Beyer, K., & Klingenberg, M. (1983) *Biochemistry 22*, 639-645.
Bogner, W., Aquila, H., & Klingenberg, M. (1982) *FEBS Lett. 146*, 259-261.
Brandolin, G., Doussiere, J., Gulik, A., Gulik-Krzywicki, T., Lauquin, G. J. M., & Vignais, P. (1980) *Biochim. Biophys. Acta 592*, 592-614.
Chen, P. S. (1956) *Anal. Chem. 28*, 1756-1758.
Dennis, E. A., & Plückthun, A. (1984) in *Phosphorus-31 NMR, Principles and Applications* (Gorenstein, D. G., Ed.) pp 423-446, Academic Press, Orlando, FL.
Fry, M., & Green, D. E. (1980) *Biochem. Biophys. Res. Commun. 93*, 1238-1246.
Fry, M., & Green, D. E. (1981) *J. Biol. Chem. 256*, 1874-1880.
Hackenberg, H., & Klingenberg, M. (1980) *Biochemistry 19*, 548-555.
Handbook of Chemistry and Physics (1969) Chemical Rubber Co., Cleveland, OH.
Herzfeld, J., Griffin, R. G., & Haberkorn, R. A. (1978) *Biochemistry 17*, 2711-2718.
Hull, W. E., & Sykes, B. D. (1975) *J. Mol. Biol. 98*, 121-253.
Klingenberg, M., Aquila, H., & Riccio, P. (1979) *Methods Enzymol. 56*, 407-414.
Krämer, R. (1977) Thesis, Universität München.
Krämer, R., & Klingenberg, M. (1980) *FEBS Lett. 119*, 257-260.
London, E., & Feigenson, G. W. (1979) *J. Lipid Res. 20*, 408-412.
Lowry, O. R., Rosenbrough, N. J., Farr, A. L., & Randall, A. J. (1951) *J. Biol. Chem. 193*, 265-275.
Plückthun, A., & Dennis, E. A. (1982) *Biochemistry 21*, 1743-1750.
Riccio, P., Aquila, H., & Klingenberg, M. (1975) *FEBS Lett. 56*, 129-132.
Robinson, N. C., Strey, F., & Talbert, L. (1980) *Biochemistry 19*, 3656-3661.
Wells, M. A., & Hanahan, D. J. (1969) *Methods Enzymol. 14*, 178-184.
Wennerström, H., & Lindman, B. (1979) *Phys. Rep. 52*, 1-86.
Yeagle, P. L. (1982) *Biophys. J. 37*, 227-239.
Yeagle, P. L., & Romans, A. Y. (1981) *Biophys. J. 33*, 243-252.

^{1}H NMR Characterization of Metastable and Equilibrium Heme Orientational Heterogeneity in Reconstituted and Native Human Hemoglobin†

Gerd N. La Mar,* Yasuhiko Yamamoto, Thomas Jue,‡ Kevin M. Smith, and Ravindra K. Pandey
Department of Chemistry, University of California, Davis, California 95616
Received March 25, 1985

ABSTRACT: A proton nuclear magnetic resonance study of the reaction of apohemoglobin A with both oxidized and reduced hemes reveals that at least two slowly interconverting species are initially formed, only one of which corresponds to the native proteins. Reconstitutions with isotope-labeled hemes reveal that the hyperfine-shift patterns for heme resonances in the metazido derivatives differ for the two species by interchange of heme environment characteristic of heme orientational disorder about the α,γ-meso axis, as previously demonstrated for myoglobin [La Mar, G. N., Davis, N. L., Parish, D. W., & Smith, K. M. (1983) *J. Mol. Biol. 168*, 887-896]. Careful scrutiny of the ^{1}H NMR spectrum of freshly prepared hemoglobin A (Hb A) reveals that characteristic resonances for the alternate heme orientation are present in both subunits, clearly demonstrating that "native" Hb A possesses an important structure heterogeneity. It is observed that this heterogeneity disappears with time for one subunit but remains unchanged in the other. This implies that a metastable disordered state in vivo involves the α subunit and an equilibrium disordered state both in vivo and in vitro is involved within the β subunit. The presence of metastable disorder in fresh blood suggests an in vivo hemoglobin assembly from apoprotein and heme that is similar to the in vitro reconstitution process. The slow equilibration and known lifetimes for erythrocytes provide a rationalization for the presence of detectable metastable states. The implications of such heme disorder for Hb function are discussed.

Human adult hemoglobin (Hb A)[1] is at present the best, albeit incompletely, understood allosteric protein in terms of its biosynthesis, structure, and function (Dickerson & Geis, 1983). It is frequently taken as the model protein on which to test hypothetical structure–function relationships because of the detailed knowledge of its properties. The X-ray structures have provided details on the unique structures for the two functional forms of the proteins as well as for numerous nonfunctional derivatives (Perutz, 1970, 1976; Fermi, 1975; Baldwin & Chothia, 1979). In each case, a single form of the protein is obtained, at least with respect to functional consequences. Rapid interconversions among various substrates differing only slightly from that of the ground-state structure are likely, but do not significantly alter the picture derived from X-ray diffraction (Karplus & McCammon, 1981).

