ADP, Chloride Ion, and Metal Ion Binding to Bovine Brain Glutamine Synthetase†

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ABSTRACT: The binding of divalent cations and nucleotide to bovine brain glutamine synthetase and their effects on the activity of the enzyme were investigated. In ADP-supported γ-glutamyl transfer at pH 7.2, kinetic analyses of saturation functions gave $[S]_{0.5}$ values of ~1 μM for Mn^{2+}, ~2 mM for Mg^{2+}, 19 nM for ADP·Mn, and 7.2 μM for ADP·Mg. The method of continuous variation applied to the Mn^{2+}-supported reaction indicated that all subunits of the purified enzyme express activity when 1.0 equiv of ADP is bound per subunit. Measurements of equilibrium binding of Mn^{2+} to the enzyme in the absence and presence of ADP were consistent with each subunit binding free Mn^{2+} ($K_A \simeq 1.5 \times 10^5$ M^{-1}) before binding the Mn·ADP complex ($K_A' \simeq 1.1 \times 10^6$ M^{-1}). The binding of the first Mn^{2+} or Mg^{2+} to each subunit produces structural perturbations in the octameric enzyme, as evidenced by UV spectral and tryptophanyl residue fluorescence changes. The enzyme, therefore, has one structural site per subunit for Mn^{2+} or Mg^{2+} and a second site per subunit for the metal ion–nucleotide complex, both of which must be filled for activity expression. Chloride binding ($K_A' \sim 10^4$ M^{-1}) to the enzyme was found to have a specific effect on the protein conformation, producing a substantial (30%) quench of tryptophanyl fluorescence and increasing the affinity of the enzyme 2–4-fold for Mg^{2+} or Mn^{2+}. Arsenate, which activates the γ-glutamyl transfer activity by binding to an allosteric site, and L-glutamate also cause conformational changes similar to those produced by Cl^- binding. Anion binding to allosteric sites and divalent metal ion binding at active sites both produce tryptophanyl residue exposure and tyrosyl residue burial without changing the quaternary enzyme structure.

Glutamine synthetase catalyzes the following biosynthetic reaction: L-Glu + NH_3 + ATP ⇌ L-Gln + ADP + P_i. The enzyme is found in large amounts in the mammalian brain (>100 mg/kg wet tissue), primarily in astrocytes. Irreversible inactivation of glutamine synthetase in vivo with L-Met-*S*-sulfoximine leads to convulsions and death of the animal, indicating that the activity of the enzyme is essential (Meister, 1974); however, the primary function of the glutamine synthetase reaction in brain has not been clearly defined. Two possible roles have been proposed: (1) the removal of NH_4^+, which is toxic if allowed to accumulate, and (2) modulation of the levels of L-glutamate and, indirectly, γ-aminobutyric acid, both neurotransmitters (Norenberg, 1979). In vitro studies with the purified enzyme from ovine brain have shed little light on the metabolic regulation of brain glutamine synthetase. Activation (e.g., by α-ketoglutarate) or inhibition (e.g., by glycine) is relatively slight and requires high concentrations of the effectors. Also, regulatory properties of the brain enzyme in vitro are very similar to those of the liver enzyme, which would be expected to play a different metabolic role.

Recently, attention has been focused on the possible regulatory effects of metal ions on glutamine synthetase. The enzyme is active in vitro with a number of divalent metal ions, although the specific activity and the pH optimum vary considerably depending on the metal ion used (Monder, 1965). Binding of metal ions may also affect the state of aggregation of the enzyme in vitro (Denman & Wedler, 1984). Our studies with glutamine synthetase from *Escherichia coli* have shown that there are two essential metal ion sites per subunit that must be saturated for activity expression (Hunt et al., 1975) and that metal ions and active-site ligands greatly stabilize intersubunit bonding domains (Maurizi & Ginsburg, 1982b). Although glutamine synthetases of bacterial origin differ from those obtained from mammalian tissues in oligomeric structure, comparisons of catalytic activities suggest marked similarities in active sites (Meister, 1974; Gass & Meister, 1970). This prompted us to examine the roles of metal ion and nucleotide binding in the activity expression and the effects of these ligands on subunit interactions of the brain enzyme. We chose to isolate the enzyme from bovine brain because that tissue was readily available to us and we expected that the bovine brain enzyme would be fundamentally similar in structure and activity to the enzymes from ovine and porcine brains, the sources for most previous studies. Our initial studies (Maurizi et al., 1986) indicated three major differences between the enzyme we purified and those previously reported: (a) the purified enzyme had no tightly bound Mn^{2+}, whereas it had been reported that the ovine enzyme contained four Mn^{2+} per octamer with $K_D < 10^{-8}$ M; (b) the enzyme could bind up to 1 equiv each of ADP and L-Met-*S*-sulfoximine phosphate and two divalent metal ions per subunit, whereas others had reported four to six ligands per octamer in autoinactivated ovine brain or rat liver enzyme (Ronzio et al., 1969; Tate et al., 1972); and (c) the specific activity was 2–4-fold higher than that reported for other enzymes both in the biosynthetic reaction and in the γ-glutamyltransferase reaction. It has recently been confirmed that the previous finding of tightly bound Mn^{2+} (Denman & Wedler, 1984) was due to contamination (Wedler & Toms, 1986). The differences in the stoichiometry of inactivation with L-Met-*S*-sulfoximine have not been satisfactorily explained, although variations in enzyme

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specific activity could have considerable bearing on the issue. A stoichiometry of <1 equiv/subunit for L-Met-*S*-sulfoximine phosphate binding has been presented as evidence for negative cooperativity in active-site ligand binding to various glutamine synthetases (Rhee et al., 1981; Wedler & Toms, 1986).

This report describes the interactions of bovine brain glutamine synthetase with metal ions, nucleotides, and other ligands. We have studied these interactions by kinetic analyses of the enzymatic reaction, by direct binding measurements, and by spectrophotometric and fluorometric analyses of ligand-induced conformational changes.

Materials and Methods

4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes),[1] L-glutamate, ATP, ADP, L-Met-*S*-sulfoximine, 5,5′-dithiobis(2-nitrobenzoic acid), and protease inhibitors were from Sigma; Chelex 100 (100–200 mesh) was from Bio-Rad; pyruvate kinase, lactate dehydrogenase, and dithiothreitol were from Boehringer; ultrapure $(NH_4)_2SO_4$ was from Schwarz/Mann; all water was distilled and further purified on a Millipore-Q2 reagent grade system. Buffers and L-glutamate were freed of divalent metal ions by passage through a column of Chelex 100 (K^+ form) as described by Hunt and Ginsburg (1980). [8-^{3}H]ADP was purchased from New England Nuclear.

