PROTON MAGNETIC RESONANCE STUDY OF THE INFLUENCE OF HEME 2,4 SUBSTITUENTS ON THE EXCHANGE RATES OF LABILE PROTONS IN THE HEME POCKET OF MYOGLOBIN

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ABSTRACT Four exchangeable protons with large hyperfine shifts are assigned in the heme pocket of sperm whale met-cyano myoglobin reconstituted with heme possessing acetyl groups, ethyl groups, bromines, and hydrogens at the 2,4 position, using both relaxation and chemical-shift data. The four protons arise from the ring NH's of the proximal (F8), distal (E7), and FG2 histidines, and the peptide NH of His F8. The similarity of all chemical shifts to those of the native protein as well as the invariance of the relaxation rates of the distal histidyl ring NH dictate essentially the same structure for the heme cavity of both native and reconstituted proteins. The exchange rates with bulk water of the four labile proteins in each modified protein were determined by saturation-transfer and line width methods. All four labile protons were found to have the same exchange rate as in the native protein for acetyl and ethyl 2,4 substituents; the two resolved labile protons in the derivative with 2,4 bromine were also unchanged. The reconstituted protein with hydrogens at the 2,4 position exhibited slower exchange rates for three of the four protons, indicating an increased dynamic stability of the heme pocket in the absence of bulky 2,4 substituents.

INTRODUCTION

The absence of a clear channel for the passage of molecular oxygen into or out of the heme pocket in myoglobins and hemoglobins (1–4) is direct evidence for the importance of dynamic properties of hemoproteins in completely understanding their functions. Various physical methods have been brought to bear on delineating the nature of the fluctuations in protein structure (5–14). One of the most versatile techniques is monitoring the rate of exchange with bulk water of labile protons buried in the protein interior (5, 6, 13–17). In cases where the signals can be resolved and identified, ^{1}H NMR provides the ideal spectroscopic window for following such exchange processes (13–16). The very sizable reduction of labile proton exchange rates in globular proteins has been associated primarily with their participation in hydrogen bonds (18), and the observed rates are interpreted in terms of the local unfolding model (5) or the solvent penetration model (12). In the local unfolding model, an internal portion of the protein is transiently exposed to solvent (12). The simplest case would have a single unfolded intermediate from which there is rapid exhange between a labile proton subset and the bulk water. Such a situation has been proposed for an allosterically sensitive subset of protons in hemoglobin (6).

We have shown recently (13, 14) that a number of the hyperfine-shifted exchangeable protons of paramagnetic forms of hemoproteins can be assigned on the basis of their differential relaxation by the iron spin and that their exchange behavior can be quantitatively characterized by the analysis of both line width and saturation transfer data for individual resonances. In met-cyano myoglobin, the local hyperfine field has permitted the identification of four labile proton resonances arising from the ring NH of the F8 (proximal), E7 (distal), and FG2 histidines, as well as from the F8 peptide proton (14). While it is possible to envision a single unfolded intermediate that would expose the proximal side of the heme to solvent, it is likely that the exposure of the distal side of the heme involves another "breathing" mode or unfolded intermediate. Information about the number of such intermediates needed to account for the exchange rates of the four assigned protons can be obtained by perturbing the equilibrium constant(s) for unfolding (12) and determining changes in the individual proton exchange rates.

While the importance of the ubiquitous vinyl groups of protoheme (Fig. 1) in determining the equilibrium structure of the heme cavity is well established (1–4, 19–22), much less is known about the role of these substituents in determining the dynamic stability of the heme pocket. Whether or not the vinyl groups are important in influencing overall protein structural fluctuations can be evaluated by reconstituting myoglobin with hemes possessing altered substituents at the 2,4 positions. We report herein on a study of the exchange behavior of the four labile protons in

