PROTON MAGNETIC RESONANCE CHARACTERIZATION OF THE DYNAMIC STABILITY OF THE HEME POCKET IN MYOGLOBIN BY THE EXCHANGE BEHAVIOR OF THE LABILE PROTON OF THE PROXIMAL HISTIDYL IMIDAZOLE

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ABSTRACT The assigned exchangeable proton signals in the proton nuclear magnetic resonance spectra of sperm whale deoxy and Met-cyano myoglobin in H_2O solution were found to exhibit pH-dependent saturation transfer from the bulk water, which allowed determination of the kinetics and mechanism of the labile proton exchange with solvent. The exchange rates are base catalyzed for both protein forms, with the rate eight times faster in Met-cyano than in deoxy myoglobin. The exchange rate is taken as a measure of the magnitude of the fluctuation in the protein conformation near the heme cavity. On the basis of tritium exchange methods, the greater stability of the unligated relative to the ligated state in myoglobin has also been reported for hemoglobin. The present study, however, localizes the differential kinetic stability on the F helix whose flexibility has been implicated in the mechanism of cooperativity. The observation that filling the hydrophobic vacancy on the proximal side of the heme near the proximal histidine in Met-cyano myoglobin with cyclopropane increases the proton lability argues against a role for this hole in facilitating the flexibility of the F helix in the native protein.

INTRODUCTION

The traditional interpretation of x-ray diffraction data on hemoglobin and myoglobin has yielded static pictures of the molecules in which most atoms have unique and well-defined positions (1–3). However, compelling support for highly fluctuating protein structures comes not only from a number of other physical studies (4–10) but also from the static x-ray structures themselves. Thus neither the crystal structure of hemoglobin nor that of myoglobin reveals obvious channels for the passage of the O_2 molecule in and out of the heme cavity without rearrangement in the position of a number of side chains (2, 3). The existence of multiple conformation substates at low temperature has in fact been suggested as one possible explanation for low-temperature flash photolysis results on carboxymyoglobin (7). A theoretical treatment has shown (11) that sizable fluctuations in the orientations of side chains can result in the lowering of the potential barrier to penetration of the O_2 molecule from ~100 kcal for the static structure of myoglobin to values consistent with the experimental results (7). Hence the side chains blocking access to the distal side of the heme are thought to act as

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dynamic gates for the O_2. Recent analyses of the mean-square displacements of both side chains and backbone nuclei obtained from variable temperature Mössbauer (10) and x-ray diffraction (8, 9) data on single crystals have also provided direct support for a relatively dynamic myoglobin structure.

Some of the strongest support for the fluctuating behavior of biopolymers has resulted from the study of the rates of exchange of the labile peptide protons with solvent water, using the traditional tritium exchange method and N-^{2}H/N-^{1}H infrared band intensities, as well as the more recently emerging proton nuclear magnetic resonance (NMR)[1] method (4–6, 12–14). Application of the first two methods has revealed that hemoglobin possesses subsets of labile protons whose exchange rates differ by ~10 to 10^3 between ligated and unligated states and are sensitive to subtle protein conformational changes (4–6, 15). The peptide NH exchange rates in myoglobin, on the other hand, were found to be much faster than in hemoglobin, and no ligation-sensitive subsets of protons were observed (4, 5). The changes in the rates with ligation in hemoglobin have been suggested to reflect allosteric influences that control dioxygen access to, and exit from, the heme cavity (5, 6).

Monitoring labile proton exchange by NMR offers the advantage that rates can be measured for individual sites that can often be unambiguously identified (12–14, 16), even though lack of spectral resolution may severely limit the number of sites that can be probed. Moreover, because of the unique features of the NMR experiment, it is possible to monitor exchange rates several orders of magnitude faster than by other methods (16). Although several highly informative proton NMR studies have been reported for very small proteins (12–14, 16), no such studies have been reported for the oxygen-binding hemoproteins. Because the hyperfine interaction in the paramagnetic forms of hemoproteins invariable leads to resolution of resonances from exchangeable protons in the heme cavity, particularly for the proximal ligand (17), exchange rates could serve as potentially sensitive probes of localized structural fluctuations near the heme. We report here an initial proton NMR investigation of the exchange behavior of the proximal histidyl imidazole exchangeable proton, N_1H, in unligated (deoxy) and ligated (Met-cyano) sperm whale myoglobin.

