the met-azido form and stored in this metastable form for many months. With kinetically controlled heme orientationally disordered Hb, unambiguous assignment of ^{1}H NMR resonances to individual subunits has been made for the met-azido derivative, which demonstrates ~2% and 10% equilibrium heme disorder in the α- and β-subunits, respectively. Comparison of the ^{1}H NMR spectra of various heme rotationally disordered Hb derivatives indicates that this disorder is observable in all forms studied, but is most easily recognized as heme disorder and most conveniently monitored in the met-azido complex. Structural consequences of heme disorder appear to manifest themselves much more strongly in peripheral than axial interactions at the heme. Preliminary studies reveal that both the rate of autoxidation of oxy-Hb and the azide affinity of met-aquo-Hb depend on the orientation of the heme.

The reaction between apohemoglobin and heme does not yield the pure native holoprotein within a few milliseconds, as originally thought (Gibson & Antonini, 1960; Rose & Olson, 1983), but affords initially a ~1:1 mixture of the holoprotein with the heme rotationally disordered with respect to the α,γ-meso axis (Figure 1) (La Mar et al., 1985). The equilibration of this metastable heme orientation to yield the structure essentially as defined by single crystal X-ray diffraction (Perutz, 1970; Fermi, 1975; Baldwin & Chothia, 1979) takes several hours to many days. Most importantly, the intermediate does not completely disappear with time, and the identification of ^{1}H NMR spectral characteristics of this intermediate in preparations of native Hb[1] A indicate that a significant degree of equilibrium heme orientation disorder exists within at least one of the subunits (La Mar et al., 1985).

The heme orientation disorder was identified in the ^{1}H NMR spectrum of freshly reconstituted Hb A in both the met-aquo and carbonyl form using the met-azido complex as the spectroscopic probe (La Mar et al., 1985). Thus the hyperfine-shifted portion of the ^{1}H NMR spectrum of freshly reconstituted metHbN_3 exhibited twice the number of peaks as observed in the native protein (Davis et al., 1969; Neya & Morishima, 1981), and assignments of the individual heme resonances using isotope labeling revealed the characteristic interchange of environments, 5-CH_3 ↔ 8-CH_3 and 1-CH_3 ↔ 4-vinyl (La Mar et al., 1978, 1980a, 1983; La Mar, 1979; Lecomte et al., 1985), predicted for the two orientations differing by a 180° rotation about the α,γ-meso axis (Figure 1). While the spectral assignment of peaks to the two heme orientations was direct and unambiguous, it was possible to

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[1] Abbreviations: Hb, hemoglobin; metHb, ferric hemoglobin; Mb, myoglobin; metMb, ferric myoglobin; NMR, nuclear magnetic resonance; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; IHP, inositol hexaphosphate; ppm, parts per million.

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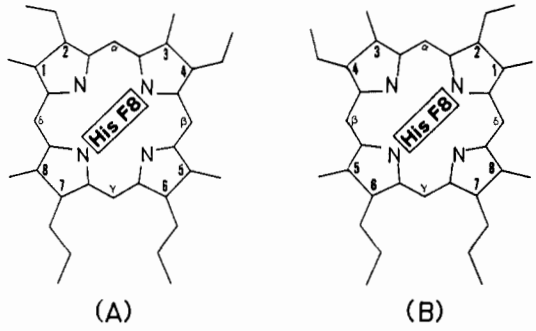

FIGURE 1: Orientation of heme relative to the proximal (F8) histidyl imidazole plane viewed from the proximal side: (A) as found in the crystal structure ("normal") (Fermi, 1975) and (B) rotated by 180° about α,γ-meso axis ("reversed").

make only tentative assignments of peaks to individual subunits in the disordered hemoglobin. Since the ^{1}H NMR spectrum of native Hb A indicates that residual disorder exists within one of the subunits (La Mar et al., 1985), it is important to unambiguously identify the subunit origin of individual peaks in freshly reconstituted Hb A.

The importance of vinyl–protein contacts in the molecular mechanism of cooperativity (Gelin & Karplus, 1977; Gelin et al., 1983) makes it desirable to determine a variety of functional properties of Hb A possessing the heme in the alternate orientation. In order to prepare such metastable species, however, we first require an understanding of the solution conditions that afford the disordered Hb A, as well as the dynamics of the equilibration to the native protein. Inasmuch as random heme orientation disorder will yield 10 nonequivalent isomeric hemoglobins with potentially distinct functional properties, it would also be desirable to generate partially equilibrated derivatives with heme orientation disorder essentially restricted to one or the other subunit. Alternatively, the desire to incorporate an isotope-labeled heme, replace the iron with another metal ion, or introduce a chemically modified heme into the native Hb tetramer (Rossi Fanelli et al., 1958; Ascoli et al., 1981) dictates that the reaction be carried out under conditions where both subunits equilibrate most rapidly.

While initial ^{1}H NMR studies clearly demonstrated some advantages of characterizing heme orientation disorder in the low-spin met-azido complex (La Mar et al., 1985), it is desirable to know if and how much heterogeneity manifests itself in the ^{1}H NMR spectra of other oxidation/ligation states of Hb (Morrow & Gurd, 1975). This is important both from the point of view of providing diagnostic methods for recognizing the phenomenon in other Hb derivatives and for potentially yielding information on the structural consequences of the reversed heme orientation. Finally, since residual heme disorder has been identified in native metHbH_2O at equilibrium (La Mar et al., 1985), it is of interest to inquire whether the currently available ^{1}H NMR methods for detecting heme disorder can also provide some direct evidence for functional consequences of heme disorder.

We present herein the results of a ^{1}H NMR investigation of reconstituted Hb A that unambiguously assign heme resonances to individual subunits in both native and disordered metHbN_3 and clearly identify the β-subunit as the origin of the dominant equilibrium heme orientation heterogeneity in Hb A. The dynamics of equilibration are shown to be pH-dependent in both subunits in a fashion that allows selective equilibration in either subunit. Furthermore the rates are found to be very different in met-aquo and met-azide Hb, so that nonequilibrium mixtures of isomers can be kinetically trapped for periods of several months. Data are presented for the influence of heme orientation on both the rate of autoxidation of HbO_2 and the azide affinity of metHbH_2O. Comparisons of the manifestation of heme disorder in various other derivatives yield information on the importance of equatorial vs. axial perturbations and confirm the advantages of monitoring heme disorder in the met-azido complex.

Experimental Procedures

Sample Preparation. Hb A was isolated and purified in the carbonyl form from whole blood obtained from a local blood center according to standard procedure (Nagai et al., 1979). Met-aquo-Hb was prepared from carbonyl Hb under a stream of O_2 with strong illumination in the presence of a 5-fold molar excess of potassium ferricyanide (Mallinckrodt Inc.). The protein was separated from the residual reagents with a Sephadex G-25 (Sigma Chemical Co.) column equilibrated with 20 mM Tris (Sigma Chemical Co.), pH 8.0, and 20 mM NaCl.

Apo-Hb was prepared from met-aquo-Hb and reconstituted with hemin (Sigma Chemical Co.) as previously described (La Mar et al., 1984, 1985). Reconstituted carbonyl Hb was prepared from met-aquo-Hb by the injection of CO gas and the addition of a 2-fold molar excess of sodium dithionite (Nakarai Chemical Ltd.). Reconstituted deoxy-, met-azido-, and met-cyano-Hb complexes were prepared by the addition of 1.5-fold molar excess of sodium dithionite, a 10-fold molar excess of sodium azide (Mallinckrodt Inc.), and a 10-fold molar excess of potassium cyanide (Mallinckrodt Inc.), respectively.

Oxy-Hb Autoxidation. For the preparation of partially autoxidized Hb samples (Mansouri & Winterhalter, 1973), oxy-Hb (or reconstituted oxy-Hb) was first prepared from carbonyl Hb (or reconstituted carbonyl Hb) in a rotary evaporator under a stream of O_2 and strong illumination. A small degree of autoxidation ($\sim$3%) was permitted to take place in the oxy-Hb sample for 30 min at 5 °C and was then quenched by the injection of CO gas into the protein solution. A 2-fold molar excess of sodium azide was added to the partially autoxidized Hb sample in order to convert only oxidized protein, namely the met-aquo subunits, to the met-azido form for NMR measurements.