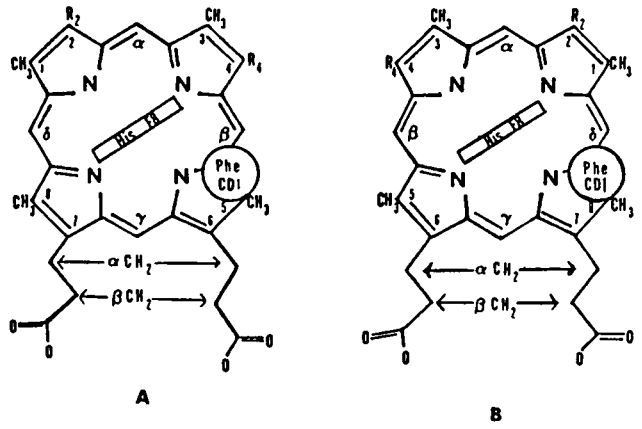

FIGURE 1 Orientation of the heme relative to the proximal histidyl imidazole plane. *A*, as found in the crystal structure, and *B*, as rotated 180° about the α-γ-meso axis from that in *A*. The heme is viewed from the proximal side.

the heme cavity of sperm whale met-cyano myoglobin reconstituted with heme where the vinyl groups have been replaced by acetyl groups, bromide, ethyl groups, and hydrogen as 2,4 substituents. Oxygen-binding studies have already shown that such substitutions influence the protein function (23,24).

The ^{1}H NMR spectra of the met-cyano myoglobin complexes containing the five hemes of interest have been analyzed for ^2H_2O solutions (19, 25, 26). Individual heme methyl assignments have been utilized to demonstrate that the major protein component at equilibrium has the same heme orientation as the native proteins (Fig. 1 *A*). All proteins exist primarily in one form, except the complex with R = bromine, for which an appreciable fraction exists with the heme rotated by 180° about the α-γ-meso axis. The hyperfine shifts for the nonexchangeable proton resonances have been rationalized in terms of an essentially invariant structure of the heme cavity for different 2,4 substituents, with the variable methyl shift patterns arising wholly from the variable rhombic electronic perturbation of the various substitutents (La Mar, G. N., N. L. Davis, and K. M. Smith, in preparation). An analysis of the hyperfine shifts and relaxation properties of the expected four labile proton resonances in the various met-cyano complexes will not only allow comparison of the relative dynamic properties, but will also serve as a way to check the degree of similarity in the detailed equilibrium structure of the heme cavities for different 2,4 substituents more quantitatively.

EXPERIMENTAL PROCEDURES

Sperm whale myoglobin was purchased from Sigma Chemical Co. (St. Louis, MO) as a salt-free, lyophilized powder. The protein was used as such. Deuterohemin, diacetyl-deuterohemin, dibromo-deuterohemin, and mesohemin were prepared from hemin according to standard literature methods (27–30) and reconstituted into apo myoglobin according to our modified procedure (T. Jue, R. Krishnamoorthi, and G. N. La Mar, submitted for publication). The reconstituted protein solutions were dialyzed against distilled water, lyophilized, and stored at −4°C. Solutions were prepared by dissolving ~30 mg of protein (3 mM) in 0.5 ml of 90% H_2O/10% ^2H_2O containing 0.2 M NaCl, centrifuging to remove any precipitate, and adjusting the pH to the acid side by adding 0.2 M 90% HCl/10%^{2}HCl. The pH was subsequently adjusted to the alkaline side by sequential addition of 0.2 M 90% NaOH/10% NaO^{2}H. The reported pH values are meter readings obtained in a 5-mm NMR tube, using an Ingold microcombination electrode (Ingold Electrodes, Amover, MA) and a Beckman model 3500 pH meter (Beckman Instruments, Inc., Fullerton, CA).