EXPERIMENTAL

Sperm whale myoglobin was purchased from Sigma Chemical Co. (St. Louis, Mo.) as a salt-free lyophilized powder, and was used without further purification. Solutions were prepared by dissolving 26 mg of protein (~4 mM) in 0.4 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 addition of 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. Identical solutions of Met-cyano myoglobin (MetMbCN) in 0.2 M 10% NaCl/90% H_2O were prepared at pH 9.54, with one of the solutions equilibrated with cyclopropane. The reported pH values are meter readings obtained in the NMR tube (5 mm), using an Ingold micro-combination electrode and a Beckman model 3500 pH meter (Beckman Instruments, Fullerton, Calif.)

Proton NMR spectra in 90% H_2O were recorded at 40°C on a 360 MHz ^{1}H FTNMR spectrometer (Nicolet Magnetics Corp., Mountain View, Calif.). operating in the quadrature mode. Spectra over the

[1] *Abbreviations used in this paper:* DSS, di-sodium 2,2-dimethyl-2-silapentane-t-sulfonate; MetMbCN, Met-cyano myoglobin; NMR, nuclear magnetic resonance.

complete bandwidth were recorded using 90° pulses of 10 μs over 35 kHz (deoxy myoglobin) and 12 kHz (MetMbCN) windows using 8K points. The strong H_2O solvent resonance was suppressed by a 30-ms presaturation pulse from the homonuclear decoupler.

Determination of the degree of solvent saturation transfer to the protein resonances was carried out using the Redfield 2-1-4 pulse sequence (18), which for deoxy Mb required at 3.8-μs pulse with 8K points over a 35-kHz bandwidth, whereas MetMbCN required a 19.9-μs pulse over a 10-kHz bandwidth. The carrier frequency was in each case placed within 750 Hz of the imidazole N_1H peak of interest. The saturation factor, F, was obtained by comparing two Redfield 2-1-4 spectra recorded under identical conditions except for the frequency of the homonuclear decoupler. In one spectrum the decoupler was set on the nonexcited H_2O signal, and this signal was saturated before data acquisition. In the companion experiment the H_2O signal was not presaturated, because the decoupler frequency was chosen so that it and the H_2O resonance were symmetrically positioned on opposite sides of the proximal histidyl imidazole resonance, the decoupler being in a spectral region were there are no resonances. The resulting pair of spectra yielded identical intensities for all nonexchanging resonances and those exchangeable peaks that experienced no saturation transfer. F for an exchangeable peak is given by (16)

$$F = I/I_0 = T_1^{-1}/(T_1^{-1} + \tau_p^{-1}) \quad (1)$$

where I, I_0 are peak heights with and without H_2O saturation, respectively, τ_p is the lifetime of the exchangeable proton in the protein environment, and T_1 is the spin-lattice relaxation time, ignoring any contribution from exchange. Overhauser effects are assumed to be negligible (16). Peak heights were found reproducible to >5%, so ratios are reliable to >10%.

Under conditions used to derive Eq. 1 a determination of spin-lattice relaxation by the conventional 180°-τ-90° pulse sequence yields an effective relaxation time $T_1^{-1} + \tau_p^{-1}$ when the solvent H_2O resonance is presaturated, and our data were obtained using this method (19). Relaxation times were calculated via nonlinear regression using an exponential decay function and 9–13 points on the decay curve to describe the relaxation kinetics. With the known effective relaxation times, determination of F directly leads to the exchange rate, τ_p^{-1}, via Eq. 1.

