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# Nuclear Magnetic Resonance Study of the Complexes of Manganese(II) and Fully Adenylylated Glutamine Synthetase (*Escherichia coli* W). Frequency, Temperature, and Substrate Dependence of Water Proton Relaxation Rates†

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ABSTRACT: A study of the longitudinal, $(1/T_{1p})_b$, and transverse, $(1/T_{2p})_b$, proton relaxation rates (prr) of water was conducted as a function of frequency (6–48 MHz) and temperature (1–40°) for the binary complex of Mn(II) and fully adenylylated glutamine synthetase ($E_{\overline{12}}$) isolated from *Escherichia coli* W. The prr data display a maximum in plots of $(1/T_{1p})_b$ *vs.* the reciprocal of the absolute temperature $(1/T)$, with the maximum shifting to lower temperatures as the frequency is lowered from 48 to 6 MHz. When these data were analyzed according to the Solomon–Bloembergen–Morgan (SBM) scheme the data are in accord with the predicted dipolar relaxation mechanism for proton ($H_2O$)-electron (Mn(II)) relaxation involving a correlation time, $\tau_c$, in the range (2–10) $\times 10^{-9}$ sec. The $(1/T_{1p})_b$ values were higher at 24 MHz than at 48 MHz as expected from the SBM scheme but the values at 12 and 6 MHz were lower than the 24-MHz relaxivity data. The explanation for this observation is that the $\tau_c$ values are themselves frequency dependent which establishes that the electron spin relaxation time, $\tau_s$, is the dominant correlation time for the electron–proton dipolar interaction. The $(1/T_{2p})_b$ data also fit the SBM scheme with contributions from dipolar relaxation processes and from $\tau_m$, the exchange of water molecules from the primary coordination sphere of $E_{\overline{12}}$-bound Mn(II). The overall analysis led to the conclusion that there are three water molecules exchanging from the $E_{\overline{12}}$-Mn(II) complex with a lifetime of $\tau_m = 1.3 \times 10^{-7}$ sec (300°K). In the presence of L-glutamate the same features are seen in the $(1/T_{1p})_b$ and $(1/T_{2p})_b$ data for the ternary $E_{\overline{12}}$-Mn(II)-glutamate complex as in the binary $E_{\overline{12}}$-Mn(II) complex with the exception that all relaxivity values are lower. Analysis by the SBM scheme was consistent with a reduction in the number of exchanging water molecules ($\tau_m = 1.0 \times 10^{-7}$ sec) from three to two upon formation of the ternary complex. These data suggest that L-glutamate is coordinated to the enzyme-bound Mn(II). The substrate ATP does not produce the same effect as L-glutamate but instead the major effect of ATP seems to be to decrease the concentration of $E_{\overline{12}}$-Mn(II) by formation of an ATP-Mn(II) complex. The following activators or inhibitors of glutamine synthetase activity, L-tryptophan, L-alanine, L-histidine, glycine, glucosamine-6-P, and carbamyl-P, had no effect on the $(1/T_{1p})_b$ values in solutions of $E_{\overline{12}}$-Mn(II). GDP and CTP both lowered the relaxivity values in solutions of $E_{\overline{12}}$-Mn(II) in a similar manner to ATP and an interpretation was made that ATP, GDP, and CTP did not interact with enzyme-bound Mn(II) in the same manner as did L-glutamate. The role of the metal ion in the high affinity and intermediate affinity Mn(II) binding sites is discussed in relation to previous kinetic, binding, and isotope exchange data.

Recognition of the central role for glutamine synthetase in nitrogen metabolism has produced considerable recent interest in all aspects of this key enzyme. Regulation by feedback mechanisms of the reaction

$$\text{L-Glu} + NH_3 + \text{ATP} = \text{L-Gln} + \text{ADP} + P_i \quad (1)$$

is observed to varying degrees in enzymes from various sources, most notably the case of *Escherichia coli.* The catalytic mechanism as well as the modes of modifier action have been investigated in some detail (Wedler and Boyer, 1972a,b) for the *E. coli* enzyme. In addition to feedback inhibition, other regulato-

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ry mechanisms include pH optima, metal ions, and states of adenylylation (Shapiro and Stadtman, 1970).

