Manganese(II) and Substrate Interaction with Unadenylylated Glutamine Synthetase (*Escherichia coli* W). II. Electron Paramagnetic Resonance and Nuclear Magnetic Resonance Studies of Enzyme-Bound Manganese(II) with Substrates and a Potential Transition-State Analogue, Methionine Sulfoximine†

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ABSTRACT: The enhancement of the longitudinal proton relaxation rate of solvent water protons which occurs when Mn(II) is bound to the "tight" metal ion site of unadenylylated glutamine synthetase (GS) was used to determine the binding constant of L-methionine (*SR*)-sulfoximine to GS-Mn(II) complexes. The binary enhancement for GS-Mn(II) is 22 at 24 MHz, 25 °C. The enhancement is lowered in the presence of the sulfoximine and the computed dissociation constant is 30 μM with ϵ_t, the enhancement for the ternary complex, equal to 3.0. Titration curves for the sulfoximine were also obtained in the presence of Mg-ADP, Mg-ADP plus P_i, and Mg-ATP. The dissociation constants were 9, 5, and 0.8 μM, respectively. The progressive tightening of the dissociation constants is symptomatic of conformational changes at the active site as the total subsite occupied by ATP is filled. The number of rapidly exchanging water molecules drops from 2 to ~0.1 when saturating concentrations of L-methionine (*SR*)-sulfoximine and nucleotide are present. The kinetically determined K_I value of ~4 μM for the sulfoximine is about three orders of magnitude tighter than the K_m' value of ~3 mM for L-glutamate. The previously mentioned dissociation constants obtained by enhancement titrations are also orders of magnitude tighter than K_m'. These data suggest that L-methionine (*SR*)-sulfoximine is a "transition-state" analogue for the glutamine synthetase reaction. The electron paramagnetic resonance (EPR) spectrum (at both 9 and 35 GHz) for Mn(II) bound at the "tight" metal ion site is dramatically sharpened in the presence of L-methionine (*SR*)-sulfoximine. The EPR spectrum thus obtained is isotropic and the "forbidden" transitions are observed. This result is in concert with the reduced accessibility of enzyme-bound Mn(II) for water molecules in the presence of sulfoximine and suggests that this inhibitor is near or bound to this metal ion. In contrast, the Mn(II) EPR spectrum is much more anisotropic when sulfoximine and Mg-ATP are present together, and this may reflect protein conformation changes which result in ligand distortions about the Mn(II). The weak inhibitor L-methionine sulfone (K_3 = 0.45 mM) and the substrate L-glutamate each produce subtle changes in the environment of bound Mn(II). More dramatic changes result from the addition of Mg-ATP to either of these two enzyme complexes. The changes in the EPR spectra for various complexes are seen in more detail at 35 GHz and demonstrate that most of the distortions about the Mn(II) ligand environment are rhombic in nature. These data lead to a model for the active site of glutamine synthetase in which Mn(II) at the "tight" metal ion site may be involved in binding the substrate L-glutamate and substrate analogue inhibitors. This binding is influenced by addition of the substrates Mg-ATP and NH_4^+ since progressive changes in the EPR spectra are observed. Thus the active site of the *Escherichia coli* enzyme may require a full complement of substrates for catalytic function to occur and the tetrahedral geometry of methionine sulfoximine may represent a transition-state structure for the enzymic reaction.

Glutamine synthetase has a central role in the metabolism of nitrogen in *Escherichia coli*. The enzyme can be modified enzymatically by adenylylation of each of the 12 subunits (Stadtman et al., 1970). Of these two enzyme forms, the adenylylated enzyme requires Mn(II) for the biosynthesis of glutamine. Additionally, the transfer of the γ-glutamyl group from glutamine to hydroxylamine is catalyzed in the presence of Mn(II) with both unadenylylated and adenylylated enzyme. The γ-glutamyl transfer reaction requires the presence of ADP, arsenate, and Mn(II) suggesting that two divalent metal ion sites and the nucleotide site must be occupied for this partial reaction to occur (Ginsburg, 1972).

Hunt et al. (1975) recently reported metal ion binding and kinetic studies for the transferase reaction with unadenylylated glutamine synthetase. These authors report that "... (a) each of the 12 subunits of glutamine synthetase catalyzes the γ-glutamyl transfer reaction in the presence of ADP-Mn; (b) there is an absolute requirement for Mn(II) binding to two subunit sites in the expression of activity; (c) the binding of Mn(II) to these two subunit sites is random (i.e., independent); (d) glutamine increases the ap-

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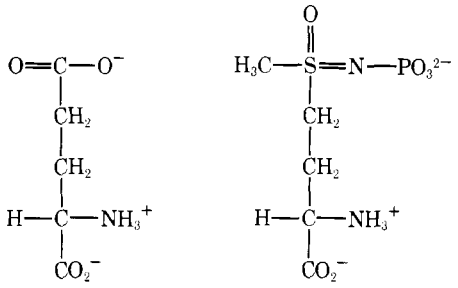
parent affinity for Mn(II) at the second site ("active site") without influencing the binding of Mn(II) to the tightening site, which induces a conformational change to an active configuration ..." The studies reported in this paper deal with spectroscopic changes detected by observing the electron paramagnetic resonance (EPR) spectrum of enzyme-bound Mn(II). These studies suggest that Mn(II) bound at the high affinity "tight" metal ion site may be near or at the L-glutamate (I) binding region of the active site.

In addition to substrate binding experiments, two inhibitors of the enzymic reaction, viz., L-methionine (*SR*)-sulfoximine and L-methionine sulfone, were studied extensively. Extensive studies by Meister's group showed that the irreversible inhibition of glutamine synthetase in the presence of L-methionine (*SR*)-sulfoximine and ATP was due to formation of the sulfoximine phosphate (II) (Ronzio and Meister, 1968; Ronzio et al., 1969; Rowe et al., 1969; Weisbrod and Meister, 1973). The tetrahedral geometry at the sulfur atom of the sulfoximine was suggested by modeling studies (Gass and Meister, 1970) to be a "mimic" of the "active structure" of the adduct of γ-glutamyl phosphate and ammonia (III). Data in this paper provide spectroscopic evidence that L-methionine (*SR*)-sulfoximine and the sulfoximine phosphate are transition-state analogues of the glutamine synthetase reaction. Earlier work with adenylylated enzyme (Villafranca and Wedler, 1974) and the work in the previous paper with the unadenylylated enzyme (Villafranca et al., 1976) have suggested that both metal ions (per subunit) are involved in binding and possibly catalysis at the active site of glutamine synthetase. A preliminary report of nuclear magnetic resonance (NMR) and EPR data with enzyme-Mn(II) and the sulfoximine has appeared (Villafranca et al., 1975).



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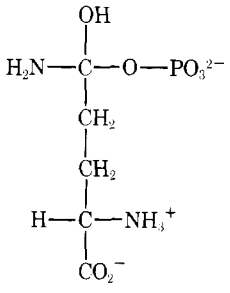


III

Experimental Section

Materials. Glutamine synthetase was isolated from *Escherichia coli* W in a state of low adenylylation ($E_{1.7}$ or $E_{4.8}$) as described previously (Woolfolk et al., 1966). Imidazole, L-glutamine, L-glutamate, ADP, ATP, L-methionine (*SR*)-sulfoximine, and L-methionine sulfone were products of Sigma. Miles Laboratories, Inc. provided the β,γ-methyleneadenosine 5′-triphosphate, App(CH_2)p, and P-L Biochemicals supplied the adenylyl imidodiphosphate App(NH)p. All other chemicals were reagent grade. The enzyme activity and Mn(II) content were determined as described in the previous paper (Villafranca et al., 1976). L-Methionine (*SR*)-sulfoximine phosphate was prepared by the enzymic method of Ronzio et al. (1969). The subscripts 1.7 and 4.8 refer to the average state of adenylylation of the 12 subunits.