All the pairs of Redfield 2-1-4 spectra (31) were collected as described before (14) on a Nicolet NT-360 quadrature FT NMR spectrometer (Nicolet Instrument Corpl., Madison, WI). In one trace, the nonexcited H_2O resonance was saturated by the proton decoupler, in the companion

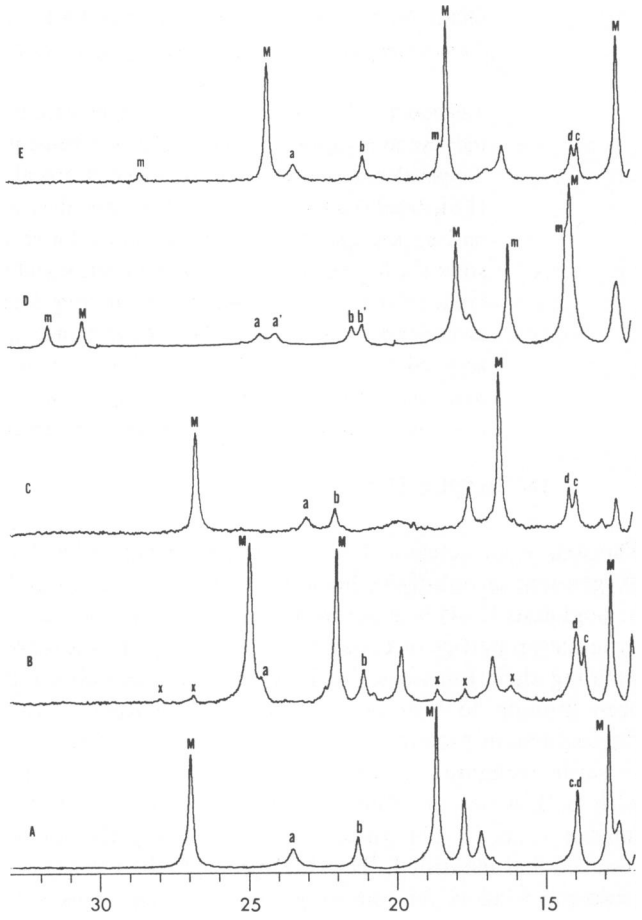

FIGURE 2 The low-field hyperfine shifted portion of the 360 MHz ^{1}H NMR spectra of native and reconstituted (R = vinyl group) met-cyano myoglobins in 90% H_2O/10% ^2H_2O, 25°C, and pH 8.6. The spectra were collected using the Redfield 2-1-4 pulse sequence (31) with the transmitter located at ~20 ppm in each sample. The relative intensities of resonances drop dramatically with distance from the carrier frequency. *M*, *m* designate the methyl groups corresponding to the heme orientations in Fig. 1 *A* and *B*, respectively, as taken from reference 26. *A*, native metMbCN (R = vinyl group). *B*, mesohemin-metMbCN (R = ethyl group). *C*, diacetyldeuterohemin-metMbCN, (R = acetyl group). *D*, dibromodeuterohemin-metMbCN (R = bromine). *E*, deuterohemin-metMbCN (R = H). Peaks *a*, *b*, and *c*, represent the ring NH's of His-E7, F8, and FG2, respectively, while *d* arises from the peptide NH of His F8; unprimed and primed peaks *a*, *b* correspond to the heme orientation as in Fig. 1 *A* and *B*, respectively. *x* indicates impurity peaks.

reference spectrum, the decoupler frequency was offset downfield from the resonance of interest to where there are no protein peaks. A typical Redfield (31) spectrum required 2,000 scans using a 20-μs pulse over a bandwidth of 10 KHz with 8,000 points; peak positions are given in parts per million (ppm) from internal 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). The T_1 values for the exchangeable peaks, *a* and *b*, were determined in H_2O solution at pH 8.6 (saturation factor = 1.00) by the conventional 180°-τ-90° pulse sequence (32), with the H^2O resonance saturated at all times except during acquisition (14).

The exchange rate, k, is obtained from the standard equation (13 14, 33)

$$SF = I/I_o = T_1^{-1}/(T_1^{-1} + k), \quad (1)$$

where the saturation factor, SF, is given by the ratio of intensity with (I) and without (I_o) saturating the solvent resonance, and T_1 is the intrinsic spin-lattice relaxation time determined above.

