Glutamine Synthetase from Ovine Brain Is a Manganese(II) Enzyme†

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ABSTRACT: The activation of ovine brain glutamine synthetase by Mn(II) or Mg(II) was studied by steady-state kinetics. The metal ion concentration was varied at several fixed concentrations of ATP, and vice versa, and the resultant kinetic curves were analyzed according to the method of London and Steck [London, W. P., & Steck, T. L. (1969) *Biochemistry 8*, 1767–1779]. The data for Mg(II) indicated optimal activation at Mg:ATP = 2:1, whereas that for Mn(II) occurred at Mn:ATP = 1:1. This was interpreted as indicating formation of Mg·E·Mg·ATP as the subunit complex of optimum activity with Mg(II) (pH_{opt} 7.5). With Mn(II) (pH_{opt} 5.0), the complex of optimum activity may be either E·Mn·ATP or Mn·E·Mn·ATP, where the Mn·E complex is very tight. So that the latter two possibilities could be distinguished, titrations of the enzyme with Mn(II) were performed, electron paramagnetic resonance being used to determine free Mn(II). Four moles of Mn(II) ions was found to bind per mole of octameric enzyme, with an apparent $K_d \simeq 0.54$ μM. Addition of either HCl or Nd(III) ions to Chelex-treated enzyme results in the release of 3.7 ± 0.2 Mn(II) ions. Thus, it appears that four Mn(II) are very tightly bound per octamer and that four more Mn(II) can bind tightly. Neither Mg(II) nor Ca(II) at 50 mM can displace Mn(II) from the Mn_4·E complex, but Mn(II) still binds tightly to apoenzyme or Mn_4E in the presence of 50 mM Mg(II). As one proceeds from apo-E to Mn_4·E to Mn_4·E·Mn_4 (±ATP), the intensity of the fluorescence emission of protein tryptophan residues decreases strongly and successively. The specific activities of the apo-E, Mn_4·E, and Mn_4·E·Mn_4 complexes were found to be 0, 50, and 200 units/mg, respectively. If apoenzyme is added to a continuous coupled assay system with Mg(II), buffer, and with or without Mn(II) present, a time-dependent activation is observable with $t_{1/2} \simeq 0.5$–1.0 min. The total intracellular concentration of Mn(II) in ovine brain tissue was determined to be 1.9–2.6 μM, whereas the free [Mn(II)] was below 0.5 μM. Since the enzyme binds Mn(II) in preference to other divalent ions, it appears that the enzyme may exist as a manganoenzyme in vivo.

Glutamine synthetase from ovine brain has been known for many years to require divalent cations for catalytic activity (Meister, 1962). The enzyme is composed of eight identical subunits of M_r 45 000 each, arranged approximately at the vertices of a cube (Haschenmeyer, 1970; Strahl & Jaenicke, 1972). Although Monder (1965) has examined the pH activity profiles produced by activation with various divalent metal ions, the mechanism of interaction among metal ion, enzyme, and substrate(s) has not been studied in depth.

In contrast, for the dodecameric *Escherichia coli* enzyme the metal–enzyme–substrates interactions have been elucidated in great detail by a variety of elegant physicochemical experiments (Ginsburg, 1972, and references cited therein). There are two classes of sites: the (tight) n_1 site plays both a major structural role in the protein and lies within 5–6 Å of the C-5 of L-glutamate bound in the active site (Villafranca et al., 1976; Villafranca, 1978); the n_2 (looser) site apparently bridges ATP and enzyme. In the case of octameric porcine brain glutamine synthetase, Jaenicke & Jesiõr (1978) have explained the sigmoidal saturation curves for activation by Mg(II) by a multiequilibrium scheme involving enzyme, Mg(II), and several ionized states of ATP. In addition, two distinct ATP binding sites per subunit have been proposed, one in the site for Mg·ATP and one at a separate modifier site for ATP alone (Jaenicke & Berson, 1977). For the octameric rat liver enzymes, only 4 mol of L-methionine (*S*,*R*)-sulfoximine or ca. 5 mol of Mn·ATP were bound per mol of enzyme (Tate et al., 1972; Tate & Meister, 1971). These findings may be relevant to the half-site stoichiometry of the present results.

London & Steck (1969) have derived a general model for the interaction of enzyme, metal modifier, and ATP, shown in Scheme I. This complex scheme was subdivided into three kinetically distinguishable submodels: model I involves the lower tier of reactions, model II involves the upper tier of reactions plus conversion of E to M·E, and model III involves the diagonal set of equilibria. Also developed were methods for interpreting the steady-state kinetics with these models. Distinctive kinetic responses were shown to arise from each model as one varies the [M] at various fixed concentrations of ATP and then varies the [ATP] at various fixed [M] values. Their "isovelocity" and other analytical replot methods provided diagnostically useful tools that have been applied to advantage in the present work.

These current results are the first for the ovine brain enzyme that combine kinetic and binding data, to quantitate the stoichiometry and relative tightness of binding of Mn(II). Two different classes of metal sites have been found, with very different characteristics from those of sites on the *E. coli* system. The data also indicate that brain enzyme probably exists as a Mn(II) enzyme in vivo, for which there are few examples in mammalian systems. These results provide the basis for future studies on the interaction of enzyme–metal and for active site metal–metal and metal–ATP distances.

Materials and Methods

Materials. Ovine brain glutamine synthetase was purified by the procedures of Ronzio et al. (1969) with slight modifications. In place of the acid precipitation step, the ammonium sulfate precipitate fraction between 20% and 40% saturation was taken. DEAE-Sephacel[1] (Pharmacia) was used in place of DEAE-cellulose, and the enzyme was eluted batchwise with 0.01 M phosphate buffer (pH 6.4) containing 2 M KCl. As a final purification step, filtration on Bio-Gel

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[1] Abbreviations: DEAE, diethylaminoethyl; PEP, phosphoenolpyruvate; EPR, electron paramagnetic resonance; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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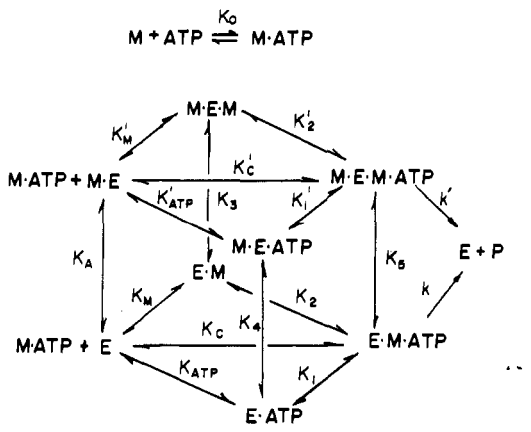
Scheme I



A-0.5M (Bio-Rad) was used, if needed. Enzyme purity was determined by disc gel electrophoresis at pH 6.8 (Orr et al., 1972). The specific activity of the purified enzyme was ca. 200 μmol of product (15 min)$^{-1}$ (mg of protein)$^{-1}$ for the Mg^{2+}-stimulated transferase assay (Ronzio et al., 1969) at pH 7.2, either before or after storage in 50% glycerol/buffer.[2]

Pyruvate kinase and lactate dehydrogenase were of the highest purity available from Sigma. All lanthanide salts were obtained from the Rare Earth Division of the American Potash and Chemical Corp. or the Kerr-McGee Chemical Corp. Chelex 100 resin was purchased from Bio-Rad Laboratories. All other chemicals and biochemicals were ACS reagent grade or of the purest grade commercially available (Sigma Chemical Co.).