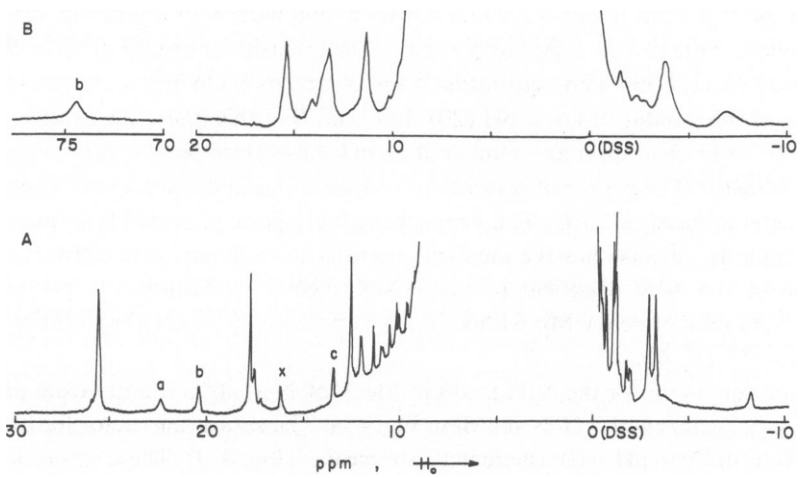

FIGURE 1 360 mHz ^1H NMR spectra of MetMbCN (*A*) at pH 8.56 and deoxy Mb (*B*) at pH 6.97 in 90% H_2O/10% ^2H_2O 0.2 M in NaCl at 40°C. No additional peaks are resolved in the region ±150 ppm from DSS for either protein form, and the intense diamagnetic regions 0 + 10 ppm are omitted at this amplification. The proximal histidyl imidazole-exchangeable N_1H resonances are labeled *b* in both traces (20, 22); peaks *a* and *c* are due to the distal histidyl imidazole N_3H and the proximal histidine peptide NH (20), and peak *x* is a single-protein nonexchangeable heme resonance, probably a propionic acid α-CH.

RESULTS

The normal proton NMR traces of MetMbCN and deoxy Mb at 40°C are illustrated in Fig. 1 *A* and *B*, respectively. The pH of these solutions was selected to minimize any saturation transfer from the H_2O. The three exchangeable proton peaks *a-c* in MetMbCN have been reported previously (20), and *a* and *b* have been tentatively assigned to the exchangeable imidazole proton of the distal and proximal histidines, respectively. Recent determination of their spin-lattice relaxation times, T_1, has quantitatively confirmed these assignments.[2] The only low-field exchangeable resonance in deoxy Mb has been assigned to the imidazole of the proximal histidine on the basis of its unique resonance position in high-spin ferrous models and hemoproteins (21, 22).

For deoxy Mb a value of T_1 in Eq. 1 was found to be 15 ± 1 ms for the imidazole N_1H peak from measurements[3] under conditions where the peak exhibited no saturation transfer. Since the line width of this resonance is found to be 256 ± 11 Hz, independent of pH over the range 6–10, we infer that T_1 is pH independent over the range of interest here. These results are consistent with the absence of any detectable conformational change in the heme pocket in the pH range 7–10 (21, 22). For comparison, a value of 25 ± 2 ms at 40°C has been reported[2,3] for the T_1 in Eq. 1 for the N_1H in MetMbCN under conditions where the peak exhibited no saturation transfer. The absence of any conformation change (20) and the invariance of the line widths[2] of several nonexchangeable resonances in the pH range 7-11 indicates that T_1 for N_1H may also be assumed to be pH independent in MetMbCN. In two instances, one each for deoxy Mb and MetMbCN, the effective relaxation rate, $T_1^{-1} + \tau_p^{-1}$, was independently measured, and its value was shown to be quantitatively consistent with the separately determined T_1 and τ_p. Thus, our assumption that T_1 is pH independent is tenable.

The region 15–24 ppm from di-sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) for MetMbCN at two pH values at 40°C is depicted in Fig. 2; the proximal histidyl imidazole peak is labeled *b*. Traces *A* and *B* are taken without and with H_2O saturation, respectively, at pH 8.56, where there is only a slight decrease in the intensity of peak *b* in trace *B*. The distal histidine N_1H also exhibits some saturation transfer, and its exchange has been ascribed to the protonation of this residue at lower pH (20). In *C* and *D* of this figure the spectra without (*C*) and with (*D*) H_2O saturation are compared at pH 9.84. Here peak *b* experiences extensive saturation transfer. The degree of saturation transfer is markedly decreased when the sample temperature is lowered to 25°C. The nonexchangeable peak *x*, probably originating from a heme propionic H_α, always has the identical intensity in each pair of spectra. Fig. 3 presents the portion of the 40°C Redfield proton NMR spectra containing the proximal histidyl imidazole N_1H peak of deoxy Mb without (*A*, *C*) and with (*B*, *D*) H_2O saturation at pH 9.92 (*A*, *B*) and 10.62 (*C*, *D*).