† This work was supported by Grants HL-16087 and HL-22252 from the National Institutes of Health.
‡ Present address: Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06511.

[1] Abbreviations: NMR, nuclear magnetic resonance; Hb A, human adult hemoglobin; Tris, tris(hydroxymethyl)aminomethane; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; DSS, 4,4-dimethyl-4-silapentane-1-sulfonate; ppm, parts per million; met-Hb, ferric hemoglobin; Mb, myoglobin.

0006-2960/85/0424-3826$01.50/0 © 1985 American Chemical Society

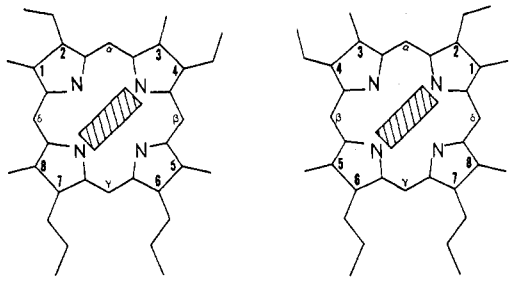

FIGURE 1: Two possible orientations of heme relative to the proximal histidylimidazole plane: (A) that found in the X-ray of Hb A (Fermi, 1975) and (B) that with the heme rotated 180° about the α,γ-meso axis from that of (A). The shaded rectangle indicates the orientation of the proximal histidylimidazole plane.

The apparently simple and instantaneous (~1 ms) in vitro reconstitution of heme and apo-Hb to yield the unique holoproteins (Gibson & Antonini, 1960; Rose & Olson, 1983) has led to the view that the last step of the biosynthesis is a similarly rapidly folding process that yields the same species as found in single crystals. Originally, similar views were held for a rapid and unique reconstitution process to yield a unique holoprotein form for mammalian myoglobin (Mb) (Gibson & Antonini, 1960; Adams, 1976, 1977). Recent ^{1}H NMR studies, however, have demonstrated that the in vitro reconstitution of Mb proceeds via a random isomeric incorporation with respect to rotation about the heme α,γ-meso axis (Figure 1) and that the alternate isomer, though less stable, was definitely present to a small degree at equilibrium (La Mar et al., 1983, 1984; Jue et al., 1983). Oxygen binding studies of the two Mb heme rotational isomers revealed distinguishable oxygen affinities (Livingston et al., 1984).

In the case of Hb A, the molecular basis of cooperativity has been proposed to involve transmission of structural information between the two types of subunits by altering important contacts between specific heme and amino acid side chains (Val FG5) (Baldwin & Chothia, 1979; Gelin & Karplus, 1977; Gelin et al., 1983). Thus the question of the uniqueness of the heme orientation in Hb A may have relevance not only for the inherent oxygen affinity for a given subunit but also for the nature of the interaction between subunits.

We present herein the results of ^{1}H NMR studies of the reaction of apo-Hb A with heme, which demonstrate that, like Mb (Jue et al., 1983) and in contrast to results of recent optical studies (Rose & Olson, 1983), the holoprotein is formed initially with disordered heme and that the equilibration is exceedingly slow. Moreover, freshly drawn blood yields Hb A indicative of a detectable population of a metastable state involving heme rotational isomerism, and, even at equilibrium, Hb A exhibits ~10% heme disorder within one of its subunits.

Experimental Procedures

Fresh human blood was drawn from one of the authors and was immediately saturated with CO to convert Hb A to the carbonyl complex to stabilize against autoxidation and to inhibit Hb isomer interconversion (see below). Carbonyl-Hb A was isolated and purified according to a standard procedure (Nagai et al., 1979). Met-azido-Hb A was prepared from HbCO under a stream of O_2 with strong illumination in the presence of a 5-fold molar excess of both potassium ferricyanide (Mallinckrodt Inc.) and sodium azide (Mallinckrodt Inc). The residual ferri- or ferrocyanide was removed on a Sephadex G-25 (Sigma Chemical Co.) column equilibrated with 20 mM NaCl, 20 mM Tris buffer (Sigma Chemical Co.), pH 8.0, and 1 mM sodium azide. All above procedures were carried out at 5 °C and completed within 36 h.

Apo-Hb A was prepared from whole blood obtained from a local blood center. Hb A was prepared and purified as the met-aquo complex by the same procedure (Nagai et al., 1979), except that azide ion was omitted. The purified met-aquo-Hb A was deionized by passage through an AG-50 1-X8 column (Bio-Rad Laboratories). The heme extraction was carried out according to the method described by Teale (1959). Apo-Hb was dialyzed exhaustively against chilled water, then once against 2 mM sodium bicarbonate (Mallinckrodt Inc.), and finally once against 30 mM Bis-Tris buffer (Sigma Chemical Co.), pH 6.9. Any precipitate was removed by centrifugation.