A variety of physical techniques have been applied toward characterizing the structural and binding properties of this enzyme. These studies, with particular emphasis on the interactions and effects of metal ions, have been reviewed in depth by Ginsburg (1972). Three types of sites, each with different affinities for Mn(II), are known to exist per dodecamer (Denton and Ginsburg, 1969): $n_1$ (12 sites), of high affinity, responsible for producing a conformationally tightened, catalytically active protein from the relaxed metal ion free protein; $n_2$ (12 sites), of moderate affinity, assumed to be involved in active site activation; and $n_3$ (48 sites) of low affinity, probably involved in gross stabilization of enzyme structure. The state of adenylylation of the protein alters the metal ion specificity and affinities: adenylylated subunits bind Mn(II) very tightly and are activated thereby, whereas unadenylylated protein is specifically activated by Mg(II). Unadenylylated enzyme binds Mn(II) more tightly than does adenylated enzyme, however. Mn(II) binding to the $n_1$ site is quite pH dependent (Mg(II) binding is less so) and metal ions displace protons in the process, providing a means of observing the kinetics of metal ion–protein association (Hunt *et al.*, 1972). Binding of divalent metal ions such as Co(II), Zn(II), Ca(II), and Ba(II) have also been studied. Other parameters observed in relation to metal ion binding have been ultraviolet difference spectral perturbations, optical rotatory dispersion, and circular dichroism (Hunt and Ginsburg, 1972), changes in hydrodynamic properties (Shapiro and Ginsburg, 1968), fluorescent probes (Wulff *et al.*, 1967; Shapiro and Ginsburg, 1968), sulfhydryl group reactivity (Shapiro and Stadtman, 1968), and calorimetric changes (Hunt *et al.*, 1972).

## Experimental Section

*Materials.* Fully adenylylated glutamine synthetase ($E_{\overline{12}}$)[1] was isolated as previously described (Woolfolk *et al.*, 1966). In all calculations the molecular weight is assumed to be 600,000 (Woolfolk *et al.*, 1967). All buffers and substrates were purchased from standard biochemical supply houses and were the highest purity available.

*Mn(II) Binding Experiments by Epr.* The concentration of free Mn(II) was determined from the peak heights of the electron paramagnetic resonance (epr) spectra obtained on a JEOL epr spectrometer equipped with a variable-temperature attachment. Samples of 35 $\mu$l were placed in quartz capillaries and allowed to come to thermal equilibrium for 5 min prior to measurement of the signal. The temperature was maintained to ±1°. Standard Mn(II) samples were run for calibration.

*Measurements of Proton Relaxation Rates (Prr) of Water.* The longitudinal, $1/T_1$, and transverse, $1/T_2$, relaxation rates of solvent protons were measured on an NMR-Specialties variable frequency pulsed nuclear magnetic resonance (nmr) spectrometer. Measurements were made from 6 to 48 MHz and from 1 to 40°. A 180°-$\tau$-90° pulse sequence was used to measure the $1/T_1$ values. The transverse relaxation rates were measured by the Meiboom–Gill (1958) modification of a Carr–Purcell pulse train $90°x'$, $\tau$, $180°y'$, $\tau$, (echo), $\tau$, $180y'$, $\tau$, (echo), $\tau$, etc.

The paramagnetic contribution to the observed relaxation rate, $1/T_{1p}$, is obtained by subtracting the $1/T_1$ value for a solution of enzyme and buffer in the absence of Mn(II) from the $1/T_1$ value of a solution of buffer, enzyme, and Mn(II). The amount of free Mn(II) can be obtained for each solution at each temperature from an epr experiment as described above, and the contribution due to bound Mn(II), $(1/T_{1p})_b$, was calculated from eq 2 and 3, where subscripts b, f, and t refer to

$$1/T_{1p} = \frac{[Mn]_f}{[Mn]_t T_{1p}(i)} + \frac{[ENZ-Mn]}{[Mn]_t T_{1p}(j)} \quad (2)$$

$$(1/T_{1p})_b = [H_2O]/[Mn]T_{1p} = n/(T_{1m} + \tau_m) \quad (3)$$

bound, free, and total Mn(II), i and j to the relaxation rate of the free and bound Mn(II) complexes ($1/T_{1p}(j)$ becomes equal to $(1/T_{1p})_b$ therefore), $n$ is the number of water molecules interacting with bound Mn(II), $T_{1m}$ is the relaxation time of water molecules in the first coordination sphere, and $\tau_m$ is their residence time. In the presence of substrate, eq 4 is used and

$$1/T_{1p} = \frac{[Mn]_f}{[Mn]_t T_{1p}(i)} + \frac{[ENZ-Mn]}{[Mn]_t T_{1p}(j)} + \frac{[Mn-S]}{[Mn]_t T_{1p}(k)} + \frac{[ENZ-Mn-S]}{[Mn]_t T_{1p}(l)} \quad (4)$$

the $(1/T_{1p})_b$ value corresponding to $1/T_{1p(l)}$ is used to calculate $n$ and $\tau_m$ for the ternary complex of enzyme, Mn(II), and substrate. Analogous equations are used to calculate the value of $(1/T_{2p})_b$ for the various bound species.

$$(1/T_{2p})_b = [H_2O]/[Mn]T_{2p} = n/(T_{2m} + \tau_m) \quad (5)$$

The Solomon–Bloembergen (1956) equations for the electron-nuclear dipole–dipole interaction of $1/T_{1m}$ and $1/T_{2m}$ are

$$1/T_{1m} = A\left[\frac{3\tau_c}{1 + \omega_I^2\tau_c^2}\right] \quad (6)$$

$$1/T_{2m} = A\left[2\tau_c + \frac{1.5\tau_c}{1 + \omega_I^2\tau_c^2}\right] \quad (7)$$

where $\omega_I$ is the nuclear Larmor frequency; $A$ is $(2/15)S(S + 1)\gamma_I^2 g^2\beta^2 r^{-6}$ where $S$ is the electronic spin angular momentum in $h/2\pi$ units, $g$ is the electronic $g$ value (approximated by 2.0), $\gamma_I$ is the nuclear gyromagnetic ratio, $\beta$ is the Bohr magneton, and $r$ is the metal ion–nuclear distance.

