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A Nuclear Magnetic Resonance Study of the Heme Environment in Beef Liver Catalase†

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ABSTRACT: The effect of high-spin heme iron in beef liver catalase on the longitudinal and transverse proton relaxation rates of the solvent has been used to probe the environment of the paramagnetic center. The longitudinal proton relaxation rates were measured as a function of temperature (5–31 °C), frequency (5–100 MHz), and pH. T_{1p} was found to be pH independent in the range 6–11, indicating that no significant difference occurs in the heme surrounding within this pH range. The ligands formate and acetate, which preserve the spin state of the heme iron upon ligation, displace a water molecule from the sixth coordination position. This reaction is pH independent, while the binding measured by optical spectroscopy is pH dependent. The electron donors methanol and ethanol essentially do not change the proton relaxation rates. The temperature and frequency dependencies indicate that the relaxation times are governed by the electronic relaxation time of the high-spin ferric iron, τ_s. τ_s, which was found to be frequency independent, could not be determined from the T_{1p}/T_{2p} ratio, but only from the frequency dependence of the longitudinal relaxation rate at low frequencies. The results of the least-squares fit of the data to the theory indicate that there is one iron-bound rapidly exchanging water molecule. For the Fe^{3+} ion it was determined that $\tau_s = 7 \times 10^{-11}$ s.

In spite of the extensive data on various physical properties of catalase and its derivatives (Nicholls and Schonbaum, 1963; Deisseroth and Dounce, 1970), the nature of the ligand at the sixth coordination position is not definitely known. Three different proposals have been made for the identity of this ligand. The prevalent assumption is that a water molecule is bound to the catalase heme iron (Nicholls, 1962). This hypothesis was critized on the basis of two experimental facts. First, in contrast to metmyoglobin and methemoglobin, which possess a water molecule at the sixth coordination position (Brill and Williams, 1961; Fabry et al., 1971; Perutz, 1970), catalase does not form alkaline hematin derivatives up to nearly pH 12 (Chance, 1952a), nor are the catalatic and peroxidatic reactions of catalase sensitive to pH changes (Chance, 1952a; Jones and Suggett, 1969). Second, an apparent uptake of a proton results from the reaction between the enzyme and the anions F^- and CN^- (Chance, 1952a,b). These experimental facts were explained by postulating a hydroxide group (Theorell and Paul, 1944; Chance, 1952b) or an amino acid residue of the protein with a high pK (George and Lyster, 1958) as the relevant ligands of the iron in catalase. However, none of the three suggested possibilities was substantiated by direct experimental evidence. In view of these uncertainties an investigation was conducted in order to identify the sixth iron ligand in beef liver catalase by the pulsed NMR technique.[1] It should be noted that there seems to be no other way, including x-ray evidence, of choosing between water and an hydroxyl anion as the species occupying the sixth coordination position.

NMR spectroscopy has the potential of counting the hydration number of a paramagnetic metal ion bound to a biological macromolecule (Navon, 1970; Mildvan and Cohn, 1970; Dwek, 1972). If the various parameters which are responsible for the proton relaxation mechanisms can be precisely evaluated, it is possible to decide whether OH^- or H_2O groups are coordinated to the metal ion (Lanir et al., 1975). The proton relaxation technique has been used several times in attempts to study the accessibility of solvent molecules to hemes in various heme proteins (Maricic et al., 1966; Fabry et al., 1971; Pifat et al., 1973; Lanir and Aviram, 1975; Lanir and Schejter, 1975a) and to probe the immediate environment of the Fe^{3+} ion (Fabry and Eisenstadt, 1974; Lanir and Aviram, 1975; Gupta and Mildvan, 1975). A preliminary account of part of this work has been published (Lanir and Schejter, 1975b).

Materials and Methods

Beef liver catalase was obtained from Boehringer Mannheim GmbH. Three milliliters of the enzyme suspension (1 g/50 ml)

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[1] Abbreviations used are: NMR, nuclear magnetic resonance; zfs, zero field splitting; EPR, electron paramagnetic resonance.

was centrifuged and the enzyme was dissolved in 1 ml of 10% NaCl. At the concentrations required for the NMR experiment (above 1×10^{-4} M catalase), below pH 6 the enzyme was partly precipitated. Heme concentrations were determined from measurements of the Soret band intensity at 403 nm using a molar absorptivity of 1.485×10^5 cm^{-1} M^{-1}/heme (Deiseroth and Dounce, 1967). All the concentrations specified in this paper are given in terms of catalase heme. Spectra were recorded on a Cary 118C spectrometer.