The saturation factor for the N_1H peaks in MetMbCN at 40°C is unity from pH 4.8 to pH ~8, where it decreases with pH as shown in Fig. 4 *A*. The saturation factor for the N_1H peak in deoxy Mb is unity to pH ~10, where it also decreases (Fig. 4 *A*). These saturation factors in

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[2]Cutnell, J. D., G. N. La Mar and S. B. Kong. *J. Am. Chem. Soc.* In press.

[3]The uncertainties denote two or more standard deviations, as provided by the nonlinear regression analysis from which this value was calculated.

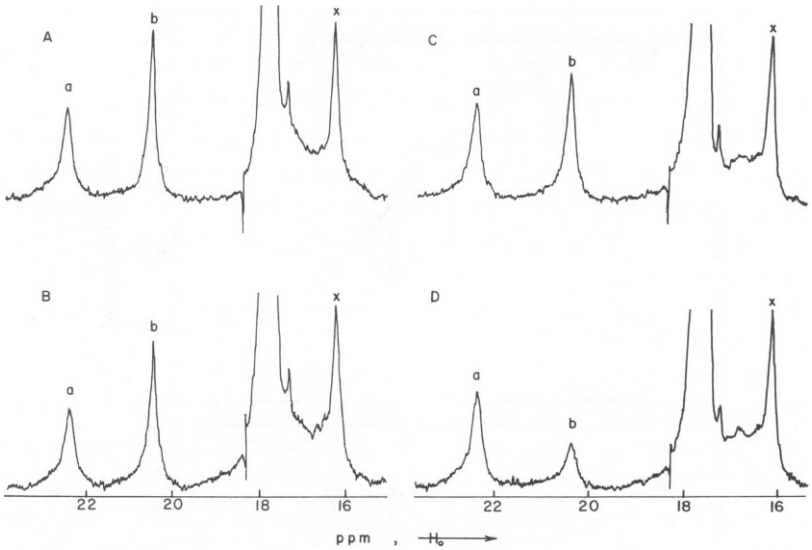

FIGURE 2 The 13- to 26-ppm regions of Redfield 2-1-4 ^{1}H-NMR spectra (18) of MetMbCN in 90% H_2O 0.2 M in NaCl at 40°C at pH 8.56 with no H_2O saturation (*A*) and with water saturation (*B*), and at pH 9.84 without (*C*) and with (*D*) saturation of the H_2O signal. The intensities of peak *x*, probably a heme propionic acid H_α, as well as those of other nonexchangeable peaks, are independent of the H_2O saturation. The glitch near 18.0 ppm is due to the carrier frequency. See caption to Fig. 1 for peak assignments.

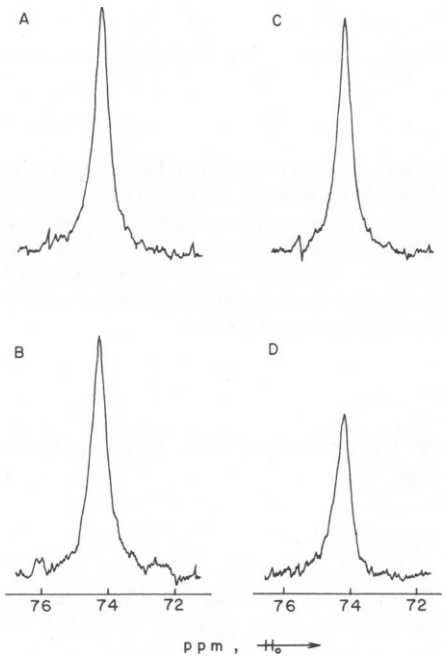

FIGURE 3 The 70- to 80-ppm portion of the Redfield ^{1}H NMR spectra exhibiting the proximal histidyl imidazole N_1H resonance of deoxy Mb (22) in 90% H_2O 0.2 M in NaCl at 40°C, pH 9.92, without (*A*) and with (*B*) H_2O saturation, and pH 10.62 without (*C*) and with (*D*) H_2O saturation.