Purification of Bovine Brain Glutamine Synthetase. The procedure was based on that of Ronzio et al. (1969). Nine hundred grams of fresh or frozen bovine brains was blended for 3 min in the cold in 2 L of 20 mM phosphate, pH 7.2, 0.15 M KCl, and 2 mM EDTA, with the following protease inhibitors added immediately before homogenization: phenylmethanesulfonyl fluoride (5 mM); L-1-(tosylamido)-2-phenylethyl chloromethyl ketone (50 mg); N^{α}-(*p*-tosyl)-L-lysine chloromethyl ketone (25 mg) and soybean trypsin inhibitor (5 mg). After centrifugation for 1 h at 9000 rpm in a GSA rotor (Sorvall), the supernatant solution was brought to 30% saturation with $(NH_4)_2SO_4$, and the pH was lowered to 4.1 by addition of 1 M acetic acid. The precipitated protein was collected by centrifugation, dissolved in 270 mL of 10 mM potassium phosphate buffer, pH 7.2, with 10 mM β-mercaptoethanol, and clarified by centrifugation. If the solution was not clear at this stage, the precipitation step was repeated. Alternatively, the solution can be centrifuged at 100000*g* for 1 h to remove suspended material. The clarified solution was dialyzed against the same buffer and passed through a hydroxyapatite column (4.5 × 10 cm); the fractions with enzyme were combined, dialyzed against 5 mM potassium phosphate, pH 8.0, with 2 mM EDTA, and run over a DEAE column (2.5 × 10 cm). These two columns were run as described by Ronzio et al. (1969). The fractions from DEAE with glutamine synthetase were combined, and the enzyme was precipitated by addition of $(NH_4)_2SO_4$ to 30% saturation and acetic acid to give pH 4.1. The precipitated enzyme was dissolved in 50 mM potassium phosphate, pH 7.2, containing 2 mM EDTA, dialyzed against this buffer, and stored at 4 °C in aliquots of 1 mL at a concentration of 2–4 mg/mL. Alternatively, the enzyme could be stored at 4 °C in 40 mM Hepes/KOH, pH 7.0, for as long as 6–8 months with ≤18% activity loss.

The purified enzyme had a specific activity of 400 ± 20 units/mg (where units are expressed as micromoles of γ-glutamyl hydroxamate formed per minute at 37 °C) and showed a single band after slab gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate with an apparent subunit molecular weight of 45 000 (Maurizi et al., 1986). Sedimentation velocity measurements in 50 mM potassium phosphate–2 mM EDTA, pH 7.2, buffer indicated that the protein was homogeneous and had an octameric structure ($s_{20,w}$ = 16.0 S), as reported for other mammalian glutamine synthetases (Meister, 1974). Quantitative amino acid analyses by the procedure described by Maurizi et al. (1986) gave the composition presented in Table I.

Table I: Amino Acid Composition of Bovine Brain Glutamine Synthetase

amino acid	residues/ 45 026 g	amino acid	residues/ 45 026 g
Asp/Asn	48	Leu	21
Thr	18	Tyr	15
Ser	18	Phe	20
Glu/Gln	42	His	9
Gly	43	Lys	21
Ala	32	Arg	27
Val	15	Pro[a]	(25)
Met	11	Cys[b]	11
Ile	20	Trp[c]	8

[a] Proline was not determined; value shown was determined for the ovine brain enzyme (Meister, 1974). [b] Cysteine was determined with 5,5′-dithiobis(2-nitrobenzoic acid) as described by Habeeb (1972). [c] Tryptophan was measured by deconvolution of the ultraviolet absorption spectrum by the method of Federici and Levine (1982).

Activity and Concentration of Glutamine Synthetase. The γ-glutamyl transfer activity of the enzyme was assayed at 37 °C in the presence of Mn^{2+} at pH 6.8 as previously described (Maurizi et al., 1986). Kinetic measurements of the Mn^{2+}, Mg^{2+}, and ADP dependence of activity were performed in 50 mM Hepes, pH 7.2, in otherwise standard assay solutions. Biosynthetic activity of the enzyme was measured at 30 °C in a coupled assay with pyruvate kinase and lactate dehydrogenase (dissolved in 0.10 M KNO_3) and 1 mM phosphoenolpyruvate essentially as described previously for the *E. coli* enzyme (Ginsburg et al., 1970), with 100 mM Hepes buffer, pH 7.1, 50 mM $MgSO_4$, 40 mM $(NH_4)_2SO_4$, 5 mM ATP, and the desired concentrations of L-glutamate and KCl under which conditions the brain enzyme had a specific activity of ~16 units/mg. Glutamine synthetase concentrations were measured by ultraviolet absorption at 280 nm using the absorption coefficient of 1.50 ± 0.05 $(mg/mL)^{-1}$ without correction for light scattering (Maurizi et al., 1986); for calculations, a subunit molecular weight of 45 000 was used.

Binding Measurements. Direct binding of [^{3}H]ADP or Mn^{2+} was measured by incubating the appropriate concentrations of the ligands with enzyme (0.07–0.43 mg/mL) for 10-30 min prior to separating the unbound ligand by ultrafiltration. Aliquots of the enzyme–ligand solutions were placed in an Amicon MPS-1 micropartition system with YMT filters that had been prerinsed with buffer. The test solution was centrifuged for 5–30 min in a swinging bucket to allow 70–80% of the solution to pass through the filter. Correction for the slight (~5%) dilution caused by residual buffer in the filter was made by centrifuging standard solutions of ligand without enzyme through the washed filters. [^{3}H]ADP was determined by scintillation counting. Mn^{2+} was measured by atomic absorption as previously described (Maurizi et al., 1986). Filtrates were determined to be free of protein.

Ligand or metal ion binding was also determined by measuring the quench of enzyme tryptophanyl fluorescence upon addition of ligands to solutions of glutamine synthetase (25–60 μg/mL, $A_{300nm} \leq 0.02$). Measurements were made

[1] Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; EDTA, ethylenediaminetetraacetic acid; GS, glutamine synthetase.

at constant temperature (21 °C) with 0.6–1.0 mL of enzyme solution in standard 3-mL fluorescence cuvettes. Measurements were corrected for dilution caused by added ligand solutions. Excitation was at 300 nm (4-nm slit), and emission was at 337 or 360 nm (6-nm slit). Light scattering was checked at the start and during titrations by setting excitation and emission wavelengths to 300 or 360 nm. Ultraviolet difference spectra produced by ligand or metal ion binding were measured with solutions of enzyme (1 mL containing 0.66–1.4 mg/mL) in matched sample and reference semimicro cuvettes. For titrations of the enzyme, ligands in increments of 2 μL were added to the sample, and identical aliquots of solutions without ligands were added to the reference. Spectra were recorded by using a ±0.02 absorbance scale after at least a 20-min incubation of enzyme with ligand at 21 °C. Peak–trough differences (with the largest amplitude difference occurring at 291–299 nm) from two spectral scans were averaged and corrected for dilution to calculate the fractional change in absorbance. Hill plots of the kinetic, direct binding, and spectral perturbation data were constructed, and data were fit by a linear least-squares program.

