This is a very large rate constant. Its size shows that the activating effect which a negatively charged oxygen substituent exerts upon electrophilic attack of H_3O^+ on carbon–carbon double bonds is very powerful indeed. This activation is many orders of magnitude greater than that of a nonionized hydroxyl group, as shown by the ratio $k_{H^+}'/k_{H^+} = (3.3 \pm 0.9) \times 10^7$.

Although this ratio is very large, it is nevertheless an order of magnitude smaller than those we have observed for other enolate ion–enol pairs.[9,30] This suggests that in this case k_H' is reduced because the enolate reaction here might be a diffusion-controlled process, an idea reinforced by the very large value of this rate constant. This rate constant is, in fact, so great as to imply that proton transfer occurs via hydrogen-bonded solvent bridges between the substrate and H_3O^+. Such a Grotthuss chain mechanism is the accepted mode of proton transfer between oxygen and nitrogen acids and bases in aqueous solution,[31] but it has heretofore been generally believed that such solvent bridges do not play a role in proton transfers involving carbon.[32]

It is possible, on the other hand, that the uncatalyzed term observed here is due to reaction by a non-diffusive process, for example, a concerted cyclic proton switch down a chain of water molecules which moves H^+ directly from enol oxygen to the β-carbon atom in a single reaction step. Another possibility which retains the general stepwise character of eq 5, but yet avoids diffusive encounter, involves ionization of the enol and then protonation on carbon of the enolate ion by the solvated proton before these ions, once formed, can diffuse apart; a "one-encounter" mechanism of this kind has been proposed for the corresponding uncatalyzed term in the ketonization of acetone enol.[33]

Acknowledgment. We are grateful to Professor William P. Jencks for pointing out the possibility of a one-encounter mechanism and to the Natural Sciences and Engineering Research Council, the donors of the Petroleum Research Fund, administered by the American Chemical Society, the Swiss National Science Foundation (Project No. 2,470-82), and the Ciba Stiftung for their financial support of this work.

Registry No. Acetophenone enol, 4383-15-7; acetophenone, 98-86-2; acetophenone enolate ion, 34172-40-2; γ-hydroxybutyrophenone, 39755-03-8.

Supplementary Material Available: Tables of rates of ketonization and bromination of acetophenone (2 pages). Ordering information is given on any current masthead page.

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Proton NMR Investigation of the Rate and Mechanism of Heme Rotation in Sperm Whale Myoglobin: Evidence for Intramolecular Reorientation about a Heme Twofold Axis

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Abstract: The characterization of the initial product upon reacting apomyoglobin with hemin is shown to be the holoprotein with the hemin 1:1 rotationally disordered about the α-γ-meso axis. Determination of the rate of equilibrium of the disordered state as a function of pH and hemin 2,4 substituents has shown that the rate is highly pH dependent with a minimum near neutral pH and that the relative rates at any pH depend critically on the 2,4 substituent; the slowest rate is observed for native hemin (2,4-vinyl), the fastest for deuterohemin (2,4-hydrogen), and intermediate for mesohemin (2,4-ethyl). Competitive reconstitution for two different hemins reveals that the forward rate is independent of the 2,4 substituent, and hemin replacement reactions yield the relative binding constants for the various hemes. These two sets of data yield the relative dissociation rates for the various hemes which are shown to be semiquantitatively the same as the heme reorientation rates, indicating that disruption of the heme cavity is the rate-determining step in the reorientation. However, competition experiments between heme reorientation and heme replacements reveal that reorientation occurs faster than replacement, dictating that the reorientation occurs by an intramolecular mechanism, i.e., without leaving a "protein cage".

Both the equilibrium structure and the mechanism of formation of myoglobin from heme and apoprotein have long been considered to be well understood. X-ray studies[2,3] have revealed a highly folded protein with a unique orientation of the heme and optical stopped-flow studies.[4,5] had indicated a single bimolecular reaction between heme and apoprotein which yielded the native holoprotein within 1 ms.

Both of these views have recently been shown to be untenable on the basis of high-resolution ^1H NMR data on sperm whale myoglobin.[6,7] First, the solution ^1H NMR spectrum clearly shows that there are two slowly interconverting protein forms in solution at equilibrium which differ in the orientation of the heme by a 180° rotation about the α-γ-meso axis[6,8] (Figure 1). The dominant (~90%) component has the same heme orientation as found

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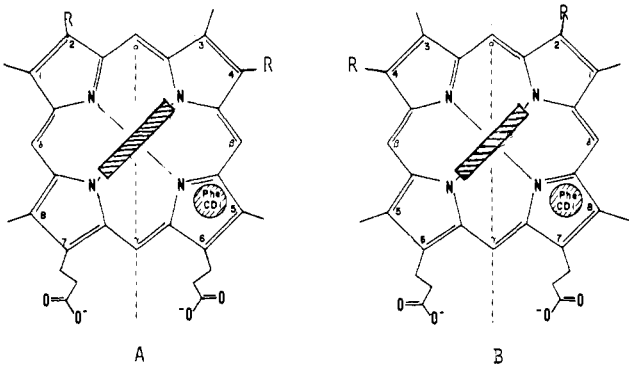

Figure 1. Orientation of heme relative to the proximal histidyl imidazole plane as (A) found in the X-ray structure[2,3] of sperm whale Mb and (B) with 180° rotation about the α-γ-meso axis.