Kinetic Analysis of Heme Reorientation Reaction. The time course of the heme reorientation reaction for met-azido-Hb was followed by recording ^{1}H NMR spectra as a function of time at various pH values. In order to follow the kinetics for the met-aquo form, reconstituted met-aquo-Hb was converted to met-azido-Hb by adding a 10-fold molar excess of sodium azide to quench the reaction at various time intervals at 25 °C after the reconstitution. The ^{1}H NMR spectra at 35 °C of the resulting met-azido derivative were recorded because it is the most inert form in terms of the heme reorientational process (see below). The very long half-life for equilibration in the metHbN_3 form (several months) allowed the recording of 35 °C ^{1}H NMR spectra in 15 min without allowing significant equilibration during the measuring time.

The kinetics were following by using the methyl peak areas of peaks a, A and b, B for the α and β subunits, respectively (see Figure 6), based on a reversible first-order reaction model. Peak areas were measured by fitting the experimental data to a Lorentzian line shape by utilizing the line shape fitting program, Nicolet NTCCAP. For a reversible first-order reaction, the heme reorientational reaction is expressed by

$$\mathrm{Hb(r)} \underset{k_b}{\overset{k_f}{\rightleftharpoons}} \mathrm{Hb(n)} \qquad (1)$$

where k_f and k_b are the first-order forward and reverse rates

for the reorientation, and where Hb(r) and Hb(n) represent Hb with a given heme with the "reversed" (B in Figure 1) and "normal" (A in Figure 1) heme orientations, respectively. The experimentally observed rate, k_{obsd}, is related to the population of the reversed heme orientational state by the equation (La Mar et al., 1984a)

$$\ln [(R_t - R_e)/(R_0 - R_e)] = -k_{obsd}t \qquad (2)$$

where R_0, R_t, and R_e are the molar fractions of the reversed heme orientational states at time zero, at time t, and at equilibrium (time = ∞), respectively ($R_i = [a]_i/([A]_i + [a]_i)$ for the α subunit and $R_i = [b]_i/([B]_i + [b]_i)$ for the β subunit, where $[a]_i$, $[A]_i$, $[b]_i$, and $[B]_i$ are the areas at time = i of the corresponding peaks as labeled in Figure 6. We determined 0.02 ± 0.005 and 0.10 ± 0.01 as the values of R_e for the α and β subunits, respectively (see Figure 5). The desired rate constant, k_f, is calculated from $k_{obsd} = k_f + k_b$ and $R_e = k_f/k_b$ for each subunit.

Characterization of Heme Disorder in Other Hb Derivatives. A single reconstituted met-aquo-Hb sample was split into two portions immediately after reconstitution. Excess azide was added to one portion to determine the degree of disorder using the assigned heme peaks (La Mar et al., 1985) as shown in traces A of Figures 9–11. The other portion was immediately converted to another Hb derivative of interest. After the ^{1}H NMR spectra of this Hb derivative was obtained (duration 3–72 h depending on the derivative (traces B in Figures 9–11), the Hb derivative was converted back to the met-azido complex, which yielded the ^{1}H NMR traces C in Figures 9–11. Deoxy-Hb was prepared by the addition of dithionite and reconverted to the met-azido complex by oxidation via ferricyanide in the presence of excess azide. Carbonyl-Hb was prepared by reduction with dithionite in the presence of CO. This reversion to met-azido-Hb was effected by adding ferricyanide in the presence of excess azide under strong illumination. Met-cyano-Hb was prepared by adding excess cyanide to the disordered met-aquo-Hb. The conversion back to met-azido-Hb involved first reduction by dithionite in the presence of CO to yield the carbonyl-Hb complex and removal of cyanide by chromatography, followed by oxidation with ferricyanide in the presence of excess azide under strong illumination. In the case of met-cyano-Hb, the detectable degree of equilibration (particularly in the α subunit) observed upon comparing A and C of Figure 11 can be accounted for by the equilibration that is expected in the met-azido derivative at the low pH where the sample was held for 5 h prior to recording the spectrum in Figure 11C.

NMR Measurements. All Hb samples for NMR measurements were ~0.5 mM in protein in nominal ^2H_2O (90% ^2H_2O/10% H_2O) or nominal H_2O (10% ^2H_2O/90% H_2O). The pH of a sample was determined with a Beckman 3550 pH meter equipped with an Ingold microcombination electrode; no correction was made for isotope effects in ^2H_2O solutions. ^{1}H NMR spectra of all paramagnetic complexes were recorded on a Nicolet NT-360 FT-NMR spectrometer operating in the quadrature mode at 360 MHz. Typical met-azido- and met-cyano-Hb spectra required a 12-kHz bandwidth, 8192 data points, a 7-ms 90° pulse, and ~2000 scans: The spectra for the met-azido subunits of partially autoxidized Hb samples required ~10^5 scans. The water resonance was suppressed with a 30-ms presaturation decoupler pulse. Deoxy-Hb spectra were collected with a 40-kHz bandwidth, 4096 data points, and ~4000 scans; a 100-ms presaturation decoupler pulse was used to suppress the water resonance. ^{1}H NMR spectra of carbonyl Hb were recorded on a Nicolet NT-500 FT-NMR spectrometer operating in the quadrature mode at 500 MHz, using a 7-kHz spectral width, 8192 data points, a 11-ms 90° pulse, and 2000 scans; the water resonance was suppressed with a 500-ms presaturation decoupler pulse. The signal-to-noise ratio of each spectrum was improved by apodization, which introduced 5–100 Hz line broadening, depending on the sample. Chemical shifts are given in parts per million (ppm) from 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), with ^{2}HOH as internal reference.

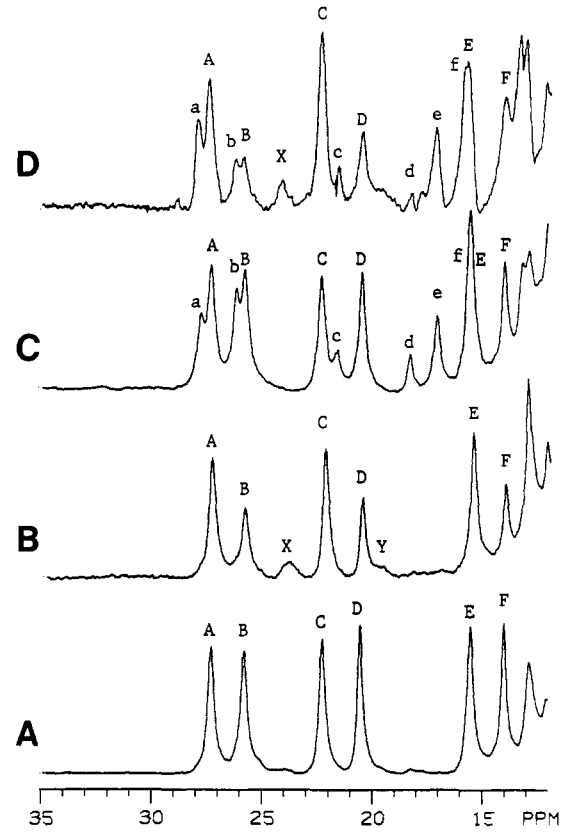

FIGURE 2: Hyperfine-shifted region of the 360-MHz ^{1}H NMR spectrum of the met-azido derivative of native and reconstituted Hb A in ^2H_2O (A, C) or H_2O (B, D), pH 7.2 at 35 °C. (A) Native HbO_2 completely converted to metHbN_3 by oxidizing with ferricyanide and adding excess azide. (B) Native HbO_2 allowed to partially autoxidize before quenching the reaction with CO by converting the remaining oxy subunit to the carbonyl derivative and the oxidized subunit to the met-azido derivative; note the larger intensity of the set of peaks A, C, and E relative to B, D, and F which assigns the former set to the more rapidly autoxidized α subunit (Mansouri & Winterhalter, 1973). (C) Freshly reconstituted Hb A sample was oxidized to metHb via ferricyanide, and azide was immediately added to yield disordered metHbN_3; peak assignments are as described in Figure 2. (D) The same reconstituted sample used to record trace C was allowed to partially autoxidize as HbO_2 before the reaction was quenched with CO by converting the remaining oxy subunits to the carbonyl derivatives; the oxidized subunits were converted to the met-azido derivatives by addition of azide. Note that the set of peaks a, c, and e is more intense than the set b, d, and f. Peak X, Y in trace B are exchangeable protons for the two subunits (Yamamoto, 1986).