Instrumentation. Ultraviolet absorption measurements and spectra were recorded with a Perkin-Elmer 320 spectrophotometer equipped with a thermostatable cell holder. Fluorescence measurements were made with a Perkin-Elmer 650-40 spectrofluorometer with water-jacketed cuvette holders. Mn^{2+} and Mg^{2+} analyses were performed with a Perkin-Elmer 603 atomic absorption spectrophotometer equipped with a HGA-2100 graphite furnace.

RESULTS

Kinetic Parameters. Figure 1 shows saturation functions for Mn^{2+} and for Mg^{2+}, respectively, in the γ-glutamyl transfer assay at pH 7.2. Free divalent metal ion concentrations were calculated as described by Hunt et al. (1975), who used similar assay conditions for the *E. coli* enzyme. The half-saturation, $[S]_{0.5}$, value for Mn^{2+} was ~1 μM and for Mg^{2+} was ~2 mM. The $[S]_{0.5}$ value for Mg^{2+} is in agreement with that reported by Denman et al. (1982) for the Mg^{2+}-dependent biosynthetic reaction with the ovine brain enzyme.

Hill plots are shown as insets in Figure 1, and the corresponding Hill coefficients obtained were 2.1 for Mn^{2+} and 1.2 for Mg^{2+}. As described below, one Mn^{2+} binds to glutamine synthetase as the Mn–ADP complex and has a very low K_m of 19 nM when Mn^{2+} is in an excess of ADP. Such tight binding of Mn–ADP would not be expected to affect the slope of a Hill plot in the range of the second Mn^{2+} binding affinity which is 2 orders of magnitude weaker (≥1 μM). That the titration curves are sigmoidal and the Hill number is 2 suggest that the apparent affinity of the enzyme for Mn–ADP is lower under the condition of the Mn^{2+} titration with excess ADP present, probably because of competition between free ADP and Mn–ADP. For Mg^{2+}, there is also >100-fold difference between the binding affinities at the Mg^{2+} and Mg–ADP sites such that the Mg–ADP sites are saturated throughout the titration of the Mg^{2+} site and hence do not influence the slope of the Hill plot. Unlike the Mn^{2+} case, free ADP was not in excess because in the titration range of 0.5–20 mM Mg^{2+}, ADP was present as Mg–ADP throughout the titration.

Double-reciprocal plots of velocity vs. ADP concentration in γ-glutamyl transfer assays at pH 7.2 and 37 °C with either 0.4 mM Mn^{2+} or 50 mM Mg^{2+} as the divalent metal ion gave K_m values for ADP–Mn and ADP–Mg of 19 nM and 7.2 μM, respectively (data not shown). Hill plots of the saturation functions for ADP–Mn and for ADP–Mg gave Hill coefficients of approximate unity. With either ADP–Mn or ADP–Mg, the K_m value was much lower (>100-fold) than the corresponding $[S]_{0.5}$ value for the free metal ion (Figure 1).

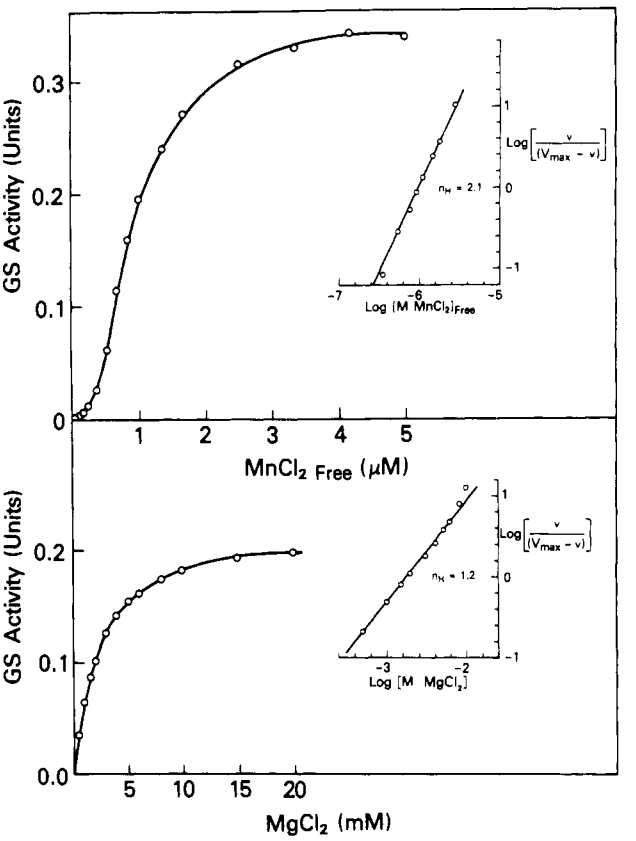

FIGURE 1: Saturation functions for Mn^{2+} and Mg^{2+} in the γ-glutamyl transfer reaction. Glutamine synthetase was assayed at 37 °C in 40 mM Hepes buffer, pH 7.2, with varying amounts of $MnCl_2$ (upper panel) or $MgCl_2$ (lower panel). Other assay components are described under Materials and Methods. Enzyme concentrations in assays were 2–5 nM; free metal ion concentrations were calculated as by Hunt et al. (1975). The insets show Hill plots of the data for each metal ion. The Hill numbers (n_H) were obtained from the slope of the line fitted to the data by linear regression.

Since ADP is a nonconsumable substrate in the γ-glutamyl transfer reaction and since the affinity of the enzyme for ADP–Mn is very high in the presence of the assay components, the stoichiometry of ADP–Mn binding to purified glutamine synthetase could be determined by the method of continuous variation (Huang, 1982). Figure 2A shows a Job plot (Vosburgh & Cooper, 1941) of ADP–Mn-supported activity at pH 7.2 in which the concentration of enzyme subunits plus ADP was kept constant at 396 nM. In this treatment, the enzyme activity was assumed to be directly proportional to GS·ADP complex formation. Maximum complex formation occurs when ADP and enzyme subunits are mixed in the molar ratio corresponding to their combining ratio in the complex (Huang, 1982). In Figure 2A, the maximum in transferase activity was obtained at ~1.0 equiv of ADP/subunit or at a 1:1 combining ratio. The same result was obtained with combined concentrations of ADP and enzyme subunit of 208 and 91 nM. This indicates that each subunit of the enzyme combined with one ADP for the expression of γ-glutamyltransferase activity.