These simplified equations will adequately describe the relaxation processes for Mn(II) bound to a macromolecule (Mildvan and Cohn, 1970). The correlation time, $\tau_c$, can have contributions from $\tau_m$, $\tau_s$, and $\tau_r$ ($\tau_c^{-1} = \tau_m^{-1} + \tau_s^{-1} + \tau_r^{-1}$) where $\tau_r$ is the tumbling time of the macromolecular complex. The $\tau_c$ value to be used in eq 6 and 7 is the electron spin relaxation time, $\tau_s$, which for Mn(II) is in the $10^{-9}$–$10^{-8}$ sec. Rigorously, the longitudinal electron spin relaxation time $T_{1e}$ should be used in the expression for $\tau_c$ in eq 6 and the transverse electron spin relaxation time, $T_{2e}$, used in eq 7. However, we are assuming that $\tau_s = T_{1e} = T_{2e}$ for Mn(II) as outlined by Reuben *et al.* (1970). The tumbling time for the macromolecular complex of $E_{\overline{12}}$ (MW 600,000) is $\sim 2 \times 10^{-7}$ sec and does not contribute significantly to the value of $\tau_c$. $\tau_r$ was calculated from Stokes law. $\tau_m$ is $\sim 10^{-7}$ sec (see Table I).

It is necessary for an evaluation of the pertinent correlation time for eq 6 and 7 to study the effect of frequency on the $1/T_{1p}$ and $1/T_{2p}$. Figure 1 shows a plot of $\tau_c$ *vs.* the functions in brackets on the right-hand side of eq 6 and 7 for the frequencies used in this study. For a correlation time of $10^{-8}$ sec, the $1/T_{1p}$ values will be different in the frequency range of 6–48 MHz and the $T_{1p}/T_{2p}$ ratio will be different for each frequency. Under these conditions a good estimation of the correlation time can be obtained from a replot of $T_{1p}$ *vs.* frequency if the correlation time itself is not frequency dependent.

Anticipating the analysis of the prr of Mn(II) bound to $E_{\overline{12}}$ the $1/T_{1p}$ data are frequency dependent but the data at 6 and

[1] Abbreviation used is: $E_{\overline{12}}$, fully adenylylated glutamine synthetase.

TABLE I: Comparison of Constants for Water Interacting with Free Mn(II), Pyruvate Kinase Bound Mn(II), and Glutamine Synthetase Bound Mn(II).

| Constant | Free Mn(II)[a] | Pyruvate Kinase–Mn(II)[a] | Glutamine Synthetase–Mn(II) | Glutamine Synthetase–Mn(II)–L-Glutamate |
|---|---|---|---|---|
| $B$, (rad/sec)$^2$ | $0.10 \times 10^{20}$ | $(0.146 \pm 0.002) \times 10^{20}$ | $(0.198 \pm 0.002) \times 10^{20}$ | $(0.199 \pm 0.002) \times 10^{20}$ |
| $\tau_v$ (300°K), sec | $2.1 \times 10^{-12}$ | $6 \times 10^{-12}$ | $6.6 \times 10^{-12}$ | $7.2 \times 10^{-12}$ |
| $E_v$, kcal/mol | 3.9 | $1.5 \pm 0.1$ | $1.4 \pm 0.1$ | $1.3 \pm 0.1$ |
| $\tau_m$ (300°K), sec | $(2.7 \pm 0.5) \times 10^{-8}$ | $(0.5 \pm 0.1) \times 10^{-8}$ | $(1.3 \pm 0.2) \times 10^{-7}$ | $(1.0 \pm 0.2) \times 10^{-7}$ |
| $\Delta H^*$, kcal/mol | $7.8 \pm 0.3$ | $6.6 \pm 0.35$ | $6.50 \pm 0.30$ | $6.5 \pm 0.30$ |
| $\Delta S^*$, eu | $2.1 \pm 0.8$ | $1.65 \pm 0.35$ | $-0.12 \pm 0.30$ | $0.38 \pm 0.30$ |
| $n$ | 6 | 3.0 | 3.0[b] | 2.1[b] |

[a] From Reuben and Cohn (1970), Table I, and references therein. [b] This represents the number of water molecules with a Mn(II) to proton distance of 2.8 Å. The number of water molecules at 2.62 Å would be 2.0 and 1.1 for $E_{\overline{12}}$–Mn(II) and $E_{\overline{12}}$–Mn(II)–L-glutamate, respectively.

12 MHz are lower than the 24-MHz data. This situation could only arise if the correlation time itself was frequency dependent. This is consistent with $\tau_s = \tau_c$ at the magnetic field values used in this study.