Longitudinal relaxation rates at 5–60 MHz were measured with a Bruker Type B-KR-322 pulsed spectrometer, and at 100 MHz were measured using a spin-echo attachment to a Varian HA-100 NMR spectrometer (Ginsburg et al., 1970), by the 180°–τ–90° null method. Values of T_2 were obtained at 100 MHz from the spectral line width, using the expression $1/T_2 = \pi\nu$ where ν is the full line width at half-maximum peak height. Corrections were made in the null method for imperfections of the π pulse. This was made by plotting log $(M_\infty - M_\tau)$ *vs.* τ where M_∞ is the equilibrium value of the magnetization and M_τ the magnetization at a time τ after the 180° pulse. The error in a T_1 determination is ±5%. The values of T_2 are accurate only to 10%.

The net contributions to the paramagnetic relaxation rates were calculated as the difference between the relaxation rates observed in solutions of native catalase and those observed in equimolar solutions of its cyanide derivative. Cyanide replaces the sixth ligand of the iron and reduces the spin state of ferric catalase from pure high-spin to $S = \frac{1}{2}$, but causes only small conformational changes in the protein moiety (Samejima and Kita, 1969) and probably no change in the content and magnetic properties of possible impurities of free paramagnetic ions in the solution. Since the longitudinal electronic relaxation time of the low-spin ferric iron is very short, $\tau_{1e} \leq 2 \times 10^{-12}$ s (Eisinger et al., 1962; Wuthrich, 1970), T_1^{-1} in solution of catalase–cyanide may be considered as being very close to the contribution of apocatalase to the relaxation. Any other method to account for the contribution to the relaxation other than that originated from the high-spin hematin iron (i.e., the diamagnetic contribution of the protein and the contribution of possible paramagnetic impurities) is considered as less reliable, since it causes either gross conformational changes in the protein or changes in the spin state of possible paramagnetic impurities.

Theoretical Background

The theory and application of proton magnetic relaxation rates of water in solutions of macromolecules that contain paramagnetic ions are now well documented (Mildvan and Cohn, 1970; Dwek, 1972, 1973). Hence, only the essential relationships and some comments related to high-spin ferric ion embedded in a heme protein are presented here. The net proton relaxation rates for water in solutions of the heme protein are given by eq 1 (Luz and Meiboom, 1964; Swift and Connick, 1962):

$$\frac{1}{T_{ip}} = \frac{nq}{55.6} \frac{1}{T_{iM} + \tau_M} \qquad i = 1,2 \qquad (1)$$

where q is the number of water molecules in the first coordination sphere of the heme iron of concentration n. T_{1M} and T_{2M} are the longitudinal and transverse relaxation times of the bound water molecules, respectively, and τ_M their mean residence time. The relaxation times can occur through both dipolar interaction and scalar coupling to the electron magnetic moment of the metal ion (Solomon, 1955; Bloembergen, 1957). In high-spin ferric systems, where $1 \gg \omega_I^2\tau_c^2$ even at high frequencies, and assuming $\omega_s \gg \omega_I$, proton relaxation rates are given by simplified Solomon–Bloembergen equations:

$$1/T_{1M} = B\left[6\tau_c + \frac{14\tau_c}{1 + \omega_s^2\tau_c^2}\right] + C\left[\frac{2\tau_e}{1 + \omega_s^2\tau_e^2}\right] \qquad (2)$$

$$1/T_{2M} = B\left[7\tau_c + \frac{13\tau_c}{1 + \omega_s^2\tau_c^2}\right] + C\left[\tau_e + \frac{\tau_e}{1 + \omega_s^2\tau_e^2}\right] \qquad (3)$$

In (2) and (3), $B = \gamma^2 g^2 \beta^2 S(S+1)/15r^6$, where r is the metal–proton distance, and $C = \frac{1}{3} S(S+1)(A/\hbar)^2$. Here $A/\hbar$ is the contact coupling constant in rad s^{-1}. All other symbols have their usual meaning (Dwek, 1973). τ_c is a correlation time for the dipolar interaction and given by $\tau_c^{-1} = \tau_r^{-1} + \tau_e^{-1}$, and τ_e is the correlation time for the hyperfine exchange mechanism, which can be written $\tau_e^{-1} = \tau_M^{-1} + \tau_s^{-1}$. τ_s is the electron spin relaxation time. Here we have assumed that there is only one electronic relaxation time. In high-spin ferric heme proteins τ_r, $\tau_M \gg \tau_s$ and $\tau_c = \tau_e = \tau_s$. It was shown that the electronic relaxation in solution of ferric ($S = \frac{5}{2}$) ions is controlled by modulation of the quadratic zero-field splitting interaction. The modulation is caused by collisions of the complex with the bulk solvent molecules (Rubinstein et al., 1971). The expression for τ_s is given by eq 4 (Bloembergen and Morgan, 1961; Rubinstein et al., 1971):

$$\tau_s^{-1} = D\left[\frac{\tau_v}{1 + \omega_s^2\tau_v^2} + \frac{4\tau_v}{1 + 4\omega_s^2\tau_v^2}\right] \qquad (4)$$

where τ_v is a frequency-independent correlation time, which is related to the rate at which the zero-field splitting is modulated, and D is a constant. In cases where $\omega_s^2\tau_v^2 \geq 1$ the frequency dependence of the experimental relaxation rate is explained and analyzed by eq 1–4. This is the case in macromolecules containing manganese (Peacock et al., 1969; Reuben and Cohn, 1970; Navon, 1970; Reed et al., 1972; Lanir et al., 1975). If, however, τ_v is very short (as will be shown to be in the case for high-spin ferric ion) $1 \gg \omega_s^2\tau_v^2$, and $\tau_s^{-1} = 5B\tau_v$, that is, τ_s is frequency independent. In such a case the frequency dependence of T_{1p}^{-1} is explained by eq 1 and 2.