RESULTS

Assignment of Peaks to Individual Subunits. The low-field portion of the 360-MHz ^{1}H NMR spectrum of native metHbN_3 at 35 °C is illustrated in spectrum A of Figure 2. In spectrum B of Figure 2, a sample of native HbO_2 was permitted to partially autoxidize for 30 min at 5 °C to form partial met-aquo-Hb (Mansouri & Winterhalter, 1973); addition of azide converted the oxidized subunits to the met-azido form, and bubbling CO through the solution to replace residual O_2 yielded the diamagnetic carbonyl derivatives of the reduced

subunits. It is readily apparent that, of the six methyl peaks, A–F (La Mar et al., 1985), three (A, C, and E) are much more prominent. Since it has previously been demonstrated by chain separation that the α subunit autoxidizes much more rapidly than the β subunit (Mansouri & Winterhalter, 1973), the assignment of peaks A, C, and E and B, D, and F to the α and β subunits, respectively, is here unambiguously confirmed.

The low-field ^{1}H NMR trace for freshly reconstituted met-aquo-Hb, trapped as the azide complex, is reproduced in Figure 2C. As discussed previously (La Mar et al., 1985), the number of peaks doubles due to heme orientational disorder about the α,γ-meso axis, with four methyl peaks, a, b, e, and f and two vinyl H_αs, c and d, resolved below 12 ppm. When a freshly reconstituted sample is converted to oxy-Hb (pH 7.2), allowed to partially autoxidize, and treated with azide and CO, the spectrum results as illustrated in Figure 2D. It is clear that the peaks due to the reversed heme orientation, a, c, and e, have larger intensities than b, d, and f in a pattern similar to that observed for A, C, and E vs. B, D, and F. This indicates that the peaks a, c, and e and b, d, and f may be associated with the two contributing subunits, with the subunit giving rise to a, c, and e autoxidizing faster. In the case of partial autoxidation, the relative intensity of peak b to B or a to A is larger (Figure 2D) than in a sample completely oxidized prior to addition of azide (Figure 2C). This greater intensity of the peaks due to the reversed heme orientation in Figure 2D suggests that the rate of autoxidation depends on heme orientation.

The influence of adding azide to native metHbH_2O is illustrated in spectra A and B of Figure 3. At low [N_3^-], the set of peaks B, D, and F is dominant, thus independently confirming the preferential azide binding to the β subunit (Gibson et al., 1969; Davis et al., 1969; Neya & Morishima, 1981). When azide is similarly added to a sample of freshly reconstituted metHbN_3 (traces C and D of Figure 3), the trace with the low [N_3^-] indicates that the set of peaks b, d, and f is more intense than the set a, c, and e for the reversed heme orientation, again indicating that the sets of peaks a, c, and e and b, d, and f are associated with the two subunits. If the reactivities of the hemes are independent of orientation (which they are not, as shown below), it would follow that a, c, and e and b, d, and f arise from the α and β subunits with reversed heme orientation.

The unambiguous assignment of the reversed heme orientation peaks can be achieved by taking advantage of the differential pH dependence of the subunit heme reorientation rates (see below). A freshly reconstituted metHbN_3 sample at pH 8.0 is shown in spectrum A of Figure 4. When a portion of the same sample is kept at pH 6.5 for 4 h, the native heme orientation peaks, A, C, and E, become larger than B, D, and F, and the reversed heme orientation peaks a, c, and e have lost intensity relative to peaks b, d, and f (Figure 4B). In contrast, if the sample is maintained at 25 °C and pH 9.2 for 4 h, peaks b, d, and f are considerably reduced in intensity compared to a, c, and e, while B, D, and F are more intense than A, C, and E (Figure 4C). In each case, the relative areas of peaks within a set remain constant (i.e., 3:3:3 for A, C, and E and B, D, and F and 3:1:3 for a, c, and e and b, d, and f), indicating that we are monitoring the differential reorientation of the heme in the two subunits. Simulation of the spectra in A, B, and C and of Figure 4 in terms of the component peaks is illustrated in A′, B′, and C′ of the same figure. The resulting composite simulation is given in A″, B″, and C″ and may be compared with the experimental traces in A, B, and C. The simulations indicate that the sum of the areas of peaks

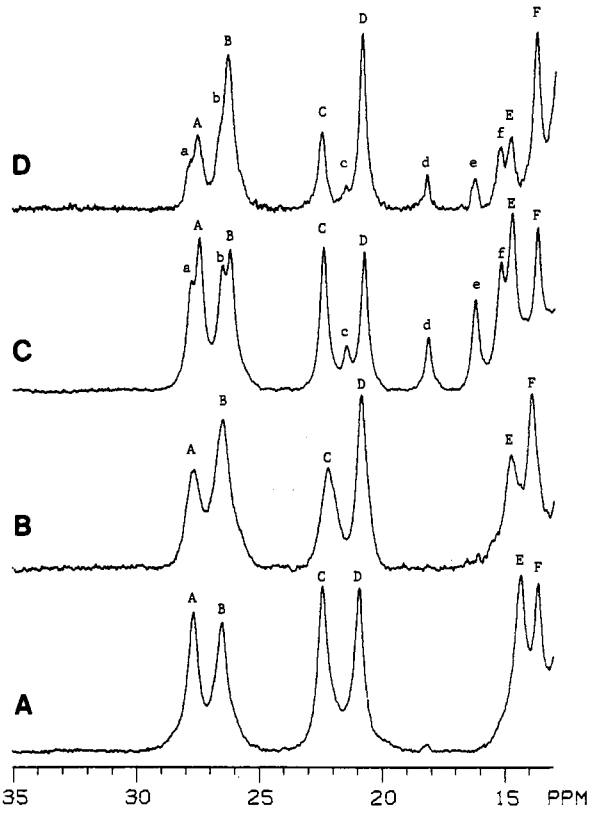

FIGURE 3: Hyperfine-shifted region of the 360-MHz ^{1}H NMR spectrum of met-aquo-Hb in the presence of azide ion in ^2H_2O, pH 8.0 at 25 °C. (A) Native met-aquo-Hb A in the presence of a 10-fold excess of azide to yield complete conversion to metHbN_3. The peaks A, B (5-CH_3), C, D (1-CH_3), andd E, F (8-CH_3) have been assigned by isotope labeling (La Mar et al., 1985). (B) Native met-aquo-Hb in the presence of 0.19 molar equiv of azide leading to partial conversion to the met-azido complex: note the greater intensity of the set of peaks B, D, and F relative to A, C, and E, allowing distinction of the subunits on the basis of the proposed greater azide affinity of the β subunit (Gibson et al., 1969; Davis et al., 1969). (C) Freshly reconstituted met-aquo-Hb A in the presence of a 10-fold excess of azide to yield the complete conversion to disordered metHbN_3. The new peaks attributed to the reversed heme orientation have been assigned a, b (8-CH_3), c, d (4-H_α), and e, f (5-CH_3) (La Mar et al., 1985). (D) Freshly reconstituted met-aquo-Hb A in the presence of 0.15 molar equiv of azide to yield partial conversion to the disordered met-azido complex: note the greater intensity of peaks b, d, and f relative to peaks a, c, and e.

a and A (or b and B, e and E, f and F) remain constant during equilibration. This combined evidence thus clearly demands that peaks A, C, and E and a, c, and e arise from one (the α) subunit and B, D, and F and b, d, and f from the other (the β) subunit. This completes the unambiguous assignment of the resolved heme peaks to individual subunits for both the native and reversed heme orientation.

