Res. Commun. 46, 2121–2126.
Paulsen, J. N., and Lane, M. D. (1966), *Biochemistry 5,* 2350–2357.
Pon, N. G., Rabin, B. R., and Calvin, M. (1963), *Biochem. Z. 338,* 7–19.
Rose, I. A., O'Connell, E. L., Litwin, S., and Tarra, J. B. (1974), *J. Biol. Chem. 249,* 5163–5166.
Siegel, M. I., Wishnick, M., and Lane, M. D. (1972), *Enzymes, 3rd Ed. 6,* 169–172.
Sugiyama, T., Nakayama, N., and Akazawa, T. (1968a), *Biochem. Biophys. Res. Commun. 30,* 118–123.
Sugiyama, T., Nakayama, N., and Akazawa, T. (1968b), *Arch. Biochem. Biophys. 126,* 737–745.
Tabita, F. R., and McFadden, B. A. (1972), *Biochem. Biophys. Res. Commun. 48,* 1153–1158.
Trown, P. W., and Rabin, B. R. (1964), *Proc. Natl. Acad. Sci. U.S.A. 52,* 88–93.
Umbreit, W. W., Burris, R. H., and Stauffer, J. F. (1972), in Manometric and Biochemical Techniques, 5th ed, Minneapolis, Minn., Burgess Publishing Co.
Walker, D. A. (1973), *New Phytol. 72,* 209–235.
Wilkinson, G. N. (1961), *Biochem. J. 80,* 324–332.

Manganese(II) and Substrate Interaction with Unadenylylated Glutamine Synthetase (*Escherichia coli* W). I. Temperature and Frequency Dependent Nuclear Magnetic Resonance Studies†

Joseph J. Villafranca,* David E. Ash,‡ and Frederick C. Wedler

ABSTRACT: A comprehensive study of solvent interaction with unadenylylated glutamine synthetase ($E_{1.7}$) has been conducted using the enzyme isolated from *Escherichia coli* W. The longitudinal, $(1/T_{1p})_b$, and transverse, $(1/T_{2p})_b$, proton relaxation rates were measured with various enzyme samples as a function of frequency (6–48 MHz) and temperature (1–40 °C). With Mn(II) bound at the "tight" metal ion site approximately two water molecules are rapidly exchanging with bulk solvent. This number is reduced to approximately one in the presence of glutamine. All data were successfully analyzed according to the Solomon–Bloembergen–Morgan (SBM) scheme for dipolar relaxation of water protons interacting with enzyme-bound Mn(II). The correlation time for this process varies from 1 to 3×10^{-9} for the complexes described above. Significant contributions to the correlation time arise from both $1/\tau_m$, the exchange rate for water molecules bound at the metal site, and from $1/\tau_s$, the electron spin relaxation rate for Mn(II) with the latter rate showing a frequency dependence at the magnetic field strengths used in this study. A study of Mn(II) binding to $E_{1.7}$ at 25 °C revealed two classes of metal ion sites, a "tight" set of one per subunit with $K_D = 5.0 \times 10^{-7}$ M and a "weak" set of one per subunit with $K_D = 4.5 \times 10^{-5}$ M. In the presence of glutamine the affinity of the first site for Mn(II) was unchanged but the K_D value for the weak site changed to 3×10^{-6} M. In $E_{1.7}$ samples with Mn(II) bound at both the tight and weak metal ion sites the data are interpretable with two rapidly exchanging water molecules interacting with *each* bound Mn(II) ion. With saturating amounts of glutamine or of ADP or of glutamine plus ADP plus arsenate, the proton relaxation rates progressively decreased suggesting that the substrates or inhibitors used were interacting with the bound Mn(II) ions resulting in diminished solvent accessibility to these bound ions. These results are interpretable in terms of ligand substitution into the coordination sphere of the bound Mn(II) ions. Indeed this is probably the case for Mn(II) at the weak metal ion site since Hunt et al. ((1975), *Arch. Biochem. Biophys. 166,* 102) showed that Mn(II) can bind as the Mn(II)–ADP complex to the second metal ion site. Results of proton relaxation rate data on $E_{1.7}$ with Mn(II) bound at both the tight and weak metal ion sites led to the conclusion that these metal ion sites are >6 Å apart. In comparison with proton relaxation rate data on fully adenylylated glutamine synthetase ($E_{11.8}$) as studied by Villafranca and Wedler ((1974), *Biochemistry 13,* 3286), the first "tight" metal ion site in $E_{11.8}$ has three rapidly exchanging water molecules. Mn(II) has a weaker binding constant to $E_{11.8}$ ($K_D \sim 5 \times 10^{-6}$ M) at the pH value used in both studies and a suggestion is made that an additional protein ligand is binding to Mn(II) in glutamine synthetase when the subunits are not adenylylated.

The interaction of metal ions with glutamine synthetase purified from *Escherichia coli* has been reviewed by Ginsburg (1972). Two sets of Mn(II) binding sites are observed that have important effects on the protein. Metal ion binding to 12 high affinity binding sites (one per monomer of the dodecamer) produces an ultraviolet spectral change at 290 nm in the protein (Shapiro and Ginsburg, 1968) and the release of two protons (Hunt and Ginsburg, 1972). Binding of a second set of 12 metal ions with weaker affinity than the first set releases one proton (Hunt and Ginsburg, 1972) with no accompanying ultraviolet spectral change. Full catalytic activity is seen when the second set of metal ion sites are saturated. A third much weaker set of

† From the Chemistry Departments of The Pennsylvania State University, University Park, Pennsylvania 16802 (J.J.V.), and Rensselaer Polytechnic Institute, Troy, New York 12181 (F.C.W.). *Received September 16, 1975.* This work was supported in part by grants from the National Science Foundation, GB-34751 (F.C.W.), instrument grants awarded to The Pennsylvania State University for the purchase of an EPR spectrometer and an on-line computer system, and the Petroleum Research Fund (F.C.W.), administered by the American Chemical Society.

‡ Present address: Chemistry Department, University of Illinois, Urbana, Illinois 61801.

metal ion sites exist which are apparently not needed for catalytic activity.