Stoichiometry of ADP binding to glutamine synthetase was confirmed by a direct binding assay of [^{3}H]ADP to the enzyme in the presence of 0.4 mM $MnCl_2$ and 10 mM phosphate (Figure 2B). Since binding ADP to the enzyme was very tight ($K_D' < 10^{-7}$ M) under these conditions, the concentration of ADP binding sites added with each aliquot of enzyme was approximately equal to the concentration of ADP bound. The reciprocal of the slope of the line in Figure 2B indicates a

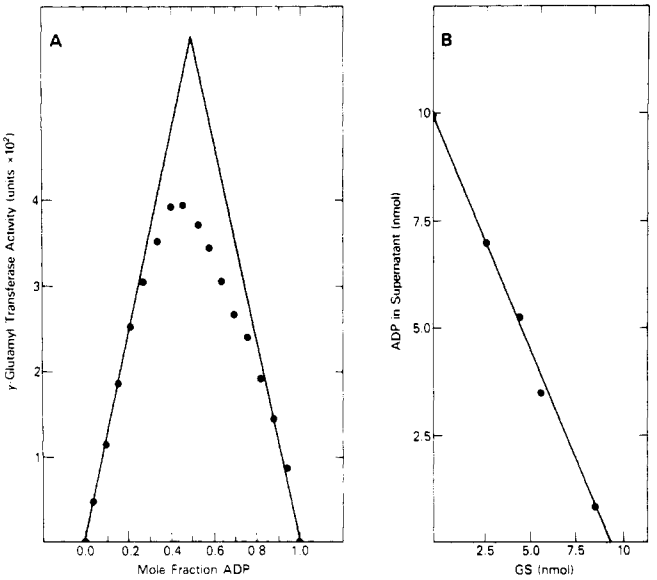

FIGURE 2: Stoichiometry of ADP binding at the active site of glutamine synthetase. (A) Job plot of glutamine synthetase activity in the γ-glutamyl transfer reaction as a function of the mole ratio of enzyme subunits and ADP. Assays were run at 37 °C in 40 mM Hepes, pH 7.2, 1.0 mM $MnCl_2$, 1.0 mM arsenate, and other components as described in Maurizi et al. (1986). The total concentration [enzyme subunit + ADP] was held constant at 396 nM in all assays. (B) [8-^{3}H]ADP (10 nmol; 400 cpm/nmol) was prepared in 40 mM Hepes, pH 7.2, with 1 mM $MnCl_2$, 10 mM potassium phosphate, and 100 mM KCl. Equal volumes (0.50 mL) of this solution and the same buffer (without ADP) containing 0–10 nmol of glutamine synthetase subunits were mixed and incubated at 23 °C for 10 min. Unbound ADP was separated from glutamine synthetase and bound ADP by ultrafiltration using the Amicon MPS-1 system with YMT membranes. The radioactivity in the ultrafiltrate was measured by scintillation counting. Glutamine synthetase concentration was determined by enzymatic assay using a specific activity of 400 units/mg for the fully active enzyme.

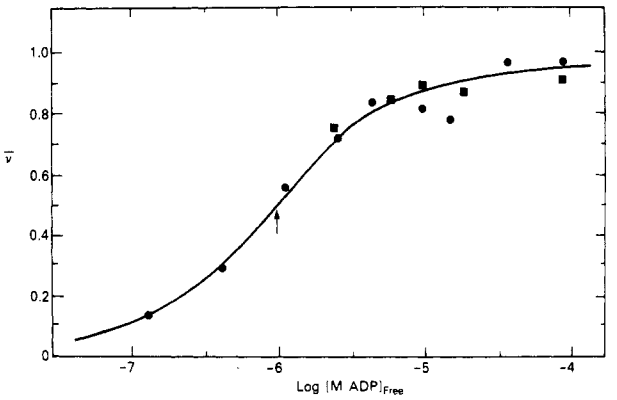

FIGURE 3: Direct binding of ADP to glutamine synthetase. Glutamine synthetase [6.7 (●) and 13.4 (■) μM subunits] in 40 mM Hepes, 100 mM KCl, and 0.40 mM $MnCl_2$ was incubated at ~23 °C with varying amounts of [8-^{3}H]ADP (18 900 cpm/μmol). Unbound ADP was separated by ultrafiltration (see Materials and Methods). The bound ADP was calculated as the difference between free ADP and total ADP added. The arrow shows the estimated K_D' value of 0.91×10^{-6} M.

stoichiometry of 0.95 ADP site/subunit at saturation.

Direct Measurement of ADP and Mn^{2+} Binding. Binding of [^{3}H]ADP to the enzyme was measured in 40 mM Hepes, pH 7.2, with 100 mM KCl and 0.40 mM $MnCl_2$ present (Figure 3). A simple saturation curve was obtained that reached a maximum at ~1 equiv of ADP bound per subunit of enzyme with a K_D' value of 0.91×10^{-6} M. Comparison of this constant with the data showing stoichiometric binding when ADP is present with Mn^{2+} and phosphate (Figure 2B)

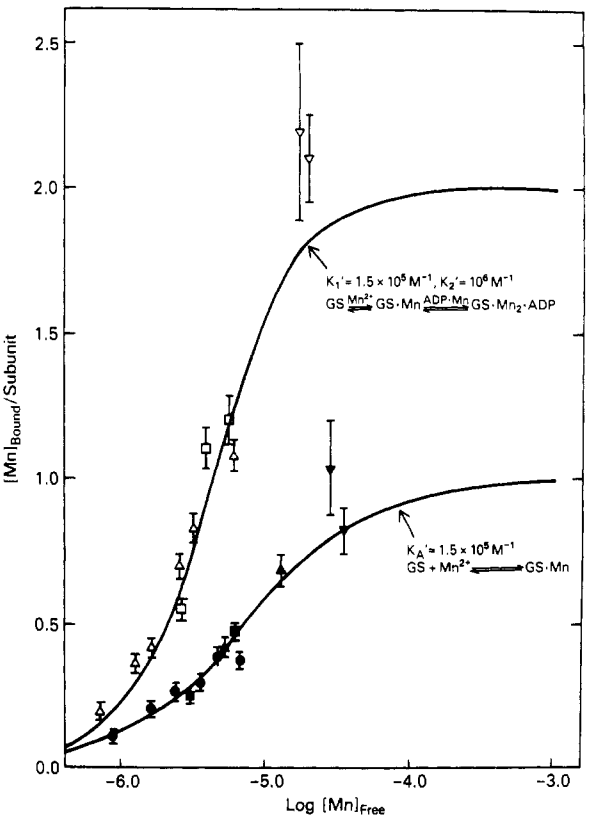

FIGURE 4: Reversible binding of Mn^{2+} to bovine brain glutamine synthetase in the absence (closed symbols) and in the presence (open symbols) of saturating ADP at pH 7.0 and ~23 °C. Protein solutions (~1 mL) ● 50 μM ADP at concentrations of ~10 (△, ▽, ▲, ▼) ~6 (●), and ~3 μM (□, ■) glutamine synthetase subunit in Chelex-treated 40 mM Hepes/KOH buffer, pH 7.0, from three different enzyme preparations were mixed with varying amounts of $MnCl_2$ ($[Mn^{2+}]/[GS\ subunit] \geq 1$) and incubated for 0.5–1.0 h at 4 °C and then for ~20 min at ~23 °C before ultrafiltration. Concentrations of Mn^{2+} in protein-free filtrates were determined by atomic absorption measurements; in the presence of ADP, the free concentrations of Mn^{2+} were calculated by using the stability constant $K = 7.6 \times 10^3$ M^{-1} for Mn–ADP formation (O'Sullivan, 1969). The curves are theoretical for $K_A' = 1.5 \times 10^5$ M^{-1} for Mn^{2+} binding to one subunit site in the absence of ADP and for $K_1' = 1.5 \times 10^5$ and $K_2' = 10^6$ M^{-1} for the sequential binding of Mn^{2+} to two sites per subunit in the presence of 50 μM ADP (see text).

and with the K_m value of 19 nM for Mn–ADP indicates that arsenate or phosphate increases the affinity of the bovine brain enzyme ~100-fold.