The analysis of the ^{1}H NMR spectrum of native (equilibrated) metHbH_2O trapped as the azido complex (spectrum A in Figure 5) in terms of the degree of heme disorder within each subunit can now be effected by a quantitative simulation of all assigned resonances. The component sets of peaks (La Mar et al., 1985) [A(5-CH_3^α), C(1-CH_3^α), E(8-CH_3^α)], [B-(5-CH_3^β), D(1-CH_3^β), F(8-CH_3^β)], [a(8-CH_3^α), c(4-CH^α), e(5-CH_3^α)], and [b(8-CH_3^β), d(4-CH^β), f(5-CH_3^β)] in the relative ratios 0.98:0.90:0.02:0.10 (trace B) yield the simulated spectrum as illustrated in spectrum C of Figure 5. The broad peaks, X and Y, arise from exchangeable protons of the two subunits (Yamamoto, 1986).

Dynamics of Heme Reorientation. The evolution over time of the hyperfine-shifted portion of the 360-MHz ^{1}H NMR of

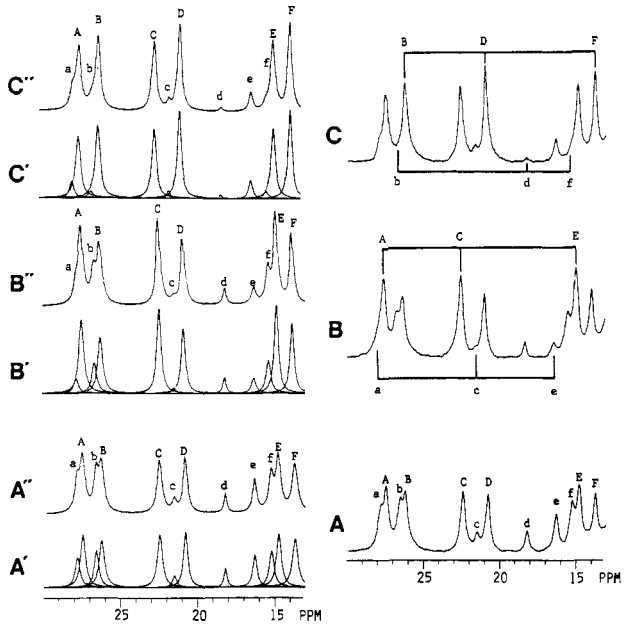

FIGURE 4: Hyperfine-shifted region of the 360-MHz ^1H NMR spectra of reconstituted Hb allowed to partially equilibrate in ^2H_2O at 25 °C as the met-aquo complex at different pH values before the reaction is quenched by adding azide to yield partially disordered metHbN_3. (A) Freshly reconstituted met-aquo-Hb A completely converted to the met-azide complex at pH 8.0 within 5 min to yield the disordered protein. (B) The same reconstituted sample allowed to equilibrate 4 h at pH 6.5 before adding azide and readjusting the pH to 8.0. Note the much larger change in the relative intensities of a, c, and e vs. A, C, and E (the α subunit) than for b, d, and f vs. B, D, and F (β subunit). (C) Same reconstituted sample allowed to equilibrate 4 h at pH 9.2 before adding excess azide and readjusting the pH to 8.0. Note the much larger change in the intensity ratio of peaks b, d, and f vs. B, D, and F (β subunit) than for a, c, and e vs. A, C, and E (α subunit). The traces in A′, B′, and C′ represent the component peaks and A″, B″, and C″ the resultant computer-simulated spectra to fit the experimental spectra in A, B, and C. In each case an excellent fit was obtained on the basis of fixed relative intensities within the sets of peaks A, C, and E and B, D, and F (each 3:3:3) and a, c, and e and b, d, and f (each 3:1:3) and the same line widths in each trace. The relative proportions of the four sets of peaks a, c, and e to A, C, and E and b, d, and f to B, D, and F are 0.38:0.62 and 0.44:0.46 in traces A → A″, 0:12:0.88 and 0.38:0.62 in traces B → B″, and 0.19:0.81 and 0.08:0.92 in traces C → C″.

freshly reconstituted Hb A in the met-azido derivative at pH 9.2 and 25 °C indicates that the β subunit equilibrates faster at alkaline pH (Figure 6). The simulation of the peak areas for A, B, a, and b as a function of time are given to the left of each trace. Plots analyzing the rate of conversion of a → A (or b → B) on the basis of reversible first-order kinetics (eq 2) are illustrated in Figure 7; similar data are included for metHbN_3 equilibration at pH 6.5 and 25 °C. The same measurements were made on samples of reconstituted Hb A allowed to equilibrate as metHbH_2O at 25 °C, to which was added N_3^- prior to recording the ^1H NMR spectrum. The forward rates of heme reorientation for both Hb derivatives are plotted as a function of pH in Figure 8. The rates are slowest near neutral pH, accelerating rapidly at both acidic and alkaline pH. The minimum rates for the two subunits are essentially the same in each derivative, but the pH value at which this is observed is lower in the β than α subunit.

The addition of 10 equiv of inositol hexaphosphate (IHP) to a freshly reconstituted metHbH_2O sample at pH 6.5 yields a rate for equilibration in the α subunit that is similar to that in the absence of IHP. The rate of equilibration in the β subunit, on the other hand, is reduced by approximately a factor of 10 as illustrated by a comparison of the rate data in Table I with and without IHP.

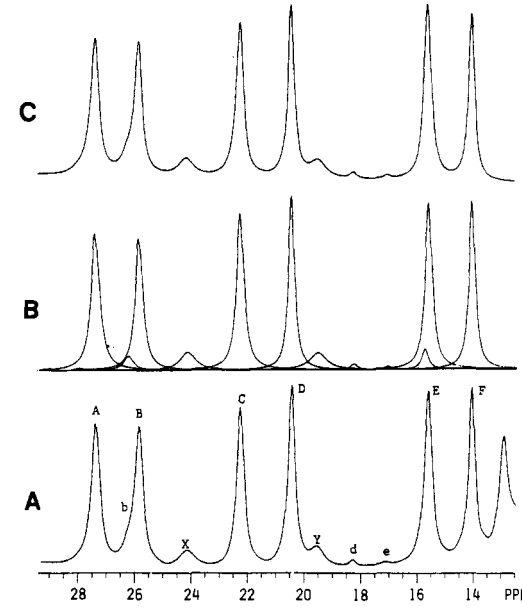

FIGURE 5: Hyperfine-shifted spectra of native (equilibrated) metHbH_2O converted to the met-azido form in H_2O at pH 7.8 and 33 °C. (A) Experimental spectrum with very high signal-to-noise. (B) Component sets of peaks A, C, and E (native heme orientation for α subunit; relative areas 3:3:3), B, D, and F (native heme orientation of β subunit; relative areas 3:3:3), a, c, and e (reversed heme orientation for α subunit; relative areas 3:1:3), and b, d, and f (reversed heme orientation for β subunit; relative areas 3:1:3). (C) Composite simulation of the experimental spectrum on the basis of the component peaks in B. The ratios of intensities of set of peaks are a, c, and e to A, B, and C, 0.02:0.98, and b, d, and f to B, D, and F, 0.10:0.90. Peaks X and Y are exchangeable protons for the two subunits (Yamamoto, 1986).