Recently Hunt et al. (1975) have shown that Mn(II) binds randomly to the two subunit metal ion sites and that an active enzyme can be formed by sequential binding of Mn(II) and ADP to the second metal ion sites or the direct binding of the Mn-ADP complex. The affinity of the second set of sites for Mn(II) is increased by the simultaneous presence of glutamine and reciprocally the K_m' value for glutamine is decreased by increasing Mn(II) concentrations. The higher affinity for Mn-ADP ($K \simeq 10^{-8}$ M) in the presence of glutamine (Rhee et al., 1974; Rhee and Chock, 1975) provides a rationale for the lack of activity of the unadenylylated enzyme in the biosynthetic assay when Mn(II) is present.

The present studies involve the use of proton nuclear magnetic resonance (^{1}H NMR) studies to probe the environment of the Mn(II) sites on the unadenylylated glutamine synthetase and the effect of substrates on these sites. This represents a continuation of our studies with glutamine synthetase in both the adenylylated and unadenylylated states (Villafranca and Wedler, 1974).

Experimental Section

Materials. Glutamine synthetase was isolated from *E. coli* W in a state of low adenylation ($E_{1.7}$) as described previously (Woolfolk et al., 1966). Imidazole, glutamine, and ADP were products of Sigma. All other chemicals were reagent grade of the highest quality available. The subscript 1.7 refers to the average state of adenylylation of the 12 subunits.

Methods. Enzyme dissolved in 0.01 M imidazole buffer containing 0.1 M KCl (pH 7.0) was dialyzed vs. buffer containing 0.1 mM $MnCl_2$. The enzyme was then dialyzed vs. buffer devoid of $MnCl_2$ until approximately 1 equiv of Mn(II) per subunit was obtained. The Mn(II) content was determined in two ways: (1) by atomic absorption spectroscopy using a Jarrell-Ash spectrophotometer and (2) by electron paramagnetic resonance (EPR) using a Varian E-12 spectrometer operating at 9 GHz. In the latter method the protein was precipitated by perchloric acid and centrifuged and the amount of Mn(II) determined as described previously (Cohn and Townsend, 1954). The two methods agree to within ±2%. When necessary enzyme solutions prepared for NMR experiments were concentrated in a collodion bag apparatus.

The enzyme was assayed before and after NMR experiments using the γ-glutamyl transferase assay. The specific activity was 80 μmol of γ-glutamylhydroxamate formed per min per mg under the following conditions: 20 mM Tris-arsenate, 20 mM NH_2OH, 0.05 mM ADP, 30 mM L-glutamine at pH 7.0, 37 °C, in a final volume of 0.5 ml. Other conditions of this assay are given in Hunt et al. (1975). The enzyme had the same activity before and after all experiments and retained activity after months of storage at 4 °C.

The longitudinal or spin-lattice relaxation time, T_1, of the protons of water in various samples containing enzyme, Mn(II), and substrates was measured at 6, 12, 24, 35, and 48 MHz using a π, τ, $\pi/2$ pulse sequence generated by an NMR Specialities pulsed spectrometer equipped with a SEIMCO broad band RF unit and receiver. The variable temperature unit utilized a heated or cooled flow of N_2 and maintained the temperature to within ±1 °C.

Transverse or spin-spin relaxation times, T_2, were measured using the Meiboom-Gill (1958) modification of a Carr-Purcell pulse train. All other details of relaxation measurements with the aforementioned pulse spectrometer have been given elsewhere (Villafranca and Colman, 1974; Villafranca and Wedler, 1974).

The method of analysis of T_1 and T_2 data for the protons of solvent interacting with protein systems containing bound Mn(II), substrates, and inhibitors has been given in detail elsewhere (Villafranca and Colman, 1974; Villafranca and Wedler, 1974) and only a brief outline will be presented. Equations for the paramagnetic contributions to the longitudinal and transverse relaxation rates due to solvent protons interacting with enzyme-bound Mn(II) are given in

$$(1/T_{1p})_b = n/(T_{1m} + \tau_m) \quad (1)$$

$$(1/T_{2p})_b = n/(T_{2m} + \tau_m) \quad (2)$$

These relaxation rates depend upon the number of interacting water molecules, n, their lifetime in the complex, τ_m, and the longitudinal and transverse relaxation times in the coordinated complex, T_{1m} and T_{2m}, respectively. As was the case for the adenylylated enzyme (Villafranca and Wedler, 1974) the electron-proton dipolar terms predominate for $1/T_{1m}$ and $1/T_{2m}$ with

$$1/T_{1m} = A\left[\frac{3\tau_{c1}}{1 + \omega_I^2\tau_{c1}^2}\right] \quad (3)$$

$$1/T_{2m} = A\left[2\tau_{c1} + \frac{1.5\tau_{c1}}{1 + \omega_I^2\tau_{c1}^2}\right] \quad (4)$$

$$1/\tau_{c1} = 1/\tau_r + 1/\tau_m + 1/\tau_{s1} \quad (5)$$

as given by Reuben et al. (1970) and Dwek et al. (1974). Since the protein complex has a molecular weight of 600 000 the rate of reorientation, $1/\tau_r$, is negligible; a major contribution to $1/\tau_{c1}$ is the longitudinal electron spin relaxation rate, $1/\tau_{s1}$, for enzyme-bound Mn(II) (see full equations for dipolar relaxation in Dwek et al. (1974)).

For the magnetic field strengths used in this study (1.4–11.2 kG) the electron spin relaxation rate is itself frequency dependent and this phenomenon was taken into account by use of the Bloembergen-Morgan (1961) treatment for the frequency dependence of $1/\tau_{s1}$

$$1/\tau_{s1} = B\left[\frac{\tau_v}{1 + \omega_s^2\tau_v^2} + \frac{4\tau_v}{1 + 4\omega_s^2\tau_v^2}\right] \quad (6)$$

where τ_v is the correlation time that is related to the rate at which the zero-field splitting is modulated by solvent impact on the enzyme-Mn(II) complex. The terms in B are given in Rubinstein et al. (1971). The use of eq 6 for macromolecular complexes gives a satisfactory fit to the experimental data although the equation was developed for evaluation of the frequency dependence of the proton relaxation rates in aqueous Mn(II) solutions.