EPR Measurements. A Varian E-12 spectrometer was used to record spectra at both 9 GHz (X-band) and 35 GHz (K-band). The spectrometer was equipped with an E-257 variable temperature accessory unit and the temperature was maintained within ±1 °C by heating precooled nitrogen gas which was passed through the Dewar assembly placed in a E-231 cavity operating at 9 GHz. Aqueous samples of 25–50 μl were placed in quartz capillary tubing of 1.0 mm i.d. and the end of the tubing was closed by using polyethylene tubing and a Teflon plug. Samples run at K-band were placed in quartz tubing which had been drawn to 0.1–0.2 mm i.d. A polyethylene plug was used to close the end after 1–3 μl of solution was drawn into the capillary tube. Temperature was controlled at 35 GHz in the same manner as at 9 GHz except that the Dewar assembly surrounded the E-266 cavity. A copper–constantan thermocouple was used to monitor cavity temperature throughout the runs.

For spectra in which the line widths were quite narrow, the modulation amplitude was varied so that the transitions were not artifically broadened by over-modulation. The microwave power was controlled to avoid saturation of the individual spectral lines and the gain and filtering controls were varied to produce the best possible spectra.

NMR Measurements. The details for gathering and analyzing the NMR data are given in the previous paper (Villafranca et al., 1976). The use of NMR to evaluate equilibrium constants requires a few additional considerations, however. For enzyme–substrate systems in which metal ions can bind to a variety of species it is important to know binding constants to each species. Since relative concentrations can change at each point when a titrant is added, the multiple equilibria involved must be solved throughout the titration and the unknown equilibrium constant(s) evaluated. The general outline for this procedure has been presented by Reed et al. (1970) and Buttlaire et al. (1975) and was the method used to evaluate the data in this paper. The CRAMS computer program written at The Pennsylvania State University was used to solve the multiple simultaneous equilibria (Butler and deMaine, 1975).

The equilibrium constants defined for this work are as follows:

$$K_1 = [M][S]/[MS]$$

$$K_D = [E][M]/[EM]$$

$$K_2 = [EM][MA]/[EMMA]$$

$$K_Q = [EMMA][S]/[EMMAS]$$

$$K_1' = [M][A]/[MA]$$

$$K_s = [E][S]/[ES]$$

$$K_3 = [EM][S]/[EMS]$$

The terms are defined as metal ion, M, ADP or ATP, A, enzyme, E, and substrate or inhibitor, S. All titrations re-

ported herein were conducted by varying S at saturating concentrations of metal ions and adenosine nucleotides. The characteristic enhancements for MS, EM, EMS, and EMMAS are defined as ϵ_a, ϵ_b, ϵ_t, and ϵ_q, respectively. Under the conditions of these experiments, the metal ion in the MS and EM complexes was Mn(II) whereas the metal ion in MA is Mg(II). Of course, only the paramagnetic Mn(II) produces the enhanced proton relaxation rates. This will be further delineated in the Results section.

The dissociation constant for methionine sulfoximine from the ternary complex was also determined by using computer programs supplied by Dr. George H. Reed of the Johnson Research Foundation, the University of Pennsylvania. We are grateful for his help in obtaining and implementing these programs and for many helpful discussions of Mn(II) EPR spectra.

Interpretation of Mn(II) EPR Spectra. The divalent manganese ion ($S = 5/2$) contains five unpaired electrons. When these ions are placed in an external magnetic field, H_0, six possible spin-energy levels arise. These six levels correspond to the six values of the electron spin quantum number, M_s, and are $5/2, 3/2, 1/2, \ldots, -5/2$. The EPR spectrum of Mn(II) is complicated by the fact that the manganese nucleus has a spin of $5/2$ ($I = 5/2$) which splits each fine structure transition into six components. Allowed EPR transitions involve a change of $\Delta M_s = \pm 1$, $\Delta m_I = 0$ leading to 30 allowed transitions. M_s and m_I are the electron and nuclear spin quantum numbers. The appearance of six lines in the spectrum of aquo-manganese(II) is due to the large (~90 G) nuclear-electron splitting and each "line" represents the near superposition of the five allowed electronic transitions.

Two further considerations need to be discussed to understand and interpret Mn(II) EPR spectra: (1) axial and rhombic distortion parameters of the zero field splitting (ZFS) interaction, and (2) electron-spin relaxation mechanisms.

The usual spin-Hamiltonian equation written for Mn(II), neglecting nuclear interactions, is

$$H_s = g\beta\mathcal{H}_s \cdot S + D[S_z^2 - (1/3)S(S+1)] + E[S_x^2 - S_y^2] \quad (1)$$

where D and E are the axial and rhombic distortion parameters of the ZFS interactions (Abragam and Bleaney, 1970). The first term is written for an isotropic g tensor and is called the Zeeman interaction. The ZFS interactions are anisotropic and the effect of these terms on the energy levels is orientation dependent. The two terms involving D and E are effectively averaged to zero in the rapidly tumbling aquo-manganese(II) complex and the following situation holds, i.e., $\Delta\omega\tau_r < 1$ where τ_r is the rotational correlation time and $\Delta\omega$ is the ZFS in rad/s. Knowledge of the ZFS in Mn(II) complexes can give information on the extent of distortion from cubic symmetry of a Mn(II)–ligand complex. The ZFS provides one source for electron-spin relaxation via rotational modulation of anisotropic magnetic interactions (Hudson and Luckhurst, 1969). For the case of Mn(II) complexes with macromolecules, $\Delta\omega\tau_r$ is often greater than one (τ_r is $\sim 10^{-8}$ to 10^{-7} s). The result will be incomplete averaging of anisotropic interactions.

The magnetic field positions for the fine structure components depend upon the angular relationships between the magnetic field vector and the crystal field axis of the ion. A detailed explanation of the predicted field positions for Mn(II) ions in a randomly oriented sample (producing a "powder spectrum") is discussed in detail by Reed and Ray (1971). Additional characteristics of Mn(II) spectra arise from a small part of the spectral intensity being shared between the normal ($\Delta M_s = \pm 1$, $\Delta m_I = 0$) and forbidden ($\Delta M_s = \pm 1$, $\Delta m_I = \pm 1$) transitions and the resultant poor resolution of the forbidden transitions. The forbidden transitions can often be resolved in well-ordered crystals, and a good example of this is Mn(II) doped in the cubic lattice of MgO (Wolga and Tseng, 1964).

Large deviations from cubic symmetry (i.e., $D \neq 0$, $E \neq 0$) produce transitions outside the isotropic $g' = 2$ region and give rise to "diminished" spectra since the spectral intensities may now be spread over a wide range and appear over several thousand gauss (e.g., in Mn(II) doped in KN_3 (King and Miller, 1964; Buttlaire et al., 1975)). Examples of all of the above distortions of Mn(II) complexes have been seen in various protein systems (Reed and Ray, 1971; Reed and Cohn, 1973; Haffner et al., 1974, Buttlaire et al., 1975).

The electronic spin relaxation arises from transient distortions of the electronic symmetry of the complex and is modulated by impact of solvent molecules on the Mn(II) complex (Wangsness and Bloch, 1955; Redfield, 1957; Lewis and Morgan, 1968; Hudson and Luckhurst, 1969). The line widths can qualitatively be expected to be "broad" (short τ_s value[1]) when bound Mn(II) is accessible to efficient solvent collision and "narrow" (long τ_s value) when Mn(II) is inaccessible to this rapid fluctuating motion. The magnetic field strength at which EPR spectra are taken will also determine the line width of the transitions since τ_s is field dependent (Bloembergen and Morgan, 1961; Rubinstein et al., 1971; Reed et al., 1971). Complexes in which the water exchange rate is slow give the sharpest transitions and isolation of the bound Mn(II) from bulk solvent will be expected to produce the narrowest lines. Thus, when narrow lines are observed the most plausible explanation would involve diminished effectiveness of the solvent collision mechanism for electron-spin relaxation. This could of course arise most directly if a water molecule is replaced by a substrate or protein ligand on the enzyme-bound Mn(II).