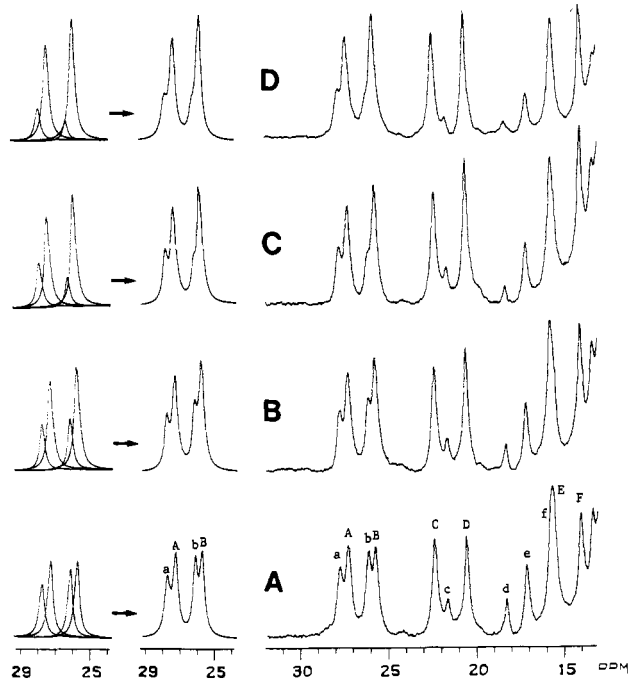

FIGURE 6: Hyperfine-shifted portion of the 360-MHz ^1H NMR spectrum of freshly reconstituted met-azido-Hb, pH 9.2, recorded at 35 °C. The sample was equilibrated at 25 °C and the spectra were recorded at various time intervals after reconstitution; (A) 5 min; (B) 3 days; (C) 9 days; (D) 29 days. Computer-simulated spectra (individual peak simulation and composite simulation) for the four peaks a, A, b, and B are shown to the left of each spectrum. The β subunit equilibrates faster than the α subunit at alkaline pH.

Plotting the rates of equilibration of metHbH_2O (trapped as metHbN_3) at pH 7.2 and 5, 25, and 37 °C yields linear

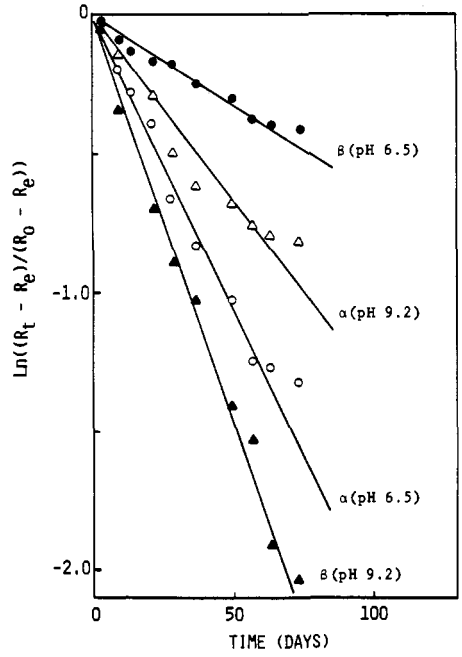

FIGURE 7: Plot of ln $[(R_t - R_e)/(R_0 - R_e)]$ vs. time for the equilibration of the heme reorientation reaction of freshly reconstituted met-azido-Hb at 25 °C, pH 6.5 (α (○) and β (●) subunits) and pH 9.2 (α (△) and β (▲) subunits). The slope yields k_{obsd} (eq 2).

Table I: Influence of IHP on Rate of Heme Reorientation in Reconstituted Met-aquo-Hb, pH 6.5, at 25 °C

	k_f (h^{-1})		k_f (with IHP)/k_f (without IHP)
	without IHP	with IHP[a]	
α-subunit	$(2.5 \pm 0.4) \times 10^{-1}$	$(2.4 \pm 0.5) \times 10^{-1}$	1.0 ± 0.3
β-subunit	$(7.2 \pm 1.0) \times 10^{-2}$	$(7.5 \pm 2.0) \times 10^{-3}$	0.10 ± 0.04

[a] IHP was added in a 10-fold molar excess relative to Hb.

Arrhenius plots (not shown) with apparent activation energies of 19 ± 4 and 12 ± 3 kcal/mol for the α and β subunits, respectively.

Detection of Heme Disorder in Other Oxidation/Ligation States. The manifestations of heme disorder in other Hb derivatives can be conveniently monitored by converting a portion of a given freshly reconstituted metHbH_2O sample to metHbN_3 to characterize the degree of heterogeneity. Another portion of the same sample is converted to the Hb derivative of interest in a time very short compared to the rate of equilibration. After a period of time, the Hb derivative is reconverted back into metHbN_3; this allows verification that a negligible degree of equilibration has occurred during the time in the state of interest.

Figure 9B illustrates the ^{1}H NMR trace of disordered deoxy-Hb A, with the degree of disorder clearly visible in the trace of metHbN_3 (Figure 9A). After 5 h at 25 °C as deoxy-Hb, the sample was oxidized and azide added to yield trace C; the degree of disorder has changed insignificantly. The spectrum of the disordered deoxy-Hb (Figure 2B) is compared to that of native deoxy-Hb (Figure 2D). While no clean doubling of peaks can be recognized in the region 25–10 ppm, considerable broadening and a loss of the distinct methyl features at 22, 15, and 12 ppm (Davis et al., 1971; Lindstrom et al., 1972) is observed. The shifts of the exchangeable protons of the proximal (F8) histidine rings in the region 60–80 ppm (La Mar et al., 1977, 1980b; Takahashi et al., 1980) are unaltered in the disordered sample, although the signals appear slightly broader (~10%) in the freshly reconstituted sample.

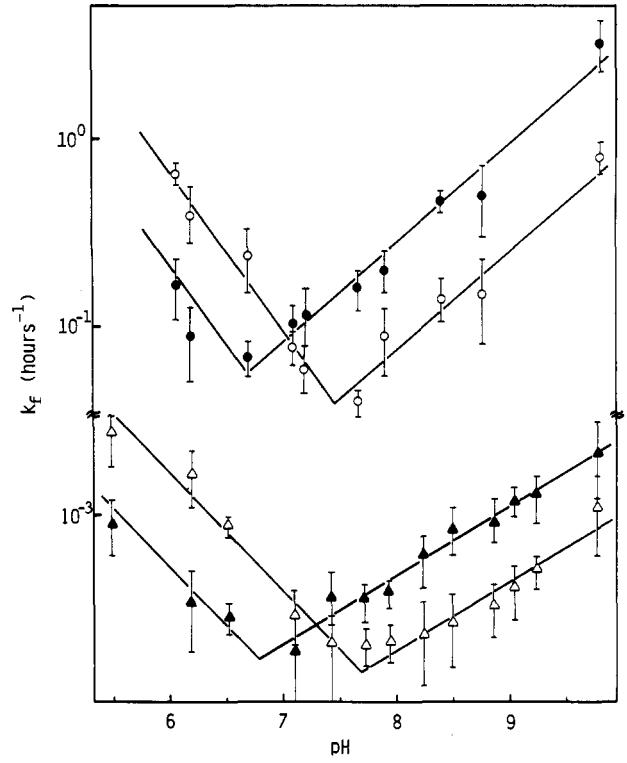

FIGURE 8: pH–rate profiles for the heme reorientation of the α (○) and β (●) subunits in reconstituted met-aquo-Hb at 25 °C and the α (△) and β (▲) subunits for reconstituted met-azido-Hb at 25 °C. The lines through the data points only indicate the qualitative trend of the pH–rate profiles.

The ligation of CO to a disordered deoxy-Hb A sample yields the ^{1}H NMR spectral features of the heme meso-Hs (9.5–11.0 ppm) and ring-current-shifted distal Val E11 region (0 to −2 ppm) illustrated in Figure 10B (Lindstrom et al., 1972a,b; Dalvit & Ho, 1985). Regeneration of metHbN_3 from HbCO after 3 h at 25 °C indicates that negligible equilibration has occurred (Figure 10C). Comparison of the disordered HbCO (trace B) with native HbCO (trace D) reveals significant changes in the region 10.5–9.0 ppm, featuring several new peaks in the disordered sample not observed in the native protein (Dalvit & Ho, 1985). There is no simple doubling of peaks in the disordered sample. The disordered sample exhibits no new peaks in the upfield region, although the peak at −1.8 ppm is broadened ~20% and there is a general loss of resolution in the region upfield of DSS as compared to native HbCO.