The temperature dependencies of $1/T_{1m}$ amd $1/T_{2m}$ are not easily predicted from inspection of eq 3, 4, and 6. The temperature dependence of τ_v is expected to be

$$\tau_v = \tau_v^0 \exp(Ev/RT) \quad (7)$$

and of $1/\tau_m$ is

$$1/\tau_m = (kT/h)\exp\left[-\frac{\Delta H^\ddagger}{RT} + \frac{\Delta S^\ddagger}{R}\right] \quad (8)$$

For the case where τ_{s1} contributes significantly to the correlation time, τ_c, the temperature dependence of eq 1 is dramatically different from the situation where τ_c is not frequency dependent (see Figure 2 of James and Cohn,

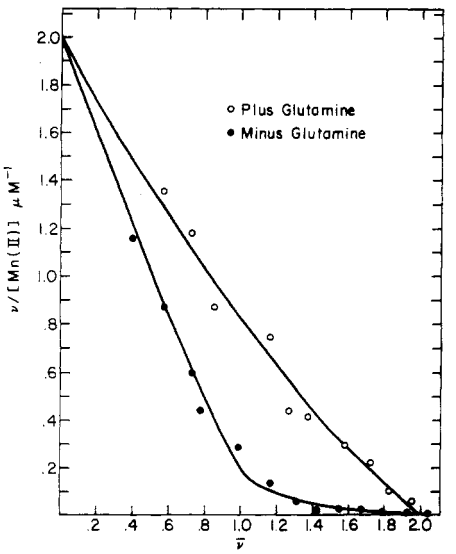


FIGURE 1: Scatchard plot of the binding of Mn(II) to glutamine synthetase ($E_{1.7}$). The experimental conditions are given in Methods. 50 mM glutamine was included for the experimental data designated by open circles. T = 25 °C.

1974). For values of B and τ_v that are larger than 0.1 × 10^{20} rad^2/s^2 and 2. 0 ps (the values for free Mn(II)), the $(1/T_{1p})_b$ values at lower frequencies (6 and 12 MHz) are actually less than the corresponding values at 24 MHz. A plot of eq 3 does not predict this for a frequency independent correlation time. Thus, whenever the above mentioned situation is observed, τ_{s1} is a predominant contributor to τ_c. There are numerous examples of this phenomenon in the literature (Peacocke et al., 1969; Reuben and Cohn, 1970; Lanir et al., 1975; Koenig et al., 1973).

The data were fit to the various NMR equations by the previously outlined method (Villafranca and Wedler, 1974). When the smallest percent error between calculated and experimental $(1/T_{1p})_b$ values was obtained by computer analysis, the fit to the data was deemed acceptable. One restriction was that the proton to metal ion distance was in the range 2.75–2.90 Å.

Results

Binding Constant of Mn(II) to Unadenylylated Glutamine Synthetase ($E_{1.7}$). Using the technique of electron paramagnetic resonance (EPR) to measure the free Mn(II) concentration (as described for Mn(II) binding to fully adenylylated glutamine synthetase (Villafranca and Wedler, 1974), the binding constant of Mn(II) to unadenylylated enzyme was measured at pH 7.0 (0.01 M imidazole–0.1 M KCl) and 25 °C. The binding constant of Mn(II) to the tight metal ion site thus determined is 5.0 (±1.0) × 10^{-7} M compared to a value of 5.3 × 10^{-7} M determined by Hunt et al. (1975). It was noted during the course of the EPR binding experiments that if a spectrum was recorded within 1 min of sample preparation, a slow decrease in the amplitude of free Mn(II) signal was observed. To avoid the complications of any time-dependent changes in Mn(II) binding to apoenzyme (Kingdon et al., 1968) an incubation period of 10 min was routinely employed before measuring free Mn(II). After this period of incubation the amount of free Mn(II) present in each sample could reproducibly be determined.

The Mn(II) binding data were plotted in the Scatchard manner (Figure 1) and a biphasic binding curve was obtained. These results are similar to those presented by Denton and Ginsburg (1969). The biphasic behavior was analyzed using the method outlined by Villafranca and Mildvan (1971) (see eq 2 in this reference) for two thermodynamically noninteraction *sets* of Mn(II) binding sites.[1] The K_D value for the second (weak) set of 12 sites was 4.5 (±0.5) × 10^{-5} M in good agreement with the value of Hunt et al. (1975) of 5.0 × 10^{-5} M. The agreement in binding constants between this work and that of Hunt et al. (1975) is remarkable, considering the fact that the protein concentrations used for the binding measurements in the two laboratories differ by up to three orders of magnitude. This result is reassuring and means that no gross changes occur in metal ion binding when very concentrated solutions of enzyme are prepared and used for magnetic resonance experiments. One therefore has some assurance that these data are relevant to the results obtained in the dilute solutions, e.g., spectrophotometric assays which are performed for kinetic and activity measurements.

The binding of Mn(II) to $E_{1.7}$ was also determined in the presence of 50 mM glutamine (Figure 1). While there is greater scatter in the experimental data, it is clear that glutamine tightens the binding of Mn(II) to the second (weak) set of metal ion binding sites (K_D = 3 × 10^{-6} M). The first (tight) set of binding sites is unaltered. This is in accord with the data of Hunt et al. (1975).[2] Although our Mn(II) binding data described above were in the presence of glutamine with no ADP added, Hunt et al. (1975) reported that the presence of 10^{-6} M ADP in the binding experiments produced little effect on the binding constant of Mn(II) to the second set of metal ion sites. Our data confirm that added glutamine produces a tightening of Mn(II) bound to the second set of sites.