The following assumptions are made in the derivations of eq 2–4 and in the estimation of the hydration number of the catalase iron: (1) The usefulness of the Solomon–Bloembergen equations for analyzing the frequency dependence of $1/T_{1p}$ in the present system is not self-evident. As it was noted by Dwek (1973), eq 2 and 3 may be used as long as zfs $\ll \omega_s$, since the zfs was neglected in deriving these equations. While in solutions of high-spin Fe^{3+} ions this assumption is justified (for example, Levanon et al. (1970), from the ESR line width in solutions of $Fe(H_2O)_6^{3+}$, derived a value of 0.127 cm^{-1} for Δ, where Δ^2 is the trace of the square of the zfs tensor), in heme proteins (Tasaki et al., 1967; Alpert et al., 1973) and heme compounds (Brackett et al., 1971; LaMar et al., 1973; LaMar and Walker, 1973; Lang et al., 1970) the zfs parameter is much greater and is of the order of 6–10 cm^{-1}. These values were obtained using NMR, EPR, far-infrared, and Mossbauer spectroscopies. Such high magnitude of the zfs, which is comparable with ω_s even at high frequencies, puts the validity of the Solomon–Bloembergen equations for analyzing the present system into question. However, since there is no valid theory at the present time for the range where the Zeeman interaction of the electronic spin with the external field is comparable with the zero-field splitting energy, we shall use eq 2 for analyzing the frequency dependence of T_{1p}.

(2) The effect due to an anisotropic rotation of the coordinated water molecules about their Fe–O axis is neglected. However, this effect, which may be important in Mn(II)

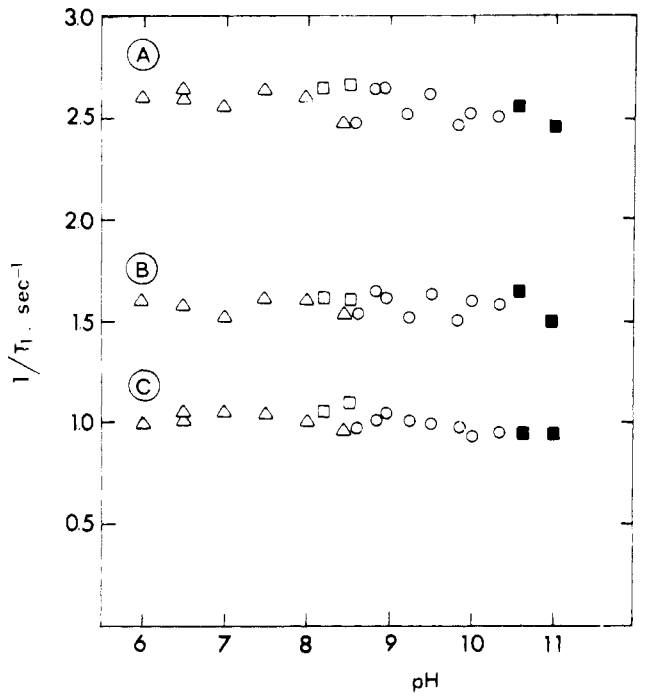


FIGURE 1: The pH dependence at 100 MHz of the longitudinal relaxation rate for solutions of 3.46×10^{-4} M beef liver catalase heme in 0.1 M buffers; (△) phosphate; (□) Tris; (○) NaOH–carbonate; (■) NaOH–phosphate. (A) Native enzyme. (B) Cyanide–catalase. (C) Net longitudinal relaxation rate, $1/T_{1p}$.

macromolecular systems, where τ_r makes an important contribution to τ_c (Dwek, 1972), is not relevant to the present system where the correlation time is dominated by the electron spin relaxation time.

(3) In eq 2 and 3 we assume that the paramagnetic ion exhibits isotropic g value. Justification of this assumption is based on the fact that the $^6A_1$ ground state of high-spin Fe(III) exhibits negligible g tensor anisotropy (Abragam and Bleaney, 1971) with $g_\parallel = g_\perp = 2.0$. Moreover, the direction of g_z in heme proteins was found to lie along the heme normal (Bennett et al., 1961). If we assume the water site in catalase to be along the z direction (normal to the heme plane), then we can take $g = 2$ even for partly anisotropic g value.