Assays. During the purification procedures, glutamine synthetase activity measurements used the transferase assay system of Ronzio et al. (1969), in which γ-glutamyl hydroxamate synthesis was measured colorimetrically at 540 nm after addition of acidic $FeCl_3$. Samples containing the assay mix were preincubated at 37 °C for 2 min prior to reaction initiation by addition of enzyme. The enzyme form added to these assays was Mn_4·E. One unit of activity is defined as the formation of 1 μmol of γ-glutamyl hydroxamate/min at 37 °C. Kinetic measurements with pure enzyme utilized the biosynthetic assay (P_i release) of Fiske & Subbarow (1925). The incubation conditions for all assays was 15 min at 37 °C. The reaction was stopped with 1.8 mL of acidic $FeSO_4$; then 0.15 mL of acid molybdate was added and inorganic phosphate determined at 660 nm. The coupled assay for ADP release involving PEP, pyruvate kinase, and lactate dehydrogenase was performed according to the procedures of Kingdon et al. (1968) with a Gilford spectrophotometer. Protein was determined during purification by a microbiuret assay (Layne, 1957) performed in a total volume of 0.5 mL. With pure enzyme, differential UV absorption spectra were recorded on a Cary Model 17 spectrophotometer with buffer as the reference. Extinction coefficients of 0.444 absorbance unit·mL/mg at 290 nm and 0.613 absorbance unit·mL/mg at 280 nm were determined by reference to biuret measurements.

A Varian E9 spectrometer was used to record 9-GHz (X-band) EPR spectra. The spectrometer was equipped with a variable temperature accessory that maintained the temperature to ±1 °C with precooled nitrogen gas that was passed through the Dewar assembly in an E-238 cavity. Aqueous samples of 50–75 μL were placed in 50-μL quartz capillary tubes, sealed at one end. These tubes were used as cuvettes for recording the X-band spectra.

A Perkin-Elmer Model MPF-44B spectrofluorometer was used to observe protein fluorescence: λ_{ex} = 295 nm, λ_{em} = 335 nm. Sample volumes of 0.4 mL were used with semimicro quartz cuvettes (Precision Cells) with a path length of 5 mm. The sample temperature was maintained at ±0.1 °C with a (Lauda) circulating water bath and was determined directly in the cuvette with a microprobe attached to a digital thermometer.

Preparation of Metal-Free Enzyme: $E(Mn)_0$. A total of 250 μL of 1.05 mg/mL ovine brain glutamine synthetase was placed in a collodian bag apparatus (Sartorius) and was dialyzed at 4 °C for 18–20 h against a Chelex-treated buffer consisting of 100 mM imidazole, 5 mM β-mercaptoethanol, and 40% glycerol (v/v), pH 6.5. The specific activity of the sample after dialysis was zero, from the standard transferase assay at 37 °C, pH 7.2, without any added divalent metal ion.

EPR Titrations for Free Mn(II). A 0.50-mL aliquot of native ovine brain glutamine synthetase (S.A. 200) was passed through a Sephadex G-25F column (1.0 cm × 3.0 cm), equilibrated with a 10 mM Hepes/100 mM KCl/5 mM β-mercaptoethanol buffer, pH 7.2, to remove glycerol and then a Chelex 100 column (1.0 cm × 20.0 cm), equilibrated with the same buffer, to remove trace metal ions. Samples were prepared by mixing 100 μL of the enzyme solution with 10 μL of various standard Mn(II) solutions. After approximately 2 h of incubation at 4 °C, EPR spectra were obtained at 8.975 GHz over the range 3200–3700 G at 120 mW with a modulation amplitude of 20 G. The free Mn(II) concentration was determined by the method of Cohn & Townsend (1954) with a Mn(II) standard curve that was linear over the range of 0–50.0 μM Mn(II). The [Mn(II)] could be determined accurately down to 0.5 μM.

Determination of Mn(II):E Ratio in Crude Brain Extracts. In order to determine whether there was sufficient free Mn(II) in vivo for the formation of a manganoenzyme, it was necessary to perform the following experiment. To a known weight of fresh ovine brain tissue was added an equivalent weight of extraction medium (0.15 M KCl, 5.0 mM β-mercaptoethanol, and 1.0 mM EDTA, pH 7.2), and the mixture was homogenized and stirred at room temperature for 30 min. The sample was centrifuged at 10 000 rpm for 20 min to remove all insoluble material, and the supernatant was then assayed for activity by the transferase assay. Aliquots of 1.0 mL of this supernatant also were brought to 10% perchloric acid and centrifuged at 10 000 rpm for 10 min to remove the precipitated protein. Samples of 50 μL of this protein-free solution were analzyed for free Mn(II) by EPR.

Results

(*A*) *Steady-State Kinetics.* According to London & Steck (1969), the qualitative shapes of the activity profiles with metal

[2] The enzyme used in Figure 4 was routinely passed through Sephadex G-25 in the presence of 20 mM phosphate buffer, pH 7.0/50 mM $MgCl_2$, in order to remove glycerol from the storage buffer. This treatment produced an enzyme species with a specific activity (S.A.) = 50 units/mg (proven by acid denaturation to be Mn_4·E). The enzyme prior to Sephadex treatment had a S.A. = 200 units/mg (presumably Mn_4·E·Mn_4). Addition of Mn(II) to non-Sephadex-treated enzyme caused the transferase activity in the presence of 0.1 mM ADP/20 mM $MgCl_2$, pH 7.2, to increase from a S.A. = 200 to ca. 800 units/mg with half-saturation near 10 μM $MnCl_2$. The pH–rate profiles for the transferase assay, stimulated by 0.1 mM Mn(II) or 1.0 mM Mg(II) with 0.1 or 0.5 mM ADP present, respectively, show maxima at pH 6.5 and 7.4, respectively, and the Mn activity > Mg activity by 4 times at pH 7.2. These data disagree with previously published profiles at different metal ion and ADP concentrations (Monder, 1965) but explain our present results. The data also suggest that a S.A. > 200 units/mg may result from binding of more than eight Mn(II) per octamer. Binding data for Mn(II) in the presence of ATP (unpublished results, this laboratory) support this view and are the subject of current research.