Frequency and Temperature Dependence of Water Proton Relaxation Rates in Solutions of $E_{1.7}$. Proton longitudinal relaxation rates of $E_{1.7}$–Mn(II) solutions (Mn(II) at the tight metal ion sites) measured in the temperature range 1–40 °C and the frequency range 6–48 MHz are plotted in Figure 2. When these data are compared to theoretical plots, good initial estimates of the parameters in eq 1–8 can be obtained by inspection. Salient features of the data are (1) the frequency dependence of $(1/T_{1p})_b$, (2) the fact that the relaxation rates are lower at 6 and 12 MHz than at 24 MHz, (3) the maxima exhibited at a number of frequencies as the temperature is increased, and (4) the decrease in $(1/T_{1p})_b$ shown in Figure 2B when 50 mM glutamine is present (compared to Figure 2A with no glutamine added). Explanations for the above characteristics follow. (1) The frequency dependence of the data is attributable to the frequency dependent part of eq 3. This must mean that $T_{1m} > \tau_m$ since no frequency dependence is predicted for τ_m dominated longitudinal relaxation. (2) A dominant process contributing to τ_{c1} must be τ_{s1}, the relaxation time of

[1] Each set of binding sites represents the binding of Mn(II) to the 12 subunits. Thus the first set of 12 sites (per dodecamer) has a binding constant of 5.0 × 10^{-7} M and the second set of 12 Mn(II) sites (per dodecamer) has K_D = 4.5 × 10^{-5} M. No cooperativity was detected in the binding of the first or second set of Mn(II) ions from the limited amount of data obtained. Denton and Ginsburg (1969) and Hunt et al. (1975) also noted a lack of cooperativity in the Mn(II) binding.

[2] While this NMR and EPR work was in progress, Drs. A. Ginsburg and E. Stadtman very kindly supplied us with unpublished results on unadenylylated glutamine synthetase. These data were of invaluable assistance in understanding and interpreting our results presented in this paper.

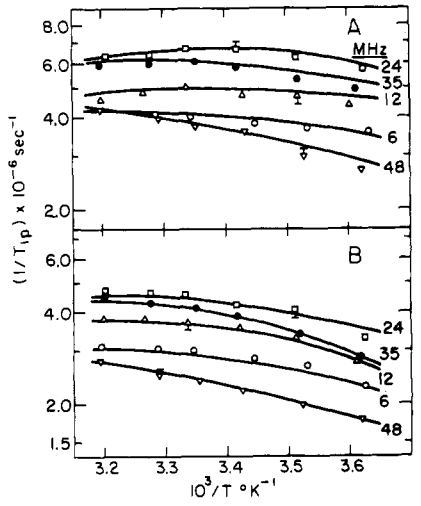


FIGURE 2: The longitudinal proton relaxation rates of solvent in solutions of $E_{1.7}$ as a function of the absolute temperature. (A) Solutions of $E_{1.7}$ (0.20 mM) in 10 mM imidazole buffer (pH 7.0) containing 0.1 M KCl and 0.187 mM $MnCl_2$; (B) identical with solution A but also containing 50 mM glutamine. The error bars are indicated at a few points for clarity. The lines represent theoretical curves based on the equations outlined in the text and the values in Tables I and II.

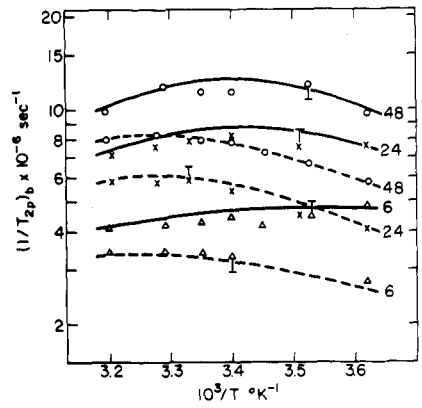


FIGURE 3: The transverse proton relaxation rates of solvent in solutions of $E_{1.7}$ as a function of the absolute temperature. The solid lines are for data taken at 6, 24, and 48 MHz on solutions identical with those described in Figure 2A. The dashed lines are for data on enzyme solutions plus glutamine (as in Figure 2B). The lines are theoretical curves for transverse relaxation processes derived from the values listed in Tables I and II and the appropriate equations listed in the text.

the electron spin. This feature of proton relaxation rate data has been seen previously in many enzyme systems (Butlaire and Cohn, 1974; Villafranca and Wedler, 1974; Lanir et al., 1975) and produces the frequency dependence of τ_{c1} via eq 6. The result is that the $(1/T_{1p})_b$ values are lower at 6 MHz than at 24 MHz for this system. (3) The maximum exhibited in the data at 24 MHz, Figure 2A, is due to a maximum in T_{1m} in eq 3 that occurs when $\omega_I\tau_c = 1$. This maximum should shift to the left in plots of $(1/T_{1p})_b$ vs. $1/T$ as the frequency increases. This feature is clearly seen for the data at 24 and 48 MHz, verifying the inequality $T_{1m} > \tau_m$ and the predictions[3] of eq 1 and 3. (4) The predictions of eq 1-4 for the case where T_{2m}, $T_{1m} > \tau_m$ are that $T_{1p}/T_{2p} > 1$ for 48 MHz and $\tau_c = 10^{-9}$-10^{-8}. This is the situation as described below for the $(1/T_{2p})_b$ data.

Transverse relaxation rates for solutions of $E_{1.7}$-Mn(II) in the absence and presence of 50 mM glutamine are presented in Figure 3. The data were taken as a function of temperature (1-40 °C) and frequency (6-48 MHz). $(1/T_{2p})_b$ values in the absence of glutamine at 6 MHz have nearly the same values as the $(1/T_{1p})_b$ data while at 48 MHz the transverse relaxation rate data are larger at all temperatures than the corresponding longitudinal relaxation rate data. The observations for the 48 MHz data are consistent with the conditions T_{1m}, $T_{2m} > \tau_m$ and $\omega_I^2\tau_{c1} > 1$ in eq 1-4 while $\omega_I^2\tau_{c1} \leq 1$ for 6 MHz.

In the presence of 50 mM glutamine, all $(1/T_{2p})_b$ values are lower than the corresponding values in the absence of glutamine. The data at 48 MHz have a negative slope. This condition arises if $T_{2m} \leq \tau_m$ and predicts that $(1/T_{2p})_b \sim n/\tau_m$. Thus the maximum in the plot of $(1/T_{2p})_b$ vs. $1/T$ reflects the condition where $T_{2m} = \tau_m$. Arrhenius plots of the log of the turnover rate vs. $1/T$ are linear from 10 to 50 °C (F. C. Wedler, unpublished data) which precludes the possibility of temperature-dependent changes in the structure of the complexes giving rise to the behavior of the NMR data in Figures 2 and 3.