Results and Discussion

Ternary and Quaternary Enzyme–Mn–Inhibitor–Nucleotide Complexes. The ratio of $1/T_{1p}$, the paramagnetic contribution to the longitudinal relaxation rate, in enzyme–metal ion complexes to $1/T_{1p}$ in aqueous buffered Mn(II) solutions is called the observed enhancement, ϵ^*. When corrected for free Mn(II) concentrations, binary, ϵ_b, ternary, ϵ_t, and quaternary, ϵ_q, enhancements can be computed (Buttlaire et al., 1975).

In the preceding paper (Villafranca et al., 1976) the binding constant of Mn(II) to the tight metal ion sites of unadenylylated glutamine synthetase was reported as 5 ×

[1] In general there will be two electron spin relaxation times to consider T_{1e} and T_{2e}. For macromolecular complexes $T_{1e} \geq T_{2e}$. Assuming $T_{1e} = T_{2e} = \tau_s$, the T_{2e} estimated from the linewidth of the EPR transition will be a lower limit for τ_s. In addition there can be up to three different T_{2e} values associated with the $\pm 5/2 \leftrightarrow \pm 3/2$, $\pm 3/2 \leftrightarrow \pm 1/2$ and $-1/2 \leftrightarrow 1/2$ transitions (Reed et al., 1971). Since τ_s can change with the strength of the applied magnetic field, comparisons between correlation times evaluated from NMR and EPR must be made under comparable conditions, viz., 3.2 kG (X-band, EPR) and 2.82 kG (12 MHz, NMR), 12.5 kG (K-band, EPR) and 11.3 kG (48 MHz, NMR). Additional narrowing can occur if π-electron overlap occurs between ligands and d electrons of Mn(II). The ligands or aromatic groups from the protein would have to be quite close to the metal ion to produce this effect, however.

10^{-7} M. This constant was not appreciably different for enzyme with an average state of adenylylation of 1.7 or 4.8. There was no detectable free Mn(II) in a solution containing 0.50 mM $E_{1.7}$ (subunit concentration) and 0.40 mM Mn(II) in 10 mM imidazole buffer (pH 7.0) containing 0.1 M KCl. Both the observed and binary enhancements were therefore 22. Titration of this solution with L-methionine (*SR*)-sulfoximine gave a reduction in the observed enhancement which was not due to release of Mn(II) from the enzyme as will be shown later. Typical titrations are given in Figure 1 (top) and identical results were obtained with solutions of $E_{1.7}$ or $E_{4.8}$. The observed enhancement drops from 22 to ~3 at 24 MHz, 25 °C. The binding constant for the sulfoximine obtained from these titrations is ~30 μM with $\epsilon_t = 3$.

Titrations of enzyme with the sulfoximine in the presence of Mg-ADP (0.7 mM ADP and 0.7 mM Mg(II); $K_1' \sim 2 \times 10^{-4}$ M, Hunt et al., 1975) (Figure 1, top) gave a dissociation constant of 9 μM and this value was reduced to ~5 μM if P_i (30 mM) was present. Thus, progressive tightening of the binding of L-methionine (*SR*)-sulfoximine has been demonstrated by these experiments and this tightening is mediated by nucleotide binding. The final value of ϵ_q was 1.0 in both cases.

In the presence of 0.70 mM ATP and 0.70 mM Mg(II) the titrations were repeated and the results given in Figure 1 (top) also. A sharper drop in enhancement is seen in the presence of Mg-ATP ($K_1' \sim 2 \times 10^{-4}$ M, Ginsburg, 1972) and also a lower final value of ϵ^* is achieved. After each addition of Mg-ATP, an incubation period of 2-3 min was allowed before measurement of the $1/T_1$ values. This was found to be necessary to obtain reproducible results in duplicate and triplicate titrations. The phosphorylation of the sulfoximine by ATP (Ronzio et al., 1969) is known to take place under the conditions of these titrations and this reaction is apparently complete in the time allotted since no change in $1/T_1$ is seen if the incubation period is longer than 3 min.

Analysis of the NMR titration data with the sulfoximine in the presence of ATP gave $\epsilon_q \sim 1$ and a dissociation constant of 0.8 μM. The K_I value from kinetic determinations for L-methionine (*SR*)-sulfoximine is 4 μM with inhibition being linearly competitive with respect to L-glutamate.[2] These kinetic and spectroscopic approaches provide the first direct measurement of the equilibrium binding constant for L-methionine (*SR*)-sulfoximine to the Mn-enzyme. Interestingly, the conversion of the γ position atom from trigonal to tetrahedral symmetry results in a decrease in the binding constant by three orders of magnitude (from 3 mM to 30 μM) comparing L-glutamate to the L-sulfoximine. The binding of ADP and P_i to the L-sulfoximine-enzyme complex tightens the binding another tenfold, approximately, whereas the phosphorylation of the inhibitor by ATP lowers the binding constant by only about fivefold. This suggests that the tight binding of the sulfoximine is due primarily to tetrahedral geometry at the γ position and conformational effects (synergism of binding) by bound nucleotide rather than to formation of a phosphorylated intermediate.

Titrations were performed using L-methionine sulfone and enzyme-Mn(II). The dissociation constant obtained was 0.45 mM. When these titrations were performed in the presence of 2 mM Mg(II), 2 mM ADP, and 30 mM P_i, the

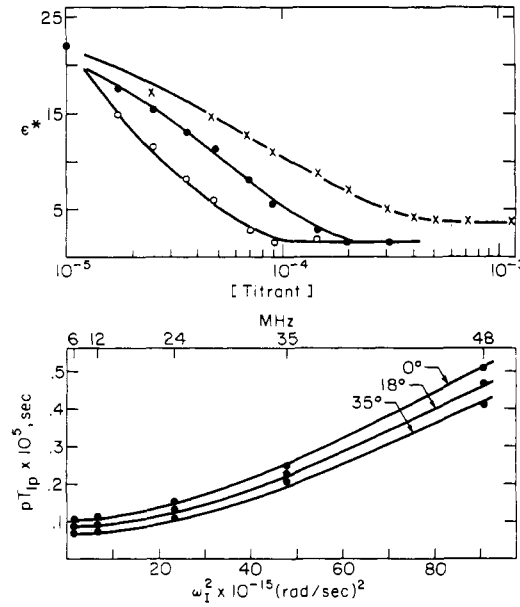


FIGURE 1: NMR titrations and a frequency and temperature dependence of the longitudinal relaxation times of glutamine synthetase-methionine sulfoximine complexes. (Top) Proton relaxation rate enhancement titrations of glutamine synthetase with methionine sulfoximine (X), methionine sulfoximine in the presence of Mg-ADP (●), and methionine sulfoximine in the presence of Mg-ATP (○). Solutions contained 0.1 mM $MnCl_2$, 0.12 mM enzyme sites in 10 mM imidazole buffer (pH 7.1) containing 0.1 M KCl. T = 25 °C, 24 MHz. Where indicated above, the solutions also contained 5 mM $MgCl_2$ with 10 mM ADP or 5 mM ATP. The solid curves are drawn with the following constants: (X), K_D = 0.5 μM, K_s = 30 mM, K_1 = 1.5 mM, K_3 = 30 μM, ϵ_a = 1.1, ϵ_b = 22.0, ϵ_t = 3.0; (●) the same constants as above and K_2 = 0.1 mM, K_1' = 5 mM, K_Q = 9 μM, ϵ_q = 1.0; (○) same as above with K_2 = 0.17 mM, K_1' = 32 μM, K_Q = 0.8 μM, ϵ_q = 1.0. (Bottom) Frequency dependence of pT_{1p} at three temperatures. The solution was identical with the one described above with the addition of 8 mM methionine sulfoximine.

dissociation constant was lowered to 0.17 mM and agrees well with the kinetically determined K_I value of 0.24 mM[2]. The ϵ_q value for this final complex was 4.0.

