PROTON MAGNETIC RELAXATION DISPERSION IN SOLUTIONS OF THE CUPROPROTEIN DIAMINE OXIDASE

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ABSTRACT Proton nuclear magnetic resonance relaxation measurements were made over the range 4.7–220 MHz for aqueous solutions of hog kidney diamine oxidase. The values of $1/T_1$ give rise in two distinct dispersions, at 16 and 75 MHz, whereas $1/T_2$ displays a minimum at 20 MHz. The temperature dependence of relaxation rates in all cases yield apparent activation energies <0.6 kcal/mol. These data indicate to us that the two Cu(II) ions of diamine oxidase are intrinsically different in terms of their electronic relaxation characteristics and hence, chemical environments. Low field limits of the two electronic relaxation times are 2 and 10 ns, with one of these correlation times being frequency dependent. The value of the frequency-dependent electronic relaxation time is governed by interactions that are modulated by a process having a correlation time of 5 ps.

INTRODUCTION

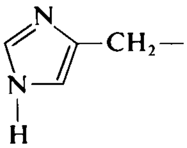
Hog kidney diamine oxidase (diamine: O_2 oxidoreductase [deaminating]; EC 1.4.3.6) catalyzes the oxidative deamination of diamines, such as putrescine and cadaverine, as well as histamine. The general reaction is:

$$R\text{—}CH_2\text{—}NH_2 + O_2 + H_2O \rightarrow R\text{—}CHO + H_2O_2 + NH_3,$$

where R =

$NH_2\text{—}CH_2\text{—}CH_2\text{—}CH_2\text{—}$ putrescine

$NH_2\text{—}CH_2\text{—}CH_2\text{—}CH_2\text{—}CH_2\text{—}$ cadaverine



histamine.

The enzyme contains two firmly bound Cu(II) ions, presumably one on each of its two subunits. We have been particularly interested in the role of the Cu(II) in this system. In

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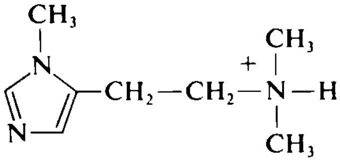
another nuclear magnetic resonance (NMR) study (Kluetz and Schmidt, 1977*a*), we partially mapped the active center of diamine oxidase with respect to these paramagnetic centers to determine the nature of the substrate and product binding and, knowing the location of the Cu(II) in the active site, to elucidate its function.

We previously found that these paramagnetic centers have a large effect on the nuclear relaxation rates of the bulk water protons. Our preliminary analysis indicated that there is at least one rapidly exchanging water which binds within an inner sphere ligand distance from Cu(II), ~3 Å. We also determined that at high concentrations, substrate analogs such as methylhistamines, in particular 3-methyl-4-(β-dimethylammonium)ethyl imidazole chloride (MH*I*), bind with the imidazole ring nitrogen within 4 Å of the Cu(II).



MH*I*

Upon doing so, the MH*I* displaces H_2O from its coordination position, decreasing the longitudinal ($1/T_1$) and transverse ($1/T_2$) relaxation rates of the water protons. However, the paramagnetically enhanced relaxation rates normally observed are not reduced to zero, as they would be if all of the water were competed off by the substrate analog, nor are they reduced to one half of the original value, as would be the case if only one of two equivalent waters were being displaced. This result suggests to us that there is more than one water molecule bound near a Cu(II) ion and that these waters are not equivalent. We therefore undertook to determine more precisely the nature of the interaction between water molecules and the Cu(II) ions in diamine oxidase by measuring T_1 and T_2 over a frequency range of 4.7–220 MHz at a variety of temperatures. The relaxation results are not characteristic of copper proteins previously studied and require a new approach to elucidate the mechanisms involved.