According to Bloembergen and Morgan (1961), and using an approximation used by Reuben and Cohn (1970), the electron spin relaxation rate is given by

$$1/\tau_s = 5B\tau_v/(1 + 2.5\omega_s^2\tau_v^2) \quad (8)$$

where $B = 12C^2/5S(S + 1)^2$, $C$ is a constant defined by Bloembergen and Morgan (1961) and $\tau_v$ is the time constant for symmetry distortions of the complex. As outlined in the Appendix of the paper by Reuben and Cohn (1970), initial estimates of $\tau_v$, B, and $A$ can be obtained by a combination of eq 3, 6, and 8 taken over a range of frequencies. The final expression which one manipulates to obtain these estimates was solved by a computer program, followed by iterations of $\tau_v$, $B$, and $A$ to give the *smallest % error* between the calculated $(1/T_{1p})_b$ and the actual values. The final refinement of the data required that the proton–metal ion distance be in the range 2.75–2.9 Å.

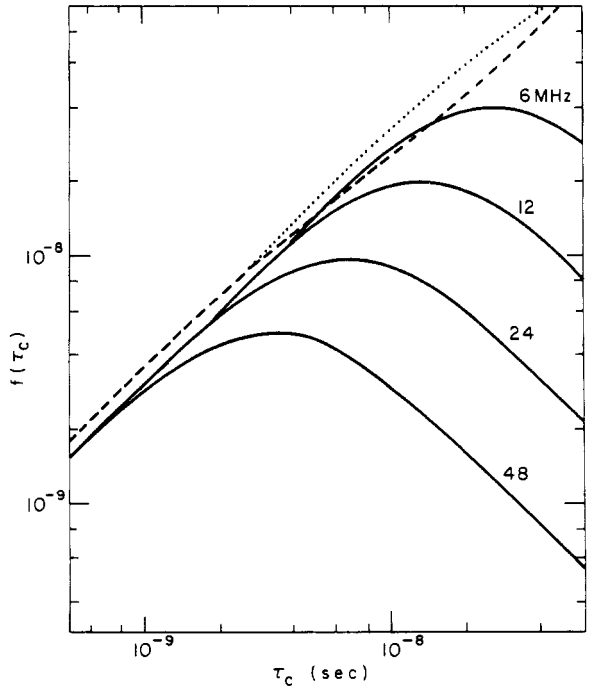



FIGURE 1: Function of the correlation time, $f(\tau_c)$, *vs.* the correlation time, $\tau_c$. The solid lines represent the curves for the function in brackets in eq 6 at values of 6, 12, 24, and 48 MHz. The dashed lines represent the curves for the function in brackets in eq 7 at 6 MHz (. . . .) and 24 MHz (- - - -).

The procedure outlined above was used to analyze the $(1/T_{1p})_b$ data at all temperatures with the assumption that $\tau_v$ obeys an exponential relationship of the form.

$$\tau = \tau^0 \exp(E_a/RT) \quad (9)$$

Once $\tau_c$ was determined, $T_{1m}$ and $T_{2m}$ were calculated from eq 6 and 7, and these values were used in eq 3 and 5 to determine values of $n$ and $\tau_m$. This procedure works best, however, when the $T_{1p}/T_{2p}$ ratio is greater than 1.2 which is the case for 12, 24, and 48 MHz in our analysis. It was assumed that $\tau_m$ obeyed Eyrings equation (1935) for a chemical rate process.

$$1/\tau_m = (kT/h) \exp\left(\frac{\Delta H^*}{RT} + \frac{\Delta S^*}{R}\right) \quad (10)$$

## Results

*Binding Constant of Mn(II) to Glutamine Synthetase.* The binding constant of Mn(II) to the high affinity sites of fully adenylylated glutamine synthetase ($E_{\overline{12}}$) was determined at 24° and pH 7.0 to be $4 \times 10^{-6}$ M (Denton and Ginsburg, 1969). This value was reconfirmed by our epr studies (using 0.01 M imidazole, buffer (pH 7.0) containing 0.1 M KCl) and these studies were extended in the temperature range of 1–40°. The dissociation constants at the extremes of this temperature range are $5.3 \times 10^{-6}$ (1°) and $3.4 \times 10^{-6}$ M (40°). The samples used for the prr studies were identical with the ones used for the epr experiments.

The binding constant of Mn(II) to $E_{\overline{12}}$ was essentially unchanged in the presence of the substrate L-glutamate (10 mM) throughout the temperature range 1–40°. The binding constant of Mn(II) to L-glutamate was also determined ($K_D$ = 20 mM) so that the concentration of the various Mn(II) containing species in solutions of $E_{\overline{12}}$, Mn(II), and glutamate could be calculated. The correction for Mn(II)-glutamate was 5% or less in most of these experiments. Using this approach, the amount of bound Mn(II) was determined for all relevant species in the prr study. The weak binding of Mn(II) by imidazole ($K_D$ = 56 mM) was neglected.