Figure 1 (bottom) shows plots of pT_{1p}, the paramagnetic contribution to the longitudinal relaxation time of water protons in solutions of $E_{1.7}$-Mn(II)-L-methionine (*SR*)-sulfoximine, as a function of ω_I^2, the Larmor precession frequency squared. The data at three temperatures were fit by using the same procedure given in the preceding paper (Villafranca et al., 1976) using eq 1, 3, 5, 6, 7, and 8. The data are frequency dependent from 6 to 48 MHz but unlike the $E_{1.7}$-Mn(II)-substrate complexes described in the previous paper the 6 MHz pT_{1p} values are not larger than the 12 or 24 MHz values. In addition, the pT_{1p} values are larger ($1/pT_{1p}$ is lower which is a restatement of the observed decrease in ϵ^*) than for $E_{1.7}$-Mn(II) suggesting that solvent water molecules are replaced or hindered from rapid exchange when a saturating concentration of L-methionine (*SR*)-sulfoximine is present. The results of NMR analysis on this complex gave the following parameters: τ_c equals 7 $\times 10^{-8}$ s at 12 MHz and 1.6×10^{-8} s at 48 MHz; $\tau_M = 0.8 \times 10^{-7}$ s (18 °C) and n = 0.2 water molecule. The correlation time is frequency dependent and this will be discussed later in this paper. Most significant from this analysis is that n equals a fraction of one first coordination sphere water molecule and suggests total occlusion of the tight metal ion site in the presence of L-methionine (*SR*)-sulfoximine. Qualitatively similar results are obtained when en-

[2] F. C. Wedler, unpublished data.

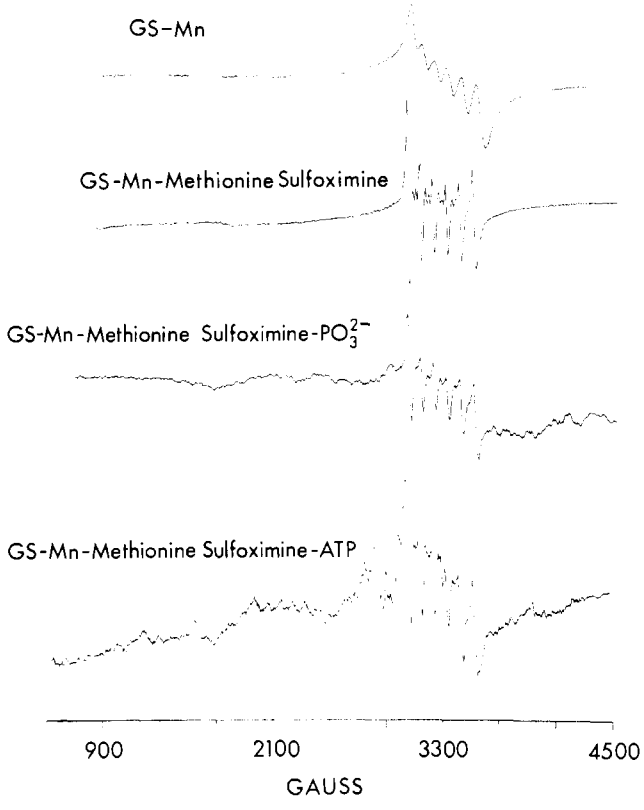


FIGURE 2: X-band EPR spectra for complexes of glutamine synthetase and methionine sulfoximine. All solutions contained imidazole, 10 mM; KCl, 100 mM; enzyme subunit concentration, 0.79 mM; $MnCl_2$, 0.7 mM; pH 7.1; T = 1 °C. The solution prepared to obtain the second spectrum also contained 12.0 mM L-methionine (*SR*)-sulfoximine The solution was incubated for 10 min at 25 °C, and cooled to ~1 °C, and the spectrum was recorded. The third spectrum from the top contained 3.3 mM L-methionine (*SR*)-sulfoximine phosphate. For the bottom spectrum, 4 mM $MgCl_2$ and 4 mM ATP were added to a solution containing 10 mM sulfoximine and the solution was incubated for 10 min at 25 °C.

zyme, Mn(II), L-methionine (*SR*)-sulfoximine, and ATP are combined with the value of n dropping to ~0.1.

X-Band EPR Spectra of Glutamine Synthetase, Mn(II), Substrates, and Inhibitors. Since the binding constants of Mn(II) to the tight and weak metal ion sites per subunit of $E_{1.7}$ are 5.0×10^{-7} and 4.5×10^{-5} M, respectively (Villafranca et al., 1976), the tight site can be selectively populated under conditions where [enzyme] > [Mn(II)]. The top spectrum in Figure 2 was obtained with a solution of 0.79 mM enzyme subunit concentration and 0.7 mM Mn(II) concentration. This spectrum represents Mn(II) bound only at the "tight" sites with no free Mn(II) present and shows that bound Mn(II) is in a relatively isotropic environment, i.e., the zero field splitting is small.

In the presence of saturating concentration of L-methionine (*SR*)-sulfoximine, a dramatic change in the line width and resolution of the EPR spectral intensities of bound Mn(II) are noted. The line widths change from about 30 G peak to peak to about 12 G (middle spectrum, Figure 2). In addition to six sharp lines representing the $-\frac{1}{2}$ to $\frac{1}{2}$ electronic transition, lines of lower intensity are resolved between each major transition. These smaller transitions are in the correct field positions for the $\Delta M_s = \pm 1$, $\Delta m_I = \pm 1$ forbidden transitions and nine of the expected ten transitions are resolved. Two observations can be made about this spectrum: (1) the narrow line widths are symptomatic of Mn(II) ions that are occluded from solvent bombardment and this is supported by the precipitous drop of ϵ^* found with enzyme solutions containing saturating concentrations of L-methionine (*SR*)-sulfoximine; (2) the six $-\frac{1}{2}$ to $\frac{1}{2}$ transitions centered near $g = 2$ with resolved forbidden transitions and no apparent transitions at higher or lower field strengths are indicative of a nearly cubic environment. Thus L-methionine (*SR*)-sulfoximine may have induced a distortion of the metal ion environment as a result of displacement of solvent by the sulfoximine on Mn(II). Alternatively close proximity of sulfoximine to the Mn(II) ion (~5 Å) may have immobilized a water molecule in the coordination sphere producing the observed distortion of the Mn(II) ion.

The value of D, the axial splitting parameter, may be estimated when the forbidden transitions are resolved (Kuska and Rogers, 1968) from the intensity ratio (IR) of the forbidden to allowed lines

$$\mathrm{IR} = \frac{8}{15}\left[\frac{3D}{4g\beta\mathcal{H}}\right]^2 \frac{1 + S(S+1)}{3M(M-1)}\left[I(I+1) - m^2 + m\right] \quad (2)$$

where $M = \frac{1}{2}$, $m = \frac{1}{2}$, and the other symbols have their usual meaning. The value of D thus calculated[3] is 0.014 cm^{-1} and is quite close to D values for Mn(II) doped in crystals possessing a cubic lattice (Kuska and Rogers, 1968).