Figure 4 shows measurements of the reversible binding of Mn^{2+} (added as $MnCl_2$) to bovine brain glutamine synthetase at pH 7.0 in the absence (closed symbols) and presence (open symbols) of a saturating concentration of ADP (50 μM). In the absence of ADP, the octameric enzyme appeared to have eight equivalent and noninteracting sites for binding Mn^{2+}. An apparent affinity constant (K_A') of $(1.5 \pm 0.6) \times 10^5$ M^{-1} was calculated from the experimentally determined values of $\bar{\nu}$ [moles of Mn^{2+} bound per mole (45 000 g) of enzyme subunit] at various free concentrations of Mn^{2+} (c), where

$$\bar{\nu} = nK_A'c/(1 + K_A'c) \quad (1)$$

and the number of intrinsic binding sites per subunit (n) was assumed to be 1. The lower curve in Figure 4 was calculated from eq 1 for $n = 1$ and $K_A' = 1.5 \times 10^5$ M^{-1}. The agreement between the experimental data and the theoretical curve suggests that there is no second subunit site for binding Mn^{2+} in the concentration range of Mn^{2+} used.

When 50 μM ADP was present, the binding of Mn^{2+} to the enzyme was more complex than in the absence of ADP (Figure

4), and the number of Mn^{2+} bound per subunit increased to 2. The Mn^{2+} binding data obtained in the presence of ADP could be fitted by assuming a sequential binding scheme in which the binding of free Mn^{2+} to a first subunit site must precede the binding of the Mn·ADP complex to a second subunit site. We assumed, therefore, that the Mn^{2+} bound to the second subunit site was present only as the Mn·ADP complex. The equilibrium expressions for the postulated sequential binding scheme can be written:

$$E \xrightleftharpoons{Mn^{2+}\ (K_1')} E{\cdot}Mn \xrightleftharpoons{Mn{\cdot}ADP\ (K_2')} E{\cdot}Mn_2ADP$$

and

$$Mn^{2+} + ADP \xrightleftharpoons{K_3'} Mn{\cdot}ADP$$

where E is the enzyme subunit. The fractional saturation of the enzyme subunit with Mn^{2+} at equilibrium can be expressed in terms of the free Mn^{2+} and ADP concentrations and the apparent affinity constants K_1', K_2', and K_3':

$$\frac{[Mn^{2+}]_{bound}}{[E]_{total}} = \frac{K_1'[Mn^{2+}]_{free} + 2K_1'K_2'K_3'[Mn^{2+}]_{free}^2[ADP]}{1 + K_1'[Mn^{2+}]_{free} + K_1'K_2'K_3'[Mn^{2+}]_{free}^2[ADP]} \quad (2)$$

Equation 2 was fitted to the Mn^{2+} binding data obtained in the presence of ADP (open symbols of Figure 4) using the value of K_1' measured in the absence of ADP ($1.5 \times 10^5\ M^{-1}$), $K_3' = 7.6 \times 10^3\ M^{-1}$ (O'Sullivan, 1969), and reiterative values of K_2' until the best fit of the data was achieved with $K_2' = 10^6\ M^{-1}$. The upper curve in Figure 4 was calculated from eq 2 and these affinity constants.

The value of K_2' estimated by fitting eq 2 to the Mn^{2+} binding data obtained in the presence of ADP agreed well with the value of $K_A' = 1.1 \times 10^6\ M^{-1}$ measured for ADP binding to glutamine synthetase at pH 7.2 in the presence of 0.4 mM $MnCl_2$ (Figure 3). This agreement supports the proposed binding scheme in which K_2' is the apparent affinity constant for binding the Mn·ADP complex to the enzyme.

Despite the tight binding of ADP and ATP (Wellner & Meister, 1966), we were unsuccessful in covalently labeling ATP binding sites of the bovine brain enzyme with the ATP analogue FSBA under conditions that produced a specific labeling of lysyl residue 47 of *E. coli* glutamine synthetase (Pinkofsky et al., 1984). Possibly, an arginyl residue (which would not be reactive with FSBA) at the active site of ovine brain glutamine synthetase (Powers & Riordan, 1975) is functionally equivalent to the lysyl residue found in the *E. coli* enzyme.

Structural Perturbations Produced by Mn^{2+}, Mg^{2+}, and Cl^- Binding. Figure 5 (top) shows saturating difference UV spectra normalized to 1.0 μM octamer concentration for glutamine synthetase + $MnCl_2$ (or $MgSO_4$) vs. glutamine synthetase at pH 7.0 and 21 °C. Spectral changes occurred within 2–5 min after mixing $MnCl_2$ or $MgSO_4$ with the apoenzyme, and UV difference spectra were recorded 20 min later. Titrations of apoenzyme with $MnCl_2$ or $MgSO_4$ gave the difference spectra shown in the top panel of Figure 5 at 0.1 mM $MnCl_2$ or 20 mM $MgSO_4$ final concentrations, respectively. Saturating difference spectra produced by $MnCl_2$ and $MgCl_2$ were essentially the same. Peak–troughs were observed at ~291–299, 291–288, and 280–288 nm upon the addition of either $MnCl_2$ or $MgSO_4$. The addition of 1 mM EDTA to the Mn–GS complex (and to the reference cuvette) reversed the specific M^{2+}-promoted spectral change (Figure 5, top). EDTA by itself produced a spectral perturbation