RESULTS

The low-field portions of 360 MHz ^1H NMR Redfield spectra recorded in 90% H_2O/10% ^2H_2O at pH 8.6 are illustrated in Fig. 2 for the five complexes of interest. This pH value is optimum for observing all four resonances with minimal line broadening. In addition to the nonexchangeable peaks detected in ^2H_2O, we find four labile proton peaks, *a*, *b*, *c*, and *d*, as reported for the native protein (14, 34). For mesohemin-metMbCN, peak *a* is under the methyl peak at 26.0 ppm, while for dibromodeuterohemin-metMbCN, the numerous methyl resonances arising from the heme disorder (25) obscure the region where peaks *c* and *d* are expected; individual labile proton resonances *a*,*a'* and *b*,*b'* are resolved for the two heme orientations. Varying the ratio of heme orientation establishes that *a*,*b* arise from the heme orientation Fig. 1 *A*, while *a'*,*b'* are due to the form in Fig. 1 *B*. The chemical shifts for the exchangeable protons are listed in Table I. For native metMbCN peaks, *a*, *b*, *c*, and *d* have been assigned to the His-E7 (distal), His-F8 (proximal), and His-FG2 ring NHs, and the His-F8 peptide NH, respectively, based on the relative relaxation ratios, as compared to a heme methyl peak (14).

At pH 8.6, peaks *a* and *b* exhibit negligible saturation transfer (i.e., SF = 1.00 in Eq. 1) at 25°C, which allows direct determination of the intrinsic T_1 values. The results

TABLE I
CHEMICAL SHIFT AND T_1 DATA FOR EXCHANGEABLE PROTONS IN THE HEME CAVITY OF METCYANO COMPLEX OF RECONSTITUTED MYOGLOBINS*

2,4-*R*	Peak *a* (distal His E 7 ring NH)		Peak *b* (proximal His-F8 ring NH)		Peak *c* (His F8 peptide N-H) shift‡	Peak *d* (His FG2 ring NH) shift‡
	shift‡	T_1	shift‡	T_1		
		ms		*ms*		
Vinyl	23.50	8.7	21.30	22.4	13.90	13.90
Acetyl	22.92	8.0	21.93	22.4	13.88	14.08
Bromine	24.05§	8.3	21.08	23.1	—	—
Ethyl	24.59	—	21.20	23.0	13.66	13.80
H	23.43	8.6	21.05	23.1	13.79	14.12

*Data at 25°C and pH 8.6.
‡Shifts in ppm from DSS.
§Data for major isomer.