Table I lists the values of B, τ_v, τ_m, n, and the activation energies for the correlation times. These values are the best fit to the longitudinal and transverse relaxation rate data in Figures 2 and 3 and the lines drawn through the data points are the theoretical curves generated from eq 1 to 8 using these values. In Table II, values for $1/\tau_s$ and $1/\tau_c$ are listed at 20 °C for three frequencies. $1/\tau_c$ was calculated from eq 5 and used to compute T_{1m} and T_{2m}. The numerical value of n was then computed for the overall analysis. Compiled values of $n/(T_{1m} + \tau_m)$ and $n/(T_{2m} + \tau_m)$ are listed in Table II and compared to the $(1/T_{1p})_b$ and $(1/T_{2p})_b$ data for $E_{1.7}$-Mn(II) with and without glutamine present.

The frequency dependence of $1/\tau_s$ is apparent from Table II. As a result of the frequency dependent correlation time and the form of eq 3, the $(1/T_{1p})_b$ value at 24 MHz is larger than the value at 6 MHz. The value of $1/\tau_m$ represents a significant contribution to $1/\tau_c$ in the frequency range covered in these experiments. At 48 MHz $1/\tau_m$ is about four times $1/\tau_s$ for $E_{1.7}$-Mn(II) and about twice the value of $1/\tau_s$ for $E_{1.7}$-Mn(II)-glutamine.

In enzyme solutions which contain glutamine (Figure 1B), the $(1/T_{1p})_b$ values are lower than the corresponding values obtained in the absence of glutamine. The data are frequency dependent but the temperature dependence at each frequency has changed upon addition of glutamine to $E_{1.7}$-Mn(II). The reasons for these changes are multifarious. In Table I one can see that B, τ_v, τ_m, and n have changed. These changes lead to different values for $1/\tau_s$ and $1/\tau_c$ (Tables I and II). The overall analysis leads to the conclusion that the correlation time changes upon addition of glutamine and also the hydration number of bound Mn(II) decreases from ~2 to ~1.

As shown above, the $(1/T_{2p})_b$ data in solutions of $E_{1.7}$-Mn(II)-glutamine are reduced in value compared to $(1/T_{2p})_b$ data on $E_{1.7}$-Mn(II). The T_{1p}/T_{2p} ratio at 48 MHz for $E_{1.7}$-Mn(II) at 1 °C is 4.1 and this value is decreased to 3.3 when glutamine is added. This is due in part to the change in τ_c (Table II) but the increase in τ_m from 44 to 120 ps results in a significant contribution to $(1/T_{2p})_b$ from

[3] The shift of the predicted maximum in $(1/T_{1p})_b$ data as a function of $1/T$ (in the limit when $(1/T_{1p})_b = n/T_{1m}$) at various frequencies can more easily be seen in Figure 1 of Villafranca and Colman (1974). In Figure 5 of Dwek et al. (1974), theoretical plots of $1/T_{1p}$ and $1/T_{2p}$ vs. $1/T$ are presented for aquo-Mn(II) and are instructive for understanding the temperature and frequency dependence of relaxation rate data.

Table I: Comparison of Constants for Water Protons Interacting with Mn(II) Bound to Unadenylylated Glutamine Synthetase and Its Complexes.

Constant	$E_{1.7}$–Mn(II)	$E_{1.7}$–Mn(II)–Glutamine
B (rad/s)^2	$(0.190 \pm 0.003) \times 10^{20}$	$(0.210 \pm 0.003) \times 10^{20}$
τ_v (20 °C), s	7.5×10^{-12}	6.6×10^{-12}
E_v, cal/mol	1.20 ± 0.05	1.50 ± 0.05
τ_m (20 °C), s	$(0.23 \pm 0.10) \times 10^{-7}$	$(0.48 \pm 0.20) \times 10^{-7}$
$\Delta H^{\ddagger}$, cal/mol	6.60 ± 0.10	6.60 ± 0.10
$\Delta S^{\ddagger}$, eu	-1.0 ± 0.4	-2.4 ± 0.6
n	1.9[a]	1.1[a]

[a] This is the number of water molecules at 2.85 Å.

Table II: Comparison of Computed Relaxation Rates and Experimental Relaxation Rates.

				Computed		Experimental	
Complex[a]	ν (MHz)	$1/\tau_s$[b] $\times 10^{-9}$ (s^{-1})	$1/\tau_c$[c] $\times 10^{-9}$ (s^{-1})	$[n/(T_{1m} + \tau_m)]$ $\times 10^{-6}$ (s^{-1})	$[n/(T_{2m} + \tau_m)]$ $\times 10^{-6}$ (s^{-1})	$(1/T_{1p})_b \times 10^{-6}$ (s^{-1})	$(1/T_{2p})_b \times 10^{-6}$ (s^{-1})
$E_{1.7}$–Mn(II)	6	0.634	1.098	4.00	4.66	3.96	4.40
	24	0.269	0.933	6.70	8.76	6.65	8.20
	48	0.102	0.566	3.60	12.5	3.60	11.4
$E_{1.7}$–Mn(II)–glutamine	6	0.632	0.861	2.66	3.03	2.86	3.30
	24	0.300	0.529	4.10	5.15	4.25	5.32
	48	0.122	0.351	2.24	7.32	2.25	7.75

[a] Values at 20 °C. [b] Calculated from eq with B and τ_v values in Table I. [c] Computed from $1/\tau_c = 1/\tau_m + 1/\tau_r + 1/\tau_s$ with $1/\tau_r = 0.4 \times 10^7$; $1/\tau_m = 0.460 \times 10^8$ for $E_{1.7}$–Mn(II); $1/\tau_m = 0.225 \times 10^8$ for $E_{1.7}$–Mn(II)–glutamine.

τ_m (T_{2m} = 78 ps at 1 °C, 48 MHz). Thus the $(1/T_{2p})_b$ values have a negative temperature coefficient at low temperatures (Figure 2) as expected when $(1/T_{2p})_b \sim 1/\tau_m$. The binding of glutamine may lead to a conformational change in the protein which affects the number of ligands on Mn(II), or glutamine may bind directly to the Mn(II) giving these changes. These alternatives will be discussed later in this paper.