*Temperature and Frequency Dependence of Prr of Water in Solutions of Fully Adenylylated Glutamine Synthetase.* Glutamine synthetase contains 12 identical subunits per 600,000 MW. Solutions of 0.4 mM $E_{\overline{12}}$ (subunits concentration) were dialyzed *vs.* 0.1 mM $MnCl_2$ in 0.01 M imidazole buffer (pH 7.0) containing 0.1 M KCl. Under these conditions one can calculate from the $K_D$ values of Denton and Ginsburg (1969) that

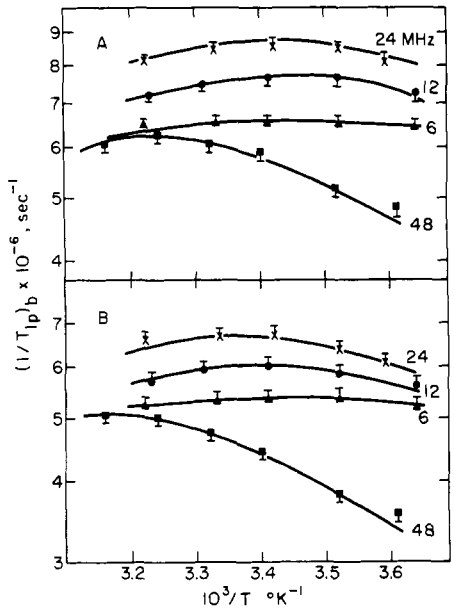



FIGURE 2: The longitudinal prr, $(1/T_{1p})_b$, of solvent due to complexes of fully adenylylated glutamine synthetase, $E_{\overline{12}}$, as a function of the reciprocal of the absolute temperature: (A) solutions of $E_{\overline{12}}$ (0.4 mM) in 10 mM imidazole buffer (pH 7.0) containing 0.1 M KCl and 0.1 mM $MnCl_2$; (B) solutions of $E_{\overline{12}}$ in the same buffer but also containing 9.9 mM L-glutamate. Error bars are standard deviations of the mean and are shown as half-flags for clarity. The lines are theoretical curves based on the equations for $(1/T_{1p})_b$ given in the text.

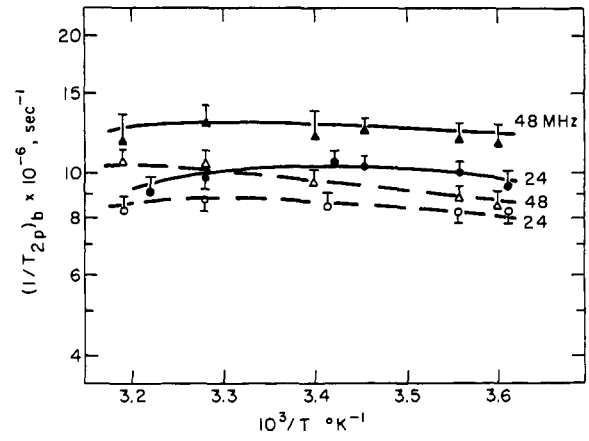



FIGURE 3: The transverse prr, $(1/T_{2p})_b$, of solvent due to complexes of fully adenylylated glutamine synthetase, $E_{\overline{12}}$, as a function of the reciprocal of the absolute temperature. The solid lines are for solutions identical with those described in Figure 2A, and the dashed lines are for solutions identical with those described in Figure 2B. The lines are theoretical curves for $(1/T_{2p})_b$ described in the text.

the tight ($n_1$) Mn(II) binding sites were fully occupied and the $n_2$ sites were ~5% occupied. Protein solutions of 0.04 mM were also used in these prr studies and were prepared by dilution of the 0.4 mM solutions with buffer which did not contain $MnCl_2$. The $1/T_1$ values for solvent in solutions containing $E_{\overline{12}}$ and Mn(II) were determined in the temperature range 1–40° and the frequency range 6–48 MHz. Using eq 2 and the dissociation constants obtained from the epr data, the amount of free and bound Mn(II) was calculated. These values and $1/T_{1p}$ values for free Mn(II) were used to calculate values for $(1/T_{1p})_b$. These values are plotted in Figure 2A.