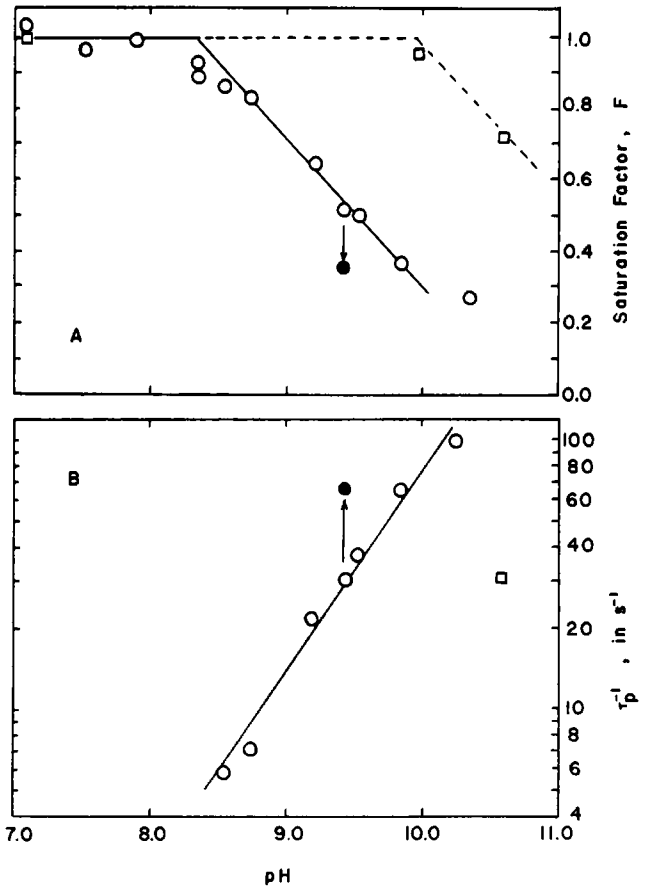

FIGURE 4 (*A*) Plot of the saturation factor, *F*, defined in Eq. 1, for the proximal histidyl imidazole N_1H as a function of pH for MetMbCN (○) and deoxy Mb (□) at 40°C. The datapoint (●) is for MetMbCN that has been equilibrated with cyclopropane. The straight lines simply indicate continuity and have no theoretical significance. (*B*) Plot of the exchange rate, τ_p^{-1}, at 40°C as a function of pH as computed from Eq. 1, using the saturation factors in *A* and the pH-invariant T_1 for MetMbCN (○) and a single data point for deoxy Mb (□). The data point (●) is for MetMbCN equilibrated with cyclopropane. The arrow refers to the change in *F* or τ_p^{-1} upon intercalating cyclopropane.

Fig. 4 *A*, in combination with the pH independent T_1 values yield the exchange rate, τ_p^{-1}, at 40°C, as illustrated in Fig. 4 *B*.

DISCUSSION

The effect of irradiating the water resonance on the intensity of the proximal histidyl imidazole N_1H resonance for both MetMbCN (Fig. 3) and deoxy Mb (Fig. 2) reveals that saturation transfer takes place only at alkaline pH. Several of the other exchangeable resonances in MetMbCN also exhibit saturation transfer at some pH values, but these resonances are incompletely assigned and at this time have no analogs in the spectrum of

deoxy Mb; these resonances are still under investigation. The detection of exchangeable imidazole NH peaks in proteins has been infrequent, largely because they are often on the surface, where exchange with the bulk water is extremely rapid (22, 24). The slow exchange rate for this proton for the coordinated imidazole in myoglobin is due to the highly buried position at the edge of one of the hydrophobic clusters (3). Exchange of the proximal histidyl imidazole N_1H with H_2O, as with internal peptide NH (4–6, 12–14), must be due to partial exposure of the hydrophobic interior to the solvent as a result of the fluctuational behavior of the molecule.

The NH exchange rate can, in general, be represented as

$$\tau_p^{-1} = k_A[H^+] + k_B[OH^-] + k_W[H_2O], \quad (2)$$

where k_A, k_B, and k_W are the rate constants for acid, base, and water catalysis, respectively; k_W is usually assumed to be negligible (12). The saturation factors for both protein forms are less than unity only at alkaline pH, indicating that the same mechanism, base-catalyzed exchange, is dominant, i.e., $k_A \ll k_B$ in Eq. 2. Though acid and base catalysis might be expected to be comparably important in free imidazole, a coordinated imidazole is not susceptible to acid attack, such that the dominance of base catalysis is to be expected. Although the plot of log τ_p^{-1} vs. pH is linear for MetMbCN, the slope of 0.8 is less than the predicted unity value in Eq. 2 by an amount that exceeds experimental error. Slopes of less than unity have also been observed for peptide NH exchange (12–14). The significance of this reaction order is not understood but is often cited in support of the EX_2 exchange mechanism (12). The reasonable slope obtained for the plot of log τ_p^{-1} vs. pH indicates that the exchange behavior observed at alkaline pH is applicable to the nature of the protein in the physiological pH range (12–14, 16). Moreover, previous NMR studies have shown that the structure of the proteins is unaltered throughout the pH range considered (20, 22).