Solvent Relaxation Rates in the Presence of Two Mn(II) Bound to $E_{1.7}$. Using the binding constants for Mn(II) to the two sets of metal ion binding sites of $E_{1.7}$, solutions were prepared in which both sets of sites were fully occupied. This required that free Mn(II) was present in these solutions. The concentration of free Mn(II) was determined in each solution by EPR measurements over the same temperature range of the NMR experiments and the paramagnetic relaxation due to free Mn(II) was substracted as described earlier (Villafranca and Wedler, 1974). The $(1/T_{1p})_b$ data for Mn(II)-$E_{1.7}$-Mn(II) are presented in Figure 4. The relaxation rates are frequency dependent and have a similar frequency and temperature dependence as solutions of $E_{1.7}$-Mn(II). Values for $(1/T_{1p})_b$ are approximately doubled for Mn(II)-$E_{1.7}$-Mn(II) suggesting that the second metal ion site has a similar "environment" as the first site in terms of access and exchange of solvent and perhaps number of coordinated water molecules.

Computations of B, τ_r, τ_m, and n were made for Mn(II)-$E_{1.7}$-Mn(II) using eq 1-8 with the assumption of no dipolar (or spin-exchange) interactions between metal ion sites. This assumption will be discussed later in this paper. The correlation times for the data in Figure 4 should be similar to the values in Figure 2 since the $(1/T_{1p})_b$ values have the same relative relationship to one another.[4]

[4] This would not be expected if τ_s was shortened appreciably (at least an order of magnitude) by close proximity of the two Mn(II) ions.

In Table III are the values for B, τ_v, τ_m, and n. These values are composites for both of the Mn(II) sites so changes in these values from the $E_{1.7}$-Mn(II) complex cannot be evaluated in a straightforward manner. It is tempting to say that since n = 4.0 for Mn(II)-$E_{1.7}$-Mn(II) that each Mn(II) has two bound water molecules rapidly exchanging with bulk solvent.

Solvent Relaxation Rates in Solutions of $E_{1.7}$ with Two Bound Mn(II) and Substrates Present. Separate solutions were prepared of Mn(II)-$E_{1.7}$-Mn(II) containing 50 mM glutamine or 0.25 mM ADP, or 50 mM glutamine plus 0.25 mM ADP plus 50 mM AsO_4^{2-}. Using a computer program developed by Dr. Paul DeMaine of the Computer Science Department of The Pennsylvania State University to solve multiple simultaneous chemical equilibria, the concentrations of all the species described below were computed. The binding constants of Mn(II) to the two metal ion sites in the presence of glutamine are 5.0×10^{-7} and 3.0×10^{-6} M. The binding constant for glutamine to the enzyme in the presence of excess Mn(II) is 2.0×10^{-3} M and the binding constant for Mn(II) to glutamine is 0.7 M (Hunt et al., 1975).

In the presence of ADP, the binding of Mn(II) to the first metal ion site was 5.0×10^{-7} M. For the second metal ion site in the presence of ADP two possible modes of binding must be considered: (1) Mn(II)-$E_{1.7}$ + Mn(II)-ADP $\rightleftharpoons$ Mn(II)-$E_{1.7}$-Mn(II)-ADP, and (2) Mn(II)-$E_{1.7}$-Mn(II) + ADP $\rightleftharpoons$ Mn(II)-$E_{1.7}$-Mn(II)-ADP. The stability constants are similar for both pathways (Hunt et al., 1975) and a value of 3.1×10^{-6} M was used. A binding constant of 1.3×10^{-4} M for ADP-Mn(II) was also included for this analysis.

When ADP, glutamine, and AsO_4^{2-} are present the binding constant for Mn(II)-ADP to Mn(II)-$E_{1.7}$ has decreased to a value of 3.5×10^{-8} M (Hunt et al., 1975). The stability constant for Mn(II)-AsO_4^{2-} is 1.3×10^{-2} M. The

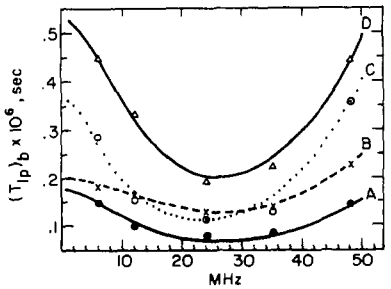


FIGURE 4: Longitudinal relaxation times plotted vs. frequency for complexes of $E_{1.7}$, Mn(II), and substrates. Solutions of $E_{1.7}$ in 10 mM imidazole buffer (pH 7.0) containing 0.1 M KCl were made up as follows: (A) 0.187 mM $E_{1.7}$, 0.43 mM, $MnCl_2$; (B) 0.187 mM $E_{1.7}$, 0.43 mM $MnCl_2$, 50 mM glutamine; (C) 0.187 mM $E_{1.7}$, 0.43 mM $MnCl_2$, 0.25 mM ADP; (D) 0.187 mM $E_{1.7}$, 0.43 mM $MnCl_2$, 0.25 mM ADP, 50 mM glutamine, 50 mM arsenate. Free Mn(II) was measured for all solutions as mentioned in Methods. The lines drawn through the points were computed using the appropriate equations from the text and the values in Table III. T = 25 °C.

constant for AsO_4^{2-} binding to the quinary enzyme complex formed from two Mn(II) ions, ADP, and glutamine and was estimated from the K_m for P_i (Denton and Ginsburg, 1970) and a value of 1.3×10^{-2} M used. The K_D for P_i was estimated as 5×10^{-3} M by Timmons et al. (1974). Using this latter value did not significantly change the results since high AsO_4^{2-} concentrations were used in the present studies.

In Table III and Figure 4 are the data and numerical results of the NMR studies on these complexes. From Figure 4 one can see that the values of $(T_{1p})_b$ are quite different for the various complexes. The curves are the computed theoretical curves for the values of constants listed in Table III. Using the stability constants outlined above and the general procedure given by Villafranca and Wedler (1974) the values of n (the number of solvent water molecules) listed in Table III are for the complexes outlined in the table. The presence of glutamine or ADP lowers the apparent n value from 3.0 to ~2.0. When AsO_4^{2-}, glutamine, and ADP are added, n is lowered further to ~1.0. This strongly suggests ligand displacement on bound Mn(II) by these substrates and arsenate. Substrate-induced changes in the ligand environment of bound Mn(II) may be indirect, however, and produce changes in $(1/T_{1p})_b$. EPR data in the accompanying paper are consistent with the latter interpretation. These complexes with $E_{1.7}$ will be discussed in a later section of this paper.