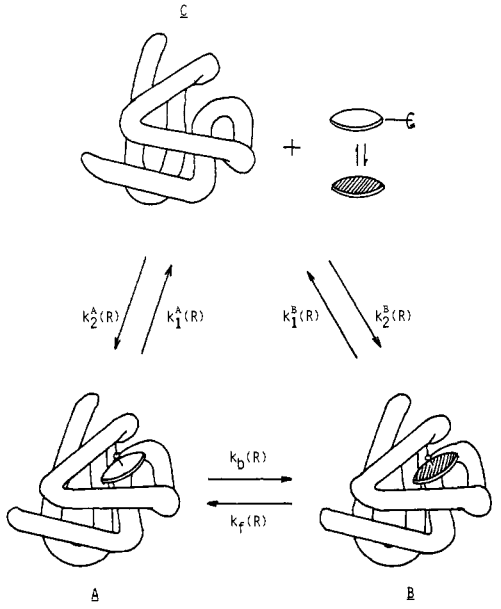

Figure 2. Schematic representation of the reversible reaction between hemin and apo-Mb. To indicate the nonequivalent sides of the heme, one side is shaded. The orientations with the unshaded (A) and shaded (B) sides showing are those depicted in A and B of Figure 1, respectively.

in the crystal (A in Figure 1). Second, the time course of the ^{1}H NMR spectrum during reconstitution of Mb with native hemin (R = vinyl in Figure 1) revealed[7] that the holoprotein is initially formed as a 1:1 mixture of the two heme orientations present at equilibrium and that the rate at which the equilibrium mixture was attained was on the hour rather than millisecond time scale.

Thus the reaction between heme and apoprotein yields two protein forms, as depicted in Figure 2, where the nonequivalent sides of the heme disc are represented as open (form A or "native") and shaded (B or "reversed"). The protein folding appears to be essentially the same for the two orientations, as indicated by their indistinguishable optical spectra[4,7] and very similar, but distinguishable, ^{1}H NMR spectra.[6,8] The apoprotein has been proposed to possess a structure quite similar to that of the holoprotein, but with reduced helical content and rigidity.[9] The reaction between apo-Mb and hemin has been shown to be second order, k_2, and the rate of dissociation can be assumed to be first order, k_1. Since forms A and B are formed initially in the holoprotein in a 1:1 ratio,[7] the forward rate constants must be the same, i.e., $k_2^A = k_2^B$. The equilibration between the two heme orientations A and B requires the rupture of both the axial histidine as well as any ligand bond, and it can be envisaged to occur either by the reversible dissociation (A $\rightleftharpoons$ C $\rightleftharpoons$ B) or by an intramolecular rearrangement (A $\rightleftharpoons$ B), as shown in Figure 2. The current view of highly flexible proteins[10] has allowed the visualization of aromatic ring reorientation[11,12] (tyrosine, phenylalanine) in folded proteins. Hemin, however, is considerably larger, and it is an intriguing question as to whether the A $\rightleftharpoons$ B conversion occurs via an intramolecular or intermolecular pathway. A second interest in the heme reorientation equilibration arises because of the potential for preparing holoproteins with nonequilibrium ratios of heme orientations which can be maintained for long enough periods so as to allow determinations of a wide variety of physicochemical properties[13] for the individual components. Preliminary results have already indicated that oxygen affinity differs significantly for the two heme orientations.[14]

We present herein the results of a ^{1}H NMR study of the mechanism of the heme reorientation reaction which demonstrates that the reaction rate is highly pH dependent, with a minimum at neutral pH which will allow maintenance of nonequilibrium mixtures for several hours. While ^{1}H NMR is capable of monitoring only the direct interconversion rate, A $\rightleftharpoons$ B, comparison of relative rates for a variety of hemins with variable 2,4 substituents, as well as monitoring the relative rates of heme equilibration vs. substitution, indicate that the pathway involves dissociation of the heme from the heme pocket, but without leaving a protein "cage".

Experimental Section

Preparation of Apo-Mb. Sperm whale myoglobin was purchased from Sigma as a lyophilized, salt-free powder. Protein (1.5 g) was dissolved in chilled water (50 mL, 4 °C), acidified to pH 2.7 with 0.1 m HCl, and treated four times with chilled 2-butanone to extract the released hemin.[15] The clear aqueous apo-Mb solution was dialyzed exhaustively against chilled water until the odor of 2-butanone could not be detected. The protein solution was then dialyzed against a chilled 50 mM phosphate buffer solution (pH 7.0) and any precipitate removed by centrifugation. The solution was again dialyzed against chilled water to remove salts and concentrated to ~30 mL by ultrafiltration. The concentrated solution was lyophilized and the powdered apo-Mb stored at −20 °C. Apo-Mb solution was prepared by dissolving the apo-Mb in chilled phosphate buffered ^2H_2O (50 mM, pH 6.6–7.2) to a concentration ~1.5 mM. Any precipitate was removed by centrifugation, and the final protein concentration was determined from the absorption intensity (15.9 cm^{-1} mM^{-1} at 280 mM).

Reconstitution of Mb. Hemin (2.0 mg) was dissolved in 0.2 M NaO^2H in ^2H_2O to yield a hemin solution of 10 mg/mL. To 0.40 mL of a 1.5 mM apo-Mb ^2H_2O solution in a 1.5-mL vial was added a molar equivalent (~40 μL) of the hemin solution (or molar equivalents of two different hemins, as needed) at 25 °C with stirring.[6,7] The pH of the solution was adjusted to the desired value by addition of either 0.1 M ^{2}HCl or 0.1 M NaO^2H. After centrifugation to remove any precipitate, the solution was transferred to a 5-mm NMR tube. The initial NMR spectrum could be collected as soon as 7 min after mixing.

Heme Displacement Reaction. The appropriate native or reconstituted and equilibrated holoprotein was dissolved (1.5 mM) in ^2H_2O containing 50 mM phosphate buffer (pH 6.9–7.0), and a molar equivalent of another hemin solution in 0.1 M NaO^2H was added.

NMR Measurements. ^{1}H NMR spectra were recorded at 25 °C on a Nicolet NT-360 FTNMR spectrometer operating in the quadrature mode at 360 MHz. For metMbH_2O samples, approximately 1000 transients (in 1.5 min) were collected over a 40-KHz bandwidth with 8192 data points; metMbCN spectra were collected over 12-KHz bandwidths with 8192 data points and a repetition rate of 1 s^{-1}. The residual water signal was suppressed in each case by a presaturation pulse from the decoupler.