(4) The longitudinal and transverse electronic relaxation times, τ_{1s} and τ_{2s}, are assumed to be equal. Such assumption is not always justified at high fields, where $\omega_s^2\tau_{c2}^2, \omega_s^2\tau_{e2}^2 \gg 1$ (where, now, $\tau_{c2}^{-1} = \tau_r^{-1} + \tau_{e2}^{-1}$; $\tau_{e2}^{-1} = \tau_{2s}^{-1} + \tau_M^{-1}$).

(5) The distance from the iron to the protons of the water in the hydration shell, r, was taken as 2.84 Å. This is based on x-ray crystallographic studies of high-spin methemoglobin (Perutz, 1970) and of some porphyrin models (Hoard, 1966). Since in these references only the distance between the iron and the oxygen atom of the water is given, we calculated the iron–proton distance assuming axial symmetry of the water molecule with respect to the iron–oxygen axis, and O–H distance of 0.965 Å.

(6) Possible errors in the interpretation of the dipolar interaction may rise from deviations from the point-dipole approximation assumed in the Solomon–Bloembergen equations. As was pointed out (Waysbort and Navon, 1975; Lanir et al., 1975), such deviations can be caused by delocalization of the electronic spin onto the ligand orbitals. This would result in an enhanced T_{1p} value and, consequently, in a somewhat larger hydration number. A method to avoid such complications is to compare the dipolar interaction of the heme protein with that of high-spin diaquo hemin, assuming an hydration number of 2 for the latter.

Results

pH Dependence of the Water Proton Relaxation Rates. Figure 1 presents the pH dependence of $1/T_1$ at 100 MHz for a 3.46×10^{-4} M solution of beef liver catalase heme, as well as for the cyanide complex. The concentration of cyanide was 0.1 M, much above the concentration which is sufficient to saturate all the heme iron (Nicholls and Schonbaum, 1963). The relaxation of water protons in solutions of catalase–cyanide, in which the heme is in its low-spin state, can be considered as being very close to the diamagnetic contribution of the protein to the relaxation (see experimental procedure), and was taken as an appropriate blank. The pH dependence of the net relaxation in the range 6–11 is also shown in Figure 1. The pH dependence of T_{1p} could be measured only above pH 6; below this pH, part of the enzyme was precipitated almost immediately.

The pH experiments were carried out in four different buffers, all of 0.1 M ionic strength, and no specific effect of a buffer on the magnetic relaxation was observed (see Figure 1).

The lack of dependence of the relaxation on pH indicates that there are no effective changes in the spin state, nor in the heme environment of catalase, upon changing the pH. Particularly, this is strong evidence for the lack of alkaline ionization of the water, later shown to occupy the sixth coordination position of the heme. This fits the facts that catalase shows neither spectral nor catalytic activity changes in this pH range.

NMR Titrations with Catalase Ligands. Catalase forms reversible complexes with anionic ligands. It has long been recognized that the inhibition of catalase by such ligands can be interpreted in terms of complex formation with the free iron. Such complexes are spectroscopically distinct from native catalase. The NMR titration of the effect of cyanide on the water proton relaxation of catalase was shown previously to agree with the spectroscopic titration of the binding of this ligand by catalase (Lanir and Schejter, 1975b). Cyanide converts the hematin iron to its low-spin state, and such a change in spin state can reduce the relaxation rate even without any alteration in the H_2O–Fe distance. Thus, this experiment is not conclusive as to the displacement of the water from the ferric iron coordination sphere.

The NMR titrations of $1/T_{1p}$ with formate and acetate in phosphate buffer at pH 7.0 are given in Figure 2. For comparison, we also present in Figure 2 the relaxation values of the catalase–cyanide complex. The catalase–formate complex retains the high-spin state of the heme iron (Nicholls and Schonbaum, 1963). Clearly, therefore, the effect of formate represents the expelling of water from the sixth coordination position of the iron. Hence, the value of $1/T_{1p}$ (in this case $(1/T_1)_{\text{catalase}} - (1/T_1)_{\text{catalase-formate}}$) should be taken for the quantitative determination of the hydration number of the catalase iron. The difference between the relaxation rates in the formate- and cyanide–catalase complexes is the second sphere water contribution to the relaxation. It can be seen that, although the relaxation of water decreases as a function of the sixth power of the distance between the iron and the relaxed protons (eq 2), still the second sphere hydration water contributes about 20% to the overall net relaxation effect.