Apo-Hb was reconstituted with stoichiometric amounts of hemin (Sigma Chemical Co.) or specifically deuterated hemins as described by La Mar et al. (1984). The concentrations of hemin and apo-Hb were determined by optical spectroscopy (Ascoli et al., 1981). The specifically deuterated hemins are the same as those described previously (Smith et al., 1983; Smith & Pandey, 1983); their ^{1}H NMR spectra indicated >90% deuteration and >95% purity. For the reaction of apo-Hb with the reduced heme–CO complex, apo-Hb and hemin solutions were deoxygenated in airtight test tubes by consecutive N_2 gas injection and evacuation. The heme–CO complex was prepared by the injection of CO gas and a 1.5-fold excess of a 1.25 M sodium dithionite (Nakarai Chemicals Ltd.) solution into the hemin solution (Rose & Olson, 1983). The apo-Hb solution was then transferred with a syringe into the heme–CO solution. The bright red reconstituted HbCO was stirred for 5 min and then freed from excess reagents by passage through a Sephadex G-25 column equilibrated with 50 mM Bis-Tris buffer at pH 7.0.

For NMR measurements, all of the Hb A samples were converted to the met-azido complex because it is both the most convenient form of Hb for which to characterize the heme orientational disorder in its NMR spectrum and kinetically the most inert form in terms of the heme reorientation process (see below). This was done for both native and reconstituted Hb A by adding azide either immediately upon oxidation to the met-aquo complex or after controlled time lapse in the met-aquo complex under nitrogen at 25 °C. Reconstituted HbCO was converted to the met-azido complex by adding ferricyanide and azide under strong illumination.

In order to follow kinetic processes involving isomer interconversion in other derivatives of Hb A, freshly reconstituted met-aquo-Hb A was converted to deoxy-Hb A (addition of dithionite) or met-cyano-Hb A (addition of excess cyanide) or freshly reconstituted HbCO was converted to HbO_2 (under a stream of O_2 with strong illumination). The new derivatives were purified by chromatography and identified by their characteristic optical spectra (Eaton & Hofrichter, 1981). After a period of elapsed time, changes in isomer composition were followed in the ^{1}H NMR spectra of met-azido-Hb A by reconverting each sample [adding fericyanide and azide to deoxy-Hb, HbO_2, and HbCO (under strong illumination)]. The met-cyano-Hb complex was converted to the met-azido by first reducing with dithionite in the presence of CO to yield HbCO.

Hb samples for NMR measurements were concentrated to approximately 1 mM in a microultrafiltration cell (8MC, Amicon) with a Diablo membrane (YM-10, Amicon). ^2H_2O contents for H_2O and ^2H_2O samples were ~10% and >90%, respectively. The pH of a sample was determined by using a Beckman 3550 pH meter equipped with an Ingold microcombination electrode; no correction was made for isotope

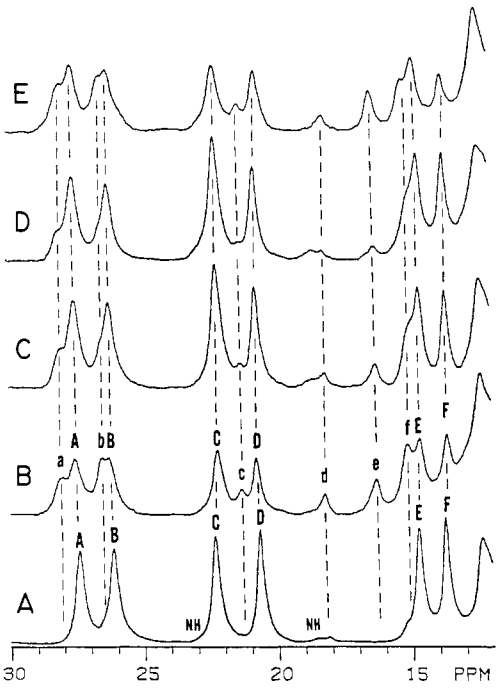

FIGURE 2: Downfield hyperfine-shifted portions of the 360-MHz ^1H NMR spectra of native and reconstituted Hb A as the met-azido complex at 25 °C. (A) Met-azido-Hb A prepared from freshly drawn blood in H_2O, pH 8.20. (B) Reconstituted met-Hb A in H_2O, pH 7.45; azide was added within 5 min of reconstitution. (C) Reconstituted Hb A in H_2O, pH 7.45; the sample was prepared as in (B), except azide was added 4 h (at 25 °C) after reconstitution. (D) Reconstituted Hb A in H_2O, pH 7.45; the sample was prepared as in (B), except azide was added 10 h (at 25 °C) after reconstitution. (E) Reconstituted Hb A in H_2O, pH 7.39; the sample was converted from reconstituted HbCO made from apo-Hb and reduced heme–CO (Rose & Olson, 1983).

effects. ^1H NMR spectra were recorded at either 25 or 35 °C on a Nicolet NT-360 FT NMR spectrometer operating in the quadrature mode at 360 MHz. Typical met-azido-Hb A spectra were obtained by using a 12-KHz bandwidth, 8192 data points, a 6-μs 90° pulse, and 3000 scans. The water resonance was suppressed with a 300-ms presaturation decoupler pulse, and the signal to noise ratio was improved by apodization, which introduced 10-Hz line broadening. Chemical shifts are given in parts per million from DSS, with ^2H_2O as internal reference.