Determination of the Heme Reorientation Kinetics. The time course of the reaction was followed either in the metMbH_2O form directly by

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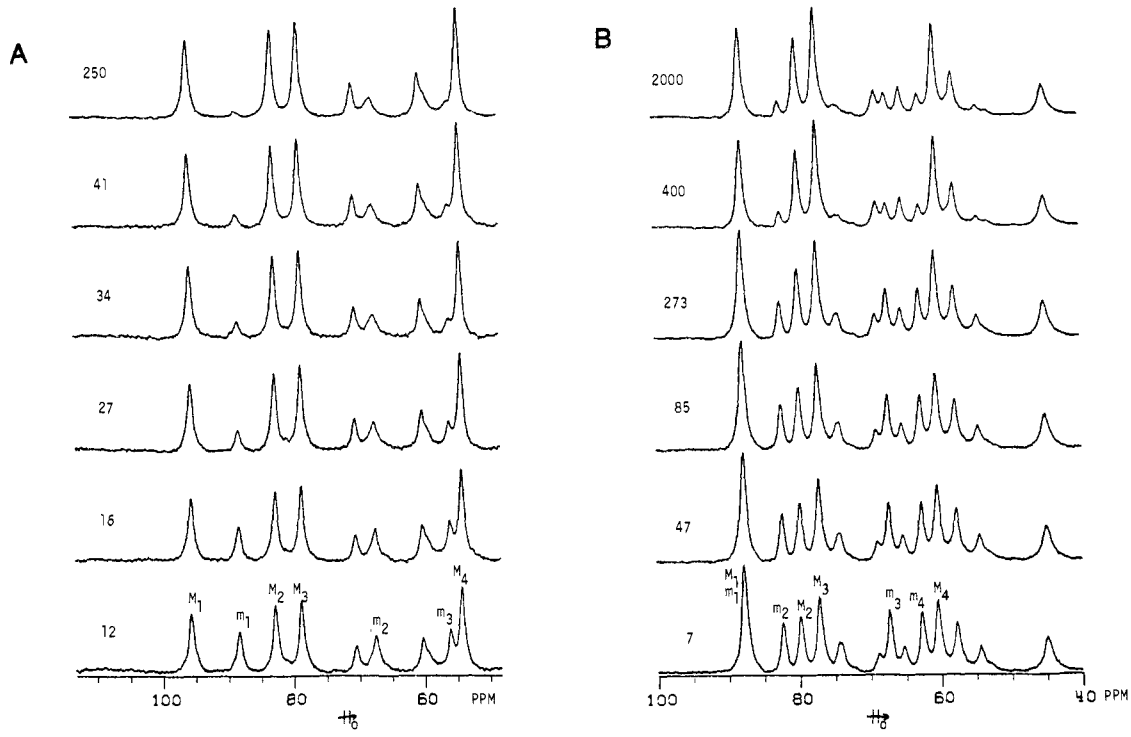

Figure 3. Low-field portions of the 360-MHz ^1H NMR spectra of the reaction products of 1 equiv each of apo-Mb and (A) deuterohemin (R = H in Figure 1) at pH 5.6 and (B) mesohemin (R = ethyl in Figure 1) at pH 6.9 in ^2H_2O. The time evolution of the heme equilibration is followed, with the elapsed time indicated in minutes at the left. The upper trace in each case is at equilibrium. The heme methyl peaks are designated M_1–M_4 and m_1–m_4 for the heme orientations[8,17] as shown in A and B of Figure 1, respectively.

detecting the previously assigned heme methyl peaks for each species,[16,17] or by rerunning a reaction repeatedly in the metMbH_2O form and then adding KCN to quench the reaction as the various metMbCN complexes. This latter "quench" method has two advantages: it leads to much more highly resolved and more completely assigned[6,8,17] ^1H NMR spectra which allow quantitative detection of four or more species, and the heme reorientation reaction in the metMbCN form is much slower (factor of ~10) than in the metMbH_2O form, allowing for the accumulation of much better spectra during a time period during which insignificant reorientation takes place. The disadvantage, of course, is that each data point requires a separate sample.

With use of the methyl peak areas for M_1 and m_1 for the native (A in Figure 1) and "reversed" (B in Figure 1) heme orientation, respectively, in the various proteins,[6,8,16,17] k_{obsd} was calculated with use of the equation

$$\ln\left(\frac{A_t - A_e}{A_o - A_e}\right) = -k_{obsd} \qquad (1)$$

where A_o, A_t, and A_e are the mole fractions of the native orientation at time zero, time = t, and at equilibrium (time = ∞), respectively ($A_i = [M_1]_i/[M_1 + m_1]_i$, where $[M_1]_i$ and $[m_1]_i$ are the intensities of the two methyl peaks at time = i). The desired k_f is related to the k_{obsd} from eq 1 via

$$k_{obsd} = k_f + k_b \qquad (2)$$

The disorder equilibrium constant for Mb reconstituted with 2,4-R_2-deuterohemin (Figure 1) is designated by $K_D(R)$ and is given by

$$K_D(R) = k_f(R)/k_b(R) = [M_1]_e/[m_1]_e \qquad (3)$$

and is obtained from the relative intensities of the methyl peaks at equilibrium. k_f is then obtained from k_{obsd} and K_D with use of eq 2 and 3.