The titration of the water at the sixth coordination position by formate and by cyanide (see also Lanir and Schejter, 1975b) is in disagreement with recent results obtained by Hershberg and Chance (1975). It should be noticed in Figure 2 that small

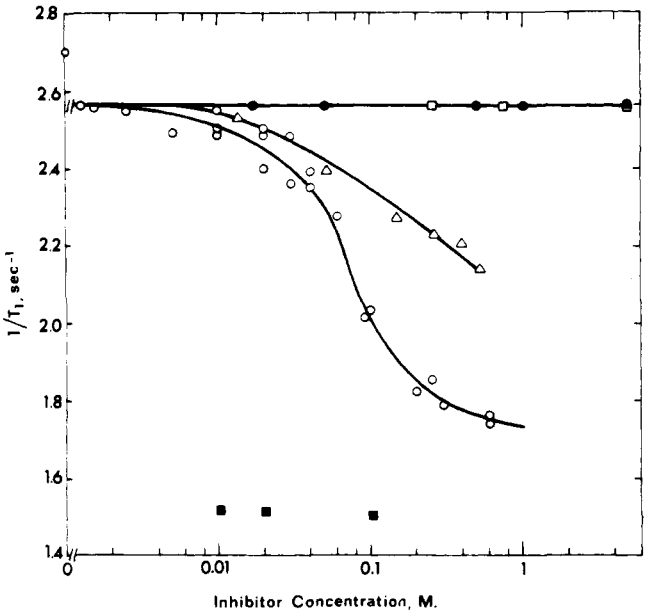


FIGURE 2: Longitudinal relaxation rates plotted against inhibitors and substrates concentrations. The effect was studied at 100 MHz, at 30 °C in solutions of 3.73×10^{-4} M of catalase heme. The solutions contained phosphate buffer 0.1 M, pH 7.0; (○) sodium formate; (△) sodium acetate; (●) ethanol; (□) methanol; (■) cyanide.

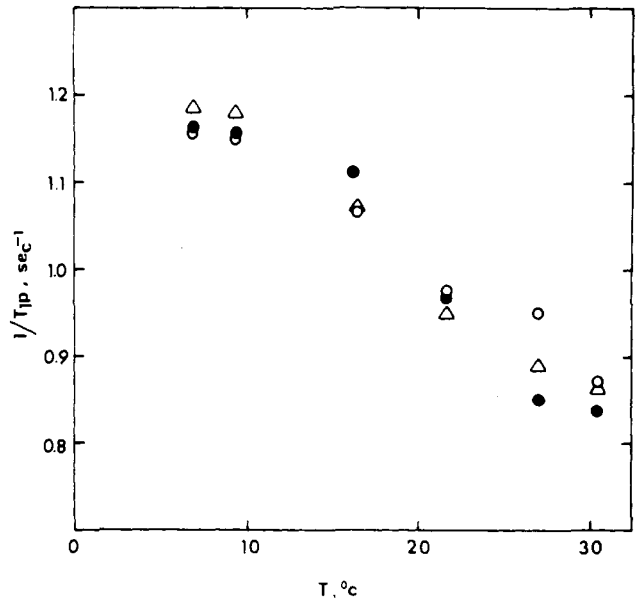


FIGURE 3: Temperature dependence of the net longitudinal relaxation rates at 100 MHz. The solutions contained 3.22×10^{-4} M of catalase heme in 0.1 M phosphate buffers; the pH values are: (△) 6.9; (○) 7.18; (●) 7.86.

concentration of anions caused a reduction of about 8% in the relaxation rate. This small but significant effect will be discussed later.

Effect of Hydrogen Donors. Catalase promotes the decomposition of H_2O_2 by catalatic and peroxidatic reactions (Nicholls and Schonbaum, 1963). In the peroxidatic reactions small organic acid anions such as formate or acetate and small alcohols such as methanol and ethanol act as hydrogen donors, reducing the "compound I" formed upon reaction between catalase and H_2O_2. As indicated in Figure 2, only a minor effect, of less than 10% reduction in the net relaxation rate, was obtained upon addition of small concentrations of alcohols, but no further reduction was obtained even at very high concentrations. Clearly, hydrogen donors do not displace the iron-bound water in beef liver catalase.

The small effect on $1/T_{1p}$, which was also observed upon addition of small amounts of formate or acetate, can be explained by the binding of the hydrogen donors to a specific site close to the heme iron (Hershberg and Chance, 1975), if such binding displaces a water molecule located in the second coordination sphere of the iron. Unfortunately, this effect is too small for precise quantitative studies.

Temperature Dependence of Longitudinal Relaxation Rates. The longitudinal relaxation rates of water in 3.22×10^{-4} M solutions of catalase heme were measured in the temperature range of 2–31 °C at three pH values (phosphate buffer, pH 6.9, 7.18, and 7.86) using a frequency of 100 MHz. The results are plotted in Figure 3. The values of $1/T_{1p}$ decrease monotonically with increasing temperature. The trend of temperature dependence unequivocally indicates that the exchange lifetime of the bound water, τ_M, is shorter than its relaxation time, T_{1M}. From Figure 1, the calculated value of T_{1M} for the water protons at 30 °C is 6.7×10^{-6} s, assuming a hydration number of 1.1 for the heme environment, as will be seen below. Since $\tau_M = k_{off}^{-1}$, where k_{off} is the dissociation constant of the water–metal complex, we obtain a lower limit of $k_{off} = 1.5 \times 10^5$ s^{-1}. This value of the lower limit of k_{off} is in agreement with the exchange lifetime values of water from the coordination sphere of many other high-spin ferric iron systems, which were found to be in the range of several microseconds in inorganic complexes (Connick and Poulson, 1959; Luz and Shulman, 1965), high-spin non-heme-iron ferric proteins (Koenig and Schillinger, 1969; Villafranca et al., 1974) and heme proteins (Pifat et al., 1973; Lanir and Aviram, 1975). A slower water-exchange lifetime was found in methemoglobin in which $k_{off} = 10^4$ s^{-1} (Gupta and Mildvan, 1975).