The replacement of azide (Figure 11A) by higher affinity cyanide ligand in disordered metHb leads to numerous new resonances (Figure 11B). In the region 15–20 ppm, where the two 5-CH_3s of the native protein resonate (Jue, 1983), we observe four peaks of similar relative intensities to those observed in the met-azide complex before (trace A) as well as after (trace C) existing as metHbCN for 72 h at 25 °C. Comparison of the trace of native metHbCN (trace D) to that of disordered metHbCN (trace B) indicates that the disorder is clearly detected as well in this protein complex. In fact, the apparent "impurity" peaks a and c in the native protein with ~0.2 proton intensity (compared to methyl peak A or B) are also observed in the disordered sample and indicate that the reversed heme orientation exists in the met-cyano complex.

Discussion

Location of the Equilibrium Heme Disorder. We have established that peaks a, c, and e, and b, d, and f originate from the α and β subunits, respectively, of metHbN_3 with the

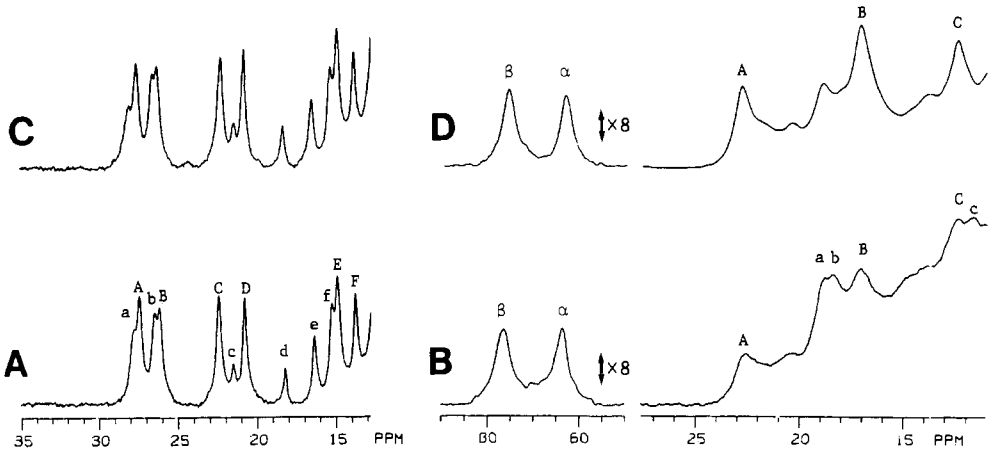

FIGURE 9: Hyperfine-shifted region of the 360-MHz ^1H NMR spectra of met-azido- and deoxy-Hbs at 25 °C. (A) Freshly reconstituted met-aquo-Hb converted immediately to met-azido-Hb in ^2H_2O, pH 7.5. (B) The same reconstituted met-aquo-Hb used in trace A but reduced immediately to yield disordered Hb A in H_2O, pH 7.5. (C) Met-azido-Hb in ^2H_2O, pH 7.2, converted from the deoxy-Hb sample used to record trace B. (D) Native deoxy-Hb in H_2O, pH 7.2.

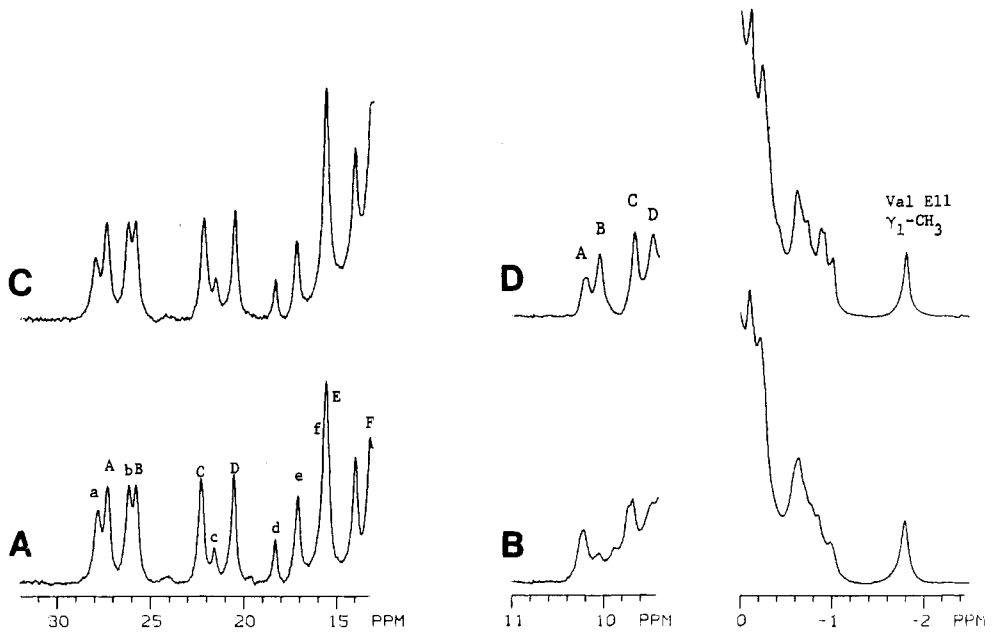

FIGURE 10: Hyperfine-shifted region of the 360-MHz ^1H NMR spectra of met-azido-Hb in ^2H_2O, pH 7.8, at 35 °C and the 500-MHz ^1H NMR spectra of carbonyl Hb in ^2H_2O, pH 7.2, at 35 °C. (A) Freshly reconstituted met-aquo-Hb immediately converted to met-azido-Hb in ^2H_2O, pH 7.8. (B) Carbonyl Hb in ^2H_2O, pH 7.2, prepared from the same reconstituted met-aquo-Hb sample used to record trace A by the immediate reduction with dithionite under CO. (C) Met-azido-Hb in ^2H_2O, pH 7.8, converted from the carbonyl Hb used to record trace B. (D) Native carbonyl Hb in ^2H_2O, pH 7.2.

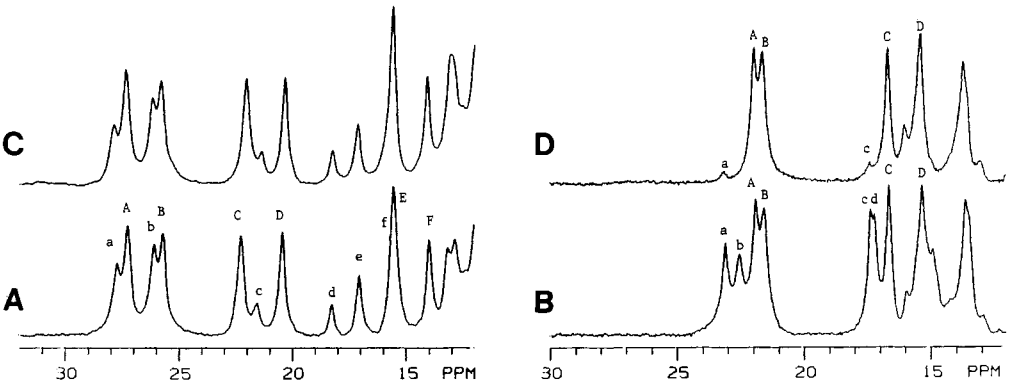

FIGURE 11: Hyperfine-shifted region of the 360-MHz ^1H NMR spectra of met-azido- and met-cyano-Hbs. (A) Freshly reconstituted met-aquo-Hb immediately converted to met-azido-Hb in ^2H_2O, pH 7.8 at 35 °C. (B) Met-cyano-Hb in ^2H_2O, pH 7.7 at 25 °C, prepared from the same reconstituted met-aquo-Hb sample used to record trace A, by adding cyanide. (C) Met-azido-Hb in ^2H_2O, pH 7.8 at 35 °C, converted from met-cyano-Hb used to record trace C (see experimental procedures). (D) Native met-cyano-Hb in ^2H_2O, pH 8.0 at 25 °C.

heme oriented as in spectrum B in Figure 1 (i.e., reversed from that found in single crystal of Hb (Fermi, 1975; Baldwin & Chothia, 1979)). Only peaks b, d, and f exhibit significant intensity in native metHbN$_3$ (La Mar et al., 1985), indicating that the major equilibrium disorder in Hb resides in the β subunit. Simulation of the metHbN$_3$ spectrum (prepared from native metHbH$_2$O) yields the heme orientational ratios favoring the crystallographically characterized form in ratios of ~50:1 and 10:1 for the α and β subunits, respectively (Figure 5). Thus the native Hb tetramer consists of the three dominant complexes, $\alpha_2\beta_2$, $\alpha\bar{\alpha}\beta_2$, and $\alpha_2\beta\bar{\beta}$ (α,β and $\bar{\alpha}$,$\bar{\beta}$ reflect heme orientations as in spectra A and B of Figure 1, respectively), in the relative proportion of 88%, ~2%, and 10%, respectively.