Direct comparison of the exchange rates for the two protein forms can be carried out at pH 10.6, where we measure τ_p^{-1} (deoxy Mb) = 30 s^{-1} at 40°C. Although the saturation factor for MetMbCN is too small to be measured accurately at that pH, the straight line in Fig. 4 *B* allows extrapolation to that pH, yielding τ_p^{-1} (MetMbCN) = 2.4 × 10^2 s^{-1} at 40°C. This yields the ratio at 40°C, τ_p^{-1} (MetMbCN)/τ_p^{-1} (deoxy Mb) = 8.0. On the premise that the exchange rates by the same mechanism in the two protein forms are based on the degree to which the site is exposed to the solvent, our kinetic data indicate that the fluctuations in deoxy Mb are smaller than those in MetMbCN. Taking MetMbCN as a reasonable model for the ligated state of the protein, we conclude that the region of the F helix near the proximal histidine is less exposed to the solvent, and hence is kinetically more stable in the unligated than in the ligated state. The mean-square displacements for the main chain atoms in deoxy Mb crystals have not yet been reported, precluding a direct comparison between ligated and unligated forms in the crystalline state.

Extensive tritium labeling studies have demonstrated that the kinetics of various subsets of exchangeable peptide protons are considerably accelerated when hemoglobin is ligated, indicating changes in fluctional behavior with ligation state (4–6). Although it has been reported that such ligation-sensitive exchange rates are absent in myoglobin (4, 5), our present results show that at least the proximal histidyl imidazole exchange rate reflects such sensitivity. Since all exchange rates in myoglobin are faster than in hemoglobin, it is highly

likely that the exchange rates of any other ligation-sensitive protons in myoglobin are too fast to have beeen detected by the much slower tritium exchange method (4, 5).

The present NMR data indicate moreover that the change in kinetic stability with ligation occurs at the proximal histidine anchored to the F helix whose flexibility has been implicated in the mechanism of cooperativity in hemoglobin (25). Detailed analyses of the mean-square displacement of the backbone and side chain atoms in myoglobin, using variable-temperature x-ray diffraction data, have also shown that the most flexible portion of the molecule involves the F helix on the proximal side of the heme (8, 9).

Detailed x-ray studies on myoglobin by Takano (3) have revealed the existence of a preformed hydrophobic vacancy on the proximal side of the heme and next to the proximal histidine, which is known to accommodate such species as cyclopropane (26) without significantly altering the molecular or electronic structure of the heme pocket (27). Although the role of this hole is not understood, it has been suggested (3) that this space may exist to allow extra flexibility or larger fluctuations for side chains on the proximal side of the heme. On this premise, filling the hole should decrease the local flexibility and slow the N_1H exchange rate. The influence of cyclopropane intercalation, which was indicated by the observation of the previously reported NMR spectral changes (27), on the N_1H resonance saturation factor at pH 9.54 for MetMbCN is also include in Fig. 4 *A* and *B*. Intercalation of cyclopropane had no detectable influence on the line width of any of the several resolved heme resonances, indicating that T_1 is unchanged. The saturation factor decreases from 0.51 to 0.37 upon the addition of C_3H_6 and therefore yields an increase in τ_p^{-1} from 38 to 68 s^{-1}. Thus, contrary to expectation, the amplitudes of the fluctuations near the proximal histidine appear to increase in filling this hole.

The present studies are being extended to hemoglobin and its isolated chains as well as other hemoproteins. Preliminary results on horseradish peroxidase[4] indicate that ligation increases the lability of the coordinated imidazole N_1H by a factor $>10^5$, suggesting that the present methods should have wide applicability for studying the dynamic behavior of the active site in a variety of hemoproteins.

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