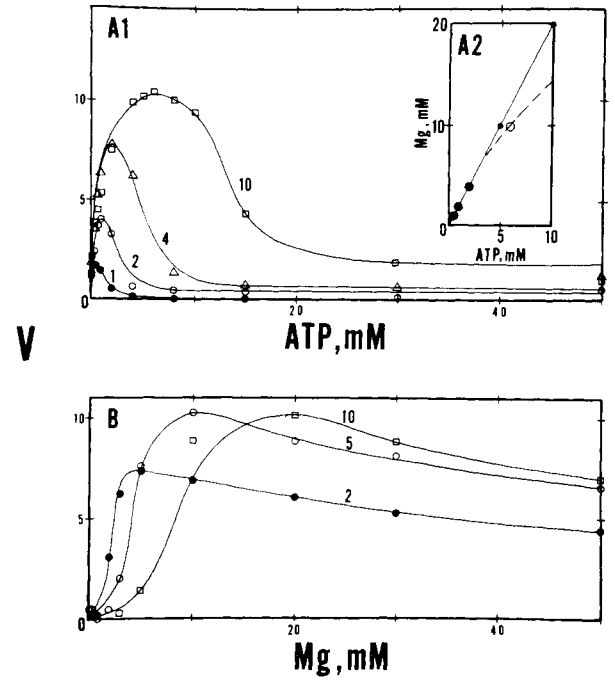


FIGURE 1: Mg(II) and ATP activity profiles for ovine brain glutamine synthetase. The biosynthetic assay was used at pH 7.0, 37 °C, with [enzyme] at 19.2 nM in 0.2 mL. V is expressed as ΔA_{660} in 15 min (see Materials and Methods). Buffer was 50 mM imidazole–HCl, with 50 mM each of L-glutamate and NH_4Cl. (A1) Activity profile, V vs. [ATP], at several fixed [Mg(II)], in mM, shown adjacent to each curve: (●) 1 mM, (○) 2 mM, (△) 4 mM, and (□) 10 mM. (A2) Replot of [ATP] and [Mg] at the maximun of the activity profiles in (A1) (●) and (B) (○). (B) Activity profile, V vs. [Mg(II)] at several fixed [ATP], in mM, shown adjacent to each curve: (●) 2 mM, (○) 5 mM, and (□) 10 mM.

and ATP can be used to distinguish among the three major submechanisms in Scheme I. These are as follows: Model I (lower tier), enzyme exists as unmodified E; model II (upper tier), enzyme exists in a modified form, M·E; and model III (diagonal), enzyme exists as either E or M·E. In model I or II, the enzyme can bind ATP, M, or M·ATP, whereas in model III it can bind only M·ATP. The criteria used to distinguish among these models are (a) whether the v vs. [S] plots are concave (hyperbolic) or sigmoidal, (b) the positions of the activity profiles relative to each other, (c) whether there is a peak velocity and its relative position, (d) whether an excess of either M or ATP gives a constant (plateau) velocity or causes inhibition, (e) the degree of sigmoidicity and the half-saturation point relative to the dissociation constant for M·ATP upon variation of M and ATP in constant ratio, and (f) the slopes of the isovelocity replots.

Isovelocity values are taken from the v vs. [M] and v vs. [ATP] plots; i.e., one can locate several pairs of metal–ATP concentrations, each of which occur at the same value of v. One can use these pairs for which [ATP] ≫ [M] or [M] ≫ [ATP] to replot as [M] vs. [ATP]. This isovelocity replot method can be used quantitatively to estimate values of K_{ATP}, K_M, $K_{M\cdot ATP}$, and V_{max}. According to London & Steck (1969), the x-axis intercept of the isovelocity replot is $-\bar{K}_s$, where $\bar{K}_s = 1/K_s$. By choosing several isovelocities, one can obtain a family of curves. The reciprocal of the slopes of these curves in the isovelocity replot can in turn be plotted as slope^{-1} vs. V_i^{-1} where V_i is the isovelocity value. For the ATP profile the slope of the curve at $1/v = 0$ obeys the equation

$$d(ATP)/d(M) = 1 - K_c\bar{K}_s + (1/v)V_mK_c\bar{K}_s$$

From the value of the x-axis intercept K_c and from the slope, one can calculate K_c and V_{max} (V_m), respectively.

Table I: Kinetic Constants Calculated from the Data of Figures 1 and 2 and Figures S-1 and S-3 (Supplementary Material), according to the London-Steck Model (Scheme I)

metal ion[a]	symbol[b]	dissociation constant (mM)
Mg(II)	$\bar{K}_0 = (Mg)(ATP)/(Mg\cdot ATP)$	0.10[c]
	$\bar{K}_{ATP} = (E)(ATP)/(E\cdot ATP)$	1.5
	$\bar{K}_{Mg} = (E)(Mg)/(E\cdot Mg)$	4.0
	$\bar{K}_0 = (E)(Mg\cdot ATP)/(E\cdot Mg\cdot ATP)$	2.3
Mn(II)	$\bar{K}_0 = (Mn)(ATP)/(Mn\cdot ATP)$	1.4[c]
	$\bar{K}_{ATP} = (E)(ATP)/(E\cdot ATP)$	2.5
	$\bar{K}_{Mn} = (E)(Mn)/(E\cdot Mn)$	6.5
	$\bar{K}_0 = (E)(Mn\cdot ATP)/(E\cdot Mn\cdot ATP)$	2.5

[a] Biosynthetic assays using 19.2 mM ovine brain glutamine synthetase were carried out at 37 °C and pH 7.0 for Mg(II) and pH 5.0 for Mn(II) to determine kinetic parameters. [b] See Scheme I. [c] Calculated values–see text.

The activity profiles for V vs. [ATP] and V vs. [Mg(II)] are shown in Figure 1. The relevant replots are presented in Figure S-1 in the supplementary material (see paragraph at end of paper regarding supplementary material). The key observations are that (1) maximal activity occurs at or very near to a Mg(II)/ATP ratio of 2:1, (2) activity is inhibited at [ATP] ≫ [Mg(II)] or [Mg(II)] ≫ [ATP], although the latter effect is weaker, (3) sigmoidicity in the V vs. [Mg] plots occurs at a value greater than $\bar{K}_0$ = [MgATP]/([Mg][ATP]) = 0.1 mM (Roby, 1978), and (4) isovelocity replots of [ATP] vs. [Mg(II)] are concave upward. The kinetic constants calculated from these plots and replots are given in Table I.

If one considers all the above facts with the theoretical plots of London & Steck (1969), the interaction of Mg(II) with ATP and enzyme at pH 7.0 and 37 °C appears to conform most closely with model II, where active enzyme is modified as Mg·E. This species then binds the Mg·ATP complex to give Mg·E·Mg·ATP. Alternative explanations will be considered under Discussion.

Activity profiles for V vs. [Mg_n·ATP] for n = 1 and 2 are presented in Figure S-2 (supplementary material). Both curves (n = 1 and 2) show sigmoidicity at a region above $\bar{K}_0$ for Mg·ATP (0.1 mM). Hill replots of the data give Hill values $n_H = 2.0 \pm 0.2$. Addition of 0.1 mM $MnCl_2$ to these reactions lowers the maximal velocity by approximately 2-fold, indicating that Mn(II) is antagonistic to the Mg(II)-stimulated activity at pH 7.0. When one considers that both Mn(II) and Mg(II) can activate ovine brain glutamine synthetase but exhibit pH optima at 5.0 and 7.0, respectively (Monder, 1965), this inhibition at pH 7.0 by Mn(II) in understandable. However, the half-saturation values of the V vs. [Mg(II)] plots are lowered by added Mn(II) also. This is clearly not consistent with a simple mechanism involving direct competition of the two metals for the same site on the enzyme but may occur by an uncompetitive inhibition mechanism, where both K_m(Mg·ATP) and V_{max} are decreased by added Mn(II).