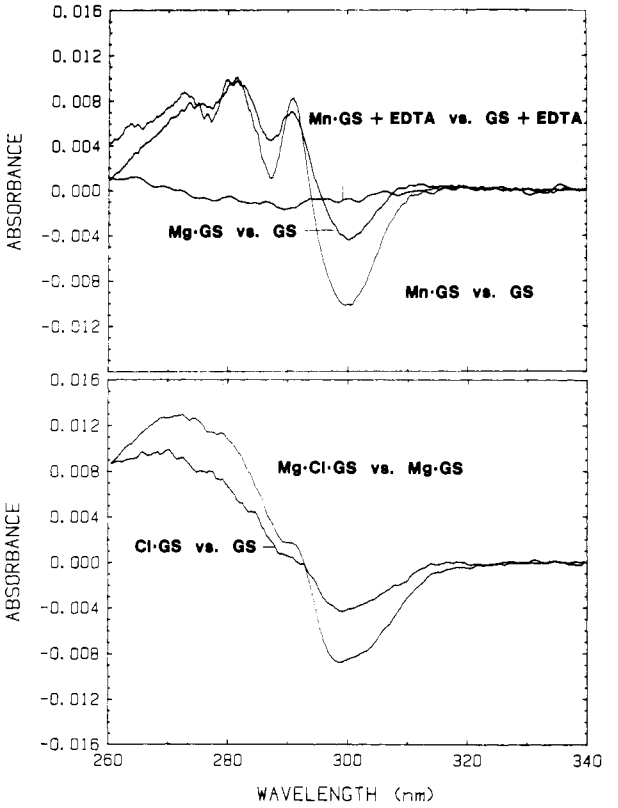

FIGURE 5: Mn^{2+}-, Mg^{2+}-, and Cl^--promoted UV difference spectral changes in glutamine synthetase (GS) at 21 °C, pH 7.0. Shown are saturating difference spectra for GS + ligand vs. GS (normalized to a protein concentration of 1.0 μM octamer) in 40 mM Hepes/KOH at pH 7.0; final ligand concentrations were 0.1 mM $MnCl_2$, 20 mM $MgSO_4$, and 1 mM EDTA (upper panel) and 6.5 mM KCl and 20 mM $MgSO_4$ (lower panel). Increments of $MnCl_2$, $MgSO_4$, or KCl were added to the sample cuvette, and identical volumes of buffer were added to the reference cuvette until no further spectral changes occurred. Time-dependent absorbance changes (monitored at 299 nm) were complete within ~2–5 min; duplicate scans were made after 20 min and averaged. The addition of 1 mM EDTA to both sample and reference cells after completion of the titration of the enzyme with $MnCl_2$ (upper panel) fully reversed spectral changes.

similar to that observed with Cl^- (see below). The peak–troughs of Mn^{2+}- or Mg^{2+}-promoted difference spectra suggest that tryptophanyl and tyrosyl residues of glutamine synthetase undergo changes in environment (Donovan, 1969) upon metal ion binding to the enzyme. The magnitude of the absorbance decrease at 299 nm ($\Delta\epsilon \simeq 1250$ M subunit^{-1} cm^{-1}) corresponds to a net exposure of ~1 Trp/subunit, whereas the amplitude of the red-shifted 280–288-nm peak–trough ($\Delta\epsilon \simeq 1060$ M subunit^{-1} cm^{-1}) suggests that ~2 Tyr/subunit are buried upon binding $MnCl_2$ or $MgCl_2$ to the enzyme.

The effect of Cl^- on protein difference spectra was measured because tryptophanyl residue fluorescence changes had been observed upon Cl^- addition to the enzyme (see below). The addition of 6.5 mM KCl to the apoenzyme produced spectral changes (Figure 5, bottom) which were similar to those obtained by adding 6.5 mM KCl to the Mg–enzyme complex (in the presence of 20 mM $MgSO_4$). Thus, the amplitude of the minimum at ~298 nm in Figure 5 (bottom) produced by Cl^- binding to the enzyme is a substantial contribution to the 292–298-nm peak–trough produced by $MnCl_2$ (Figure 5, top) or $MgCl_2$ binding. Assuming one Cl^- binding site per subunit, Hill plots of spectrophotometric titration data for KCl additions to the Mg–GS complex at pH 7.0 (21 °C) were linear and yielded a Hill coefficient of 0.8 and a half-saturation value, $[S]_{0.5}$, of 0.12 mM. These values are in excellent agreement with those obtained by fluorescence titrations.

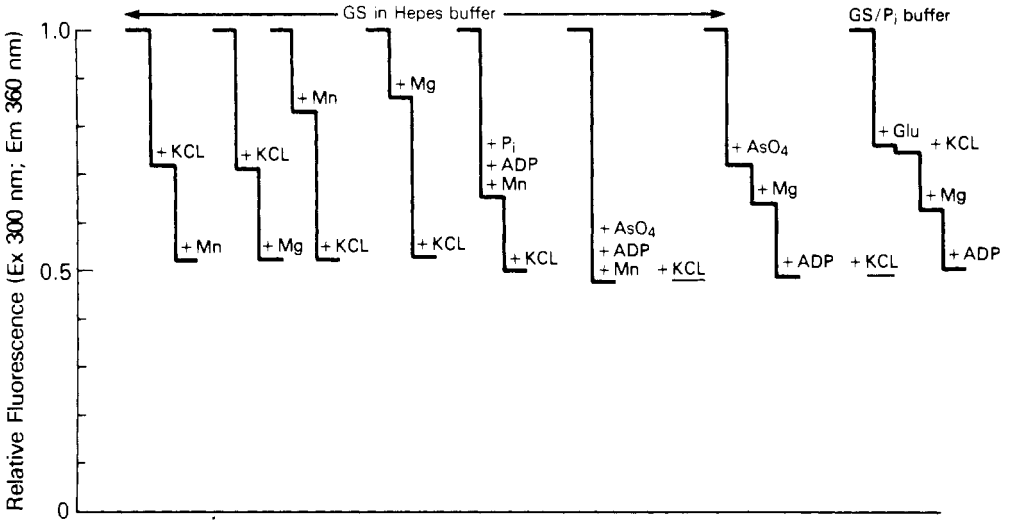

FIGURE 6: Relative protein tryptophanyl residue fluorescence changes produced by adding various effectors to glutamine synthetase at pH 7.0, 21 °C, in 40 mM Hepes/KOH or 40 mM potassium phosphate buffer. Shown are total relative quenches (corrected for dilution) at 337- or 360-nm emission with excitation at 300 nm obtained by adding ≥6 mM KCl, ≥0.1 mM $MnSO_4$, ≥20 mM $MgSO_4$, ≥20 mM potassium arsenate, and ≥90 mM L-glutamate (in the indicated order) and by adding mixtures of 0.28 mM ADP, 0.4 mM $MnSO_4$, and 1 mM potassium phosphate or ≥20 mM potassium arsenate.

Figure 6 shows effects of metal ions and other anions on enzyme fluorescence at pH 7 and 21 °C (excitation = 300 nm). The addition of $MnSO_4$, $MgSO_4$, and KCl produced fluorescent quenches that were complete within the time of mixing. The maximum fluorescence quench observed in the presence of saturating amounts of metal ion and chloride was the same with either Mn^{2+} or Mg^{2+}, or mixtures of these metal ions, but the relative changes produced by Cl^- or metal ions depended on the order of addition. Both Mn^{2+} and Mg^{2+} caused greater quenching when added in the presence of chloride. The same relative fluorescence changes were observed at 37 °C as at 21 °C. Light-scattering measurements indicated that no change in the state of aggregation of the enzyme occurred following addition of metal ions, chloride, or nucleotide.[2]

The bovine brain enzyme has a high affinity for Cl^- (see Figure 7 below). Acetate, bicarbonate, or EDTA at higher concentrations (≥1 mM) produced a tryptophanyl residue quench similar to that seen with Cl^-. Other anions tested (e.g., sulfate, nitrate, borate, and phosphate) had little or no effect on the tryptophanyl residue fluorescence at concentrations as high as 10 mM (data not shown). Arsenate, a phosphate analogue, and L-glutamate, however, did cause fluorescent quenches similar to that seen with Cl^- in the absence of metal ions. Chloride addition after arsenate or L-glutamate produced no further change. Thus, arsenate, L-glutamate, and Cl^- can produce the same or competing conformational changes in the enzyme.