There are three features of the prr data in Figure 2A which require explanation: first, the data at 12, 24 and 48 MHz show a maximum as a function of temperature. This could arise if $\tau_m$ and $T_{1m}$ in eq 3 are of the same order of magnitude. At low temperatures $\tau_m$ could dominate while at higher temperatures $T_{1m}$ could be the dominant relaxation process. If this is the case, then this feature should be present at the highest observed $(1/T_{1p})_b$ values and it is. The second feature of the data could also explain the maximum in $(1/T_{1p})_b$ values, namely, the maxima are displaced to the right upon going from 48 to 12 MHz and the 6-MHz data show little temperature dependence. If $T_{1m} \gg \tau_m$ then the maximum would reflect a maximum in $T_{1m}$ (eq 6) which would occur when $\omega_1\tau_c = 1$. The maxima due to the $T_{1m}$ relaxation process would shift to the right when progressing from higher to lower frequencies as seen in Figure 1. One can think of the plots in Figure 1 as analogous to a plot of $1/T_{1m}$ *vs.* $1/T$ since $\tau_c$ would shift to smaller values as the temperature increased. This interpretation of the data in Figure 2A is straightforward only if $\tau_c$ itself does not change with frequency. The third feature of the data in Figure 2A shows that the $(1/T_{1p})_b$ values at 24 MHz are higher than at 48 MHz as expected from Figure 1 but the values at 12 and 6 MHz are lower than the relaxivity data at 24 MHz. This could occur only if $\tau_c \simeq \tau_s$, since $\tau_s$ can itself be frequency dependent (see eq 8) but $\tau_m$ or $\tau_r$ cannot. Thus for $E_{\overline{12}}$, $\tau_c = \tau_s$, and the maxima exhibited in the relaxivity data at each frequency (12, 24 and 48 MHz) are most likely due to a maximum exhibited in $T_{1m}$.

In Figure 3 are plotted $(1/T_{2p})_b$ data which were obtained as described in the Methods section and calculated in an analogous manner to the $(1/T_{1p})_b$ values. The solid lines in Figure 3 represent data for the binary complex of $E_{\overline{12}}$ and Mn(II) at two frequencies. $1/T_2$ data were also obtained at 12 and 6 MHz but are not presented for clarity. There are two features of the $(1/T_{2p})_b$ data which will elucidate other features of the processes which dominate the electron-nuclear dipolar relaxation in this protein system. (1) The $(1/T_{2p})_b$ values at 24 and 48 MHz are larger than the $(1/T_{1p})_b$ values in Figure 2A which shows that for these two frequencies, $\omega_1^2\tau_c^2 > 1$ in eq 6 and 7. For 6 and 12 MHz the $T_1/T_2$ ratios are 1.1 and 1.25, respectively, over the entire temperature range (see Figures 2A and 3). A self-consistent explanation for this is that for 6 and 12 MHz, $\omega_1^2\tau_c^2 \leq 1$. (2) The $(1/T_{2p})_b$ data at 48 MHz (Figure 3) are essentially flat from 1 to 40°. $T_{2m}$ and $\tau_m$ are nearly equal at this frequency which leads to the observed temperature dependence since one would expect a positive slope if $T_{2m}$ dominated (as seen in Figure 1, if one considers this a plot of $1/T_{2m}$ *vs.* $1/T$). At higher temperatures the $(1/T_{2p})_b$ values do seem to be reaching a maximum which reflects the condition that $T_{2m} = \tau_m$ in eq 5.

Table I lists the values of $B$, $\tau_v$, $\tau_m$, $n$, and the activation energies for the correlation times. The values in Table I represent the best fit to the $1/T_1$ and $1/T_2$ data represented in Figures 2 and 3 and the lines drawn through the data points in Figures 2 and 3 are the theoretical curves based on the values in Table I.

*Prr of Water in Solutions of Glutamine Synthetase, Mn(II), and L-Glutamate.* In Figures 2B and 3, respectively, the $(1/T_{1p})_b$ and $(1/T_{2p})_b$ (dashed lines) values are plotted as a function of frequency and temperature for solutions which contained fully adenylylated glutamine synthetase, Mn(II), and L-glutamate. All of the relaxation data are corrected for the amount of binary L-glutamate–Mn(II) complex present as determined in control experiments. Thus these $(1/T_{1p})_b$ and $(1/T_{2p})_b$ values represent the relaxation due to the formation of a ternary complex consisting of $E_{\overline{12}}$, Mn(II), and L-glutamate.

Prior to obtaining the data in Figures 2B and 3 it was established that titration of a solution of $E_{\overline{12}}$–Mn(II) by L-glutamate lowered the $1/T_1$ values. This effect was due to the formation of a ternary complex since it was established by epr ex-

periments that the lowering of the $1/T_1$ values was not due to a decrease in the amount of protein-bound Mn(II). The change in prr was used to calculate a dissociation constant for L-glutamate from the ternary complex. This value was 2.75 mM which is very close to the $K_M'$ value of 3.2 mM determined by kinetic techniques (Denton and Ginsburg, 1970).

The $(1/T_{1p})_b$ and $(1/T_{2p})_b$ data all exhibit lower values (compare Figures 2A and 2B) in the presence of L-glutamate (9.9 mM) and the constants for the relaxation processes are listed in Table I. The major difference upon formation of the ternary $E_{\overline{12}}$-Mn(II)-L-glutamate complex is the decrease from 3.0 to 2.1 in $n$, the number of water molecules in the primary coordination sphere of $E_{\overline{12}}$-bound Mn(II). This apparent decrease in $n$ could arise from two effects, one being the actual displacement of a water ligand by a substrate ligand or a protein ligand, or the restriction of one water ligand in the bound Mn(II) so that its exchange is slow compared to the other two water ligands. The shift in the maxima in the $(1/T_{1p})_b$ values in Figure 2B compared to Figure 2A is due to a change in $\tau_s$ in the ternary complex. $(1/T_{1p})_b$ and $(1/T_{2p})_b$ data were also obtained in the presence of 25 mM L-glutamate for two frequencies (24 and 48 MHz) over the same temperature range. All the relaxivity values were ≤5% lower at the higher L-glutamate concentration indicating that the data in Figures 2B and 3 were obtained at nearly saturating levels of L-glutamate.