Results and Discussion

The time evolution of the heme methyl-containing region of the 360-MHz ^1H NMR spectra resulting for the reaction of apo-Mb with deuterohemin (R = H), and mesohemin (R = ethyl), to yield the respective met-aquo complexes[16,17] is illustrated in Figure 3, parts A and B, respectively. In each case, as previously observed for protohemin[7] (R = vinyl), the holoprotein is initially found as two species whose heme methyl signals are labeled M_1–M_4 and m_1–m_4, corresponding to conformations with the heme orientation[6,8,17] as depicted in A and B, respectively, of Figure 1. In all three cases the intensity of the subset of peaks M_i increases with time at the expense of peaks m_i, finally reaching an equilibrium ratio for which we define the heme rotational disorder constant in eq 3, where $K_D(R)$ is the disorder constant for the met-aquo Mb containing the hemin with substituent R, and $[M]_e$ and $[m]_e$ are the intensities of heme methyls at equilibrium, for orientation A and B of Figure 1. The value of K_D(vinyl) obtained from a reconstituted sample has been shown[7] to be the same as that found in native metMbH_2O. It should be pointed out that conversion to the metMbCN complexes of the disordered protein has provided direct confirmation not only of the disorder but also of the proof based on methyl assignments that the major component at equilibrium corresponds to heme for all three hemins.[6,8,17] Values for $K_D(R)$ for various R are listed in Table I.

Table I. Heme Rotational Disorder Constants for Various Hemins in Myoglobin[a]

R[b]	K_D(R) (25 °C)
vinyl	11 ± 1
H	12 ± 2
ethyl	5.5 ± 0.6

[a] Defined in eq 3 in text. [b] 2,4 substituents on hemin.

Table II. Concentration Influence on Heme Reorientation Rate[a]

R[b]	protein concn, mM	pH	k_f, min^{-1}
vinyl	1.2	6.4	$(3.1 \pm 0.4) \times 10^{-4}$
	0.3	6.5	$(2.3 \pm 0.6) \times 10^{-4}$
H	1.2	8.0	$(3.4 \pm 0.4) \times 10^{-2}$
	0.3	8.0	$(2.8 \pm 0.4) \times 10^{-2}$

[a] Obtained via eq 1 and 3; 25 °C. [b] 2,4 substituents on hemin.

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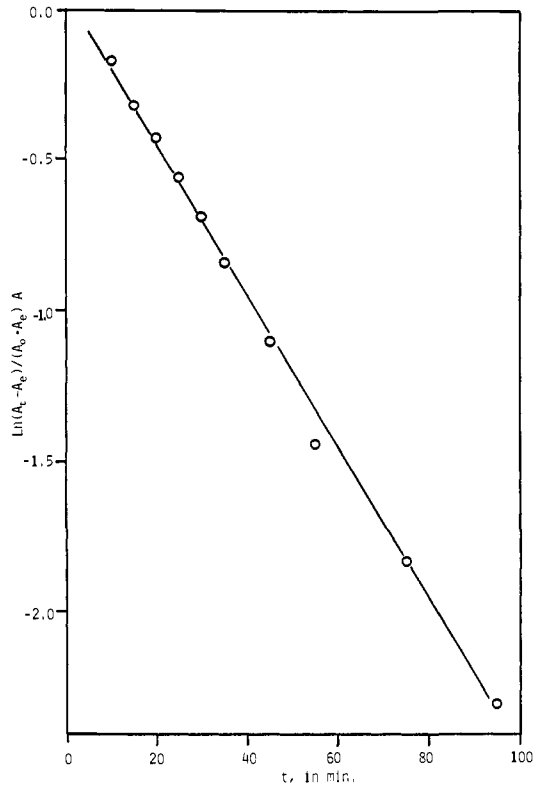

Figure 4. Plot of ln $[(A_t - A_e)/(A_0 - A_e]$ vs. time (A_0, A_t, A_e are the mole fractions in the heme orientation as in A of Figure 1, at time zero and t and at equilibrium, respectively, as determined from heme methyl intensities), for the equilibration of 1.5 mM deuterohemin–metMbH$_2$O in ^2H_2O, at 25 °C, pH 5.6. The straight line yields k_{obsd}.

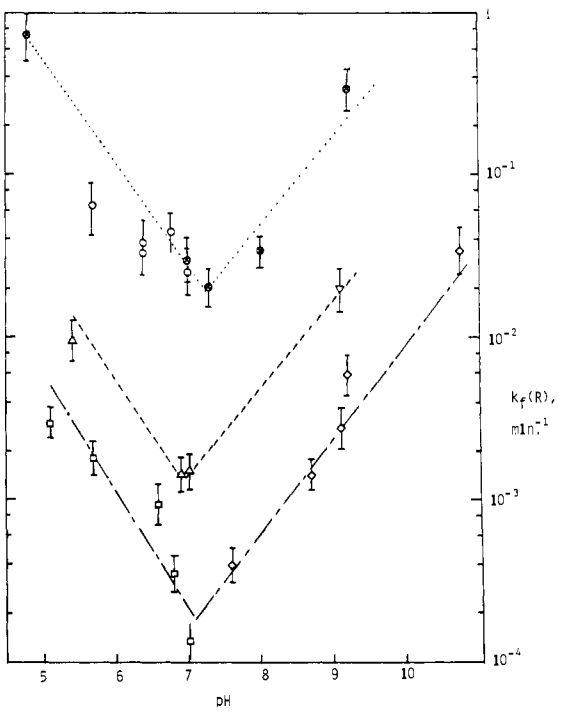

Figure 5. Graph of $k_f(R)$ vs. pH for the heme rotational equilibration of protohemins (□, ◇), mesohemin (▲, ▼), and deuterohemin (○, ⊕) in sperm whale metMbH$_2$O in ^2H_2O at 25 °C. The first symbol for each complex is for direct measurement of areas for the metMbH$_2$O complex; the second symbol is for measurements of areas on the respective metMbCN complex made by quenching the reaction with KCN. The lines through the data points have no theoretical significance but indicate the qualitative trend with pH.