Results

The hyperfine-shifted portion of the 360-MHz ^1H NMR spectrum of isolated met-azido-Hb A at 25 °C in H_2O is illustrated in trace A of Figure 2. In this essentially low-spin ferric protein form (Iizuka & Kotani, 1969), only three pairs of nonexchangeable peaks are resolved below 13 ppm, three from each subunit. The preferential azide binding to the β subunit of met-aquo-Hb A (Gibson et al., 1969) has allowed the assignment of the A, C, E and B, D, F methyl groups to the α and β subunits, respectively (Neya & Morishima, 1981). Isotope labeling of the equilibrated protein has revealed (Jue, 1983), as was also found in met-azido- and met-cyano-Mb (Mayer et al., 1974; La Mar et al., 1981, 1983), that the individual methyl assignments in the native protein are 5-CH_3 (A, B), 1-CH_3 (C, D), and 8-CH_3 (E, F), reading upfield, in each of the subunits. The exchangeable protons from the ring NH of His F8 in the two subunits resonate at 22.5 and 19.0 ppm (Y. Yamamoto and G. N. La Mar, unpublished results).

Trace B in Figure 2 illustrates the spectrum obtained when apo-Hb A is reacted with stoichiometric amounts of hemin to yield met-aquo-Hb A; azide ion is then added to yield the met-azido complex. In addition to the six peaks found in "native" met-azido-Hb A, we find six additional peaks, labeled a–f, of which a, b, e, and f are judged to have comparable intensity to the original methyl peaks A–F, immediately after reconstitution, and c and d appear to have less than half their intensity. Thus the initially formed protein is not homogeneous, and there is at least one other form of the protein present. The doubling of peaks is similar to that found for freshly reconstituted Mb (Jue et al., 1983; La Mar et al., 1984). If the reconstituted protein is allowed to remain as the met-aquo protein for 4 and 10 h at 25 °C and pH 7.85 before adding azide, we obtain traces C and D of Figure 2, respectively. Clearly the native peaks A–F gain intensity as a function of time at the expense of peaks a–f. Thus the species giving rise to peaks a–f is metastable and converts slowly to the form that generates A–F (native Hb A). The half-life for the reaction under these conditions is ~8 h, indicating very slow interconversion between the metastable and native forms.

The 35 °C ^1H NMR spectrum of Hb A quickly converted to the met-azido complex from HbO_2 prepared from freshly drawn blood (via HbCO) is given in trace B of Figure 3. It is compared to that of the freshly reconstituted protein in trace A of the same figure; at this temperature (35 °C), the labile proton peaks do not overlap nonlabile peaks. Important to notice is the fact that both peaks d and e are clearly detectable (peak b can also be seen as a shoulder to native compound peak B). *Thus freshly isolated oxy-Hb A contains some of the metastable species formed during the reconstitution; i.e., native Hb A is heterogeneous.* More remarkable is the observation in trace C of Figure 3 that if freshly prepared Hb A is allowed to remain as the met-aquo complex for 20 h at 25 °C before adding azide, peak e disappears while peak d and peaks b and f as shoulders (see Figures 2A and 3C) remain essentially unchanged. Oxy-Hb A samples kept in that form for a period of time before being transformed into the met-azido complex also yield spectra as in trace C of Figure 3; peak e is missing, but peak d and peaks b and f as shoulders (see Figures 2A and 3C) are detectable and at apparent equilibrium.

The identity of the metastable species is revealed by isotope-labeling studies. Reconstitution with hemin possessing a perdeuterated 5-methyl or 8-methyl group (Smith & Pandey, 1983) yields the spectra in traces B and C of Figure 4, respectively, which are compared with that of the same complex of the freshly reconstituted protein in trace A. In trace B, 5-methyl deuteration markedly decreases the signal intensity of peaks A and B in the native form and peaks e and f of the intermediate, establishing the origin of all four signals as 5-CH_3. Trace C shows that peaks a and b for the intermediate and peaks E and F for the native form arise from 8-CH_3. In both traces B and C, the presence of peaks c and d confirms that both forms are present in comparable amounts. The assignment of peaks c and d is performed at 25 °C. The trace of met-azido-Hb A reconstituted with hemin with the 4-vinyl deuterated at the α-position (Smith et al., 1983) is shown in trace E of Figure 4 where it can be compared to the trace of the freshly reconstituted protein at the same temperature (trace D). It is clear that both peaks c and d in the intermediate arise from the 4-vinyl $C_\alpha H$. The met-azido complex of native Hb A and the intermediate are thus related by the pairwise exchange 5-CH_3 $\leftrightarrow$ 8-CH_3 (A, B $\leftrightarrow$ a, b and E, F $\leftrightarrow$ e, f), as found for rotationally disordered heme in Mb (La Mar et al., 1983), monomeric Hbs (La Mar et al., 1980), and cytochrome b_5 (La Mar et al., 1981). The relationship between the native Hb peaks C and D (1-CH_3) (Jue, 1983) and the intermediate