Metal ion plus Cl^- produced ~45% quench, and an additional 5–10% quench was observed when ADP was added (Figure 6). When metal ion and ADP were added without Cl^-, a quench of ~20% was observed, and phosphate addition (~1–2 mM) led to a significant (~10%) further quench and produced a blue shift of ~2 nm in the fluorescence emission spectrum (with the maximum shifted from 337 to 335 nm). Thus, phosphate binding in the presence of ADP and metal ion causes a conformational change not observed with phosphate alone. Arsenate also produced an observable (2 nm) blue shift in the protein emission spectrum when added to the enzyme with ADP and M^{2+} present, but the maximum quench was greater than observed with phosphate. Since the apparent affinity of the enzyme for arsenate was not affected by the presence of ADP and metal ions (data not shown), the fluorescence titrations reflect binding of arsenate to an allosteric site. Chloride addition to the phosphate–ADP–M^{2+}–enzyme complex produced a further quench of 10–15%, indicating that phosphate does not bind to the allosteric anion site. Apparently, arsenate binds to both the phosphate site (at the catalytic site) and the allosteric site.

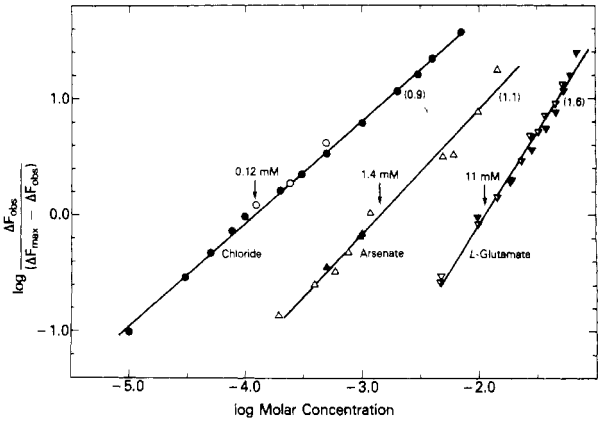

FIGURE 7: Hill plots of fluorescence titrations of glutamine synthetase with KCl (○, ●), potassium arsenate (△, ▲), and L-glutamate (▽, ▼, ▽) at 21 °C, pH 7.0, in 40 mM Hepes (open symbols), 40 mM potassium phosphate (closed symbols), or 40 mM Hepes/20 mM $MgSO_4$ (▽). The ratio of enzyme–ligand complex to free enzyme was assumed to be equal to $\Delta F_{obs}/(\Delta F_{max} - \Delta F_{obs})$ where fluorescence changes at 337- or 360-nm emission (excitation = 300 nm) were corrected for dilutions and ΔF_{obs} and ΔF_{max} were the observed fluorescence changes at a given ligand concentration and the maximum fluorescence change at a saturating ligand concentration, respectively. In each case, the Hill equation was fit by a linear least-squares program; Hill coefficients (n_H = slopes) are given in parentheses, and apparent half-saturating concentrations of free ligand are indicated at the arrows.

Saturation Functions for Anions and Metal Ions. Hill plots based on the fluorometric titrations with chloride, arsenate, and L-glutamate are shown in Figure 7. Chloride binding was not affected by the presence of metal ion or phosphate, and

[2] However, the presence of KCl in storage buffers was found to have a destabilizing effect on the enzyme, causing both losses in activity and aggregation to higher oligomeric states detected in ultracentrifugation studies. The enzyme was quite stable during storage at 4 °C in the absence of Cl^- (see Materials and Methods).

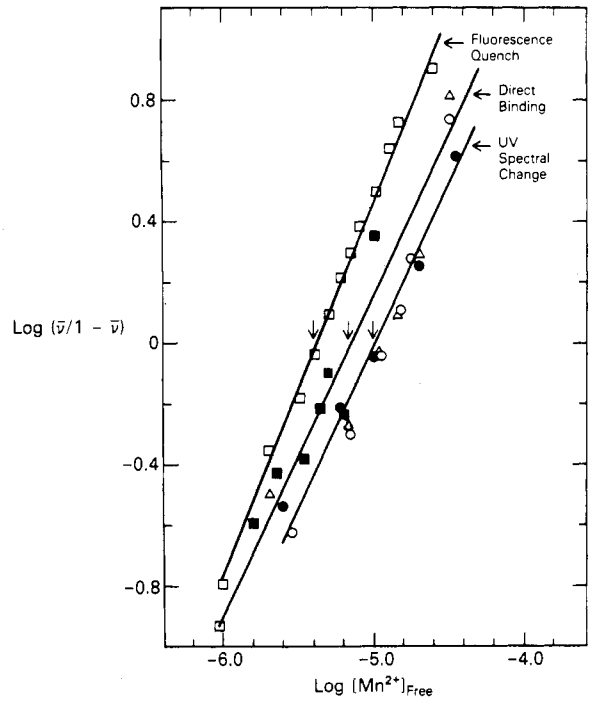

FIGURE 8: Hill plots for Mn^{2+} binding to one structural site per enzyme subunit at 21 °C. Titrations of glutamine synthetase in 20 mM Hepes–100 mM KCl buffer, pH 7.2, were monitored by fluorescence quench (□, E_m = 360 nm with E_x = 300 nm) and in 40 mM Hepes buffer, pH 7.0, by direct measurements of Mn^{2+} binding (■) and by UV absorbance changes at peak–troughs of 291–299 (○), 291–288 (●), and 281–288 nm (△) (see Figure 5). The free concentration of ligand was calculated by assuming that the fraction of enzyme subunit bound to Mn^{2+} ($\bar{v}$) was equal to $\Delta F_{obs}/\Delta F_{max}$ or $\Delta A_{obs}/\Delta A_{max}$ for fluorescence or absorbance measurements, respectively, where the observed change was at a given Mn^{2+} concentration and the maximum change was at a saturating concentration of Mn^{2+}. Fitting the Hill equation by linear least-squares analyses gave apparent half-saturation, $[S]_{0.5}$, and Hill coefficient (n_H) values of $[S]_{0.5} = 4.2 \times 10^{-6}$ M and $n_H = 1.2$ (□), $[S]_{0.5} = 6.9 \times 10^{-6}$ M and $n_H = 0.95$ (■), and $[S]_{0.5} = 1.0 \times 10^{-5}$ M and $n_H = 0.97$ (○, ●, and △).

titrations indicated an apparent binding constant for chloride of $\sim 1 \times 10^4$ M^{-1}. The Hill coefficient was essentially unity under all conditions tested. Titrations of the enzyme in either Hepes or potassium phosphate, pH 7.0, buffer with arsenate gave $[S]_{0.5}$ = 1.4 mM with $n_H = 1.0 \pm 0.1$ and with L-glutamate $[S]_{0.5} \simeq 11$ mM and $n_H = 1.6$. That L-glutamate gave a Hill number >1 suggested that L-glutamate can bind to more than one site per subunit. Since the fluorescence change is most likely due to L-glutamate binding to an allosteric site, effects of Mg^{2+} or phosphate on titrations with L-glutamate were not pronounced.