The activity profiles for V vs. [Mn(II)] at fixed [ATP], and vice versa, are shown in Figure 2. The replots relevant to the data in Figure 2 are presented in Figure S-3 (supplementary material). There are obviously some differences between the data seen for Mg(II) in Figure 1 and those for Mn(II) in Figure 2. The key observations for Mn(II) are that (1) maximal activity occurs at or very near to [Mn(II)]/[ATP] = 1.0, (2) activity is rather strongly inhibited with either [Mn] ≫ [ATP] or [ATP] ≫ [Mn], (3) sigmoidicity occurs in the V vs. [Mn(II)] curves at a value near $\bar{K}_0$ for Mn·ATP, calculated from literature data to be ca. 1.4 mM at pH 5.0, 37 °C (Roby, 1978), and (4) isovelocity replots of [ATP] vs. [Mn(II)] are concave upward. The kinetic constants calcu-

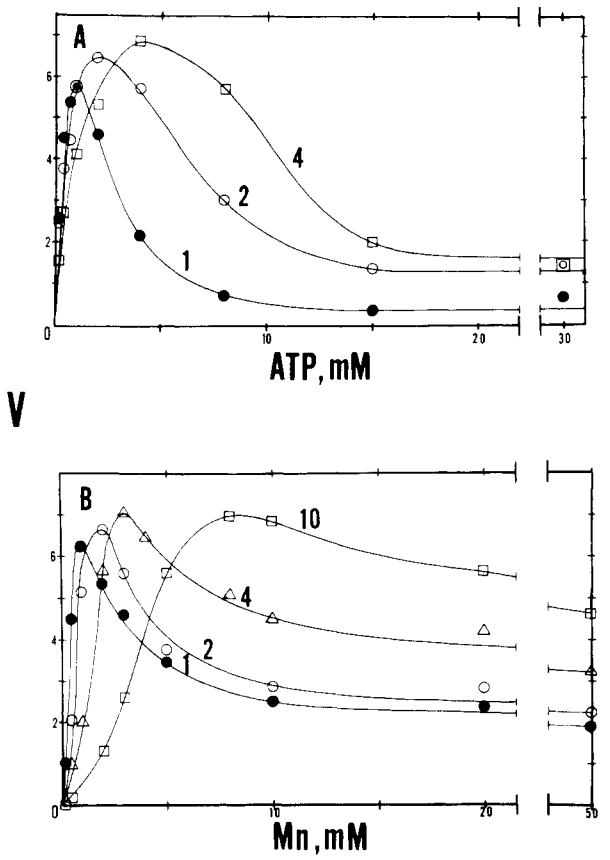


FIGURE 2: Mn(II) and ATP activity profiles for ovine brain glutamine synthetase. The biosynthetic assay was used at pH 5.0, 37 °C, with [enzyme] at 19.2 nM in 0.2 mL. V is expressed as ΔA_{660} in 15 min (see Materials and Methods). Buffer was 50 mM acetate with 50 mM each of L-glutamate and NH_4Cl. (A) Activity profile, V vs. [ATP], at several fixed [Mn(II)], in mM, shown adjacent to each curve: (●) 1 mM, (○) 2 mM, and (□) 4 mM. (B) Activity profile, V vs. [Mn(II)], at several fixed [ATP], in mM, shown adjacent to each curve: (●) 1 mM, (○) 2 mM, (△) 4 mM, and (□) 10 mM.

lated from Figures 2 and S-3 (supplementary material) are presented in Table I.

The data for Mn(II), ATP, and enzyme cannot be used to distinguish between models I and II definitively. Model III is quite unlikely for either Mg(II) or Mn(II) since either excess Mn(II) or excess ATP gave inhibition, which is totally uncharacteristic of model III. In model III excess M should give a constant V. Therefore, either (a) Mn(II) binds according to model II or (b) model I applies, with the additional requirement that K_A is very large, i.e., enzyme binds Mn(II) very tightly to form Mn·E. As London & Steck (1969) pointed out, model II behaves as model I if [M] $\gg K_A$. Thus, the nonkinetic binding experiments that follow were necessary to distinguish between these possibilities, especially since in all the above kinetic experiments [E] $\ll$ [Mn(II)].

Activity profiles for V vs. [MnATP] are shown in Figure S-4 (supplementary material) and show sigmoidicity near 0.3 mM. Hill plots gave $n_H = 1.9$. Interestingly, added Mg(II) caused an increase in the maximal velocity of the Mn(II)-stimulated activity at pH 5.0, 37 °C, in contrast to the inhibition effect observed with Mn(II) on the Mg(II)-stimulated activity at pH 7.0. Again, this suggests that Mn(II) and Mg(II) are not competing for the same site(s) on the enzyme in these experiments.

(B) Mn(II) Binding—Stoichiometry. The nature of Mn(II) binding to ovine brain glutamine synthetase was investigated by means of EPR determination of free Mn(II) at 9 GHz (see Materials and Methods). Enzyme was passed through columns of Sephadex G-25F to remove glycerol from the storage buffer and then through Chelex 100 resin to remove residual metal ions. Mn(II) was then added in increasing amounts to a fixed amount of enzyme, and the concentration of free ion was 10^{-8} by EPR. The data from these experiments are shown in Figure 3 as a Scatchard plot. From the data in Figure 3 it was calculated that $n \simeq 4$ and $K_0 = 0.54$ μM.

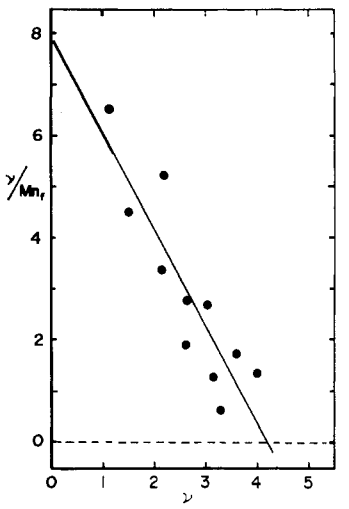


FIGURE 3: Scatchard plot of binding of Mn(II) to ovine brain glutamine synthetase, pH 7.2, 20 °C. $MnCl_2$ was added to Chelex-treated ovine brain glutamine synthetase samples in increasing concentrations. The $[Mn(II)]_f$ was determined from X-band EPR spectra (see Materials and Methods). Five separate titration experiments were performed with [E] ranging from 0.27 to 1.82 μm. Buffer contained 10 mM Hepes, 50 mM KCl, and 5 mM β-mercaptoethanol and was pretreated with Chelex. The slope of the solid line was determined by linear regression analysis.