THEORY

The longitudinal and transverse nuclear relaxation rates of a proton on a molecule in solution undergoing chemical exchange from a site on a macromolecule are given, respectively, by the equations of Luz and Meiboom (1964) and of Swift and Connick (1962). If there is no significant difference in chemical shift between free and bound states, these take the forms:

$$1/T_{1p} = 1/T_1)_{\text{obs}} - 1/T_1)_{\text{free}} = \frac{P}{T_{1m} + \tau_m} \tag{1}$$

and

$$1/T_{2p} = 1/T_2)_{\text{obs}} - 1/T_2)_{\text{free}} = \frac{P}{T_{2m} + \tau_m} \tag{2}$$

where the $1/T_i)_{\text{obs}}$ ($i = 1, 2$) and $1/T_i)_{\text{free}}$ are, respectively, the relaxation rates observed in the presence of macromolecule and those of the molecule free in solution. P is the fraction of protons bound to the macromolecule, the $1/T_{im}$ are the relaxation rates in the bound state, and τ_m is the first-order lifetime in the bound state. In the present case of water exchanging from the paramagnetic centers on the enzyme, relaxation of the water protons is dominated by the large dipolar and (generally small) scalar interaction between the nuclear spin and the unpaired electron spin. The relaxation rates of nuclei bound near a paramagnetic ion are given by (Connick and Fiat, 1966; Reuben et al., 1970; see also Koenig, 1972, and Rubinstein et al., 1971):

$$1/T_{1m} = 2B\left[\frac{3\tau_{c1}}{1+\omega_I^2\tau_{c1}^2} + \frac{7\tau_{c2}}{1+\omega_S^2\tau_{c2}^2}\right] + 2C\left[\frac{\tau_{e2}}{1+\omega_S^2\tau_{e2}^2}\right] \tag{3}$$

$$1/T_{2m} = B\left[4\tau_{c1} + \frac{3\tau_{c1}}{1+\omega_I^2\tau_{c1}^2} + \frac{13\tau_{c2}}{1+\omega_S^2\tau_{c2}^2}\right] + C\left[\tau_{e1} + \frac{\tau_{e2}}{1+\omega_S^2\tau_{e2}^2}\right] \tag{4}$$

where

$$B = (1/15)(\gamma_I^2 g^2 \beta^2 S(S+1)/r^6)$$

$$C = (1/3)(A^2 S(S+1)/h^2).$$

γ_I is the magnetogyric ratio of the proton; g is the Lande g-factor of the electron; β is the Bohr magneton; S is the total electron spin of the Cu(II); r is the distance of separation of the nuclear and electron spins; ω_I and ω_S are, respectively, the nuclear and electron precession frequencies in radians per second ($\omega_S = 658\ \omega_I$); and A is the electron-nuclear hyperfine coupling constant.

The τ_c's and τ_e's are correlation times for the dipolar and scalar interactions, respectively, and are given by the following composite quantities:

$$1/\tau_{c1} = 1/\tau_m + 1/\tau_r + 1/T_{1e}, \tag{5}$$

$$1/\tau_{c2} = 1/\tau_m + 1/\tau_r + 1/T_{2e}, \tag{6}$$

$$1/\tau_{e1} = 1/\tau_m + 1/T_{1e}, \tag{7}$$

$$1/\tau_{e2} = 1/\tau_m + 1/T_{2e}, \tag{8}$$

where τ_m is, again, the exchange lifetime of the water on the enzyme, τ_r is the rotational correlation time of the enzyme, and T_{1e} and T_{2e} are, respectively, the longitudinal and transverse relaxation times of the electron spin. For a protein of molecular weight 175,000 we expect τ_r to be $\sim$120 ns.

The sixth-power dependence of the nuclear relaxation rates on the distance of approach of protons to the paramagnetic center allows for very accurate determinations of these distances if scalar relaxation is negligible and if the correlation times $\tau_{c1,2}$ for the dipolar interaction are known. As evidenced by Eqs. 3 and 4, measurements of nuclear relaxation rates as a function of frequency will display regions where the rate decreases rapidly with frequency as the various spectral density terms "disperse"; i.e., as one passes between the limits $\omega^2\tau^2 \ll 1$ and $\omega^2\tau^2 \gg 1$, at which point the contribution from the particular term becomes negligible. The midpoint of the transition occurs when $\omega\tau_c = 1$, and a fit of the curve allows one to determine τ_c. If $\tau_{c1} = \tau_{c2}$, for example, and these remain constant at all frequencies, the ω_S terms will disperse at a frequency 658 times lower than that corresponding to dispersion of the ω_I terms, probably in the 100-kHz region. If one is confined to a higher frequency region, as we are, dispersion of the ω_S terms is not seen and one can employ simplified forms of the relaxation equations that contain only τ_{c1} and τ_{e1} and hence depend only on the longitudinal relaxation rate of the electron spin, T_{1e}. These simplified equations are given below.