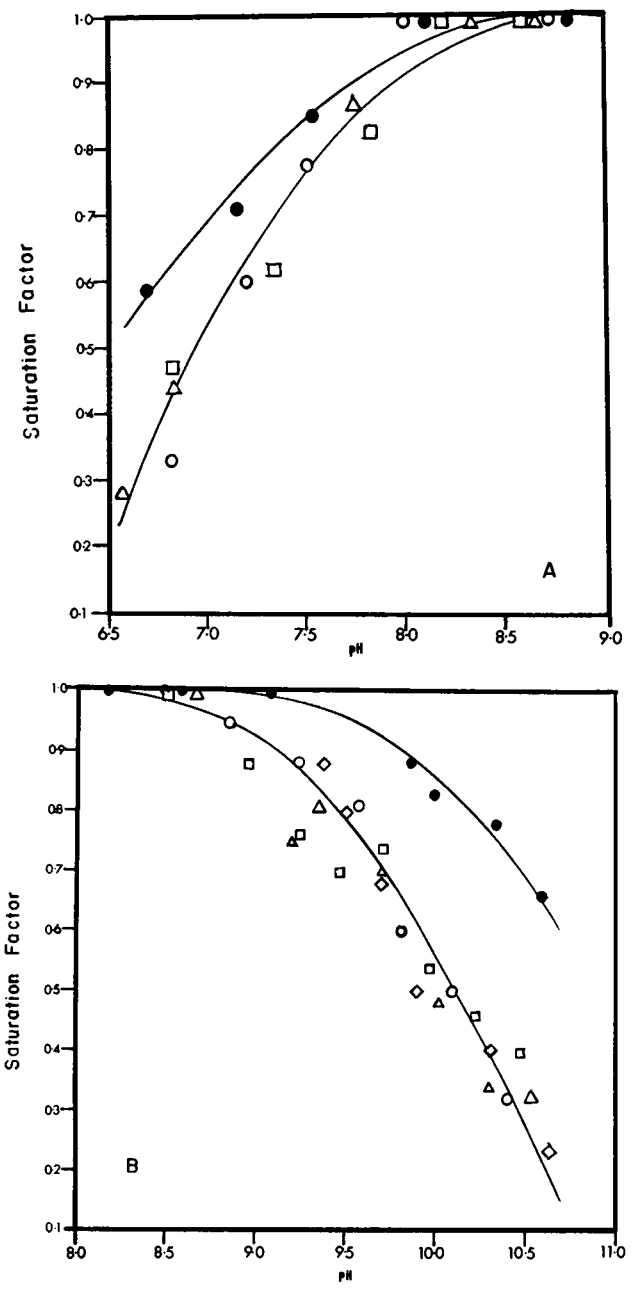

FIGURE 3 Plot of saturation (SF) factor vs. pH for (A) the distal His-E7 ring proton (peak *a*), and (B) the proximal His-F8 ring proton (peak *b*) in 90% of H_2O/10% ^2H_2O at 25°C. ○, native metMbCN (R = vinyl group); ◇, mesohemin-metMbCN (R = ethyl group); △, dibromodeuterohemin (R = bromine); □, diacetyldeuterohemin-metMbCN (R = acetyl group); ●, deuterohemin-metMbCN (R = H). The solid lines have no theoretical significance and are simply to indicate continuity of data points.

of such measurements by the inversion-recovery sequence are also included in Table I. Within experimental error, the T_1 is the same for all peaks *a* (8.6 ± 0.5 ms) and peaks *b* (23.0 ± 1.2 ms). Although T_1 could not be determined directly for peaks *c* and *d*, since they both exhibit appreciable saturation transfer under any conditions where they can be resolved, it seems reasonable to assume the intrinsic T_1 values (and line widths in the absence of exchange effects) for each of the other peaks are also independent of the 2,4 substituent.

The saturation factors for peaks *a* and *b* for each protein complex are plotted as a function of pH at 25°C Fig. 3 *A* and *B*. As found earlier for the native protein at 40°C (13, 14), *a* and *b* exhibit exclusively acid- and base-catalyzed saturation transfer, respectively. As argued earlier (14), the line width invariance with pH of nonexchangeable resonances permits the assumption that the intrinsic T_1 values are also independent of pH. The results of computing the exchange rates from the saturation factors and T_1 values using Eq. 1 are plotted in Fig. 4 *A*. As found previously (13), the exchange rates yield straight lines vs. pH on the semilogarthmic plot with slopes 0.8 ± 0.2.

Peaks *c* and *d* overlap at 25°C for several samples, so that comparison among the various proteins was carried out at 40°C. It was shown previously (14) that both peaks exhibited both acid and base catalyzed exchange, with peak *c* also broadening extensively at extreme pH values. Fig. 5 illustrates peaks *c* and *d* at 40°C and pH 9.42 for all protein complexes except with R = bromine. It is clear that the degree of line broadening (~170 Hz), and hence exchange rate, of *d* is essentially the same for all four derivatives. On the other hand, while the saturation factors (0.5) for the three derivatives with R = vinyl, acetyl, and ethyl groups are also the same and hence indicative of the same exchange rate, deuterohemin-metMbCN (R = H) shows a significantly larger saturation factor (0.75). It may be noted that the peak *c* for mesohemin-metMbCN is split into two components, probably due to a minor amino acid side-chain conformational heterogeneity that results from accommodating the bulky ethyl group, as found in x-ray studies (34); both components of peak *c* exhibit the same saturation factor. The pH dependence of the saturation factor for peak *c* and the line width for peaks *d*, both at 40°C, are compared in Fig. 6 *A* and *B*, for native metMbCN and deuterohemin-metMbCN. The exchange