T_{1p}/T_{2p} Ratio and the Contribution of the Hyperfine Term. In order to calculate the hydration number of the ferric ion in catalase according to eq 1–4, one should know precisely the correlation time, τ_c, which in our system is equal to the electron spin relaxation time of the high-spin ferric iron, τ_s. In some cases, τ_c may be uniquely determined by measuring the ratio T_{1p}/T_{2p} at a single, preferably high frequency. This technique proved to be useful in manganese-containing proteins for which only the dipolar term in eq 2–3 should be considered, and τ_c is longer than 10^{-9} s, so that $\omega_I^2\tau_c^2 > 1$ at high frequencies and the ratio T_{1p}/T_{2p} is significantly greater than 1.19 (Navon, 1970; Mildvan et al., 1971; Cohn et al., 1971; Lanir et al., 1975). In systems where the hyperfine term is negligible and τ_c is shorter than 10^{-9} s, $T_{1p}/T_{2p} = 1.19$ and no information can be obtained from this technique concerning the magnitude of τ_c.

Nevertheless, if the hyperfine terms contribute significantly to the relaxation times, then one expects $T_{1p}/T_{2p} \gg 1.19$ even for τ_c values much shorter than 10^{-9} s, and the ratio T_{1p}/T_{2p} may be used to estimate τ_c. Figure 4 illustrates the theoretical (T_{1p}/T_{2p}) hyperfine ratios at several NMR frequencies as a function of τ_c. It appears that this ratio is a very sensitive function of the correlation time even for short τ_c, particularly at high frequencies. We have measured the value of the T_{1p}/T_{2p} ratio at 100 MHz in solutions of catalase at several pH values. In all solutions $T_{1p}/T_{2p} = 1.3 \pm 0.2$. Since the fast exchange conditions hold for the present system, it appears that the hyperfine term does not contribute, or contributes rather little to the overall relaxation rates.

Frequency Dependence of Relaxation and Calculation of

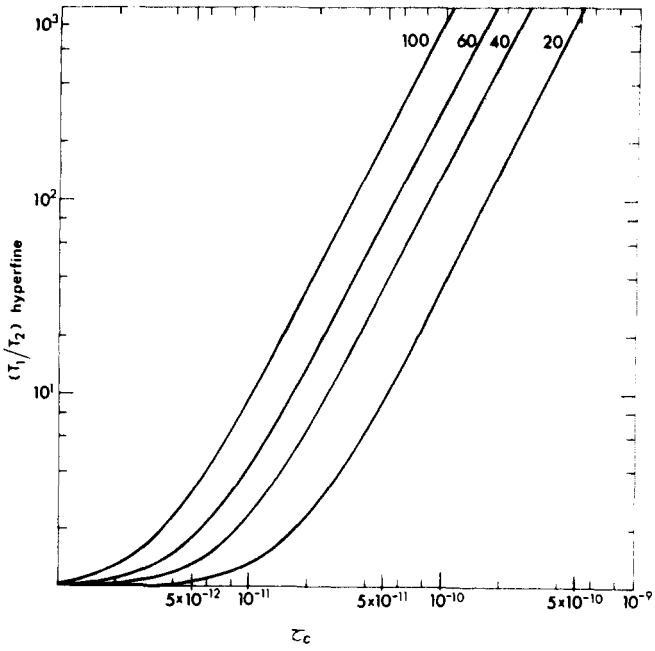


FIGURE 4: Theoretical ratios of the hyperfine contributions to T_{1M} and T_{2M} at four frequencies as a function of τ_c.

the Hydration Number in Catalase. In Figure 5 the longitudinal relaxation rates of a solution of catalase are plotted against the proton Larmor frequency. It can be seen that, above 25 MHz, the relaxation rate is frequency independent; below 25 MHz, the relaxation rate increases. Since the correlation time τ_c for the present system is dominated by τ_s, such frequency dependence of $1/T_{1p}$ is possible only if $1 \gg \omega_s^2\tau_v^2$ in eq 4, that is, τ_s for high-spin ferric ion is frequency independent. The frequency dependence of $1/T_{1p}$ below 25 MHz follows the dipolar term of the Solomon-Bloembergen equation (eq 2). In our case, therefore, τ_c itself is not a function of frequency. The results for $1/T_{1p}$ were fitted according to eq 1 and 2 for two parameters: q and τ_s. τ_c was taken as τ_s and $r = 2.84$ Å as outlined in the theoretical section. The least-squares fitting procedure yielded $\tau_s = 7 \times 10^{-11}$ s and $q = 1.3$.