When 3.3 mM L-methionine (*SR*)-sulfoximine phosphate (II) is present in a solution of enzyme–Mn(II), the resultant spectrum is quite different from that detected for the unphosphorylated sulfoximine. Additional fine structure splittings are noted around 3300 G in addition to poorly resolved resonances at about 2400 G. This indicates that the sulfoximine phosphate binds differently to the enzyme and induces different changes in the Mn(II) spectra than does either the sulfoximine itself or the methionine sulfoximine phosphate formed in situ. The addition of 10 mM $MgCl_2$ and 10 mM ADP to an enzyme solution containing the sulfoximine phosphate did not produce significant spectral changes from those shown in Figure 2.

The spectrum at the bottom of Figure 2 is obtained when Mg–ATP is added to a solution of $E_{1.7}$–Mn(II)–L-methionine (*SR*)-sulfoximine. The solution is allowed to incubate for 10 min at 25 °C and cool to 1 °C and the spectrum taken. This spectrum is quite reproducible if Mg(II) and ATP are added in a 1:1 ratio at a two- to fivefold molar excess over enzyme. Higher concentrations produced no additional changes. No detectable changes are seen in the spectrum of $E_{1.7}$–Mn(II)–L-methionine (*SR*)-sulfoximine (middle, Figure 2) with a 40-fold excess of either Mg(II) or ATP alone. This experiment demonstrates that the Mn(II) is locked into the tight metal ion site by the sulfoximine and not displaced or perturbed by Mg(II) or ATP alone. Thus, the presence of Mg–ATP produces a dramatic change in the spectrum of bound Mn(II) when this complex is bound at the metal ion–nucleotide site. This spectrum could result from the in situ formation of the sulfoximine phosphate and result in stabilization of an enzyme conformation and Mn(II) distortion unobtainable with the separate addition of sulfoximine phosphate and ADP.

The distortion produced by the addition of Mg–ATP is rhombic, i.e., both D and $E \neq 0$. A distinct six-line pattern with ^{55}Mn nuclear splitting is seen at ~2200 G and could

[3] To convert from cm^{-1} to gauss, divide the value in cm^{-1} by 9.33×10^{-5}.

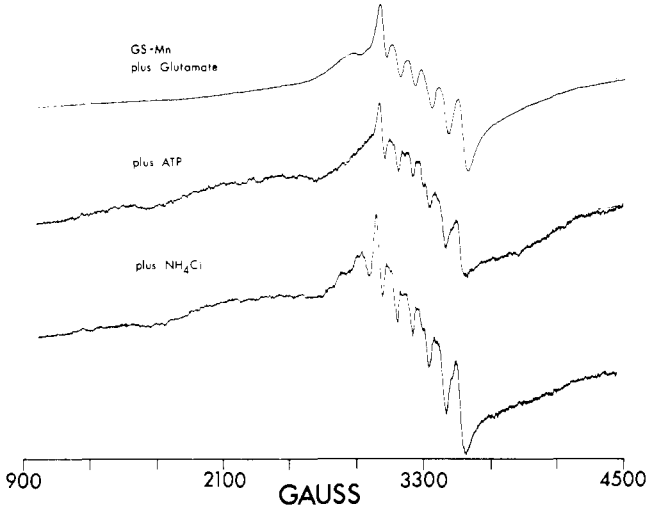


FIGURE 3: X-band EPR spectra for complexes of glutamine synthetase and substrates. The conditions for the experiment were similar to those described in the legend to Figure 2 with the following exceptions: (top spectrum) 50 mM L-glutamate; (middle spectrum) 50 mM L-glutamate, 5 mM $MgCl_2$, and 5 mM ATP; (bottom spectrum) 50 mM L-glutamate, 5 mM $MgCl_2$, 5 mM ATP, and 40 mM NH_4Cl. All solutions were prepared and manipulated at 1 °C.

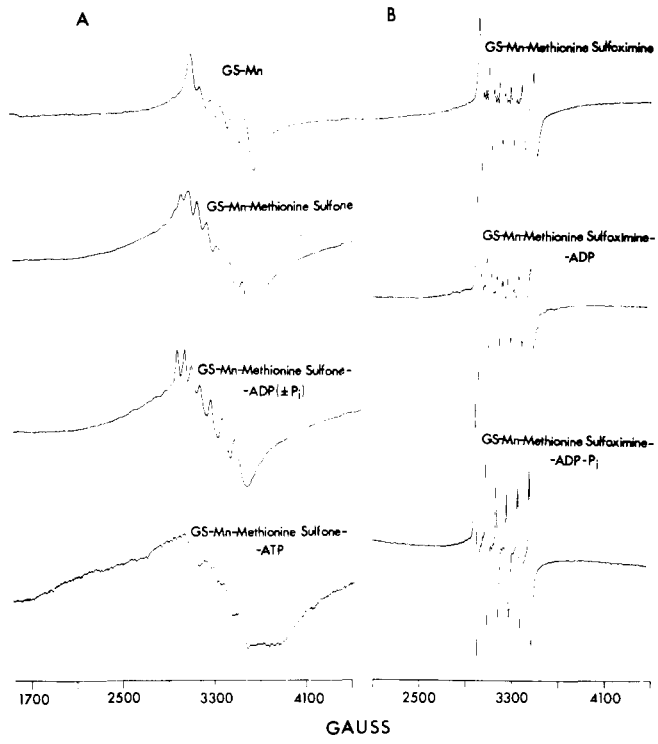


FIGURE 4: Comparison of spectra of glutamine synthetase with methionine sulfone, methionine sulfoximine, and adenosine nucleotides at X-band. Solutions contained the components described in the legend to Figure 2 with the following additions where noted on Figure 4: (A) (top) 0.60 mM enzyme sites and 0.55 mM $MnCl_2$; (2d from top) 30.0 mM L-methionine sulfone; (3d from top) 10 mM $MgCl_2$, 5 mM ADP, and 50 mM P_i; (bottom) 10 mM $MgCl_2$ and 8 mM ATP. (B) (top) 15 mM L-methionine (*SR*)-sulfoximine; (middle) 10 mM $MgCl_2$ and 5 mM ADP; (bottom) 10 mM $MgCl_2$, 5 mM ADP, and 40 mM P_i.

represent the $-3/2$ to $-1/2$ electronic transition. Using the diagrams in papers by Aasa (1970) and Dowsing et al. (1969), estimates of D and λ can be obtained where $\lambda = E/D$. These values are in the range $D = 0.07$–0.1 cm^{-1} and $\lambda = 0.1$–0.15.

The spectra in Figure 3 were taken in the presence of substrates. L-Glutamate produces a small axial distortion in the environment of enzyme-Mn(II) with unresolved fine structure and no obvious change in line width. Addition of Mg-ATP results in a diminuation of all spectral intensities when compared to the top spectrum. Unresolved broad resonances were detected at higher spectrometer gain settings at 1500, 2400, and 3900 G. This anisotropic spectrum also shows poorly resolved fine structure splitting at 3300 G in the region of the $-1/2$ to $1/2$ transitions. The appearance of these additional sets of transitions indicates that the environment of Mn(II) at the tight site is changed when metal nucleotide is bound.

The spectrum in the middle of Figure 3 was obtained immediately after addition of Mg-ATP to a solution of enzyme-Mn(II)-glutamate at 1 °C. This spectrum was unchanged after 30 min at 1 °C. It is possible that γ-glutamyl phosphate was formed enzymatically in this solution (Timmons et al., 1974) and did not dissociate from the enzyme during this period. However, a nearly identical spectrum is obtained when Mg-ADP is added in place of Mg-ATP. This suggests that binding of metal-nucleotide induces a conformational change in the protein which changes the environment of the bound Mn(II), and that there is no distinct spectroscopic change attributable to the formation of the enzyme-(Glu-P)-ADP complex.