Discussion

The binding constants of Mn(II) to the two metal ion sites per subunit have been measured using EPR techniques and are in agreement with those obtained by Hunt et al. (1975). The increased affinity of the enzyme for Mn(II) at the second set of metal ion sites in the presence of glutamine is also confirmed. The experiments reported in this paper were conducted under conditions of high enzyme concentration compared to the low enzyme concentrations employed by Hunt et al. (1975), which implies that the protein behaves similarly over a wide concentration range. This is an important consideration when one wants to compare kinetic data which are usually obtained at 10^{-6}–10^{-8} M enzyme concentration, with spectroscopic data with enzyme in the range 10^{-5}–10^{-2} M.

The analysis of the frequency and temperature dependence of the proton relaxation rates of solvent interacting with Mn(II) at the tight enzyme binding sites led to the conclusion that ~2 rapidly exchanging water molecules are in the primary coordination shell. These data appertain to the unadenylylated glutamine synthetase where the stability constant is $\sim 1.5 \times 10^7$ M^{-1}. The number of rapidly exchanging solvent molecules in the primary coordination shell of the tight Mn(II) site of the fully adenylylated enzyme is ~3 (Villafranca and Wedler, 1974) and the stability constant for this form of the enzyme is $\sim 2.5 \times 10^6$ M^{-1}. A self-consistent but not necessarily proven explanation for the above data is that an additional protein ligand is binding to the Mn(II) in the unadenylylated enzyme giving the concomitant increase in affinity for Mn(II) at the tight metal ion site. For both $E_{1.7}$ and $E_{11.8}$ solvent accessibility to the Mn(II) is diminished in the presence of glutamate or glutamine, suggesting a hindrance to rapid water exchange by proximity of these substrates or by direct coordination to the enzyme-bound Mn(II). Neither explanation has been substantiated beyond the experiments reported in this paper or our earlier paper.

Table III: Computed Constants for Mn(II)–$E_{1.7}$–Mn(II) Complexes at 25 °C.

Complex	B^a (rad/s)^2 × 10^{-20}	$\tau_v{}^b$ s × 10^{12}	$\tau_m{}^c$ s × 10^7	n^d
Mn(II)–$E_{1.7}$–Mn(II)	0.22	10.0	0.5	4.0
Mn(II)–$E_{1.7}$–Mn(II) plus Gln	0.20	6.0	0.3	2.0
Mn(II)–$E_{1.7}$–Mn(II) plus ADP	0.18	14.0	0.6	2.2
Mn(II)–$E_{1.7}$–Mn(II) plus Gln, ADP, AsO_4^{2-}	0.20	10.0	0.3	1.2

^{a} ±0.01 for all values. ^{b} ±1.0 for all values. ^{c} ±0.1 for all values. ^{d} Computed using a Mn(II) to water proton distance of 2.82 Å for all complexes.

The $(1/T_{1p})_b$ and $(1/T_{2p})_b$ data have been fit to the Solomon-Bloembergen-Morgan (SBM) scheme. The correlation time for the relaxation processes is frequency dependent thus demonstrating that the electron spin relaxation time contributes to τ_c. At higher frequencies (48 MHz, Table II), τ_m now contributes significantly to τ_c since τ_{s1} is becoming much longer as the magnetic field increases. This prediction is verified by EPR experiments in which the spectrum of bound Mn(II) was observed to narrow at increasing magnetic fields.[5]

The data presented in this paper on $E_{1.7}$ with Mn(II) bound at two sites per subunit provide some clues to the role of both Mn(II) ions in catalysis. Each bound Mn(II) appears to be accessible to solvent and the proton relaxation rate data are interpretable in terms of the SBM scheme (Table III). No drastic changes in τ_{s1} are apparent from the frequency dependence of $1/T_{1p}$ (Figure 4) and the data are consistent with a decrease in the number of rapidly exchanging water protons upon subsequent addition of glutamine, ADP, and arsenate. Only gross effects in the quantity, n, can be computed with no knowledge obtained about which metal ion is becoming less accessible to solvent. However, using previous data available on $E_{1.7}$ (Ginsburg, 1972;

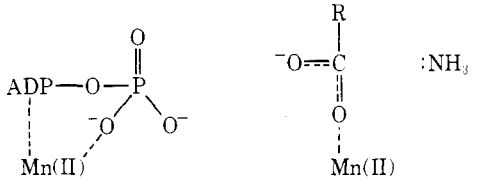
[5] The spectrum of bound Mn(II) was narrower at 35 GHz (~12 500 G) than at 9 GHz (~3200 G) (Villafranca et al., 1976).

Hunt et al., 1975) one can make reasonable interpretations of the changes seen in these relaxation rate data.

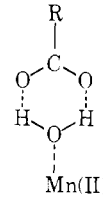
The contention that there is little or no interaction between the two $E_{1.7}$ bound Mn(II) ions is supported by data in the literature on systems where interaction was found. Spin–spin interactions between bound Mn(II) ions (Abragam and Bleaney, 1970; Owen, 1961) or dipolar interactions (which would lead to broadened EPR lines) should result in appreciable shortening of τ_s and consequently τ_c. These effects have been seen by Owen (1961) for Mn doped in MgO where nearest neighbor Mn(II) ions are 2.97 Å apart and next nearest neighbors are 4.2 Å apart. If the Mn(II) ions were this close in Mn(II)–$E_{1.7}$–Mn(II), then τ_c would be predicted to be different for this complex compared to $E_{1.7}$–Mn(II). These effects are not seen if a comparison of the tabulated constants in Tables I and III is made. Data presented in the next paper on the EPR spectra of bound Mn(II) ions also support the conclusion that the bound Mn(II) ions at the weak and tight sites are >6 Å apart. Thus the use of the SBM scheme to analyze these data seems justified.