Table II: Stoichiometry of Mn(II) Released from Ovine Brain Glutamine Synthetase

method	M/E octamer	N^a
(A) acid denaturation[b]	3.87 ± 0.126	4
	3.99 ± 0.136	3
(B) lanthanide displacement[c]	3.84 ± 0.70	3
	3.27 ± 0.70	2

[a] N = number of determinations. [b] Native enzyme was passed through a Sephadex G-25F column to remove glycerol and then through a Chelex 100 column to remove residual metal ions. X-band EPR spectra of a 50-μL sample (0.104 mg/mL E) of this solution were recorded as a base line. This sample was subsequently denatured by addition of 1 μL of 1.0 N HCl, and the X-band spectra were redetermined. The method of Cohn & Townsend (1954) was used to determine the $[Mn(II)]_f$ from the difference spectrum. [c] Native enzyme was prepared as in footnote *b*. X-band spectra of a 50-μL sample (0.48 mg/mL E) of this solution were recorded as a base line. Subsequently, 6.5 mM Nd(III) was added to the sample, and the X-band spectra were redetermined.

Since only four Mn(II) bind per octamer, this raised the possibility that the enzyme might possess more than one class of Mn(II) sites. The free Mn(II) ion was determined by EPR before and after denaturation of the Chelex-treated enzyme in HCl, to probe the possibility that additional Mn(II) ions might be very tightly bound. The results are shown in Table IIA. The data indicate clearly that the ovine brain enzyme in vivo contains four very tightly bound Mn(II) ions ($K_d \leq 10^{-8}$ M) per octamer that survive the purification scheme intact. In addition, the enzyme can bind four more Mn(II) ions with $K_d \simeq 0.54$ μM, as observed by the titration experiments of Figure 3.

Addition of excess (20 mM) Mg(II) or Ca(II) to enzyme in Hepes buffer at pH 7.2 does not cause displacement of the

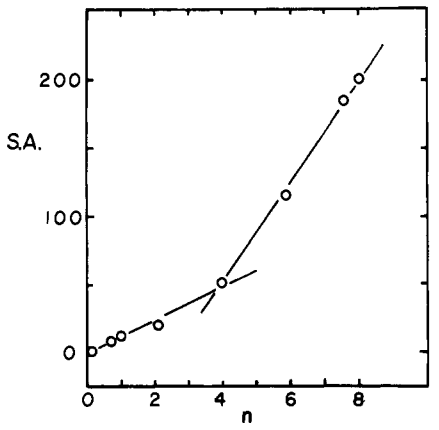


FIGURE 4: Dependence of specific activity (S.A.) on total number of Mn(II) ions, *n*, bound to ovine brain glutamine synthetase. Assays utilized the transferase assay, pH 7.2, 37 °C, with 5 mM $MgCl_2$, 15 min (see Materials and Methods). The number of Mn(II) ions bound to the enzyme was determined by EPR from Mn(II) titration (Figure 3) and acid denaturation experiments (see Figure 3 and Table I).

four very tightly bound Mn(II) ions, however. Apoenzyme could be prepared, however, by extensive dialysis of enzyme against concentrated (100 mM) imidazole buffer at pH 7.0 (see Materials and Methods). This may suggest that the very tightly bound Mn(II) ions are chelated by two or more protein ligands.

The relationship between enzyme specific activity and Mn(II) bound per octamer was studied by separate determinations of (a) Mn(II) bound per octamer by EPR after HCl denaturation and (b) specific activity by transferase and biuret assays of activity and protein, respectively. These data are shown in Figure 4. The amount of Mn(II) bound per octamer was near 4.0 for native enzyme, as isolated. This could be reduced to 0 by dialysis in buffer containing 100 mM imidazole (see above) and could be increased to values between 0 and 4.0 by readdition of Mn(II) to enzyme. Values of Mn(II) bound per octamer above 4.0 were produced by addition of Mn(II) to native enzyme.

The results in Figure 4 clearly indicate that apoenzyme has no residual activity and that native (Mn_4·E) enzyme had a specific activity of ca. 50 units/mg, whereas Mn-activated native enzyme (Mn_4·E·Mn_4) had a specific activity of ca. 200 units/mg in the transferase assay at pH 7.2 and 37 °C.[2] These data serve to further emphasize the half-molar stoichiometry of the Mn(II) activation of ovine brain glutamine synthetase. The experiments that follow were designed to probe further the nature and relationships of these two classes of sites.

(*C*) *Binding Competition of Mn(II) vs. Mg(II).* Enzyme from which all metal ions had been removed by dialysis in 0.1 M imidazole buffer, pH 7.0 (apoenzyme), was then incubated in buffer containing 1 mM $MgCl_2$, 10 mM imidazole, 50 mM KCl, and 5 mM dithiothreitol. After addition of 10 μM $MnCl_2$ to this solution, the amount of free Mn(II) ion was determined by EPR (see Table II and Figure 3). The binding of Mn(II) at pH 7.0 was virtually unaltered by the presence of 1 mM Mg(II) already present. This indicates that the enzyme preferentially binds Mn(II) much more tightly than Mg(II). As noted above, rather high concentrations of Mg(II) or Ca(II) failed to displace Mn(II) from the species Mn_4·E isolated from brain tissue. Thus, one may conclude that at pH 7 with ca. 3–4 μM Mn(II) present in vivo[3]—see Table III—along with

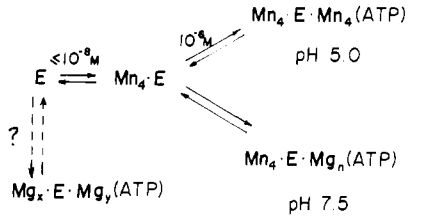
[3] The values of the [Mn(II)] in Table III are *total* Mn. We have also measured the free [Mn(II)] by EPR of fresh brain tissue, homogenized in 1:1 buffer/tissue. There was less than 0.5 μM Mn(II) detectable.

Table III: Mn(II) Content of Crude Brain Extracts (See Materials and Methods)

determination[a]	[Mn(II)][b] (μM)	[E][c] (μM)	[Mn(II)]/E octamer]
1	2.52	0.52	4.80
2	2.60	0.52	4.95
3	2.41	0.52	4.59
4	1.945	0.422	4.61

[a] All values were corrected for background concentrations of Mn(II) in the distilled, deionized water used in all these experiments. These corrections amounted to less than 20% of any of the reported values and were highly reproducible. [b] Perchloric acid extraction of brain tissue, followed by EPR determination of Mn(II). [c] Transferase assay and biuret protein assay to determine specific activity. [E] in brain tissue was calculated by using S.A. = 200 units/mg for pure enzyme.