While K_D values are very similar, the rates of equilibration depend critically on R. Analysis of the rate of change of the M_i and m_i signal intensities based on a pair of reversible first-order rate constants according to eq 1 leads to the expected straight line (Figure 4) which yields $k_f(R)$ vis eq 2 and 3. Determination of the equilibration rate at variable concentrations yields the values for k_f in Table II which indicate that the process is essentially independent of concentration. The influence of pH on $k_f(R)$ for the three hemins is illustrated in Figure 5. For each protein the rate drops as the pH is raised from the acidic side, reaches a minimum near pH 7, and increases again markedly at alkaline pH. Since each of the met-aquo complexes convert[18,19] to the met-hydroxy form above pH 7, it is not clear at this time what the relative importance is of pH on the aquo complex vs. the increased conversion to the met-hydroxy form. For the purpose of this report we will restrict ourselves to the rate data below pH 7 which hold for the met-aquo species. The pH profiles for the three hemins are very similar but with marked differences at any given pH. The data points clearly show that protohemin equilibrates slowest and deuterohemin fastest, with mesohemin intermediate but closer to the former. On the average, the relative equilibration rates near neutral pH[20] are

$$k_f(\text{vinyl}):k_f(\text{ethyl}):k_f(\text{H}) = 1:\sim 12:\sim 200 \qquad (4)$$

Insight into the likely mechanism for the reorientation can be obtained from a knowledge of the relative values of both $k_1(R)$ and $k_2(R)$ in Figure 2 as a function of R. Figure 6 illustrates the spectra of met-cyano complexes formed initially upon reacting 1 equiv each of protohemin and apo-Mb (A), 1 equiv each of deuterohemin and apo-Mb (B), and 1 equiv each of protohemin, deuterohemin, and apo-Mb (C) and trapping the initial product as the met-cyano complexes by addition of KCN. A similar set of spectra for protohemin and mesohemin are displayed in Figure 7. The fact that the 1:1 heme rotationally disordered proteins are formed for each hemin confirms[6,8,17] that the forward rate does not discriminate between the heme orientation for any R (i.e., $k_2^A(R) = k_2^B(R)$ in Figure 2). Moreover, the fact that equal amounts of native and deuterohemin–metMbCN (C in Figure 6) or mesohemin–metMbCN (C in Figure 7) are found when two different hemins are present in equal (but excess) amounts dictates that the *forward rate constant is also independent of R* (i.e., $k_2(R) = k_2(R')$).

While the off-rate, k_1, is not directly determinable by NMR, relative $k_1(R)$s can be obtained from the relative heme binding constants, $\beta(R)$, defined by

$$\beta(R) = k_2^A(R)/k_1^A(R) = k_2^B(R)/k_1^B(R) \qquad (5)$$

For simplicity we assume $\beta(R)$ does not depend on heme orientation. $\beta(R)$ has been reported for protohemin,[21] but qualitative estimates for other hemins are lacking. Optical studies of heme replacement reactions report[22] relative values $\beta(\text{vinyl}) \sim 100\beta(H)$. Relative $\beta(R)$s are readily obtained from NMR data by simply following a heme replacement reaction, i.e.

$$(R)\text{metMbH}_2O + (R')\text{-hemin} \rightleftharpoons (R')\text{-metMbH}_2O + (R)\text{-hemin} \qquad (6)$$

to equilibrium. In the case of equimolar ratios of holoproteins and R′-hemins in eq 6 and a large β so that essentially all of the protein is present as holoprotein, the relative $\beta(R)$s are related by the expression

$$\beta(R)/\beta(\text{vinyl}) = [M_i']_e^2/[M_i]_e^2 \qquad (7)$$

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(19) McGrath, T. M.; La Mar, G. N. *Biochim. Biophys. Acta* **1978**, *534*, 99.
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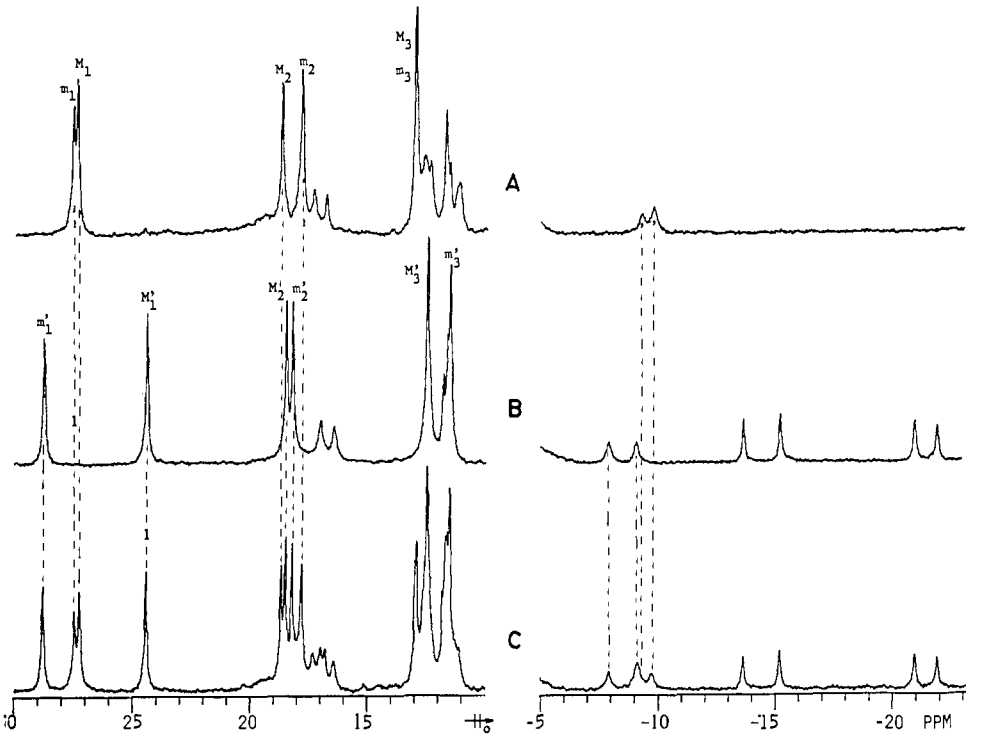

Figure 6. Hyperfine-shifted portions of the 360-MHz ^1H NMR spectra of the initial products of reaction of equimolar amounts of (A) protohemin and apo-Mb, (B) deuterohemin and apo-Mb, and (C) protohemin, deuterohemin, and apo-Mb. All reactions were carried out between the high-spin hemin and apo-Mb in ^2H_2O at 25 °C, pH 7.2, with KCN added within 10 min of mixing, to yield the respective met-cyano complexes. The heme methyl peaks for native metMbCN are designated M_i and m_i for the heme orientations A and B in Figure 1, respectively; the same signals are designated M_i' and m_i' for deuterohemin–metMbCN.