$$1/T_{1m} = 2B\left[\frac{3\tau_{c1}}{1+\omega_I^2\tau_{c1}^2}\right] \tag{9}$$

$$1/T_{2m} = B\left[4\tau_{c1} + \frac{3\tau_{c1}}{1+\omega_I^2\tau_{c1}^2}\right] + C\tau_{e1} \tag{10}$$

Just as the dipolar component of nuclear relaxation is modulated via the spectral density functions as in Eqs. 9 and 10 by processes such as exchange, rotation, or changes in electron spin orientation, the electronic relaxation time itself is affected by modulation of such interactions as zero-field splitting, hyperfine splitting, and spin-orbit coupling (Bloembergen and Morgan, 1961; Lewis and Morgan, 1968). One can account for the frequency dependence of the electronic relaxation rate by an equation of the form:

$$1/T_{1e} = \frac{D\tau_v}{1+\omega_S^2\tau_v^2}, \tag{11}$$

where D represents some interaction of the unpaired spin with its environment and τ_v in the spectral density term is the characteristic time for modulation of this interaction. In the limit $\omega_S^2\tau_v^2 \ll 1$, $D\tau_v = 1/T_{1e}^{\circ}$.

EXPERIMENTAL PROCEDURE

Diamine Oxidase

Diamine oxidase was prepared from fresh hog kidneys by a procedure developed in our laboratory (Kluetz and Schmidt, 1977*b*). The enzyme was electrophoretically pure and had a specific activity near the maximum observed in this system. Solutions were made with 0.050 M potassium P_i buffer, pH 7.2, prepared with glass-distilled water.

Our early water proton relaxation experiments indicated the presence of loosely bound extraneous Cu(II). Therefore, we routinely dialyze diamine oxidase solutions against 1 mM EDTA, followed by buffer alone, to remove this excess Cu(II). This procedure does not remove any active site Cu(II), does not affect the enzymatic activity, and is sufficient to remove all of the nonspecifically bound metal. We found, for example, that removal of this extraneous metal resulted in reductions of 14 and 25%, respectively, of the water proton $1/T_{1p}$ and $1/T_{2p}$ (in a $5.7 \cdot 10^{-5}$ M solution of diamine oxidase, at 220 MHz). Also, complete resolution of the hyperfine structure in the electron paramagnetic resonance (EPR) spectrum and a relatively narrow, symmetric g_m peak are not obtained until this excess Cu(II) is removed (see Fig. 1). Diamine oxidase treated in this way has been shown to contain only a stoichiometric amount of active site Cu(II) (Kluetz and Schmidt, 1977*b*).

NMR Relaxation Measurements

Longitudinal ($1/T_1$) and transverse ($1/T_2$) relaxation rates were measured over a 4.7–220-MHz range with a variable-frequency pulse spectrometer. The instrument employed a Varian Associates (Palo Alto, Calif.; model V-7400) 15-inch electromagnet with an operating range of up to 24 kG. A Varian flux stabilizer (model V-3508) and field homogeneity unit (model V-7530) provided adequate field stability and homogeneity. The output of the frequency synthesizer (Rockland, Inc., Gilbertsville, Pa.; model

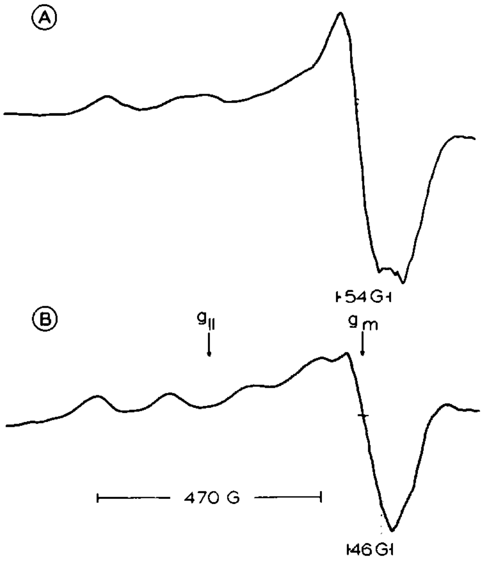


FIGURE 1 9 GHz EPR spectra of 0.25 mM hog kidney DAO before (A) and after (B) treatment with EDTA (see Experimental Procedure). $T = 77$ K. From the spectrum in frame B one obtains $A_\parallel = 0.015$ cm^{-1} (157 G).