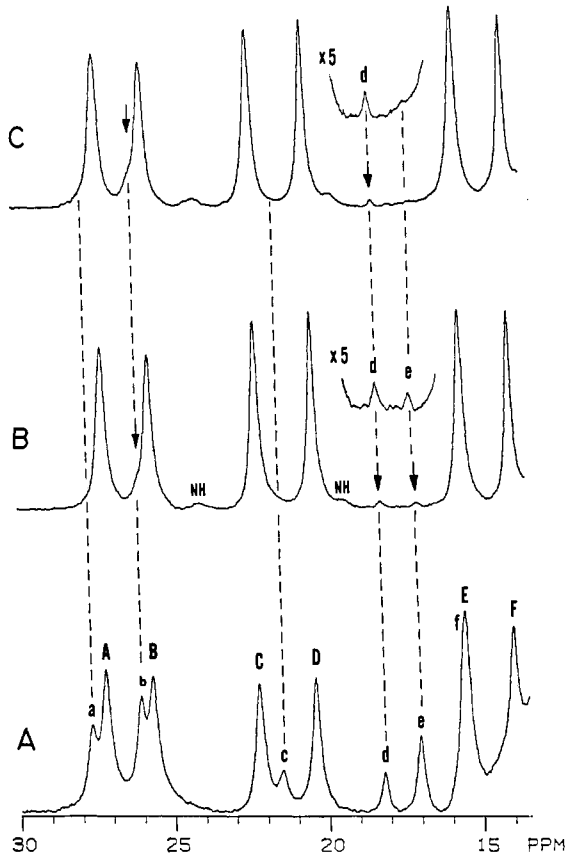

FIGURE 3: Downfield hyperfine-shifted portions of the 360-MHz ^1H NMR spectra of native and reconstituted Hb A as the met-azido complex at 35 °C. (A) Reconstituted met-aquo-Hb A converted to the met-azido complex within 5 min after reconstitution in ^2H_2O, pH 8.3 (the exchangeable proximal histidine ring NH peaks at 19.3 and 23.9 ppm are missing in ^2H_2O). (B) Met-azido-Hb A in H_2O, pH 8.03, prepared from freshly drawn blood by oxidation with ferricyanide in the presence of azide (to prevent met-aquo-Hb A formation, as described in the text). Note the obvious presence of peaks d and e and of peak b as a shoulder (arrows). (C) Met-azido-Hb A in H_2O, pH 8.03. Hb A isolated from freshly drawn blood was kept in the met-aquo form, pH 7.85, 25 °C, for 20 h, and then azide was added. Note the continued and unchanged presence of peak d (and shoulder b) but the absence of peak e.

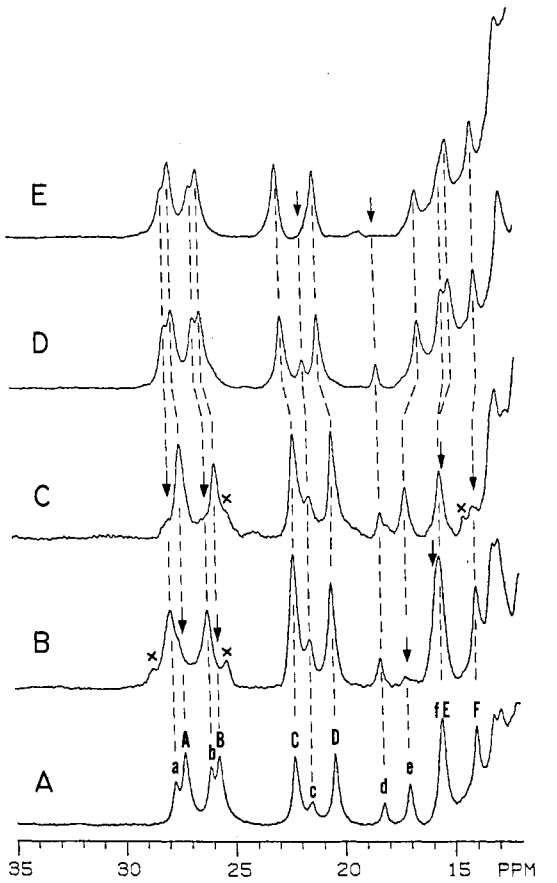

FIGURE 4: Assignments of downfield hyperfine-shifted resonances in reconstituted met-azido-Hb A using specifically deuterated hemins. (A) Reconstituted met-azido-Hb A with normal hemin, pH 8.30, at 35 °C (peaks a, b, A, and B are better resolved at that temperature). (B) Reconstituted met-azido-Hb A using 5-methyl-perdeuterated hemin in ^2H_2O, pH 8.11, at 35 °C. Decreased intensities due to deuteration are indicated by vertical arrows. Thus peaks A, B, e, and f are due to 5-CH_3. Minor peaks marked × arise from impurities. (C) Reconstituted met-azido-Hb A using 8-methyl-perdeuterated hemin in H_2O, pH 7.81, at 35 °C. Decreased intensities due to deuteration are indicated by vertical arrows. Thus peaks a, b, E, and F are due to 8-CH_3. Minor peaks marked × arise from impurities. (D) Reconstituted met-azido-Hb A using normal hemin, pH 9.24, at 25 °C (peaks c and d are optimally resolved). (E) Reconstituted met-azido-Hb A using 4-vinyl C_α-deuterated hemin, pH 9.27, at 25 °C. Decreased intensities due to deuteration are indicated by vertical arrows. Thus peaks c and d arise from 4-vinyl $C_\alpha H$.

form peaks c and d (4-vinyl $C_\alpha H$) also involves a 180° rotation about the α,γ-meso axis (see Figure 1).