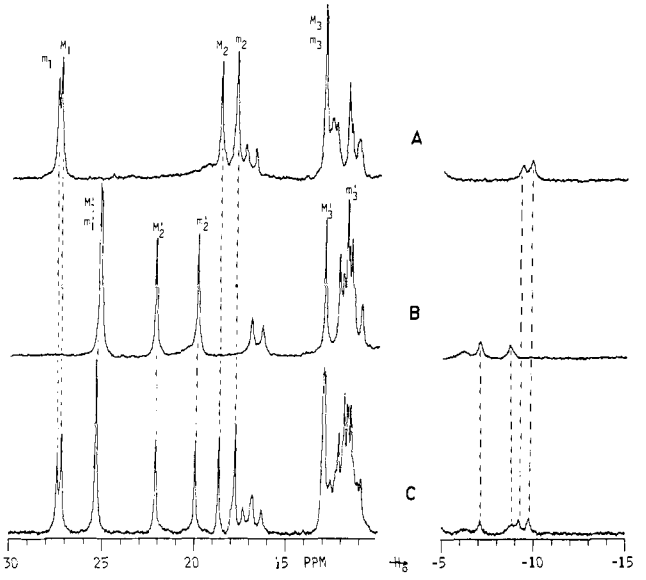

Figure 7. Hyperfine-shifted portions of the 360-MHz ^1H NMR spectra of the initial products of reaction of equimolar amounts of (A) protohemin and apo-Mb, (B) mesohemin and apo-Mb, and (C) protohemin, mesochemin, and apo-mb. All reactions were carried out between the high-spin hemin and apo-Mb in ^2H_2O at 25 °C, pH 7.2, with KCN added within 10 min of mixing, to yield the respective met-cyano complexes. The heme methyl peaks for native metMbCN are designated M_i and m_i for the heme orientations A and B in Figure 1, respectively; the same signals are designated M_i' and m_i' for mesohemin–metMbCN.

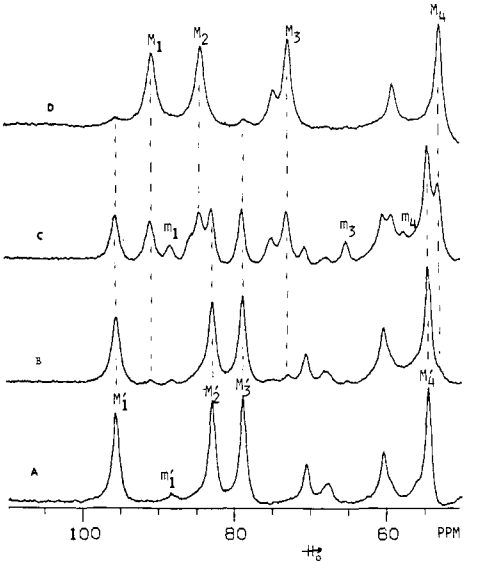

Figure 8. Low-field portion of the 360-MHz ^1H NMR spectra of the reaction of equimolar amounts of heme rotationally equilibrated deuterohemin–metMbH$_2$O (trace A, with peak M_i', m_i' for heme orientation as in A, B of Figure 1) and protohemin, in ^2H_2O at 25 °C, pH 6.3. Initially (trace B, 5 min after mixing), native metMbH$_2$O is formed with both heme orientations (M_i, m_i designated methyl peaks for heme orientation as in A, B of Figure 1), with subsequent equilibration (trace C, 250 min after mixing) to yield primarily (~90%) the native protein with the equilibrium ratio of heme orientations (trace D, 3 days after mixing). The equilibrium amount of deuterohemin–metMbH$_2$O at equilibrium (peaks M_i') relative to native metMbH$_2$O (peak M_1) yields the relative β(R)s according to eq 7 in the text.

where $[M_i']_e$ and $[M_i]_e$ are the intensities of the major component (orientation A in Figure 1) methyl peaks of R-hemin–metMbH$_2$O and native metMbH$_2$O.[23] The NMR spectra resulting from adding 1 equiv of protohemin to equilibrated deuterohemin–metMbH$_2$O in ^2H_2O are illustrated in Figure 8. First, protohemin displaces deuterohemin readily, and it is initially incorporated equally probably in the two heme orientations.[7] At equilibrium, ~8% of the protein still exists as deuterohemin–metMbH$_2$O.[16]

(23) With eq 5 rewritten as $\beta(R) = [R\text{-metMbH}_2O]/[\text{apo-Mb}][R\text{-hemin}]$, and recognizing that for starting equimolar amounts of R′-hemin and R-metMbH$_2$O requires that [R′metMbH$_2$01] = [R-hemin] and [R-metMbH$_2$O] = [R′-hemin], leading directly to eq 7.