The value of τ_s is in perfect agreement with the value obtained for τ_s of Fe^{3+} in catalase by Hershberg and Chance (1975) and is similar to the values obtained in other high-spin heme proteins (La Mar and Walker, 1973; Lanir and Aviram, 1975).

The hydration number in this experiment was found to be 1.3. This includes the effect of the water in the second sphere. If, however, we take $1/T_{1p}$ as the difference between the relaxation rates in the absence and in the presence of saturating amounts of formate, taking $\tau_c = 7 \times 10^{-11}$ s, then the hydration number, calculated from eq 2, is 1.1, indicating that one molecule of water is bound directly to the Fe^{3+} in catalase.

Effect of pH on the Binding of Formate by Catalase. It is a well-known fact that the binding of anionic ligands to catalase measured by optical spectroscopy is a pH-dependent reaction (Chance, 1952a; Hershberg and Chance, 1975). Formate is no exception to this rule. When NMR titrations of the displacement of water from the catalase iron (Figure 1) were performed at various pH's and the corresponding association constants were estimated, it was found that the latter did not change with the pH in the range of 7–8.5, and its value is 14.3 ± 2.0 M^{-1}. Obviously, the reactions of catalase with formate measured by optical spectroscopy and pulsed NMR are different.

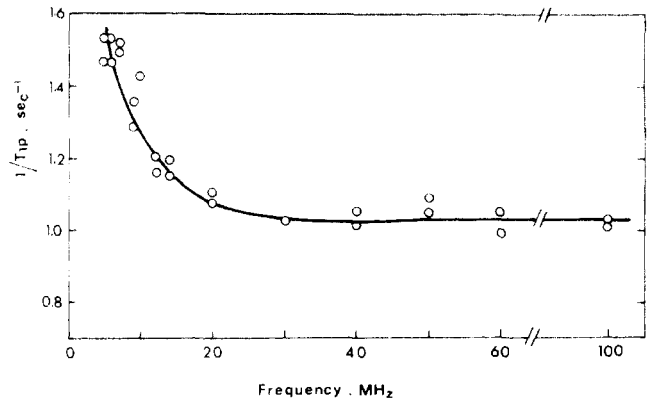


FIGURE 5: Net longitudinal relaxation rate, $1/T_{1p}$, plotted against proton Larmor frequency for solution of 3.5×10^{-4} M catalase heme. The curve was calculated from the best fit parameters: $q = 1.3$ and $\tau_c = 7 \times 10^{-11}$ s.

Discussion

From the experiments described in this article, the hydration number of the ferric iron of beef liver catalase was estimated as 1.1 ± 0.1. This value was obtained from the frequency dependence of T_{1p}, and it should be noticed that in the present case this procedure suffers from a theoretical shortcoming. Since the fitted function is not very sensitive to all the parameters at high frequencies, by necessity, the fitting procedure relies on values of T_{1p} measured at low frequencies, where the use of the Solomon-Bloembergen equations (eq 2 and 3) is not entirely justified, in view of the high value of the zfs for Fe^{3+} in heme proteins (see theoretical background).

Unfortunately, the hydration number cannot be deduced in the present case by the alternative procedure that uses the ratio T_{1p}/T_{2p}, because the electronic correlation time of high-spin ferric iron is too short.

It is also worth noticing that the hyperfine terms were neglected in the calculations. This is justified, since the experimental value of T_{1p}/T_{2p} excludes the possibility of any important contribution of the hyperfine terms to T_{1M} and T_{2M} (see Figure 4). Moreover, this can be demonstrated by calculating the hyperfine terms, using the value of $\tau_s = 7 \times 10^{-11}$ s that results from the least-squares fitting procedure, (Figure 5), and $A/\hbar = 1.9 \times 10^6$ Hz for the Fe^{3+} electron nuclear hyperfine coupling constant (Angerman et al., 1969). Introducing these values into the hyperfine terms in eq 2 and 3, it can be calculated that the hyperfine exchange term contributes less than 1% to the overall net relaxation rate.

In conclusion, therefore, the experiments are in keeping with the view that the sixth coordination position of the iron in beef liver catalase is occupied by a water molecule (Chance, 1952a; Nicholls and Schonbaum, 1963). Catalase is, therefore, an "open crevice" heme protein, in the terminology of George and Lyster (1958); and the mean residence time of the catalase water is $\tau_M < 6.7 \times 10^{-6}$ s, somewhat shorter than those observed for metmyoglobin and methemoglobin.