Functional Consequences of Heme Disorder. To date, there is no data available addressing whether heme disorder influences either intrinsic affinity of individual subunits or cooperativity for oxygen binding, although such studies are in progress (R. Weber, personal communication). The importance of the vinyl contacts with protein side chains, particularly Val-FG5, in the proposed molecular model of cooperativity (Perutz, 1970; Gelin & Karplus, 1977; Gelin et al., 1983) strongly suggests that at least the heme–heme interaction should be modified if the pertinent 4-vinyl group is replaced by the smaller 1-CH$_3$ in the reversed heme orientation (Figure 1).

Some preliminary aspects of the influence of heme orientation on heme reactivity are indicated by the data in Figures 2 and 3. The set of peaks a, c, and e is more intense than the set b, d, and f in the partially autoxidized disordered HbO$_2$ sample converted to the N_3^-/CO hybrid (Figure 2D). The independent assignment of a, c, and e and b, d, and f to the reversed heme orientations of the α and β subunits, respectively, indicates that the rate of autoxidation is faster in the α than the β subunits for both heme orientations (Mansouri & Winterhalter, 1973). However, a disordered HbO$_2$ sample allowed to partially autoxidize (Figure 2D) yields greater peak intensities for a, c, and e and b, d, and f relative to A, C, and E and B, D, and F than those observed in an identical sample of HbO$_2$ converted immediately and completely to metHbN$_3$ (Figure 2C). From this we conclude that *the rate of autoxidation is slightly faster for the reversed than the native heme orientation* for both subunits.

Similarly, the larger intensity of the met-azido subunit peaks, b, d, and f relative to a, c, and e of disordered metHbH$_2$O with low azide concentration (Figure 3D) confirms that the β subunit has the greater azide affinity for both reversed and native heme orientations (Gibson et al., 1969; Davis et al., 1969). However, the relative intensity of the β subunit native heme orientation peaks (i.e. peak D = 1-CH$_3$) is much larger relative to the β subunit peak for the reversed heme orientation (i.e. peak d = 4-H) at low azide concentration (Figure 3D) than in the same sample with high azide concentration (Figure 3C). *The azide affinity in the β subunits is therefore significantly lower for the reversed than the native heme orientation.* It is thus reasonable to predict physiological consequences of heme rotational disorder as well.

Dynamics and Mechanism of Heme Reorientation. The rates of heme reorientation for both subunits in both metHbH$_2$O and metHbN$_3$ exhibit base and acid catalysis, as previously reported for sperm whale metMbH$_2$O (La Mar et al., 1984a). The pH for the minimum rate, however, differs for the two subunits, being lower in the β (6.7–6.8) than in the α subunit (7.5–7.7) in both forms. The relationship between the relative importance of base and acid catalyses in the rate of heme reorientation has not been addressed previously; the present data may provide some insight. The isoelectric points, PIs, of the separated oxy α and β chains of Hb are 7.8 and 6.7, respectively (Winterhalter & Colosimo, 1971). While such values cannot be determined for the individual subunits in Hb A, they are unlikely to be altered significantly in the tetramer assembly. Thus the difference between the subunits in the pH required for the slowest rate may reflect their effective PIs in the tetramer. This suggests that the rate of heme reorientation is slowest when the protein (or subunit) is neutral, with the relative importance of the acid- and base-catalyzed rates determined by the net charge on the subunit. Since the rates of heme reorientation in the two subunits are essentially the same at their minimum value, we conclude that the inherent dynamic stabilities of the heme pocket are similar in the two subunits in both Hb derivatives.

The differential rate of heme reorientation observed for the two subunits thus reflects solely the differential pH profiles. The main advantage of this differential pH sensitivity of the reorientation is that it will allow preparation of samples with variable selected degrees of disorder in individual subunits. Thus partial equilibration of freshly reconstituted Hb at extreme acidic (or alkaline) pH can be made to yield samples with residual heme disorder restricted essentially to the β (or α) subunits. This will limit the number of structural isomers of Hb and allow more systematic correlations between physicochemical properties and degree of disorder in the respective subunits.

The rate of heme reorientation in metHbH$_2$O is slightly faster than in metMbH$_2$O at neutral pH (La Mar et al., 1984a). Replacement of H$_2$O with N_3^- to yield the low-spin derivative decreases the rate by $\sim 3 \times 10^2$ in Hb. This ratio of rates is essentially the same as found for the same two complexes of Mb (La Mar et al., 1984a; Yamamoto, 1986). These results indicate that quaternary structure is unimportant relative to the nature of the axial ligand in determining the rate of heme orientation. The qualitative rates of equilibration in the physiologically more relevant deoxy-Hb and HbO$_2$ (or the better characterized HbCO) are between those of metHbH$_2$O and metHbN$_3$, with half-lives exceeding 20 days at 5 °C near neutral pH, as reported earlier (La Mar et al., 1985).

The heme reorientation rate is sufficiently rapid in the met-aquo form (half-life of 1–10 h) to achieve a desired distribution of heme disorder in a time scale consistent with the stability of this derivative. Simple addition of azide and resetting the pH toward neutrality will trap this nonequilibrium mixture of isomers with a half-life ~150 days, making possible the preparation and storage of samples with controlled heme disorder for extended periods of time. The chemically as well as kinetically inert disordered metHbN$_3$ sample can be converted to any desired Hb derivative within a small fraction of the half-life of the heme equilibration reaction of most Hb derivatives.

The molecular mechanism of heme reorientation is not completely clear. In sperm whale Mb, competition experiments between heme reorientation and heme replacement indicated that the former was slightly faster (La Mar et al., 1984a). This was interpreted as indicating that reorientation of the heme may occur within some form of "protein cage", i.e., the reorienting heme does not mix with a pool of free heme in solution. In the present case, a rate of 10^{-4}–10^{-5} s^{-1} at pH 7 and 37 °C has been reported for the heme dissociation for metHbH$_2$O, as measured by the abstraction by another apo-Hb (Bunn & Jandl, 1968). This is very similar to the rate

for heme reorientation presently determined for metHbH$_2$O (2×10^{-5}, 5×10^{-5} s^{-1} for α and β subunits, respectively) under similar conditions. Thus we conclude that the rate-limiting step in heme reorientation in Hb is probably the dissociation of the subunit into heme and apoprotein. The significant decrease in the heme reorientation rate in low-spin ligated Hb is consistent with the previously reported inability to abstract heme from HbO$_2$ or metHbCN by the apoprotein method within a time compatible with the stability of the apoprotein (Bunn & Jandl, 1968).