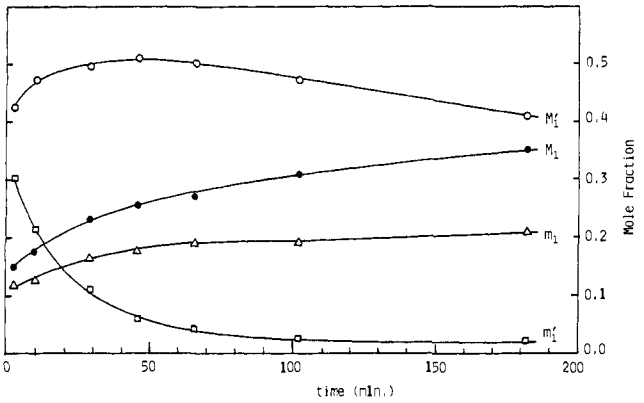

Figure 9. Plot of the mole fraction of native metMbH$_2$O with heme orientation A (peak M_1) and B (peak m_1) of Figure 1 and deuterohemin–metMbH$_2$O with heme orientation A (peak M_1') and B (peak m_1') of Figure 1, respectively, as a function of time for a sample made by initially reacting 1.0 equiv of apo-Mb with ~0.8 equiv of deuterohemin, to which we subsquently add 1.0 equiv of protohemin. The reaction is carried out in ^2H_2O at pH 6.3, and the intensity data are determined on the mixture of metMbCN complexes (see trace C of Figure 6) by quenching the reaction at various times by adding KCN. Initially, deuterohemin is found in equal amounts for the two heme orientations. With time, the rotationally disordered deuterohemin–metMbH$_2$O equilibrates and is substituted by protohemin to yield rotationally disordered native metMbH$_2$O which equilibrates on a much slower time scale.

The same trace (D in Figure 8) results at equilbrium when equivalent amounts of deuterohemin and native metMbH$_2$O are mixed. A similar displacement reaction was carried out on equilibrated mesohemin–metMbH$_2$O with protohemin (not shown), resulting in ~16% mesohemin–metMbH$_2$O at equilibrium. The relative methyl intensities (i.e., $(M_i'/M_i)^2$) at equilibrium yield the relative β(R)s, i.e.

$$\beta(\text{vinyl}):\beta(\text{ethyl}):\beta(\text{H}) = 1.0:\sim 0.04:\sim 0.01 \qquad (8)$$

Thus the native hemin yields the most stable holoprotein, with the replacement of vinyls by ethyl groups or hydrogens leading to decreases in the stability of the holoprotein by a factor of ~25 and ~10^2, respectively. The ethyl groups are known to cause some steric strain in the heme pocket,[24] possibly accounting for the decrease in β. The dramatic decrease of β for deuterohemin, however, dictates that the vinyl–protein interactions play an important role in stabilizing the holoprotein and that they influence only the dissociation rate. Since k_2(R) is independent of R, we can conclude that the relative off-rates, k_1(R), are

$$k_1(\text{vinyl}):k_1(\text{ethyl}):k_1(\text{H}) = 1:\sim 25:\sim 10^2 \qquad (9)$$

Since the heme equilibrium rate is first order and varies with R in a manner qualitatively the same as that found for k_1(R), the heme reorientation mechanism is consistent with involving dissociation of the holoprotein into the apo-protein and hemin, i.e., an apparent intermolecular process. That a substantial degree of protein unfolding must occur to allow the large heme (disc with diameter 12 Å) to rotate by 180° is to be expected. The similar trends with R for the equilibration rates, k_f(R)s, and dissociation rates, k_1(R)s, support a dissociative mechanism, but they do not indicate whether the hemin must entirely leave the protein environment. The quantitative discrepancies between trends with R of k_f(R) and k_1(R) suggest a process slightly more complicated than A ⇌ C ⇌ B in Figure 2.

Direct support for only a partial dissociation of hemin in the heme reorientation which prevents free exchange with hemin in the bulk solvent can be gained from considering rate data on simultaneous heme replacement and heme reorientation processes. Figure 9 plots the intensities of heme methyl peaks as a function of time for a sample where first ~0.8 equiv of deuterohemin is added to 1.0 equiv of apo-Mb to yield initially equal amounts of the disordered deuterohemin–metMbH$_2$O (peaks labeled M_i' and m_i' for orientations as in A and B of Figure 1). To this solution we immediately add 1.0 equiv of protohemin. The spectra are analyzed in the met-cyano form (by adding KCN at various reaction times) to facilitate resolution of the previously assigned[6,8] peaks of interest.

In addition to initially forming the small amount (~20%) of 1:1 disordered native metMbH$_2$O from the residual apo-Mb, the protohemin displaces the deuterohemin as found in Figure 8. At very short times after mixing, primarily the B (peaks m_i') orientation of deuterohemin–metMbH$_2$O will dissociate. If the dissociated deuterohemin mixes with the excess hemin in solution, then the large excess of free protohemin over deuterohemin in solution and the identical k_2(R)s dictate that we expect the loss of the signal intensity for the B orientation of deuterohemin–metMbH$_2$O to appear in the intensities for the "correct" A (peaks M_i) and "reversed" B (peaks m_i) forms of native metMbH$_2$O. If, on the other hand, the rate of deuterohemin equilibration is faster than the exchange with free hemin, then the loss of B-form deuterohemin–metMbH$_2$O intensity (m_i') will convert primarily to A-type deuterohemin–metMbH$_2$O intensity (M_i'). The data in Figure 9 clearly show that, while the intensity decrease of the B form of deuterohemin–metMbH$_2$O signal (m_1') represents a loss of ~8% of the protein in the 8-min interval 2–10 min, only ~1.0 and ~1.3% appear as native Mb in the B (m_1) and A (M_1) orientations, respectively, and the bulk (~5%) converts to the A orientation (M_1') of deuterohemin–metMbH$_2$O. Thus the initial rate of heme orientational equilibration is faster than the replacement of deuterohemin by protohemin, and it dictates that the equilibration process does not allow heme to exchange with that in the bulk solvent. We therefore conclude that the heme reorientation mechanism proceeds by a partial dissociation of the holoprotein which frees the hemin from the heme pocket but contains the heme within some sort of protein "cage".