When apo-Hb A is reacted with stoichiometric amounts of reduced heme–CO, the carbonyl-Hb A complex is formed (Rose & Olson, 1983), whose 500-MHz ^1H NMR spectrum reveals some peak broadening and a splitting of the Val E11 γ_1-CH_3 at −2 ppm (Lindstrom et al., 1972) but otherwise the same resonances as those of the native HbCO complex (not shown). However, if the initially reconstituted HbCO complex is placed under strong illumination (to eject CO), in the presence of ferricyanide (to form met-Hb) and azide (to yield the met-azido complex), the ^1H NMR trace observed is that shown in trace E of Figure 2, which can be compared with that resulting from direct reaction of hemin and apo-Hb (trace B in Figure 2). All of the peaks for the intermediate are present, indicative of complete heme disorder in both cases. Thus, the holoprotein formed is initially heterogeneous when either oxidized or reduced hemes are utilized.

The time course of the reorientation of the heme was followed in the met-aquo form (detecting the two forms as met-azido-Hb). The rates for the two subunits appear to differ slightly, and the half-life at pH 8 is ~8 h. If the freshly reconstituted met-aquo-Hb (which yields the met-azido spectra as shown in trace B of Figure 2) is converted into either deoxy-Hb, HbO_2, HbCO, or HbCN, kept in that form for 5 days at 5 °C, pH 7.1, and reconverted into the met-azido complex, the intensities of the set of a–f peaks relative to those of the A–F peaks are *essentially unchanged*. This dictates that the heme reorientation rates in the met-cyano form or in any of the reduced Hb forms are considerably longer than for met-aquo-Hb, with half-lives ≥30 days at 5 °C, pH 7 (we estimate <10% change in the ratio of peak a relative to peak A). Direct determination of peak areas in the met-azido-Hb complex as a function of time indicates the *slowest equilibration rate (half-life ~200 days at 25 °C, pH 7)*.

Discussion

The reaction of heme with apo-Hb leads to a heterogeneous holoprotein whether the heme is aggregated or monomeric. These conclusions are in contrast to the interpretations of stopped-flow optical studies, which concluded that the native holoprotein is formed on the millisecond time scale (Gibson & Antonini, 1960; Rose & Olson, 1983). As in the case of Mb, we conclude that the two forms so clearly seen resolved and identified by ^1H NMR in Hb are simply not distin-

guishable by optical spectroscopy, and this reinforces the necessity and tremendous advantage of studying the nature of reconstituted hemoproteins by ^{1}H NMR (Jue et al., 1983; La Mar et al., 1983, 1984).

The nature of the intermediate as the folded protein with the heme rotated 180° about the α,γ-meso axis relative to that found in the crystal is established by the peak assignments as determined by isotope labeling. Peaks with similar hyperfine shifts in each case arise from groups related by this rotation at the heme (A ↔ B in Figure 1), as found in various low-spin met-Mb derivatives (La Mar et al., 1983). For Mb, the physical basis of the heterogeneity has been independently and unambiguously established by protein–heme nuclear Overhauser effect measurements (Lecomte et al., 1985). Therefore, peaks a, b, e, and f are the four methyl and peaks c and d the two vinyl α protons of met-azido-Hb subunits with the heme rotated by 180° about the α,γ-meso axis when compared to that in crystals of Hb A (Fermi, 1975). Thus the heme–apo-Hb reaction also fails to distinguish between vinyls and methyls during the initial formation of the folded protein. The slower equilibration of the heme orientation in Hb A relative to that in Mb poses even more serious questions as to the exact nature of Hb A samples reconstituted with native or modified hemins and the interpretability of measurements made on such samples (Asakura et al., 1982).

The fact that only two sets of comparably intense methyl signals are observed in the initial product indicates that the hyperfine-shift pattern in each subunit is determined solely by the heme orientation within that subunit. Thus random heme disorder yields ten structural isomers involving permutation of heme orientations. The hyperfine shifts in met-azido-Hb A appear not to detect any influence on the hyperfine shifts of a given heme orientation in one subunit with that of the heme orientation in another subunit. [The presence of such permutational isomers is suggested in ^{1}H NMR spectra of met-cyano-Hb complexes converted from disordered met-azido-Hb A; however, except for the presence of more than the doubling of peaks, the spectra have so far precluded analysis at currently available ^{1}H NMR frequencies (Y. Yamamoto and G. N. La Mar, unpublished results).]

The peaks for the intermediate are consistent with arising from two methyl groups and one vinyl group from each of the two subunits. It is not possible to unambiguously assign the peaks to individual subunits on the basis of presently available data. The fact that the heme reorientation occurs slightly faster for one subunit than for the other (Figure 2, traces B → D) indicates that peaks a, c, and e arise from one subunit and peaks b, d, and f from the other subunit. On the basis of the fact that α-subunit peaks (A, C, E) always resonate on the low-field side of β-subunit peaks (B, D, F) in the native heme orientation (trace A in Figure 1), we make a tentative assignment of peaks a, c, e and b, d, f to the α and β subunits, respectively, with the heme orientation as in structure B of Figure 1.