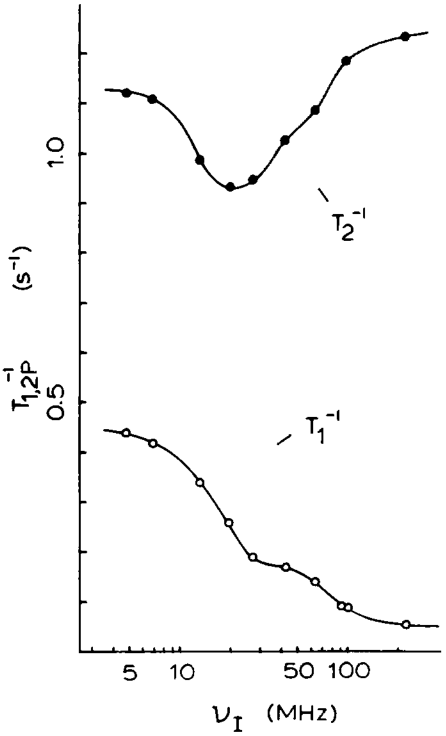


FIGURE 2 $1/T_{1p}$ (○) and $1/T_{2p}$ (●) are plotted vs. the proton Larmor frequency, ν_I. The data were obtained on a 0.1 mM solution of diamine oxidase at a temperature of 24°C.

5600; 0.1–160 MHz) was split into two channels by the gating unit and was phase-shifted for the pulse sequence of Carr and Purcell (1954) with the modification of Meiboom and Gill (1958). The A and B channels were gated by a pulse programmer through double-balanced mixers. The gated low-level radio frequency was amplified using an Arenberg Ultrasonic Inc. (Jamaica Plain, Mass.; model PG 650) turnable oscillator operated in the gated amplifier mode (this unit puts out up to 400 W). The probe design (single coil, 6.5-mm diam) featured interchangeable plug-in insert, vacuum Dewar assemblies. The 90° pulse widths were ~2 μs. The receiver was broad-banded (3–150 MHz) and employed a Watkins-Johnson Inc., Palo Alto, Calif., preamplifier (28 dB gain) and Kay Elemetrics Corp. (Pine Brook, N.J.) amplifier (40 dB). A double-balanced mixer was the phase-sensitive detector, the output of which was applied to an operational amplifier for further gain and low-pass filtering.

The $1/T_1$ measurements were made with the $180°\text{-}\tau\text{-}90°$ pulse sequence. Free induction decays (FID) after the 90° pulse were collected on a storage oscilloscope for measurement of their amplitudes as a function of τ. At least 8 τ values were sampled and acquisitions were repeated at intervals of no less than $5T_1$. The signal:noise ratio of the FID was sufficiently good even at the lowest frequency that semilog plots of the magnetization, $\ell n[M_0 - M(\tau)]$ vs. τ, showed essentially no scatter and were linear over their entire range. The T_1 values are estimated to have an experimental uncertainity of $\sim \pm 5\%$ and a deviation on a particular sample of not more than $\pm 3\%$.

The $1/T_2$ values were obtained from the decay of the echo envelope plotted on an oscillographic recorder. We used a delay of $2\tau = 1$ ms between 180° pulses. The $1/T_2$ results have approximately the same error limits as $1/T_1$.

Probe temperature was controlled by a thermostatted flow of N_2. A calibrated thermocouple encased in the NMR tube was used to measure the sample temperature before and after relaxation measurements. These temperatures varied by $<1°$C after the temperature was stabilized.

RESULTS AND DISCUSSION

Frequency Dependence of Relaxation Rates

Fig. 2 displays the results of measurements of $1/T_1$ and $1/T_2$ at 4.7, 6.9, 13, 19.5, 27, 42, 64, 92, 100, and 220 MHz obtained at 24°C. The diamine oxidase concentration was 0.1 mM. The longitudinal relaxation rate decreases monotonically with increasing frequency showing two dispersions, one near 16 MHz and another near 75 MHz. The traverse rate, on the other hand, has a minimum near 20 MHz.

Temperature Dependence

The temperature dependences of $1/T_1$ and $1/T_2$ were measured in the present work at 4.7 MHz. In a previous study (Kluetz and Schmidt, 1977*a*) we determined the variation of $1/T_1$ with temperature at 100 and 220 MHz and of $1/T_2$ at 60, 100 and 220 MHz. These results are combined with those at 4.7 MHz in Fig. 3.