The incomplete dissociation of the hemin/protein complex is supported by heme release data in the literature. Thus Smith et al.[25] have determined the rate of *complete* release of hemin (i.e., the process A → C in Figure 2) from sperm whale metMbH$_2$O by measuring the rate of abstractions of hemin by the apo-protein of horseradish peroxidase. Their value for this rate at 25 °C and pH 7 is ~1.1×10^{-5} m^{-1}, which is approximately 10 times slower than presently found for the heme equilibration under the same conditions.

The highly variable pH dependence of the heme reorientation rate indicates that reconstitution[26] aimed at yielding equilibrated proteins should include an incubation time at either pH extreme compatible with the protein stability. The very slow equilibration at neutral pH may bring into question the validity of a large number of physicochemical measurements made on reconstituted proteins. Thus the recent study[27] aimed at minimizing heme disorder in the reconstitution of apo-Mb with deuterohemin failed to discriminate between the thermodynamics of disorder and the kinetics of heme reorientation. In fact, contrary to the recent interpretation,[27] the disorder is independent of pH[17] and the equilibration profile in Figure 5 clearly dictates that pH values at either the acidic (5.5) or alkaline (10.5) are optimal. The slow equilibration at pH 7, on the other hand, readily affords nonequilibrium mixtures of the two heme orientations with lifetimes on the order of an hour, which should allow characterization of the detailed properties of the protein with the "reversed" heme orientation. Oxygen affinity studies already indicate significant differences for the two heme orientations.[14]

Studies aimed at assessing the relative importance of individual 2 and 4 substituents as well as the influence of iron oxidation/

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spin/ligation states on the equilibrium disorder and heme reorientation rate are in progress.

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Registry No. Hemin, 16009-13-5; deuterohemin, 21007-21-6; mesohemin, 21007-37-4.

Alkyldinitrogen Species Implicated in the Carcinogenic, Mutagenic, and Anticancer Activities of *N*-Nitroso Compounds: Characterization by ^{15}N NMR of ^{15}N-Enriched Compounds and Analysis of DNA Base Situselectivity by ab Initio Calculations†

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Abstract: The syntheses of certain specifically ^{15}N-labeled (*E*)- and (*Z*)-alkanediazoates and alkylnitrosamines (which have been implicated in the carcinogenic, mutagenic, or anticancer activities of *N*-nitroso compounds) together with related dinitrogen species including diazoalkanes are reported. Study of their conformational and configurational equilibria by ^{15}N and ^{13}C NMR revealed that corresponding pairs of (*E*)- and (*Z*)-alkanediazoates do not interconvert at ambient temperature in aprotic solvents. A fast interchange of metal counterion occurs between oxygen and nitrogen in the *Z* diazoates, whereas in contrast there is a slower interchange of metal ion between oxygen and nitrogen in the corresponding *E* diazotates. Interconversion of (*Z*)-arenediazoates occurs via detectable *Z* and *E* diazohydroxides to the *E* diazoates. Rapid stereoelectronically assisted conversion of (*Z*)-alkanediazoates to diazoalkane contrasts with the properties of the more stable *E* diazoates. The ^{1}H and ^{15}N NMR spectra in aprotic solvents at −50 °C of the unstable methyl nitrosamine showed averaged line positions in accord with an equilibrium between *Z* and *E* forms. SCF ab initio calculations show that the *Z*-OH (syn) conformation of the methanediazohydroxide is the highest in energy and the most reactive form with a larger LUMO coefficient on carbon than for the *E* isomer. In contrast, the *E*-OH (syn) conformation is more stable (by ~19.8 kcal M^{-1}) and with a zero LUMO carbon coefficient. SCF calculations predict a somewhat lower energy of activation for rotational inversion of *Z* and *E* configurations via the methanediazoates than by direct configurational inversion of the methanediazohydroxides. Complementary molecular orbital examinations of the main contributions of individual sites in the HOMO of guanine combined with a hard and soft acids and bases (HSAB) analysis are in accord with the view that the *Z*-I form (from methylnitrosamine) with a softer carbon may attack the softer O^6 position preferentially while *E*-I with a harder carbon would prefer to react at N_7. The results may provide a rationale for the in vivo reactions of the carcinogens such as dipropylnitrosamine with DNA. In this case propylation of G-N_7 occurs predominantly by an S_N2 process and without rearrangement and the concomitant propylation and isopropylation of G-O^6 occurs via a more S_N1-like process. The results may serve to explain the formation of the characteristic G-O^6 alkyl carcinogenic lesion by nitrosamines of DNA.

N-Nitroso compounds including nitrosamines, nitrosocarbamates, and clinically useful anticancer nitrosoureas are carcinogenic and/or mutagenic.[1-4] Several lines of evidence indicate that damage of cellular DNA by these agents may be responsible for these biological effects.[1,3,4] Certain DNA lesions, e.g., guanine O^6-alkylation,[5-7] appear to be more critical in giving rise to cell transformation than others, e.g., the relatively innocuous guanine N_7-alkylation.[8] The key reaction appears to be generation of electrophilic species, either by enzymatic action during metabolism in the case of nitrosamines[1,3] or by spontaneous decomposition in the case of (2-chloroethyl)nitrosoureas.[2,4] However, the nature of the electrophile that is ultimately responsible is by no means clear. Possible candidate electrophiles generated from nitrosamines include the (*Z*)- and (*E*)-alkanediazohydroxides, the alkanediazonium ion, diazoalkane, and the carbenium ion. Recent evidence in the cases of DNA base alkylation by N_1N_3-bis(2-chloroethyl)-*N*-nitrosourea[9,10] as well as by nitrosopropylamine[11-13] require largely S_N2 processes, which rules out a free carbenium ion.

High-Field ^{15}N NMR of specifically ^{15}N-enriched compounds has provided detailed information on the structure, conformation, and reactions of (2-chloroethyl)nitrosoureas.[14] Accordingly we

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