The presence of peaks b and d at arbitrarily long times in met-aquo-Hb dictates that *the protein is heterogeneous at equilibrium*. An estimate of the intensities, particularly peak d (4-vinyl $C_\alpha H$), leads to ~10% disorder in *one subunit*. The absence of peak e at equilibrium dictates that a single orientation dominates (estimated ≥98%) in the other subunit. Our tentative assignment to subunits suggests that the *equilibrium disorder is solely in the β subunit*. Several spectroscopic methods have detected heterogeneity of some form for individual subunits. Resonance Raman spectra (Ondrias et al., 1982) and IR CO bands (Potter et al., 1983) have been interpreted as indicating molecular heterogeneity in the α subunit of Hb. However, the molecular nature of the heterogeneity in these cases could not be ascertained. Taken together with the different degree of heterogeneity and the different resolution times involved, it is not clear that there is any relationship between the previously reported heterogeneity and the previously established heme disorder within one of the subunits.

Whether the disorder is in the α or β subunit, either case dictates that equilibrium Hb A is heterogeneous, with at least two types of molecules. With the tentatively assigned disorder in the β subunit, this dictates that there exists ~90% $\alpha_2\beta_2$ and ~10% $\alpha_2\bar{\beta}\beta$ (where $\bar{\beta}$ indicates the β subunit with a 180° rotated heme), with a negligible amount of $\alpha_2\bar{\beta}_2$. This heterogeneity has two consequences for interpreting the properties of native Hb A; the oxygen affinity of a given subunit likely depends on heme orientation, as found for Mb (Livingston et al., 1984), and the reversed heme orientation replaces the 4-vinyl that is in contact with Val FG5 with 1-CH_3. Since the repulsive interaction between 4-vinyl and Val FG5 transmits ligation information between subunits (Baldwin & Chothia, 1979; Gelin & Karplus, 1977; Gelin et al., 1983), the effective Hill coefficient n for the "disordered" Hbs, $\alpha_2\bar{\beta}_2$ or $\alpha_2\bar{\beta}\beta$, could differ significantly from that in $\alpha_2\beta_2$.

The observation of disorder in the other (α) subunit (peaks a, c, e) in freshly prepared Hb, which disappears when converted to met-aquo-Hb or stored for long periods of time as HbO_2, suggests that *more extensively disordered Hb exists in vivo or that metastable forms are functional proteins*. If true, this argues for a last step in the biosynthesis of Hb A that is very similar to the in vitro reconstitution; namely, the rotationally disordered heme protein is initially formed. Half-lives for reorientation of >30 days at 5 °C in the physiologically active forms of Hb A, together with the known ~120-day lifetime of erythrocytes, make plausible the presence of detectable amounts of the metastable states in whole blood.

Registry No. Hb A, 9034-51-9; azidomet-Hb A, 9072-23-5; aquomet-Hb A, 61008-19-3; oxy-Hb A, 9062-91-3; carbonyl-Hb A, 9072-24-6; hemin, 16009-13-5; carbonylheme, 36883-71-3; heme, 14875-96-8.

REFERENCES

Adams, P. (1976) *Biochem. J. 159*, 371–376.

Adams, P. (1977) *Biochem. J. 163*, 153–158.

Asakura, T., Lau, P. W., Sono, M., Adachi, K., Smith, J. J., & McCray, J. A. (1982) *Hemoglobin and Oxygen Binding* (Ho, C., Ed.) pp 177–184, Elsevier Biomedical, New York.

Ascoli, F., Fanelli, M. R. R., & Antonini, E. (1981) *Methods Enzymol. 76*, 72–87.

Baldwin, J., & Chothia, C. (1979) *J. Mol. Biol. 129*, 175–200.

Dickerson, R. E., & Geis, I. (1983) *Hemoglobin: Structure, Function, Elution and Pathology*, Benjamin/Cummings Publishing Co., New York.

Eaton, W. A., & Hofrichter, J. (1981) *Methods Enzymol. 76*, 175–261.

Fermi, G. (1975) *J. Mol. Biol. 97*, 237–256.

Gelin, B. R., & Karplus, M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 801–805.

Gelin, B. R., Lee, A.-W., & Karplus, M. (1983) *J. Mol. Biol. 171*, 489–559.

Gibson, Q. H., & Antonini, E. (1960) *Biochem. J. 77*, 328–341.

Gibson, Q. H., Parkhurst, L. J., & Geraci, C. (1969) *J. Biol. Chem. 244*, 4668–4676.

Iizuka, T., & Kotani, M. (1969) *Biochim. Biophys. Acta 181*, 275–286.

Jue, T. (1983) Ph.D. Thesis, University of California, Davis.