Not surprisingly, the temperature dependence of the relaxation rates is a function of NMR frequency. However, it is noteworthy that in all cases the slopes of relaxation rate vs. $1/T$ are small, with the largest apparent activation energy being 0.6 kcal/mol; also, in all cases, $1/T_2 > 1/T_1$. This indicates that $\tau_m < T_{1m}, T_{2m}$ (Eqs. 1 and 2) and that water protons are in fast exchange with the paramagnetic centers; therefore, $1/T_{1p} = P/T_{1m}$ and $1/T_{2p} = P/T_{2m}$. The small activation energies are consistent with the electronic relaxation rate being the dominant contribution to τ_{c1} (and τ_{e1}) over the whole range since activation energies of >5 kcal/mol would be expected for τ_r and τ_m. This limiting behavior simplifies analyses of the relaxation dispersion.

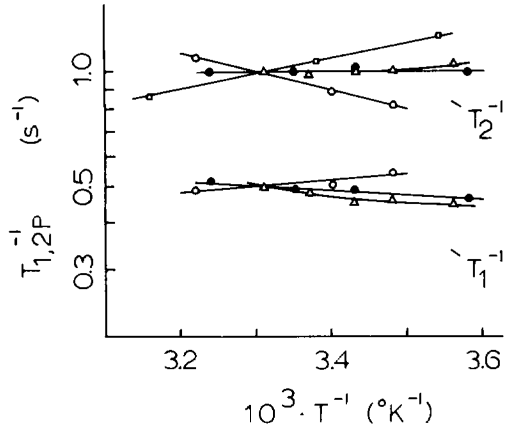


FIGURE 3 $1/T_2$ at 4.7, 60, 100, and 220 MHz, and $1/T_1$ at 4.7, 100, and 220 MHz are plotted vs. reciprocal temperature. (▵) 4.7 MHz, (☐) 60 MHz, (●) 100 MHz, (○) 220 MHz. For clarity, the data from the individual experiments have been normalized to yield $1/T_{2p} = 1.0\ s^{-1}$ and $1/T_{1p} = 0.5\ s^{-1}$ at 30°C.

Longitudinal Relaxation Rates

The data of Fig. 2 indicate the dispersion of two spectral density terms, one at 16 MHz and another at 75 MHz. We initially made an effort to fit the $1/T_{1p}$ curve to Eqs. 9 and 11. Previous work of Gaber et al. (1972) on the Cu(II)-containing enzyme superoxide dismutase is useful to consider here. They obtained T_1 measurements down to 0.05 MHz and observed dispersion of both the ω_S and ω_I terms of Eq. 3. The curve of $1/T_1$ vs. increasing frequency showed an initial decrease at very low frequency due to dispersion of the $\omega_S\tau_{c2}$ term of Eq. 3, followed by an upward deflection as τ_{c1} (T_{1e}) increased with frequency (Eq. 5) and finally a second decrease for the dispersion of $\omega_I\tau_{c1}$ (Eq. 3). From these results and others (Koenig and Brown, 1973) we expect to find for diamine oxidase that: (*a*) the $\omega_S\tau_{c2}$ term of Eq. 3 is negligible for frequencies above 4.7 MHz, and (*b*) τ_{c1} (T_{1e}) may be frequency dependent in the range 4.7–220 MHz. Again, because of point *a* above, we can use the simplified Eqs. 9 and 10.

Although there is evidence for $1/T_{1e}$ dispersion (see below), one does not obtain the type of curve in Fig. 2 even if $1/T_{1e}$ dispersion and dispersion of the ω_I spectral density term in $1/T_{1m}$ occur at approximately the same frequency. This fact has been verified by extensive computer calculations in an attempt to generate the type of monotonically decreasing biphasic curve observed.[1] The only way the curve can be fit is by including two dispersions of $\omega_I\tau_{c1}$ terms, which necessarily implies two different correlation times. This could occur if, for example, there were two water ligands per Cu(II), one whose τ_{c1} was governed by T_{1e} and one by τ_m (or possibly τ_r) (Eq. 5). However, the temperature-dependence data (Fig. 3) would seem to rule out this possibility since the activation energies are consistent with only a T_{1e} contribution to

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[1] Once $1/T_{1m}$ (Eq. 9) begins to disperse, it rapidly goes to zero (unlike $1/T_{2m}$). If τ_{c1} (T_{1e}, Eq. 11) increases after $1/T_{1m}$ begins to disperse, it only serves to enhance the rate with which $1/T_{1m}$ goes to zero as a function of frequency, and no inflection point occurs.