Scheme II



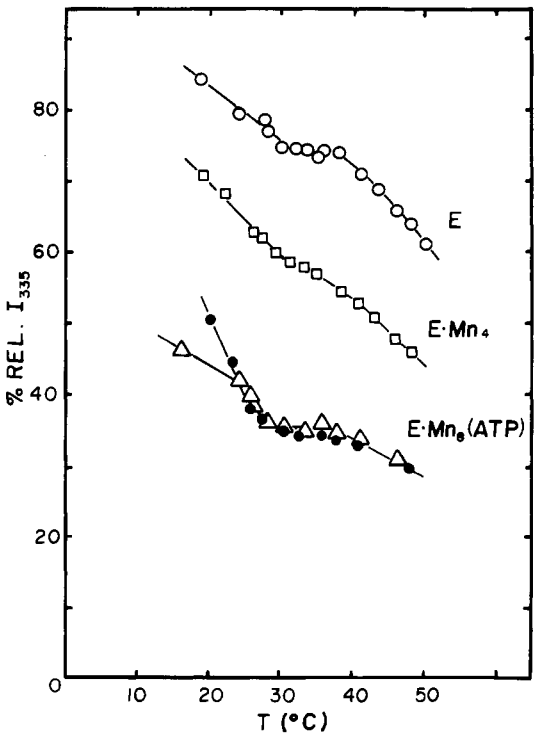


FIGURE 5: Effect of bound Mn(II) and temperature on fluorescence emission intensity (*I*) of ovine brain glutamine synthetase. λ_{ex} = 295 nm and λ_{em} = 335 nm, 10 nM Hepes buffer (pH 7.2), 100 mM KCl, and 2 mM β-mercaptoethanol. [E] = 0.27 mg/mL = 0.68 μM; [Mn_4] = 2.7 μM; [Mn_8] = 5.4 μM; [ATP] = 0.606 mM. (○) Apoenzyme, E; (□) apoenzyme plus four Mn(II); (△) apoenzyme plus eight Mn(II); (●) apoenzyme plus eight Mn(II) plus ATP. All enzyme samples were preincubated with the ligands 10 min at 37 °C.

milimolar levels of Mg(II), the predominant species will be Mn_4·E or more likely Mn_4·E·Mn_4.

Scheme II outlines the minimal number of equilibria one must consider for the metal ion activation of ovine brain glutamine synthetase. Thus, the kinetics presented above in section A plus the binding data of section B indicate that model II of London and Steck probably holds for both Mn(II) and Mg(II) with this enzyme.

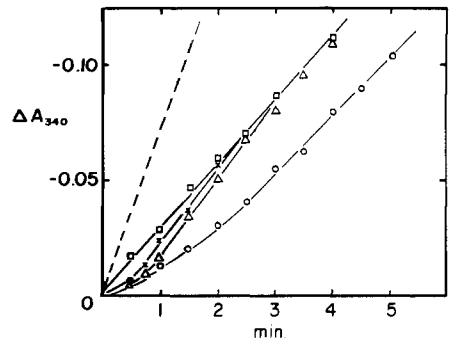


FIGURE 6: Effect of metal ions on time dependence of activation of ovine brain (apo) glutamine synthetase, pH 7.2, 37 °C (see Materials and Methods). The coupled assay mixture contained 45 mM imidazole, 81 mM KCl, 45 mM $MgCl_2$, 27 mM L-glutamate, 45 mM NH_4Cl, 3.25 mM ATP, 0.38 mM NADH, 0.9 mM phosphoenolpyruvate, 45 units of pyruvate kinase, and 324 units of lactate dehydrogenase in a total volume of 1.1 mL. The enzymic reaction was initiated by addition of 30.4 nM apoenzyme. Enzyme activity was determined by the rate of ADP release, reflected in the oxidation of NADH, measured at 340 nm. Data points shown were taken from the continuous trace of the Gilford spectrophotometer recorder. The dotted line is the rate of reaction obtained upon addition of 5 mM ADP. (○) Apoenzyme; (△) apoenzyme plus four Mn(II); (×) apoenzyme plus eight Mn(II); (□) native enzyme (E·Mn_4) plus eight Mn(II). Thus, four Mn(II) ≡ 0.121 μM, and eight Mn(II) ≡ 0.242 μM.

(*D*) *Mn(II) Binding—Fluorescence.* In order to probe whether binding of Mn(II) ions to ovine brain glutamine synthetase elicits significant conformational changes in the protein, it was necessary to observe the fluorescence emission of Trp residues. Apoenzyme (E) was observed first as a function of temperature (Figure 5). There are distinct discontinuities, i.e., a region from 30–40 °C where emission does not decrease with temperature. Enzyme with four Mn(II) ions bound per octamer, Mn_4·E, has a distinctly lower emission than E at all temperatures. Those species with eight Mn(II) bound, with or without ATP present, E·Mn_8 and E·Mn_8·ATP, had even lower relative percent intensities of emission. Thus, binding of Mn(II) at a stoichiometry of 1:4 or 1:8 causes two distinct successive changes in the fluorescence emission of protein Trp residues.

As an additional observation, in the region of 40–50 °C the *slope* of the curves in Figure 5, i.e., $\Delta I_{335}/°C$, *diminishes* as one proceeds from E to Mn_4·E to E·Mn_8 (±ATP). This may indicate that the polarity in the region of Trp residues decreases and that thermostability of these species increases as the amount of bound Mn(II) increases. If true, this would indicate a structural role for Mn(II) in the protein at both classes of sites. The time dependence for the conversion of E to Mn_4·E to E·Mn_8 (±ATP) will be the subject of future research but is examined in the following section in a preliminary manner by activity assays.

(*E*) *Mn(II) Binding—Time Dependence.* Kingdon et al. (1968) have shown that the binding of divalent cations to the n_1 site of *E. coli* glutamine synthetase was a time-dependent process, on the minutes time scale. We also have looked for such activation with the ovine brain system, as shown in Figure 6. With 45 mM Mg(II) present at pH 7.2 apoenzyme shows a time-dependent lag phase of ca. 3 min prior to attaining full activity. Enzyme from which metal ions were not removed, i.e., native enzyme, designated E·Mn_4, did not show any such lag. Apoenzyme that was added to an assay mixture containing 4 or 8 equiv of Mn(II) showed a lag phase involving activation toward a steady-state rate that was eventually equal to that for Mn_4·E. These results indicate that either Mn(II) or Mg(II) can activate apoenzyme at pH 7.2 and that enzyme preferentially binds Mn(II) over Mg(II), to produce a species with a somewhat higher activity. Mn(II) may also bind to a separate site on Mn_4·E, as suggested by the data of Figure 5. Eight Mn(II) per enzyme reduces the lag time for activation more than four Mn(II) per enzyme, which was more effective than 45 mM Mg(II).

(*F*) *Effects of Lanthanide Ions.* The use of trivalent lanthanide ions, Ln(III), as probes of metal binding sites in proteins has been well documented (Reuben, 1979; Martin & Richardson, 1979). To take advantage of the unique chemical and physical properties of the trivalent lanthanide, Ln(III), ions, one must first demonstrate that the particular ion can bind to the protein of interest. To this end, replacement experiments were performed with three Ln(III) ions. The inhibition effects of Tb(III), Nd(III), and Ho(III) upon the transferase activity of the ovine brain enzyme are shown in Figure 7. Over the concentration range 5.0×10^{-7} to 5.0×10^{-3} M, none of these ions stimulate the transferase activity, while Mn(II) exhibits a large activation (Figure 7A). Essentially identical inhibitions were obtained with all three Ln(III) ions. Earlier experiments with *E. coli* enzyme with Ln(III) ions showed both activation and inhibition phenomena (Wedler & D'Aurora, 1974). Addition of Nd(III) to transferase assays containing [Mn(II)] = [ATP] caused inhibition with increasing concentrations of Nd(III) (Figure 7B).