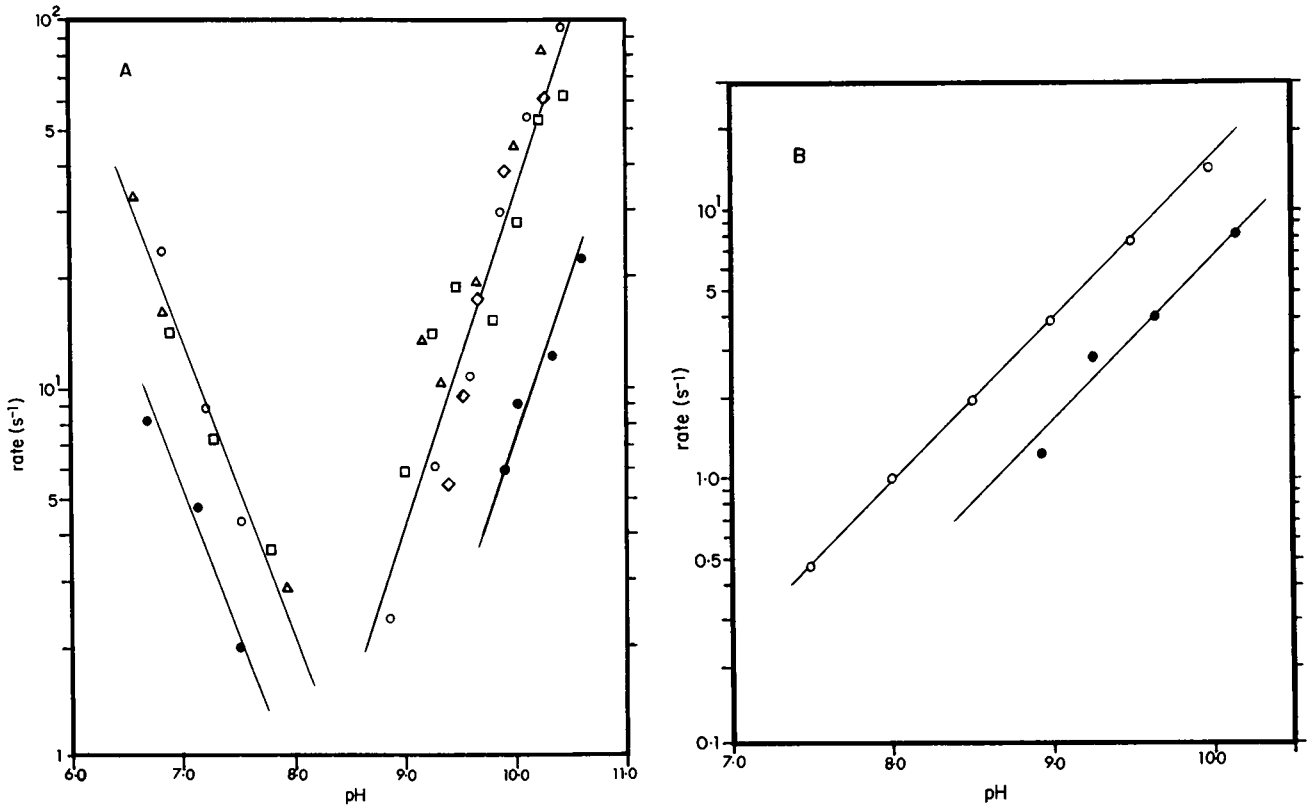

FIGURE 4 Semilogarthmic plot of the rate, as calculated by Eq. 1 from the saturation factor data. *A*, the distal His-E7 ring NH peak *a*, (*left* side of graph) and the proximal His-F8 ring NH, peak *b* (*right* side of graph), all data at 25°C. *B*, the His-F8 peptide NH, peak *c*, at 40°C. All samples in 90% H_2O/10% ^2H_2O. ○, native metMbCN (R = vinyl group); ◇, mesohemin-metMbCN (R = ethyl group); □, diacetyldeuterohemin-metMbCN (R = acetyl group); △, dibromodeuterohemin-metMbCN (R = bromine); ●, deuterohemin-metMbCN (R = H). Data for peak *c* of the native protein taken from reference 14.