The possibility that Mn(II) and Mg(II) have exchanged and are binding randomly to the two metal ion sites was considered and tested experimentally. The apparent binding constant for Mg(II) to the tight metal ion site ($\sim 5 \times 10^{-5}$ M, Hunt and Ginsburg, 1972; Hunt et al., 1975) is about two orders of magnitude weaker than the Mn(II) binding constant. Indeed, at a $E_{1.7}$-Mn(II) concentration of 0.4 mM, about 100 mM $MgCl_2$ was needed to produce approximately 30% free Mn(II) in an EPR experiment. The binding constants of Mg(II) and Mg-ADP to the second enzyme sites are $\sim 1 \times 10^{-4}$ and 2×10^{-4} M, respectively (Hunt et al., 1975), and the Mg-ADP binary dissociation constant is 5×10^{-3} M. Binding constants of Mg to ATP and of the Mg-ATP complex to the enzyme are 3.2×10^{-5} M and 1.7×10^{-4} M, respectively (Ginsburg, 1972). Since concentrations of Mg(II) and ADP were from 3 to 10 mM in the experiments, the enzyme was 80–98% saturated with metal nucleotide. In addition to the above evidence, the presence of Mn(II) at two different sites is unlikely because of the resolution of spectral lines at ~3300 G. If two or more Mn(II) ions were in different environments, a broad featureless EPR spectrum would be expected due to spectral overlap. This has been suggested for the Mn(II)-alkaline phosphatase complex (Haffner et al., 1974) and will be discussed later.

The spectrum at the bottom of Figure 3 was obtained after 40 mM NH_4Cl was added to a solution of $E_{1.7}$-Mn(II) plus glutamate and Mg-ATP at 1 °C. This spectrum represents the equilibrium mixture of reactants and products in the biosynthetic assay, and a distinct distortion of bound Mn(II) is found. This spectrum does change with time and resembles the spectrum in the middle of Figure 3 after 1 h at 1 °C.

Figure 4A presents spectra for GS-Mn(II) in the presence of L-methionine sulfone. The sulfur atom has tetrahedral symmetry and should bind similarly to the sulfoximine. The EPR spectrum suggests slight axial distortion but the line widths remain broad unlike the spectrum with methionine sulfoximine (Figure 4B). When the nucleotide site is

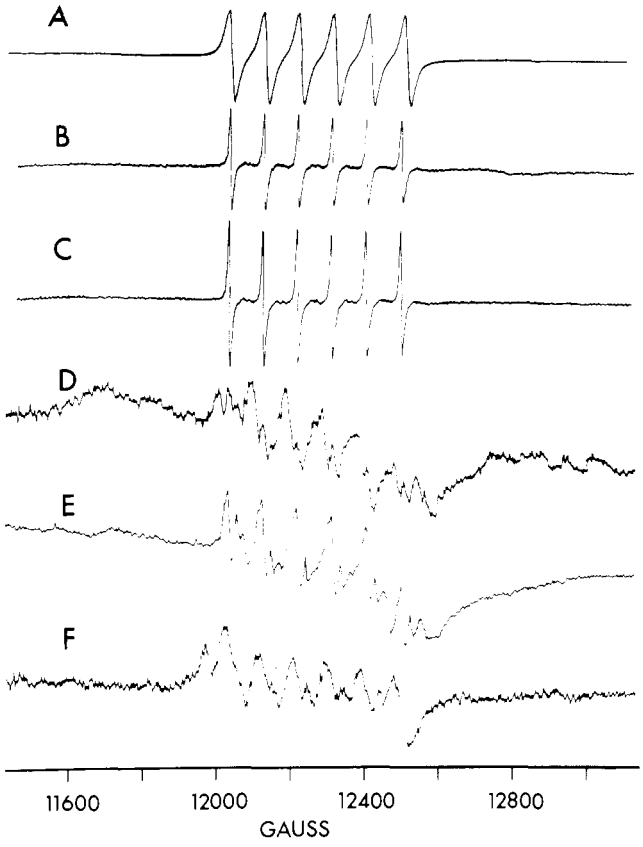


FIGURE 5: K-band EPR spectra for glutamine synthetase complexes. (A) $Mn(H_2O)_6^{2+}$. (B) 0.79 mM enzyme sites, 0.70 mM $MnCl_2$, 12.0 mM L-methionine (*SR*)-sulfoximine. (C) Same as B with the addition of 9 mM $MgCl_2$ and 5 mM ADP. (D) Same as B with the addition of 8 mM $MgCl_2$ and 8 mM ATP. The sample preparation was identical with that described in the legend to Figure 2. (E) Enzyme plus L-methionine sulfone. (F) Enzyme plus L-methione sulfone and Mg-ATP. The samples in E and F were identical with the appropriate samples described in Figure 4. T = 1 °C.

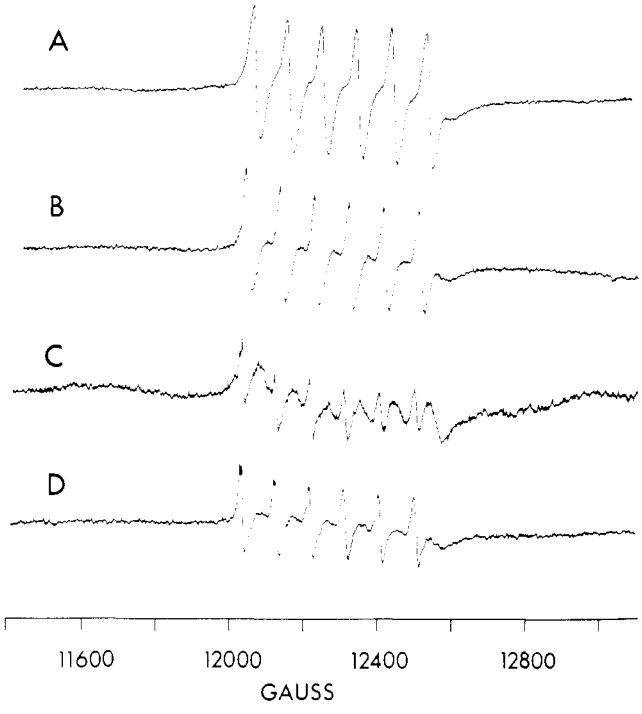


FIGURE 6: K-band EPR spectra for complexes of glutamine synthetase and substrates. (A) 0.50 mM enzyme subunits and 0.45 mM $MnCl_2$. (B) Same as A plus 40 mM L-glutamate. (C) Same as B plus 8 mM $MgCl_2$ and 8 mM ATP. (D) Same as C plus 30 mM NH_4Cl. T = 1 °C.

occupied by Mg-ADP, certain spectral lines become more intense indicating a change in ligand geometry around the Mn(II) produced by a conformational change induced by ADP. The addition of P_i or arsenate (not shown) does not change this spectrum. However, addition of Mg-ATP produces a broad featureless spectrum with spectral patterns spread from 1700 to 4500 G (Figure 4A, bottom). This type of spectrum could arise from at least two situations. (1) Conformational changes produced by binding Mg-ATP could tighten binding of L-methionine sulfone producing an enzyme-Mn(II) complex with rhombic distortion. Also, a very short electron spin relaxation rate(s) for individual transitions may arise and produce broad lines. A rhombic distortion leading to spectral spreading of $\pm 5/2 \leftrightarrow \pm 3/2$, $\pm 3/2 \leftrightarrow \pm 1/2$, and $-1/2 \leftrightarrow 1/2$ transitions could give the observed spectrum. (2) The Mn(II) ion may be in different environments in the 12 subunits or may have dissociated and reassociated at the nucleotide site. This possibility cannot be ruled out for the sulfone but almost certainly can be ruled out for the complex formed from the sulfoximine and Mg-ATP (Figure 2, bottom) since sharp well-resolved transitions are seen in the EPR spectrum due to formation of that complex.