τ_{c1}. Thus the two Cu(II) centers in diamine oxidase appear to be intrinsically different in terms of their electronic relaxation rates.

Unfortunately, the EPR spectrum (Fig. 1, frame B) does not unequivocally indicate whether or not the two Cu(II) sites are different, as the parameters describing the EPR spectrum are relatively insensitive. There are many small Cu(II) complexes, and other cuproproteins, whose EPR parameters ($A_{\parallel}$ and $g_{\parallel}$) are within a few percent of those found with diamine oxidase (DAO), despite a large variation in the nature of the donor atoms and the structure. (Malmström and Vänngård, 1960; see also Kivelson and Neiman, 1961, and Beinert and Palmer, 1965). In addition, we have recently measured the electronic relaxation rate (via water proton relaxation) of the Cu(II) in pea seedling DAO[2] and found it to be 0.7 ns, which is substantially less than the 2 and 10 ns reported here. However, the $A_{\parallel}$ and $g_{\parallel}$ are again within a few percent of those of hog kidney DAO, and the linewidths of both the g_m and hyperfine lines are indistinguishable.

Eq. 1 can be rewritten to account for two different relaxation centers:

$$1/T_{1p} = P_1/T_{1m,1} + P_2/T_{1m,2}, \tag{12}$$

where $\tau_m < T_{1m,i}$ ($i = 1,2$) as obtained in the present case. Using the T_1 data of Fig. 2 and Eq. 9, two correlation times were derived; $\tau_{c1,1} = 10$ ns for the dispersion at 16 MHz and $\tau_{c1,2} = 2$ ns for the 75 MHz dispersion. These values are within the range of previously determined electron spin relaxation times for Cu(II) in other proteins (Koenig and Brown, 1973).

Transverse Relaxation Rates

In the present study, the $1/T_2$ data have proven useful in elucidating the difference in electronic relaxation properties of the two Cu(II) ions in this system. If relaxation of water protons were produced purely by electron-nuclear, dipole-dipole interactions, we would expect that the value of T_{1p}/T_{2p} should approach 7/6 at low frequency (e.g., 4.7 MHz) since from the T_1 data it appears that $\omega_S^2\tau_{c2}^2 \gg 1$ and $\omega_I^2\tau_{c1}^2 \ll 1$ at this frequency. The actual ratio is 2.6. Clearly other processes contribute to T_2 that are unimportant for T_1; possibilities include scalar relaxation contributions, diamagnetic (nuclear-nuclear) interactions, and chemical exchange of water protons from environments of significantly different chemical shift. However, neither of the latter two mechanisms would account for the distinct behavior of $1/T_2$ with increasing frequency, namely, a decrease to ~20 MHz followed by an increase. Scalar relaxation can be included in the analysis of $1/T_2$ in a straightforward way by recognizing that $\tau_{e1} = \tau_{c1} = T_{1e}$ in the frequency region of interest. Then

$$1/T_{2m} = \tau_{c1}(4B + C) + 3B\tau_{c1}/(1 + \omega_I^2\tau_{c1}^2). \tag{13}$$

While the data display the expected dispersion near 16 MHz corresponding to that observed with $1/T_1$, the rise above 20 MHz indicates that the correlation time must be increasing with frequency, because in this region

$$1/T_{2m} = \tau_{c1}(4B + C), \tag{14}$$

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[2] KLUETZ, M. D., and K. A. ADAMSONS, unpublished results.