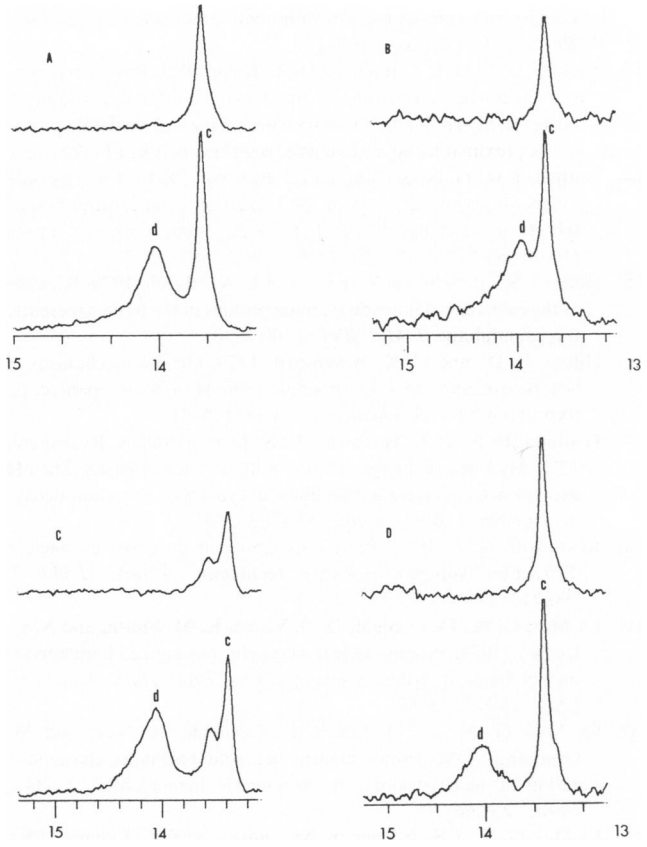

FIGURE 5 Redfield 2-1-4 spectra (31) showing peaks *c* (His FG2 ring NH) and *d* (His F8 peptide NH) in 90% H_2O/10% ^2H_2O solution at 40°C at pH 9.42 for (*A*) native metMbCN (R = vinyl group), (*B*) diacetyldeuterohemin-metMbCN (R = acetyl group), (*C*) mesohemin-metMbCN (R = ethyl group), and (*D*) deuterohemin-metMbCN. In each case, the *lower* spectrum is collected without exciting the H_2O resonance while the *upper* trace results while saturating the solvent peak with the decoupler. The saturation factor for peak *c* is 0.5 for *A*, *B*, and *C*, and 0.75 for *D*: the line width for peak *d* is ~170 Hz for all four complexes.