*Prr of Water in Solutions of Glutamine Synthetase, Mn(II), and Other Substrates and Effectors.* The $1/T_{1p}$ values in solutions of $E_{\overline{12}}$-Mn(II) were obtained in the presence of ATP. A lowering of the $1/T_{1p}$ was observed which was due in part to a decrease in the amount of $E_{\overline{12}}$-bound Mn(II). The resultant ATP-Mn(II) could be binding at another site on $E_{\overline{12}}$ with a different effect on $1/T_{1p}$ than is seen with $E_{\overline{12}}$-Mn(II). We are presently trying to differentiate these effects, but it appears that ATP does not interact with the tight Mn(II) binding site in the same manner as L-glutamate.

The following compounds, which are activators or inhibitors of $E_{\overline{12}}$ activity, were also used as titrants of $E_{\overline{12}}$-Mn(II): L-tryptophan, glycine, L-alanine, L-histidine, glucosamine-6-P, carbamyl-P, GDP, and CTP. None of these effectors gave a reduction of the $1/T_{1p}$ values which could be attributed to any effect other than a reduction of the concentration of $E_{\overline{12}}$-Mn(II). It is not clear whether this involves competitive Mn(II) complexation or direct displacement of Mn(II). GDP and CTP reduced the amount of $E_{\overline{12}}$ by competing with $E_{\overline{12}}$ for Mn(II). It appears that none of these effectors interacts with the tight Mn(II) binding site in the same manner as L-glutamate.

## Discussion

Glutamine synthetase from *E. coli* is composed of 12 identical subunits, each of which can be adenylylated. The fully adenylylated enzyme ($E_{\overline{12}}$) possesses 12 high affinity binding sites for Mn(II) in addition to a second set of 12 weaker Mn(II) binding sites and a third set of weaker metal ion binding sites (Denton and Ginsburg, 1969). The combination of nmr and epr techniques is ideally suited for probing the nature of these metal ion sites and for the determination of substrate interactions. These metal ion sites are necessary for catalytic activity and protein conformational stability (Ginsburg, 1972).

The analysis of the prr data in this paper led to the conclusion that three water molecules remain in the primary coordination sphere of the high affinity Mn(II) binding sites ($n_1$). The number of rapidly exchanging water molecules changes from 3 to 2 in the presence of L-glutamate. For both studies the $(1/T_{1p})_b$ and $(1/T_{2p})_b$ data for the protons of water have been successfully analyzed according to the Solomon-Bloembergen-Morgan scheme. The relevant correlation time in all cases was the electron spin relaxation time which was shown to change when prr data were taken at a variety of magnetic field strengths (different frequencies). The data analysis predicts values for $\tau_v$, the correlation time for symmetry distortions of enzyme bound Mn(II), which appear quite reasonable (Table I) and values of $B$ (the zero-field splitting) which predict an asymmetric environment of enzyme-bound Mn(II) and a diminution of the epr signal for Mn(II). This prediction is supported by epr experiments and permitted the Mn(II) binding experiments to be performed by epr techniques.

Equation 6 represents the dipolar contribution to the longitudinal relaxation rate of water molecules in the first coordination sphere of enzyme-bound Mn(II). The contact or scalar contribution to $1/T_{1m}$ can be neglected as discussed by Mildvan and Cohn (1970) and Villafranca and Colman (1974). The scalar contribution to the transverse relaxation rate (eq 7) was also neglected in the data analysis and this can also be justified.

In general, $1/T_{2m}$ is the sum of dipolar and scalar contributions with the scalar contribution having the form

$$\frac{S(S+1)A^2}{3\hbar^2}\left[\tau_e + \frac{\tau_e}{1+\omega_s^2\tau_e^2}\right]$$

where $\omega_s$ is the Larmor precession frequency for the electron spins, $A/\hbar$ is the hyperfine coupling constant in Hz and $\tau_e$ is the hyperfine correlation time ($\tau_e^{-1} = \tau_s^{-1} + \tau_m^{-1}$). For the work described in this paper $\tau_e = \tau_s$ and $\omega_s\tau_e \gg 1$ so that the scalar contribution to $1/T_{2m}$ varies as $\tau_s$ varies (eq 8). For the binary $E_{\overline{12}}$-Mn(II) system at 300°K, $\tau_s$ varies from ~1.6 to 9 × $10^{-9}$ sec from 6 to 48 MHz, respectively. Using an *upper* limit of $A/\hbar$ of $10^6$ Hz for aqueous Mn(II) (Bloembergen and Morgan, 1961) the scalar contribution to $1/T_{2m}$ is predicted to be from 1.9 × $10^5$ to 1.1 × $10^6$ $sec^{-1}$ from 6 to 48 MHz, respectively. These values can be compared to the dipolar contributions to $1/T_{2m}$ as calculated from eq 7 of ~4 × $10^6$ to 1.4 × $10^7$ $sec^{-1}$. Thus the scalar contribution would represent ≤10% of $1/T_{2m}$ and was neglected in the analysis. An *upper* limit to $A/\hbar$ was calculated from the 48-MHz data and this value was ~$10^6$ Hz. The extensive line broadening by enzyme-bound Mn(II) made it difficult to measure a shift in the water signal at 60 MHz and the absence of a large shift supports the estimation of a small scalar contribution to $1/T_{2m}$.