Since Mn–ADP was shown to bind to the second Mn(II) binding site, the decrease in n from 4 to 2 upon binding ADP to Mn(II)–$E_{1.7}$–Mn(II) could reflect a decrease in solvent hydration by binding of ADP to the Mn(II) bound at the second site. The addition of glutamine and arsenate to the enzyme complex formed with ADP shows a further decrease in n to ~1 which could reflect glutamine binding to the first Mn(II) binding site. The synergistic binding of Mn–ADP to the second Mn(II) site in the presence of glutamine could change the protein conformation around the metal sites and as a result lower the number of rapidly exchanging solvent water molecules. Nonetheless the fact that Mn–ADP binds to the second site would support the contention that the decrease in n in the Mn(II)–$E_{1.7}$–Mn(II)–ADP complex is due to ligation of Mn(II) by the phosphate oxygens of ADP at the second metal ion site.

At present the enzyme complex may be envisioned as



The role of Mn(II) at the first metal ion site could be to bind the γ-carboxyl of glutamate prior to nucleophilic attack by the carboxylate moiety on phosphorus of ATP (as shown by ^{18}O experiments of Boyer et al. (1956) and Kowalsky et al. (1956)). A highly immobilized water molecule between Mn(II) and glutamate is also consistent with the data presented herein and could serve as a template to "orient" the carboxyl group for attack on phosphorus followed by nucleophilic attack by ammonium on a γ-glutamyl phosphate intermediate.



Electron paramagnetic resonance data presented in the subsequent paper (Villafranca et al., 1976) provide evidence for glutamine and methionine sulfoximine binding at or near the tight metal ion site. This latter compound is known to bind tightly to glutamine synthetase and to be phosphorylated by bound ATP (Ronzio et al., 1969; Rowe et al., 1969).

The preliminary data reported in this paper suggest binding and catalytic roles for both metal ions bound to glutamine synthetase. In addition to producing a conformational change in the protein the tightly bound Mn(II) may also be intimately involved in the catalytic mechanism. Distance measurements of ~5 Å between the γ-carboxyl of L-glutamate and Mn(II) at the tight site support the role of the Mn(II) ion to orient the γ-carboxyl (J. J. Villafranca and F. C. Wedler, unpublished results). Further insight into structure–function relationships in glutamine synthetase is the aim of our future research effort.

References

Abragam, A., and Bleaney, B. (1970), Electron Paramagnetic Resonance of Transition Ions, London, Oxford University Press, p 491.

Bloembergen, N., and Morgan, L. O. (1961), *J. Chem. Phys. 34,* 842.

Boyer, P. D., Koeppe, O. J., and Luchsinger, W. W. (1956), *J. Am. Chem. Soc. 78,* 356.

Buttlaire, D. H., and Cohn, M. (1974), *J. Biol. Chem. 249,* 5741.

Cohn, M., and Townsend, J. (1954), *Nature (London) 173,* 1090.

Denton, M. D., and Ginsburg, A. (1969), *Biochemistry 8,* 1714.

Denton, M. D., and Ginsburg, A. (1970), *Biochemistry 9,* 617.

Dwek, R. A., Williams, R. J. P., and Xavier, A. V. (1974), in Metal Ions in Biological Systems, Vol. IV, Sigel, H., Ed., New York, N.Y., Marcel Dekker, p 62.

Ginsburg, A. (1972), *Adv. Protein Chem. 27,* 1.

Hunt, J. B., and Ginsburg, A. (1972), *Biochemistry 11,* 3723.

Hunt, J. B., Smyrniotis, P. Z., Ginsburg, A., and Stadtman, E. R. (1975), *Arch. Biochem. Biophys. 166,* 102.

James, T. L., and Cohn, M. (1974), *J. Biol. Chem. 249,* 3519.

Kingdon, H. S., Hubbard, J. S., and Stadtman, E. R. (1968), *Biochemistry 7,* 2136.

Koenig, S., Brown, R. D., and Brewer, C. F. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 475.

Kowalsky, A., Wyttenbach, C., Langer, L., and Koshland, D. E., Jr. (1956), *J. Biol. Chem. 219,* 719.

Lanir, A., Gradstajn, S., and Navon, G. (1975), *Biochemistry 14,* 242.

Meiboom, S., and Gill, D. (1958), *Rev. Sci. Instrum. 29,* 688.

Owen, J. (1961), *J. Appl. Phys., Suppl. 32,* 2135.

Peacocke, A. R., Richards, R. E., and Sheard, B. (1969), *Mol. Phys. 16,* 177.

Reuben, J., and Cohn, M. (1970), *J. Biol. Chem. 245,* 6539.

Reuben, J., Reed, G. H., and Cohn, M. (1970), *J. Chem. Phys. 52,* 1617.

Rhee, S. G., and Chock, P. B. (1975), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34,* Abstr. 1961.

Rhee, S. G., Luterman, D. L., Timmons, R. D., and Chock, P. B. (1974), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33,* Abstr. 106.

Ronzio, R. A., Rowe, W. B., and Meister, A. (1969), *Biochemistry 8,* 1066.
Rowe, W. B., Ronzio, R. A., and Meister, A. (1969), *Biochemistry 8,* 2674.
Rubinstein, M., Baram, A., and Luz, Z. (1971), *Mol. Phys. 20,* 67.
Shapiro, B. M., and Ginsburg, A. (1968), *Biochemistry 7,* 2153.
Timmons, R. B., Rhee, S. G., Luterman, D. L., and Chock, P. B. (1974), *Biochemistry 13,* 4479.
Villafranca, J. J., and Colman, R. F. (1974), *Biochemistry 13,* 1152.
Villafranca, J. J., and Mildvan, A. S. (1971), *J. Biol. Chem. 246,* 5791.
Villafranca, J. J., and Wedler, F. C. (1974), *Biochemistry 13,* 3286.
Villafranca, J. J., Ash, D. E., and Wedler, F. C. (1976), *Biochemistry,* following paper in this issue.
Woolfolk, C. A., Shapiro, B., and Stadtman, E. R. (1966), *Arch. Biochem. Biophys. 166,* 177.