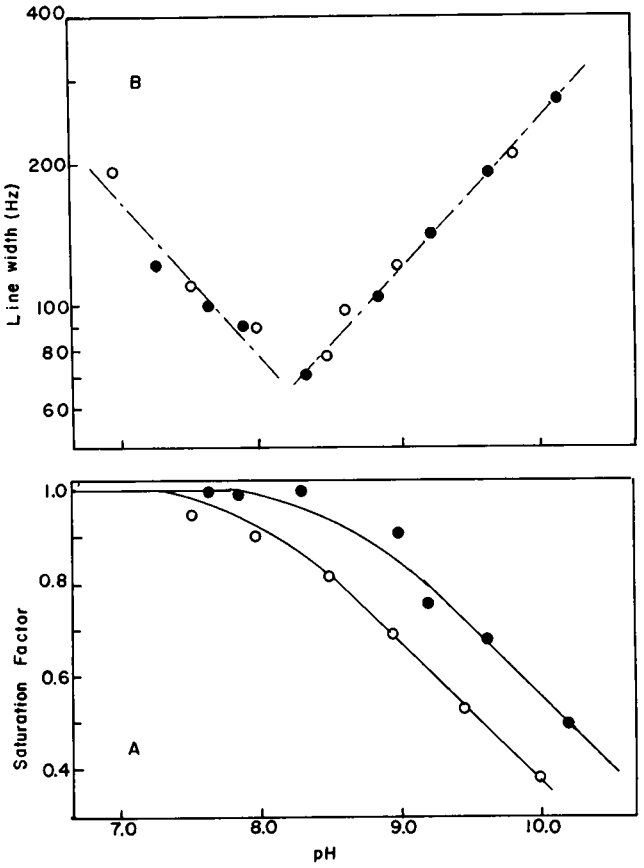

FIGURE 6 *A*, plot of saturation factor for peak *c* (His-F8 peptide NH) as a function of pH; *B*, semilogarthmic plot of line width for peak *d* (His-FG2 ring NH) as a function of pH. (○) Native metMbCN (R = vinyl group) (●) and deuterohemin-metMbCN (R = H), both at 40°C in 90% H_2O/10% ^2H_2O. Saturation factor and line width data for the native protein were taken from reference 14. The drawn lines have no theoretical significance and are included simply to indicate continuity of data points.

rate data for peak *c* for both proteins, computed using the previously reported (13) T_1 at 40°C, are included in Fig. 4 *B*.

DISCUSSION

The chemical shifts for each of the four exchangeable hyperfine shifted protons are essentially independent of the 2,4 substituent (Table I). Because these hyperfine shifts are primarily dipolar (except for peak *b*) in origin, (14, 35, 36), this dictates the same magnetic, and hence electronic structure in the heme cavity. The T_1 values for peak *a* (and also peak *b*) assigned to the ring proton of the distal histidine are also invariant, which requires the same distances and geometry for the hydrogen-bonding interaction of the distal histidine with the coordinated cyanide (14). We can therefore conclude that the assignments of peak *a–d* deduced earlier for native metMbCN (14, 35) are also valid for each of the reconstituted proteins.

As is already suggested by the same T_1 values (Table I) and the nearly superimposable saturation factor vs. pH plots (Fig. 3), the exchange rates for the His-F8 and His-E7 ring NHs are the same within experimental error (Fig 4) for the derivatives with R = vinyl, ethyl, and acetyl groups, and bromine. In dibromodeuterohemin-metMbCN, both the T_1 values and saturation factors are within experimental error for peaks *a* and *a'*, as well as *b* and *b'*, indicating that for this prosthetic group the protein fluctuations are independent of the heme orientation (19–22). The qualitative data in Fig. 5 similarly indicate an unaltered exchange rate for the protons giving rise to peak *c* (His-F8 peptide NH) and peak *d* (His-FG2 ring NH) for the derivatives with R - vinyl, acetyl, and ethyl groups (these peaks are not resolved for R = bromine).

The invariance of the exchange rates of all labile protons for the "bulky" substituents (i.e., R = vinyl, ethyl, and acetyl groups, and bromine) are in contrast to the data for deuterohemin-metMbCN (R = H). In this protein, both the proximal (*b*) and distal (*a*) histidyl ring NH's exhibit significantly reduced exchange rates, with $k(a)$, $k(b)$, and

$k(c)$ reduced by factors of 2.0, 5.7, and 2.5, respectively, in comparison to the native protein; $k(d)$, on the other hand, is the same as in the other four protein derivatives. Even the three protons on the proximal side of the heme (a, b, c) exhibit markedly different sensitivities of their exchange to the 2,4 substituent, although this subset could be expected to be exposed by a single intermediate involving, for example, unfolding of the F helix (4).

These results lead us to conclude that the dynamic stabilization of the heme pocket is increased for R = H relative to that for any sizeable 2,4 substituent (R = ethyl, vinyl, and acetyl groups, and bromine), and suggest that the vinyl substituents, while perhaps contributing to the thermodynamic stability of the equilibrium structure (1–4, 19, 22), increase the fluctuations of the protein in the heme cavity that unfold the protein enough to allow exchange of the labile protons (5, 12). The absence of any correlation between exchange rate and electron-withdrawing strength of the substituent dictates that direct *cis* electronic influences (37) are negligible. Thus, the present results are consistent with either the solvent penetration model or an unfolding model involving a number of intermediates from which proton exchange takes place.

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