In separate experiments, the concentration of Mn(II) was varied at a series of fixed concentrations of Nd(III). The Eadie–Hofstee plot of these results indicate that Nd(III) is competitive vs. Mn(II)—see Figure 8 (top). Second, the inhibition data of Figure 5B were replotted as i^{-1}, the fractional inhibition, as a function of $[I]^{-1}$, the reciprocal of the Nd(III) concentration—see Figure 8 (bottom). The biphasic curve implies that Nd(III) may interact with more than one class of sites on the enzyme. That $i_{max} = 1$ implies that Nd(III) gives complete rather than partial inhibition.

Finally, it was discovered that Nd(III) could displace Mn(II) from Mn_4·E. Table IIB shows that 6.5 mM Nd(III) causes the release of 3.58 ± 0.7 Mn(II) ions per octamer for the Chelex-treated enzyme. This implies that Nd(III) not only competes kinetically with Mn(II) for the four titratable sites but also can actually displace Mn(II) from the four tighter sites, probably on a slower time scale.

Discussion

In many enzyme systems the divalent cations Mn(II) or Mg(II) are known to be essential for either structural integrity or catalytic activity. Other ions that commonly serve in these roles include Ca(II) or Zn(II). Mg(II) is most often considered to be the ion of major physiological significance in mammalian systems, with Mn(II) viewed as a convenient replacement ion (Mildvan, 1970). Only a few true Mn(II) enzymes have been reported: superoxide dismutase from mitochondrion (Fridovich, 1979), arginase from rat liver (Hirsch-Kolb et al., 1971), and pyruvate carboxylase from chicken liver mitochondrion (Scrutton et al., 1966). The n_1 structural site as well as the n_2 ATP-chelation site of *E. coli* glutamine synthetase can bind either Mn(II) or Mg(II), with a preference for Mn(II) at pH 7.0. The results of the present research show that octameric ovine brain glutamine synthetase exists in vivo as Mn_4·E, which can then form Mn_4·E·Mn_4 or Mn_4·E·Mg_4 and other higher complexes (see Scheme II). Earlier kinetic studies—see Figure 3A of Monder (1965)—indicated that with 5 mM Mn(II) and 5 mM Mg(II) present, the pH–activity profiles were totally characteristic of those with Mn(II) alone, not Mg(II).

The significance of the unusual stoichiometry of four Mn-

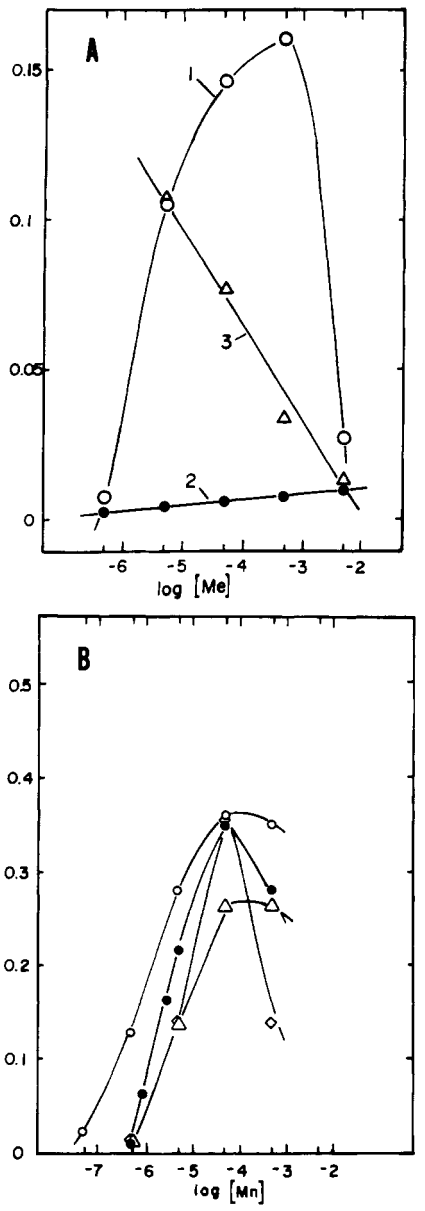


FIGURE 7: Effect of Nd(III) and other metal ions on Mn(II)-stimulated activity of ovine brain glutamine synthetase, pH 7.2, 37 °C, transferase assay (see Materials and Methods), [E] = 0.936 mg/mL. (A) (Curve 1) (○) Effect of [Mn(II)] on enzyme activity with [ADP] = 5×10^{-4} M; (curve 2) (●) effect of Nd(III) on enzyme activity [activity data with Tb(III) or Ho(III) were essentially identical with those with Nd(III)]; (curve 3) (△) inhibition by Nd(III) of Mn(II)-stimulated activity at [Mn(II)] = 5×10^{-4} M. (B) Effect of added Nd(III) on Mn(II) saturation plots, with [Nd(III)] held fixed at 0 mM (○), 5×10^{-7} M (●), 5×10^{-6} M (◇), and 5×10^{-5} M (△).

(II), then four more Mn(II) bound per octamer, compared to the *E. coli* enzyme where one sees 12 Mn per dodecomer, is not yet fully understood. Complete inhibition of enzyme with only four to five methionine sulfoximine molecules bound per octamer in the presence of ATP (Tate et al., 1972) or binding of 8–12 ATP molecules per octamer (Tate & Meister, 1973; Wellner & Meister, 1966) has been reported earlier with other mammalian glutamine synthetases. Tate & Meister (1971) also reported inhibition of the Mg(II) stimulated activity by Mn(II). The possibility of half-of-the-sites reactivity was invoked in these earlier publications but was not investigated in depth.

Indeed, the present results raise a number of questions about the exact nature and relationships of the Mn(II) and Mg(II) binding sites on the ovine brain enzyme. Although Mn_4·E