Jue, T., Krishnamoorthi, R., & La Mar, G. N. (1983) *J. Am. Chem. Soc. 105*, 5701–5702.
Karplus, M., & McCammon, A. (1981) *CRC Crit. Rev. Biochem. 91*, 293–349.
La Mar, G. N., Smith, K. M., Gersonde, K., Sick, H., & Overkamp, M. (1980) *J. Biol. Chem. 255*, 66–70.
La Mar, G. N., Burns, P. D., Jackson, T. J., Smith, K. M., Langry, K. C., & Strittmatter, P. (1981) *J. Biol. Chem. 256*, 6075–6079.
La Mar, G. N., Davis, N. L., Parish, D. W., & Smith, K. M. (1983) *J. Mol. Biol. 168*, 887–896.
La Mar, G. N., Toi, H., & Krishnamoorthi, R. (1984) *J. Am. Chem. Soc. 106*, 6395–6401.
Lecomte, J. T. J., Johnson, R. D., & La Mar, G. N. (1985) *Biochim. Biophys. Acta* (in press).
Lindstrom, T. R., Noren, I. B. E., Charache, S., Lehmann, H., & Ho, C. (1972) *Biochemistry 11*, 1677–1681.
Livingston, D. J., Davis, N. L., La Mar, G. N., & Brown, W. D. (1984) *J. Am. Chem. Soc. 106*, 3025–3026.
Mayer, A., Ogawa, S., Shulman, R. G., Yamane, T., Cavaleiro, J. A. S., Rocha Gonsalves, A. M. d'A., Kenner, G. W., & Smith, K. M. (1974) *J. Mol. Biol. 86*, 749–756.
Nagai, K., Hori, H., Morimoto, H., Hayashi, A., & Taketa, F. (1979) *Biochemistry 18*, 1304–1308.
Neya, S., & Morishima, I. (1981) *J. Biol. Chem. 256*, 793–798.
Ondrias, M. R., Rousseau, D. L., Kitagawa, T., Ikeda-Saito, M., Inubushi, T., & Yonetani, T. (1982) *J. Biol. Chem. 257*, 8766–8770.
Perutz, M. F. (1970) *Nature (London) 228*, 726–739.
Perutz, M. F. (1976) *Br. Med. Bull. 32*, 195–208.
Potter, W. T., Hazzard, J. H., Kawanishi, S., & Caughey, W. S. (1983) *Biochem. Biophys. Res. Commun. 116*, 719–725.
Rose, M. Y., & Olson, J. S. (1983) *J. Biol. Chem. 258*, 4298–4303.
Smith, K. M., & Pandey, R. K. (1983) *J. Heterocycl. Chem. 20*, 1383–1388.
Smith, K. M., Fujinari, E. M., Langry, K. C., Parish, D. W., & Tabba, H. D. (1983) *J. Am. Chem. Soc. 105*, 6638–6646.
Teale, F. W. J. (1959) *Biochim. Biophys. Acta 35*, 543.

Articles

Vicinal Coupling Constants and Protein Dynamics†

Jeffrey C. Hoch‡
The Rowland Institute for Science, Cambridge, Massachusetts 02142
Christopher M. Dobson‡
Inorganic Chemistry Laboratory, Oxford OX1 3QR, England
Martin Karplus*
Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138
Received August 22, 1984; Revised Manuscript Received February 7, 1984

ABSTRACT: The effects of motional averaging on the analysis of vicinal spin–spin coupling constants derived from proton NMR studies of proteins have been examined. Trajectories obtained from molecular dynamics simulations of bovine pancreatic trypsin inhibitor and of hen egg white lysozyme were used in conjunction with an expression for the dependence of the coupling constant on the intervening dihedral angle to calculate the time-dependent behavior of the coupling constants. Despite large fluctuations, the time-average values of the coupling constants are not far from those computed for the average structure in the cases where fluctuations occur about a single potential well. The calculated differences show a high correlation with the variation in the magnitude of the fluctuations of individual dihedral angles. For the cases where fluctuations involve multiple sites, large differences are found between the time-average values and the average structure values for the coupling constants. Comparison of the simulation results with the experimental trends suggests that side chains with more than one position are more common in proteins than is inferred from X-ray results. It is concluded that for the main chain, motional effects do not introduce significant errors where vicinal coupling constants are used in structure determinations; however, for side chains, the motional average can alter deductions about the structure. Accurately measured coupling constants are shown to provide information concerning the magnitude of dihedral angle fluctuations.

Scalar coupling constants between protons separated by three bonds have been widely used in the conformational analysis of amino acids and peptides by nuclear magnetic resonance (NMR) (Feeney, 1975; Bystrov, 1976). Both theoretical (Karplus, 1959, 1963) and experimental (Bystrov, 1976) investigations have demonstrated that the three-bond (vicinal) coupling constant between two protons can be described by an equation of the form

$$^3J_{H\text{-}H'} = A \cos^2 \theta + B \cos \theta + C \quad (1)$$

where $^3J_{H\text{-}H'}$ is the vicinal coupling constant, θ is the H–X–

† Supported in part by grants from the National Science Foundation and the National Institutes of Health and by the Rowland Institute for Science. C.M.D. is a member of the Oxford Enzyme Group.
‡ Work initiated while the author was at Harvard University.

0006-2960/85/0424-3831$01.50/0 © 1985 American Chemical Society