In Figure 4B, spectra are shown which result from progressive additions of Mg-ADP and P_i to a solution of GS-Mn(II)-L-methionine (*SR*)-sulfoximine. The line widths become much sharper with these successive additions. This can be readily seen by noting the height of the lowest field transition. The spectrometer settings were identical for all three spectra and the peak to peak line width narrows from ~13 G to ~9 G to ~6 G. The "true" line width may be narrower for the bottom spectrum in Figure 4B due to artificial broadening by instrumental over-modulation.

The progressive sharpening of transitions is accompanied by changes in the intensity and resolution of the forbidden transitions. Addition of Mg-ADP produces a larger axial distortion than is present with the sulfoximine alone and also gives weaker lines at 2800 and 3600 G. These are probably due to the $\pm 3/2 \leftrightarrow \pm 1/2$ set. This distortion disappears when P_i is added and the forbidden transitions are very weak. The environment of Mn(II) at the tight site changes upon occupancy of the metal nucleotide diphosphate and P_i sites and is indicative of distortion of the metal ion coordination sphere perhaps produced by conformational changes in the protein. The use of eq 2 permits calculation of D and these values are 0.015 and 0.0075 cm^{-1} for complexes of GS-Mn-sulfoximine with Mg-ADP and Mg-ADP plus P_i, respectively. The bottom spectrum of Figure 4B represents Mn(II) in a more highly symmetric environment (cubic symmetry) than is present in any of the other reported spectra.

K-band EPR Spectra for Glutamine Synthetase Complexes. EPR spectra were obtained at higher magnetic field strengths for many of the previously discussed enzyme complexes (Figures 5 and 6). The advantages of obtaining spectra under these conditions are threefold: (1) the $-1/2$ to $1/2$ transition will be dominant at moderate D values resulting in better spectral resolution; (2) the forbidden transitions which result in inhomogeneous broadening at 9 GHz become much less intense at 35 GHz since they are inversely proportional to the square of the applied field; (3) if the electron spin relaxation time is field dependent between

3000 (9 GHz) and 12000 G (35 GHz), the spectral lines will be sharper at the higher field strengths.

The K-band spectra in Figure 5 show marked narrowing when compared to spectra at X-band. This is attributed to changes in the electron spin relaxation time of the bound Mn(II). Figure 5A is a spectrum of $Mn(H_2O)_6^{2+}$ included for reference to demonstrate the very sharp lines obtained with GS-Mn(II)-methionine sulfoximine (Figure 5B) and this enzyme complex plus Mg-ADP (Figure 5C). The peak to peak line widths are 8 G (Figure 5B) and 6 G (Figure 5C), respectively. Very faint transitions can be found between the six major peaks and these are attributable to the forbidden transitions.

A K-band spectrum was run on a sample containing enzyme, Mn(II) bound to the tight site, L-methionine (*SR*)-sulfoximine, and Mg-ATP (Figure 5D). The conditions for sample preparation were identical with those described earlier. The spectrum in Figure 5D suggests that Mn(II) is in a distorted environment. A doublet pattern of splitting is expected for each $-\frac{1}{2}$ to $\frac{1}{2}$ transition at K-band for an axial distortion while more complex splitting is expected for rhombic distortions. The spectrum in Figure 5D demonstrates that bound Mn(II) is in a rhombic ligand environment. The simple interpretations for the X- and K-band spectra for this complex are consistent but more detailed analyses are not straightforward.

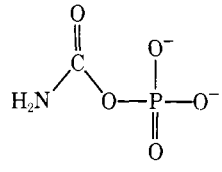
Spectra for enzyme complexes formed with methionine sulfone and methionine sulfone plus ATP are presented in Figure 5E and F. These spectra display features of rhombic distortions and demonstrate the better resolutions obtained at higher magnetic field strengths (compare with Figure 4A). Once again the suggestion is made that the addition of methionine sulfone or methionine sulfoximine influences the protein shape in a manner which distorts the ligand environment of bound Mn(II) and this environment is further changed when nucleotide is present.

In Figure 6A-C, spectra for GS-Mn(II) (6A), then the successive additions of glutamate (6B), and Mg-ATP (6C) are presented. The spectra show progressive sharpening of lines and distortions from axial symmetry. The bottom spectrum (Figure 6D) shows the equilibrium mixture formed by incubating enzyme, glutamate, Mg-ATP, and NH_4^+ for 1 h at 25 °C. A small axial distortion is apparent as a doublet splitting of the lowest field transition. A nearly identical spectrum can be obtained if L-glutamine and Mg-ADP are added to a solution of GS-Mn(II). It is notable that L-glutamine tightens the binding of Mn-ADP to GS-Mn(II) (Hunt et al., 1975), but mixed metal ion experiments have not been performed to demonstrate whether Mg-ADP binds tighter to GS-Mn(II). In any event, no free Mn(II) is present in the solution as can be easily demonstrated by comparing the line widths in the spectra in Figures 6D and 5A ($Mn(H_2O)_6^{2+}$).

Complexes of Glutamine Synthetase with Other Compounds. Nitrate has been shown to be a potent inhibitor of enzymic activity of creatine kinase, arginine kinase, and formyltetrahydrofolate synthetase. Intense changes are found in the EPR spectra of enzyme-bound Mn(II) for these enzymes in the presence of substrates and nitrate (Reed and Cohn, 1972; Buttlaire and Cohn, 1974; Buttlaire et al., 1975). By contrast, none of the spectra reported herein are altered by addition of potassium salts of nitrate, thiocyanate, or formate nor is the enzyme activity altered by these compounds. The planarity of nitrate anion was suggested as a good analogue of metaphosphate for phosphoryl transfer in the three enzymes discussed above. If the glutamine synthetase reaction goes through a metaphosphate intermediate, the three anions tested do not mimic its formation as judged by activity measurements[2] and EPR spectral data.

The nonphosphoryl transferring ATP analogues App(CH_2)p and App(NH)p were used in an attempt to show structural changes induced by the three phosphate groups of the nucleotide substrate. Addition of either analogue separately to a solution of GS-Mn(II)-methionine sulfoximine produced a spectrum identical with the one shown in Figure 4B, bottom. Thus, the presence of a triphosphate nucleotide analogue produces the same structural perturbations about the Mn(II) on the enzyme as does a diphosphate nucleotide plus P_i.

Tate and Meister (1973) showed that carbamyl phosphate and ADP in the presence of Mg(II) (or Mn(II)) and glutamine synthetase would synthesize ATP. Using these results, experiments were devised in which carbamyl phosphate was added to GS-Mn(II) and the EPR spectra recorded. Additions of up to 10 mM carbamyl phosphate gave small spectral perturbations which were intermediate between those shown in Figure 2, top and middle. Subsequent addition of ADP or L-alanine did not produce further changes. Since carbamyl phosphate (IV) has planar geometry at the carbonyl carbon, and could occupy portions of the ammonia, carboxyl and phosphate sites, the absence of large spectral changes provide further evidence that only the tetrahedral geometry found in methionine sulfoximine and methionine sulfone, not a simple acylphosphate, sufficiently resembles the transition state (or short-lived intermediate).