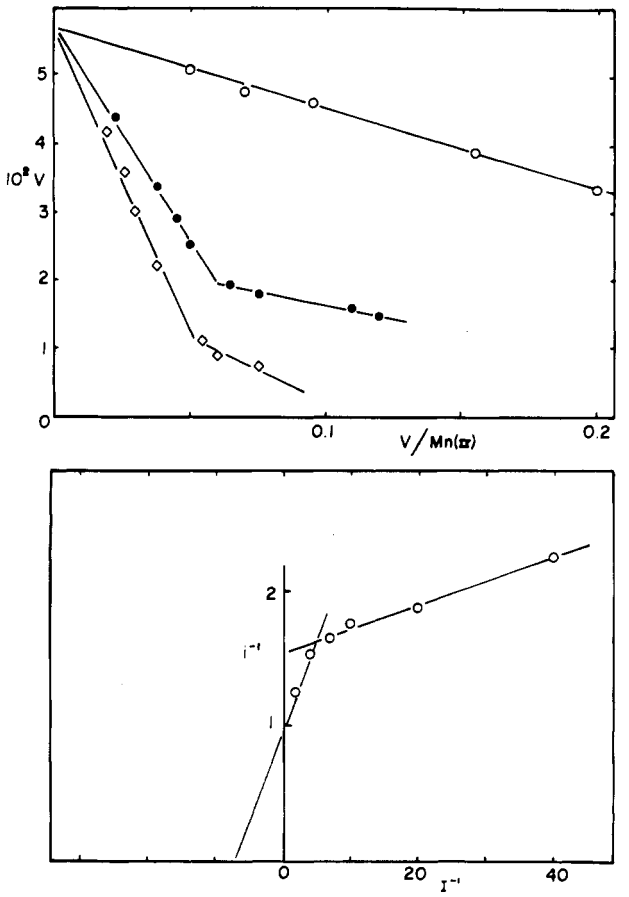


FIGURE 8: Competition of Nd(III) vs. Mn(II) with ovine brain glutamine synthetase, pH 7.2, 37 °C, by transferase assay (see Materials and Methods). (Top) Eadie–Hofstee plot of V vs. [S] data with [Nd(III)] at 0 M (○), 5×10^{-5} M (●), and 2.5×10^{-4} M (◇); [E] = 7.63×10^{-7} M. (Bottom) Fractional inhibition by Nd(III) of Mn(II)-stimulated activity replotted as i^{-1} vs. $[I]^{-1}$ where I = Nd(III) and [E] = 2.45×10^{-6} M, with conditions as in Figure 7A, curve 3.

seems to be the physiologically significant species, it is not known whether Mn_4·E·Mn_4, Mn_4·E·Mg_4, or other species predominate in vivo. Although added Mn(II) can convert Mn_4·E·Mg_4 to Mn_4·E·Mn_4, the present data suggest that Mg and Mn are not competing directly for the same site, or only do so for some sites. The fact that optimal activity with Mg(II) occurs at two Mg bound per ATP (Figure 1) suggests that a rather different complex is forming with Mg(II), such as Mn_4·E·(Mg_8·ATP_4).

The spatial and functional relationships between the sites for very tightly bound Mn(II) and the less tightly bound ones are by no means understood. The Mn(II) ions bound in Mn_4·E may have more of a structural role than a catalytic one, since the specific activity of Mn_4·E·Mn_4 is 4 times that of Mn_4·E. However, addition of ATP to Mn_4·E perturbs the 35.5-GHz spectra of the bound Mn(II) significantly (F. C. Wedler et al., unpublished results). The possibility of a dual role, structural and catalytic, for Mn(II) has already been elucidated for the n_1 site in the *E. coli* system (Villafranca et al., 1976; Villfranca, 1978).

The time dependence of Mn(II) binding studied by the coupled assay system (Figure 6) established a hierarchy for the *rates* of metal activation: Mn_4·E > (apo-E + Mn) > (apo-E + Mg). Apoenzyme showed the longest lag phase upon activation by Mg(II), an essential activator for pyruvate kinase used in this coupled assay system. Apoenzyme but not native Mn_4·E showed a marked lag phase in the activation with Mn(II). A finite amount of time (minutes time scale) is

required for the Mn(II) to bind to each of its various sites. Both these and the fluorescence results (Figure 5) suggest that metal association involves changes in protein conformation or microenvironment in the region of Trp residues.

From the present data, there are a variety of models that could predict the activation behavior of divalent cations with ovine brain glutamine synthetase. It is impossible from the present data to elucidate the function of Mn(II) or Mg(II) at each of these sites. Future research will seek to distinguish the role each site serves: structural, nucleotide binding, or modifier.

Acknowledgments

We thank Drs. J. J. Villafranca and V. L. Schramm for helpful suggestions and discussions.

Supplementary Material Available

Isovelocity replots of Figures 1 and 2 and plots of biosynthetic activity (v) of ovine brain glutamine synthetase vs. [Mn·ATP] and [Mg_n·ATP] (4 pages). Ordering information is given on any current masthead page.

References

Cohn, M., & Townsend, J. (1954) *Nature (London) 173*, 1090–1091.

Fiske, C. H., & Subbarow, Y. (1925) *J. Biol. Chem. 66*, 375–400.

Fridovich, I. (1979) in *Advances in Inorganic Biochemistry* (Eichorn, G. L., & Marzilli, G., Eds.) pp 67–90, Elsevier/North-Holland, New York.

Ginsburg, A. (1972) *Adv. Protein Chem. 27*, 1–79.

Haschenmeyer, R. H. (1970) *Adv. Enzymol. Relat. Areas Mol. Biol. 33*, 71–118.

Hirsch-Kolb, H., Kolb, H. J., & Greenberg, D. M. (1971) *J. Biol. Chem. 246*, 395–401.

Jaenicke, L., & Berson, W. (1977) *Z. Physiol. Chem. 358*, 883–888.

Jaenicke, L., & Jesiôr, J. C. (1978) *FEBS Lett. 90*, 115–118.

Kingdon, H. S., Hubbard, J. S., & Stadtman, E. R. (1968) *Biochemistry 7*, 2136–2142.

Layne, E. (1957) *Methods Enzymol. 3*, 447–454.

London, W. P., & Steck, T. L. (1969) *Biochemistry 8*, 1767–1779.

Martin, R. B., & Richardson, F. (1979) *Q. Rev. Biophys. 12*, 181–209.

Meister, A. (1962) *Enzymes, 2nd Ed. 6*, 443–468.

Mildvan, A. S. (1970) *Enzymes, 3rd Ed. 2*, 445–536.

Monder, C. (1965) *Biochemistry 4*, 2677–2686.

Orr, M. D., Blakeley, R. L., & Panagou, D. (1972) *Anal. Biochem. 45*, 68–85.

Reuben, J. (1979) in *Handbook on the Physics and Chemistry of Rare Earths* (Gschneidner, K. A., Jr., & Eyring, L., Eds.) Vol. 4, Chapter 39, pp 515–552, North-Holland, New York.

Roby, W. G. (1978) M.S. Thesis, Rensselaer Polytechnic Institute.

Ronzio, R. A., Rowe, W. B., Wilk, S., & Meister, A. (1969) *Biochemistry 8*, 2670–2674.

Scrutton, M. C., Utter, M. F., & Mildvan, A. S. (1966) *J. Biol. Chem. 241*, 3480–3487.

Strahl, J., & Jaenicke, L. (1972) *Eur. J. Biochem. 29*, 401–407.

Tate, S. S., & Meister, A. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 781–785.

Tate, S. S., & Meister, A. (1973) in *The Enzymes of Glutamine Metabolism* (Prusiner, S., & Stadtman, E. R., Eds.) pp 77–127, Academic Press, New York.

Tate, S. S., Leu, F.-Y., & Meister, A. (1972) *J. Biol. Chem. 247*, 5312–5321.

Villafranca, J. J. (1978) in *Biomolecular Structure and Function* (Agris, P. A., Ed.) pp 353–362, Academic Press, New York.

Villafranca, J. J., Ash, D. E., & Wedler, F. C. (1976) *Biochemistry 15*, 544–553.

Wedler, F. C., & D'Aurora, V. (1974) *Biochim. Biophys. Acta 371*, 432–441.

Wellner, V. P., & Meister, A. (1966) *Biochemistry 5*, 872–879.