The highly selective influence of IHP on the heme equilibration rate of the β subunit suggests that the effect is due to the stabilization of the β subunit by the direct binding of IHP in the space between the β subunits (Arnone, 1972) rather than to the quaternary structional transition, R $\rightarrow$ T, induced in metHbH$_2$O by IHP binding (Perutz et al., 1974a,b). This influence of IHP, nevertheless, has the advantage of additionally retarding the equilibration of the β vs. α subunit at acidic pH, thereby allowing the introduction of additional control of differential heme reorientation in the two subunits. The reduced rate of heme reorientation in the β subunit upon binding IHP to metHbH$_2$O is also consistent with the report that such IHP binding reduces the rate of heme dissociation (Nishikura, 1977). Our results, however, suggest that the slower rate of spontaneous heme dissociation may be restricted to the β subunit.

The dissociation of heme probably occurs from some distorted structure for a given subunit. The estimated activation energies of 12–19 kcal/mol are inconsistent with global unfolding but are similar to values obtained for exchange of buried labile protons in Hb (Englander, 1972; Woodward & Hilton, 1979; Karplus & McCammon, 1981; Han & La Mar, 1986). The structural fluctuations responsible for such isotope exchanges are generally envisaged as representing only local unfolding of the protein. Since the effect of temperature on the pH profiles for heme reorientation are not known, no significance can be placed on the difference in activation energies for the two subunits.

Detection of Heme Disorder in Other Hb Derivatives. The ^{1}H NMR spectra of Hb derivatives with essentially completely disordered heme (as monitored by the ^{1}H NMR in the met-azide form both prior and after recording these spectra) are illustrated in traces B of Figures 9, 10, and 11 for deoxy-Hb, HbCO, and metHbCN, respectively. The spectra of these three complexes prepared from native Hb are shown in spectra D of each of these figures. While the ^{1}H NMR spectrum of each of the three disordered Hb derivatives exhibit clear evidence of heterogeneity when compared to that of the native proteins, the molecular nature of the heterogeneity is more difficult to recognize.

In disordered deoxy-Hb (Figure 9B), the resolved native heme methyl peaks (Davis et al., 1969; Lindstrom et al., 1972a,b; Ho et al., 1973; Jue, 1983) A, B, and C (trace D) are obscured by the appearance of some new peaks (i.e., a, b, and c in trace B) and several shoulders causing general loss of resolution in the 25–10 ppm region associated with heme side-chain resonances (Morrow & Gurd, 1975; La Mar, 1979). Clearly, there is no simple doubling of the number of peaks as observed in metHbN$_3$. In this respect, the loss of resolution in the prepared disordered deoxy-Hb A sample is consistent with our earlier conclusion concerning carp deoxy-Hb, in which the loss of resolution relative to Hb A was attributed to the presence of extensive heme disorder (La Mar et al., 1984b).

It is noteworthy that the exchangeable proton signals at 76 and 64 ppm, assigned to the ring NH of the proximal His F8 of the α and β subunit, respectively (La Mar et al., 1977, 1980b; Takahashi et al., 1980), are essentially unaffected by heme orientation. Thus, while the change in peripheral protein–heme contacts due to heme rotation about the α,γ-meso axis obviously strongly perturbs the spin delocalization pattern in the heme, the axial interaction with the histidyl imidazole, as monitored by the NH contact shift, appears essentially unperturbed (La Mar, 1979).

The ^{1}H NMR spectrum of disordered HbCO in the region where the meso-H peak (9.0–10.5 ppm) and the ring-current-shifted distal Val-E11 γ-CH$_3$ peak are resolved is illustrated in Figure 10B. In native HbCO, four peaks are observed in the region 10.5–9.0 ppm, A–D, each consisting of two meso-Hs (Dalvit & Ho, 1985). The disordered HbCO exhibits new peaks and shoulders resulting from the heterogeneity but with variable intensities. Simple relationships of peak intensities with the known degree of disorder are obscured by the overlapping of many of the possible 16 lines that would result if each heme environment is independent of that in another subunit. The clear manifestation of the heterogeneity at the heme periphery is again contrasted to the inconsequential effects of heme orientation on the environment at the distal Val E11 (Lindstrom et al., 1972), as reflected in only a slight broadening of the signal at −1.8 ppm. The reduced resolution in the region −0.4 to −1.0 ppm is similarly suggestive of only small perturbations on the distal environment in ligand Hb.

The other Hb derivative likely to provide direct information on the degree and nature of the structural consequences of heme disorder is the met-cyano complex (La Mar et al. 1978, 1980a, 1983; Lecomte et al., 1985). Disordered metHbCN exhibits four peaks, a, b, A, and B (Figure 11B) in the region where the native protein complex yields only the two 5-CH$_3$ peaks (Jue, 1983) for the α and β subunits, A and B (Figure 11D). However, two of the four peaks, a and b, are broader than A or B in either the disordered or native protein complex. The increased line widths of apparent heme methyls in disordered metHbCN may arise from partial resolution of the environment of the 10 possible structural isomers of disordered Hb, such that the environment of a given heme orientation in one subunit may sense the heme orientation in another subunit (heme–heme interaction). Experiments are in progress using ^{13}C labels that are designed to provide improved resolution and may provide more quantitative support for intersubunit interaction as influenced by heme orientation.

The fact that two of the intense peaks in disordered metHbCN, a and c in Figure 11B, are also observed with reduced intensity in native metHbCN (Figure 11D) again demonstrates that the heme orientational heterogeneity is present at equilibrium. Since the equilibrium heme disorder could be unambiguously attributed to the β subunit in native Hb A as the met-azide complex, we can confidently assign peaks a and c to the reversed heme of the β subunit in metHbCN. Further analysis must await peak assignments via isotope labels and the preparation of samples with disorder limited to one or the other subunits. It is clear that, while heme disorder is detectable in the ^{1}H NMR spectra of all Hb derivatives studied, the clearly superior resolution and available peak assignment make the met-azide complex the probe of choice for characterizing and monitoring the phenomenon in Hb.

Conclusions

The reconstitution of Hb A from heme and apoprotein to yield the "native" protein is an exceedingly slow process even under optimal solution conditions. The range of conditions and state of the heme iron dictate that the reaction be carried

out most rapidly with the ferric heme in the absence of ligand and at the pH extreme consistent with the apoprotein stability.

We conclude that the met-aquo and met-azide derivatives serve as the ideal vehicles for implementing a comprehensive study of the consequences of heme rotational disorder for a variety of physiological as well as physicochemical properties of Hb. The met-aquo form permits rapid preparation of samples with selective degrees of disorder in individual subunits, while the met-azide form kinetically traps any nonequilibrium disordered sample in a chemically inert form for both long-term storage and convenient monitoring of sample state by ^{1}H NMR. The unique advantages of monitoring heme disorder by ^{1}H NMR in any of the Hb derivatives is underscored by the complete insensitivity to heme disorder of the more conventionally recorded optical spectra (Gibson & Antonini, 1960; Rose & Olson, 1983). The rate of heme reorientation is determined by the rate of spontaneous heme release from a given subunit of Hb. This rate is differentially pH-dependent for the two subunits in a fashion that suggest that the relative importance of base- vs. acid-assisted processes is determined by the effective charge on the subunit.

The structural implications of heme disorder appear largely restricted to changes in peripheral contacts that modulate heme electronic structure. The proximal and distal interactions, on the other hand, as monitored in disordered deoxy-Hb and HbCO, respectively, appear relatively insensitive to heme orientation. This may suggest that inherent oxygen affinity of a subunit in Hb, usually thought to be controlled by a combination of distal and proximal influences (Perutz, 1976), may be less affected by heme orientation than the communication between subunits (i.e., cooperativity), which has been proposed to directly involve protein–heme interaction at the periphery (Perutz, 1970, Gelin & Karplus 1977; Gelin et al., 1983). Detectable inherent differences in heme reactivity are witnessed by the small, but distinct influences of heme orientation on both rate at autoxidation of HbO_2 and azide affinity of metHbH_2O.

ACKNOWLEDGMENTS

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Registry No. IHP, 83-86-3; HbO_2, 9062-91-3; metHbN_3, 9072-23-5; met-aquo-HbA, 61008-19-3; HbACO, 9072-24-6; N_3^-, 14343-69-2.

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