This conclusion is reached on the basis of temperature and frequency dependencies of the longitudinal relaxation rates. It should be noticed, however, that the trend of the temperature dependence of $1/T_{1p}$ is somewhat unexpected. Since in our case, $1 \gg \omega_s^2\tau_v^2$ in eq 4, a negative temperature coefficient for τ_s is expected according to the theory of Bloembergen and Morgan (1961) which assumes that the entire temperature dependence of τ_s is due to τ_v. This is incompatible with our results as presented in Figure 3. However, the expression for τ_s^{-1} includes a term, D, which depends on the collisions be-

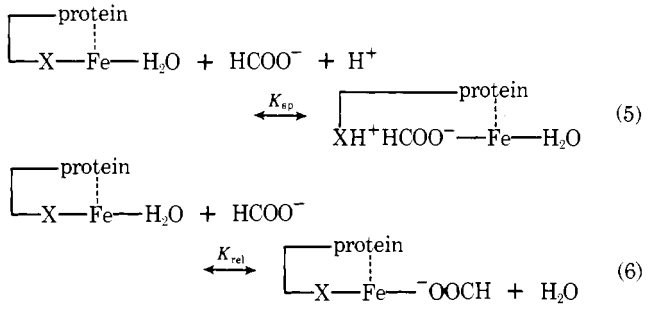
tween the hydrated ferric ion and the solvent molecules. As was noticed by Koenig and Epstein (1975) this interaction increases as T increases in opposite direction to that of τ_v. Hence, any temperature dependence of τ_s should be expected even in the case in which $1 \gg \omega_s^2\tau_v^2$.

In one important sense catalase differs from other open crevice heme proteins: the iron-bound water does not ionize up to pH 12 (Figure 1). The relaxation data of Figure 1 are thus in perfect agreement with the absence of pH effects on the spectrum and catalytic activity of catalase (Chance, 1952a).

In metmyoglobin and methemoglobin, the iron-bound water ionizes already at pH's around 9 (George and Hanania, 1952, 1953). It is not altogether clear why this ionization does not take place in catalase. It must be kept in mind, however, that the pK of the iron-bound water may well be affected by its environment. In myoglobin and hemoglobin, for example, the ionization is apparently assisted by the basicity of the distal histidine to which the iron-bound water is associated through a hydrogen bond (Shire et al., 1974; Gupta and Mildvan, 1975; Hanania and Nakhleh, 1975); this structural feature may be absent in catalase.

Water is displaced from the catalase iron by formate and acetate (Figure 2). However, this reaction is not the well-known binding of the same ligands to catalase detected by optical spectroscopy (Chance, 1952a). First, the association constants at pH 7.0 and 25 °C differ by one order or magnitude. Second, while the affinities measured by optical spectroscopy are pH dependent, those estimated from the displacement of water are not affected by the pH.

This behavior of catalase in its reactions with formate and acetate suggests that the catalase iron can bind two anionic ligands: one of them displaces the iron-bound water, while the other probably replaces an amino acid of the protein chain that is able to bind a proton from the solution (George and Lyster, 1958). The following equations represent the proposed reactions:



In these equations, X denotes the amino acid bound to the iron in the native enzyme; the dotted line represents the noncovalent forces that keep the heme group attached to the catalase protein; K_{sp} and K_{rel} are the binding constants for the reactions detected by optical spectroscopy and pulsed NMR, respectively. It should be pointed out that eq 5 is similar to the formulation proposed by George and Lyster (1958) for the binding of ligands to "closed-crevice" heme proteins.

The normal titration curves indicate that the two binding reactions of formate are independent of each other. Hershberg and Chance (1975) reported the binding of formate to the catalase protein. These authors stated that the catalase iron-bound water is not displaced by formate, and, by studying the relaxation rates of the proton in formate, estimated an average minimal distance of 7 Å between this proton and the iron atom of the enzyme. Our results indicate clearly that water is displaced by formate; there seems to be no other valid explanation for the loss of water proton relaxation shown in Figure 2, since the spin state of the iron is not changed upon formate ligation. We conclude, therefore, that the binding of formate to catalase 7 Å away from the iron is not the same reaction observed in our experiments.

It seems unlikely that the catalase iron is the binding site for the formate anion that acts as hydrogen donor in the peroxidatic activity of the enzyme. In the first place, the peroxidatic activity is not pH dependent, so that the closed site of the crevice cannot be the enzymatic active site; secondly, neither methanol nor ethanol in concentrations at which they act as donors was found to displace the iron-bound water. The possibility exists, thus, that the binding site for the hydrogen donors of catalase in its peroxidatic reaction lies at some distance from the iron; the site found by Hershberg and Chance (1975) fits this requirement. In this sense, beef liver catalase appears to be similar to horseradish peroxidase, for which a binding site for donors was estimated at a distance of 7–9 Å from the enzyme iron (Schejter et al., 1976).

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