IV

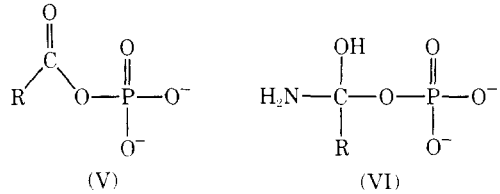
Attempts were made to study the binding of Mn-ADP to GS-Mn(II) in the presence of glutamate or methionine sulfoximine. The results of such experiments were difficult to interpret since spectral lines unique to each of the two bound Mn(II) ions were not readily apparent. In fact, it was not possible to distinguish whether certain broad unresolved resonances were due to spectral overlap or a small dipolar electron-electron relaxation (Abragam and Bleaney, 1970). However, spin-spin coupling of two adjacent Mn(II) ions can be ruled out on the basis of previously reported EPR data with model systems. Both dipolar and spin-spin exchange interactions have been seen by Owen (1961) for Mn(II) doped in MgO where nearest neighbor Mn(II) ions are 2.97 Å apart and next nearest neighbors are 4.1 Å apart. If the Mn(II) ions were this close in Mn(II)-$E_{1.7}$-Mn(II), appreciable shortening of the electron spin relaxation time, τ_s, would be predicted along with multiple line EPR spectral patterns. Neither the well-resolved EPR spectra or shortening of τ_s were found for enzyme with two metal ions bound. The latter data are presented in the NMR analyses of the first paper. A shortened τ_s value would dominate the correlation time for water interactions with Mn(II)-$E_{1.7}$-Mn(II) and this was not found to be the case in the data analysis. A lower limit can be set for the Mn(II) separation ($\leq$7-8 Å) where the two ions will have

no spin–spin interaction or a small (<10%) dipolar relaxation effect on one another.

Implications of NMR and EPR Data on the Active Site Structures of Glutamine Synthetase. Ample documentation exists for conformational changes (both inter- and intrasubunit) induced in glutamine synthetase upon binding metal ions (Hunt and Ginsburg, 1972) and substrates (Wedler, 1974; Timmons et al., 1974). The presence of two metal ions has also been demonstrated for the biosynthetic reaction and the γ-glutamyl transfer reaction both catalyzed by the enzyme (Hunt et al., 1975). In the latter reaction, the amide nitrogen of glutamine is replaced by hydroxylamine, but only in the presence of the nonconsumed reactants ADP and arsenate. Thus, for this partial reaction, the nucleotide substrate site must be occupied, implying that an obligatory enzyme conformational change induced by nucleotide is required for reaction at the ammonia site. The EPR data presented in this paper provide evidence for conformational changes in glutamine synthetase at the tight Mn(II) site induced by substrate binding. The important aspect of these experiments is that the tight metal ion site is now implicated as interacting intimately with bound L-glutamate. Changes in the geometry about the metal ion site may reflect enzyme conformational changes near the γ-carboxyl of L-glutamate which may be required for activation of this carboxyl for the biosynthetic reaction. This activation is also apparently necessary for hydroxamate displacement of the amide nitrogen of glutamine in the transferase reaction.

The mechanism of the biosynthetic formation of glutamine from glutamate, ATP, and ammonia has been studied by isotope exchange experiments. Wedler and Boyer (1972) demonstrated that all substrates were required in the active site for any exchange reactions to occur. The suggestion by Meister and co-workers (cf. Tate and Meister, 1973) that γ-glutamyl phosphate is formed as an intermediate in the overall reaction pathway is made difficult to test experimentally by the observation that the rate-limiting step in turnover is not bond formation or breaking but nucleotide release from the enzyme surface. Exchange of ^{18}O between glutamate and P_i (Boyer et al., 1956; Kowalsky et al., 1956) has been observed but does not permit delineation of an exact chemical mechanism for the reaction pathway.

The transient formation of γ-glutamyl phosphate (V) (trigonal carbon at γ position) and the ammonia adduct of this compound (VI) (tetrahedral carbon at γ position) could of course be of finite lifetime (in the msec range) and be classified as intermediates along the reaction pathway. Alternatively, the lifetime of either or both of these compounds (V and VI) could be $\sim 10^{-13}$ s, which is the "lifetime" of a transition state. The present spectroscopic data, taken together, do not indicate that the glutamyl phosphate–ADP–enzyme complex produces any unusual effects in the metal ion sites. As noted above, carbamyl phosphate with ADP and L-alanine also did not produce spectral changes nearly as dramatic as bound sulfoximine. The elegant demonstration of methionine sulfoximine phosphate as a nondissociable inhibitor of glutamine synthetase along with the modeling of the active site of this enzyme (Gass and Meister, 1970) led to the postulate of a tetrahedral center as being an intimate part of the overall reaction. The data in this and the preceding paper support the hypothesis that the active site of the enzyme has high affinity for a substrate analogue with a tetrahedral center at the γ position. This enclosure of the active site of the enzyme about a substrate analogue and the higher affinity of the analogue for the enzyme compared to the substrate is in concert with the transition-state theory of enzyme action (Wolfenden, 1972).



(V) (VI)

R = $-CH_2CH_2CH(NH_3^+)CO_2^-$

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The Interaction of Borate and Sulfite with Pyridine Nucleotides†

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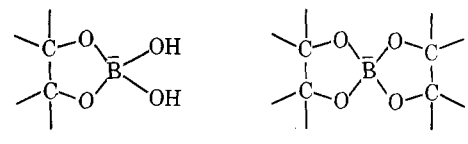
ABSTRACT: The kinetics and equilibria of the borate interaction at ribose with NAD^+ and NMN^+ have been measured using as a chromophoric probe the perturbation effect borate has on the addition of sulfite to the 4 position of the nicotinamide ring. NAD^+ and NMN^+ have more favorable borate association constants than do their corresponding sulfite addition complexes. The rate of interaction of the ribose moiety with borate at low borate buffer concentration is dependent on the concentration of both borate and boric acid. At high borate concentration the rate becomes independent of borate concentration, indicating the existence of a two-step process for the interaction of NAD–sulfite with borate with a change of rate-determining step from the interaction of the ribose hydroxyl group with borate at low borate to an elimination of sulfite at high borate concentration. A linear free energy relationship with a slope of 0.94 describes an increased reactivity of the nucleotide for sulfite as the affinity of the nucleotide for sulfite increases.

During an investigation of the rate of interaction of sulfite with NMN^+,[1] and NAD^+ as a function of pH using the technique of stopped-flow spectrophotometry, it was found that in borate buffers, two transients are observed instead of the single transient due to the addition of sulfite to the 4 position of the nicotinamide ring. That this phenomenon is due to the borate–ribose interaction was ascertained by the finding of only one reaction in nicotinamide cation analogues lacking the ribose moiety.

Borate is known to be toxic to living organisms (Thienes and Haley, 1964; Dreisbach, 1971). Few studies have been carried out on the mechanism of interaction of borate with enzymes and coenzymes. Borate is also known to competitively inhibit a number of dehydrogenases (Weser, 1968; Misawa et al., 1966; Roush and Gowdy, 1961; Deitrich, 1967). Because this inhibition is possibly due to the interaction of borate with the coenzyme we have studied the interaction of borate with NAD^+ and NMN^+ in an attempt to better understand the biological effects of borate. The interaction of borate with diols and sugars with adjacent hydroxyl groups is well known. Stable borate complexes of structure type I in dilute sugar and of structure type II, in more concentrated sugar, are formed (Weser, 1967a). The association constants[2] for the formation of I and II from borate, K_1' and K_2', are measured most conveniently by differential potentiometry in which the release of protons from diol solutions upon the addition of borate is measured (Kilpi, 1952). This method is more convenient for measuring K_2' because of the relative concentrations of reactants used.



I II

No measurement of borate–nucleotide association constants K_1' has been made, although K_2' values for two nu-

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[1] Abbreviations used are: NAD^+, nicotinamide adenine dinucleotide; NMN^+, nicotinamide mononucleotide; B, borate; BH, boric acid; NADB, borate complex of NAD^+; NAD-SO_3^-, sulfite complex of NAD^+; NADB-SO_3^-, sulfite complex of NADB; NMN-SO_3^-, sulfite complex of NMN^+; NADH, reduced nicotinamide adenine dinucleotide; ADPR, adenosine diphosphoribose.

[2] K_1' is defined as [I]/[diol][borate]; K_2' is defined as [II]/[diol]^2[borate].