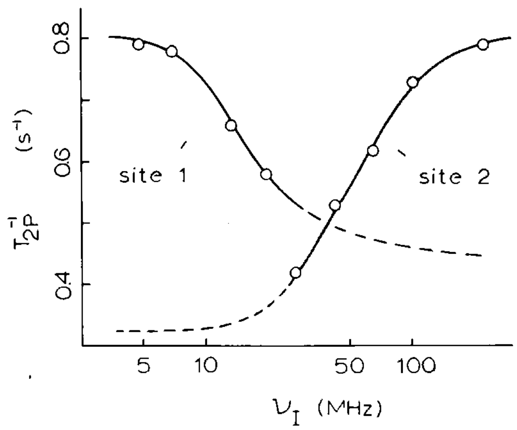

FIGURE 4 Model I for the possible individual $1/T_{2p}$ vs. frequency curves one would obtain from the two Cu(II) sites of diamine oxidase. The dashed portion of the site 1 curve was obtained by extrapolation of the data from 4.7 to 19.5 MHz according to an equation with two spectral density terms, $4\tau_{c1} + 3\tau_{c1}/(1 + \omega_I^2\tau_{c1}^2)$. The site 2 curve was obtained by subtracting that of site 1 from the original data (Fig. 2).

and changes in $1/T_{2m}$ reflect changes in τ_{c1} only. That is, we are observing dispersion of $1/T_{1e}$ itself at approximately the same frequency as one of the dispersions observed in $1/T_1$. (However, the two dispersions are too close together to be resolved in the $1/T_1$ curve, and it continues to monotonically decrease; similarly, the true high-frequency dispersion in $1/T_2$ is obscured by the changing τ_{c1}.) Estimating the $1/T_{1e}$ dispersion frequency to be ~50 MHz, we can calculate a value for τ_v of ~$5 \cdot 10^{-12}$ s. These data also indicate the $1/T_2$ begins to level off rather than continue to increase as would be the case if the increasing T_{1e} were the only

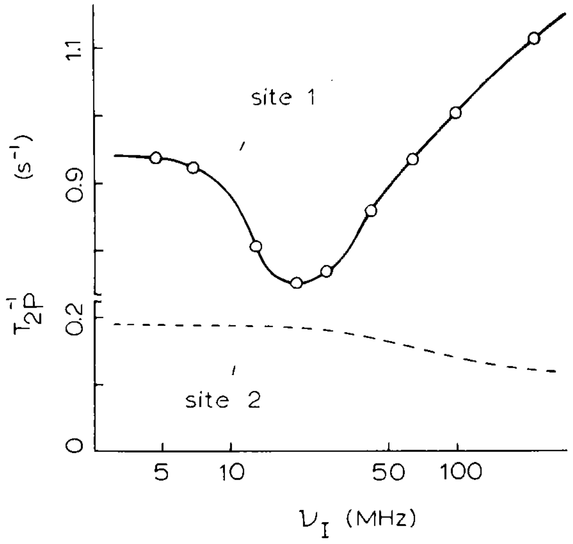

FIGURE 5 Model II for the possible individual $1/T_{2p}$ vs. frequency curves from the two Cu(II) sites. The dashed line is the calculated $1/T_2$ vs. frequency curve corresponding to the $1/T_1$ curve which dispersed at 75 MHz. The curve for site 1 was determined by subtracting that of site 2 from the original data (Fig. 2).

important time contributing to τ_{c1}. An additional time in Eq. 5 contributes to τ_{c1} as T_{1e} increases; again, this term may be $1/\tau_r$ or $1/\tau_m$.

The question remains which Cu(II), or which site, is giving rise to the frequency-dependent correlation time. There are two possibilities: (*a*) at one site there is a frequency-independent, electronic relaxation time T_{1e} = 10 ns, and at the other a T_{1e} equal to 1–2 ns at low frequency (T_{1e}°), but which disperses according to a term $D\tau_v/(1 + \omega_S^2\tau_v^2)$, where τ_v has the value quoted above, or (*b*) the electronic relaxation time which is equal to 10 ns at low frequency disperses and the other remains constant at T_{1e}° = 2 ns. For clarity, the consequences of these two models in terms of the individual $1/T_{2p}$ curves one would obtain from the two sites independently are shown in Figs. 4 and 5, respectively. Unfortunately, there is no unequivocal evidence that indicates which possibility is correct.

In light of the fact that interactions other than electron-nuclear, dipole-dipole contribute to relaxation in diamine oxidase, further quantitative evaluation of the relaxation data does not appear warranted at this time. However, the main conclusion, that Cu(II) exists in different magnetic (and chemical) environments in the enzyme, is supported by both the T_1 and T_2 frequency dependences. This result is unexpected in view of the apparent symmetry of the dimeric enzyme (Kluetz and Schmidt, 1977*b*) and must be rationalized in formulating a mechanism of action.

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