The exchange rates of water molecules, $1/\tau_m$, were determined for all complexes and represent lower limits to these values. The reason for this is that $\tau_m$ was most accurately evaluated at the highest frequency where the $T_1/T_2$ ratio was the largest.

A comparison is made in Table I between Mn(II) bound to pyruvate kinase and bound to $E_{\overline{12}}$. The only notable differences are in the values of $B$ (from eq 8 and Bloembergen and Morgan (1961)) and $\tau_m$. In contrast, the value of $B$, which contains terms representing the zero-field splitting of Mn(II), is larger for $E_{\overline{12}}$-Mn(II) and the residence time, $\tau_m$, is much longer than in pyruvate kinase-Mn(II). Both protein systems have three water molecules remaining in the primary coordination shell of bound Mn(II).

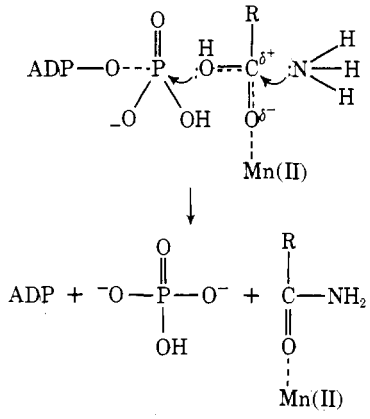
In the process of three protein ligands binding to Mn(II), the protein could undergo gross conformational changes. In fact when Mn(II) binds to apo-$E_{\overline{12}}$ the result is a change in the overall subunit interactions which produce the conformationally tightened form of the enzyme and the induction of catalytic activity (Denton and Ginsburg, 1969). The tight $n_1$ Mn(II) site is implicated in the γ-glutamyl transferase activity of gluta-

mine synthetase (Ginsburg, 1972) and might therefore be involved in binding of glutamate and/or the catalysis in eq 11.

$$\text{L-glutamine} + NH_2OH \xrightarrow[\text{ADP, arsenate}]{M^{2+}} \gamma\text{-glutamylhydroxamate} + NH_4^+ \quad (11)$$

The results of the prr analysis in the presence of glutamate suggest that one water molecule is displaced from the enzyme-bound Mn(II) in the formation of this ternary complex. Alternative explanations could be given for the reductions of $(1/T_{1p})_b$ and $(1/T_{2p})_b$ (Figures 2B and 3) such as the hindrance of one water molecule of the bound Mn(II) from fast exchange with bulk solvent or the displacement of a water molecule by another ligand from the enzyme in response to the binding of glutamate. The involvement of Mn(II) in the catalytic activity is attractive for mechanistic reasons since chelation of oxygen of the C-5 carboxyl of glutamate (and perhaps $\gamma$-phosphoryl of ATP) could result in enhanced polarization of the C–O bond.

The result of the polarization of the carbonyl in eq 12 could




(12)

be to facilitate nucleophilic attack by $NH_3$ and could also enhance the transfer of oxygen from the $\gamma$ carboxylate of glutamate to the $\gamma$ phosphoryl of ATP. The above suggestion is in line with the proposed concerted nature of the reaction catalyzed by the enzyme from *E. coli* (Wedler and Boyer, 1972a).

Our preliminary results of the effect of ATP on the prr of the tight Mn(II) binding site are not conclusive but show that under our experimental conditions, ATP and the $n_1$ binding site compete for Mn(II) and ATP does not bind to the $n_1$ site in a manner similar to glutamate. The attractive possibility exists that ATP-Mn(II) binds to the $n_2$ metal ion binding site as suggested by Ginsburg (1972) but we have no direct proof for this hypothesis.

None of the feedback modifiers tested appear to interact with the $n_1$ binding site for Mn(II) in the same manner as does glutamate. This suggests that these affectors do not chelate to the Mn(II) at $n_1$ sites and do not produce protein conformational changes which effect the interaction of protein-bound Mn(II) with solvent. Of course there are a myriad of combinations of affectors, substrates, and state of adenylylation of glutamine synthetase which could be studied to understand the role of Mn(II) in binding and catalysis. We are presently attempting to systematically study the most important of these to further unravel the incredibly complex interactions (Ginsburg, (1972) already known to occur with glutamine synthetase.

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