Figure 8 shows Hill plots of Mn^{2+} binding as measured by direct binding, spectrophotometric, and fluorometric titrations. Hill coefficients for each titration were ~ 1. The agreement between the direct binding assays and the fluorescence and spectral titrations indicates that the observed conformational changes resulted from binding of a single metal ion per subunit. Also, a stoichiometry of 1 equiv of $MnCl_2$ per subunit for producing the spectral perturbations was indicated since the first additions of $MnCl_2$ to the enzyme were not sufficient to saturate a second subunit site. The agreement obtained by monitoring the different peak–troughs in the Mn^{2+}-promoted UV spectral changes suggests that the tryptophanyl residue exposure and tyrosyl residue burial occur synchronously without apparent contributions from cooperative subunit interactions. The fluorescence titrations were performed in the presence of 100 mM KCl at pH 7.2 which may explain the slightly higher apparent affinity calculated from tryptophanyl residue quench. A K_D' value of $(7 \pm 3) \times 10^{-6}$ M for Mn^{2+} dissociation from the Mn–enzyme subunit complex is estimated from the data in Figure 8.

Table II: Half-Saturation ($[S]_{0.5}$) and Hill Coefficient (n_H) Values from Fluorescence Titrations of Bovine Brain Glutamine Synthetase (~50 μg/mL) with $MgSO_4$ in the Absence and Presence of Effectors at pH 7.1 (21 °C) in 40 mM Hepes/KOH Buffer[a]

ligand present	$[S]_{0.5}$ (mM)	n_H
none	1.4	1.6
chloride, 100 mM	0.46	2.0
arsenate, 19 mM[b]	0.24	1.0
L-glutamate, 50 mM[b]	0.45	1.3
L-glutamine, 50 mM	0.64	1.2

[a] See Figure 6 and legend to Figure 7. [b] The $[S]_{0.5}$ values were corrected to those corresponding to the free concentration of Mg^{2+} by using the published affinity constants for Mg^{2+} binding to arsenate (K = 100 M^{-1}) and to L-glutamate (K = 79 M^{-1}) (O'Sullivan, 1969).

Titrations of the bovine brain enzyme with $MgSO_4$ gave $[S]_{0.5}$ = 1.8 and 1.4 mM, monitoring spectral changes (Figure 5, top) and protein fluorescence quenches (Figure 6), respectively. Table II summarizes $[S]_{0.5}$ values together with Hill coefficients obtained from fluorescence titrations with $MgSO_4$ at pH 7.0 in the absence and presence of various ligands. The presence of saturating Cl^- increased the apparent affinity of the enzyme for Mg^{2+} ~3-fold and increased n_H from 1.6 to 2.0. Chloride also increased the affinity of the enzyme for Mn^{2+} ~2–4-fold, but in this case, Hill coefficients were ~1.2 (Figure 8). As mentioned above, the binding of Cl^- (with $n_H \simeq 1$) was not affected by the presence of either saturating $MgSO_4$ or $MnSO_4$. The decrease in the $[S]_{0.5}$ value for Mg^{2+} produced by saturating arsenate (Table II) was unexpected on the basis of observations with *E. coli* glutamine synthetase (Hunt et al., 1975). However, arsenate appeared to produce a structural perturbation in the brain enzyme that was similar to that caused by chloride (Figure 6), and both Cl^- and arsenate decreased the $[S]_{0.5}$ value for Mg^{2+}. The effects of L-glutamate and L-glutamine on $[S]_{0.5}$ values (Table II) could be on the binding of Mg^{2+} to either or both subunit sites for M^{2+} (Hunt & Ginsburg, 1980). The Hill coefficients of >1 in Table II suggest that in these cases Mg^{2+} is binding to two subunit sites, but if similar to Mn^{2+} binding (Figure 8), the binding of Mg^{2+} to one subunit site is responsible for tryptophanyl residue exposure.

Relationship between Chloride Binding and Activity Expression. Tate and Meister (1973) observed an activation of ovine brain and rat liver glutamine synthetases by high concentrations of KCl (>100 mM) at subsaturating L-glutamate concentration which was attributed to a lowering of the K_m for L-glutamate by chloride. When lower concentrations of KCl (0.5–25 mM) were used, which produced structural perturbations in the bovine brain enzyme [Figures 5 (bottom) and 6], no effect of chloride on the K_m for L-glutamate ($K_m \simeq 5$ mM) could be detected, and no effect of KCl at 5 and 10 mM L-glutamate on the rate of the Mg^{2+}-dependent biosynthetic reaction was observed. Double-reciprocal plots of activity vs. [L-glutamate] showed a slight downward curvature (data not shown). Since fluorescence titrations with L-glutamate suggested that 2 equiv/subunit of L-glutamate could bind in the concentration range of the measured K_m for L-glutamate, it would appear that binding the second L-glutamate has little effect on activity.

High concentrations of chloride were found, in fact, to inhibit the γ-glutamyltransferase activity of the enzyme, and Cl^- appeared to be competitive with arsenate ($K_i \simeq 130$ mM Cl^-). Curiously, however, the effect of chloride was observed only at concentrations above 20 mM, even when arsenate

concentrations were lowered to near the K_m value (~ 1 μM) for arsenate. The kinetics of arsenate-dependent γ-glutamyl transfer showed a biphasic dependence of activity on arsenate in the absence of Cl^-. The data are most easily interpreted in terms of two sites for arsenate: a substrate site, with a very low K_m of ~ 1 μM, and an allosteric, activating site, with a lower affinity ($K_D' \geq 1$ mM). Chloride possibly inhibits γ-glutamyl transfer activity by competing with arsenate for the activating site only. This interpretation is consistent with the results of fluorescence quenching effects caused by arsenate and chloride. The activating effect of arsenate may in part explain the very large difference in turnover number of the enzyme in the γ-glutamyl transfer reaction (~ 400 s^{-1}) compared to the biosynthetic reaction (~ 19 s^{-1}) at 37 °C.

Discussion

Starting from fresh or frozen bovine brain, we were able to purify glutamine synthetase with specific activities in the biosynthetic and the γ-glutamyl transfer reactions higher than those reported previously. The absorption coefficient ($A^{0.1\%}_{280nm,1cm} = 1.50 \pm 0.05$) was determined by refractometry and by quantitative amino acid analysis (Maurizi et al., 1986) to allow measurement of the absolute enzyme subunit concentration. We then began a systematic examination of the binding affinities and stoichiometries of binding for nucleotides, metal ions, and anions interacting with the enzyme. Kinetic titrations were conducted to relate the binding properties of the different ligands to their effects on the enzymatic activity of the enzyme. We found no evidence for negative cooperativity or for half-of-the-sites reactivity with any of the ligands examined.

The purified enzyme is free of metal ions and can bind reversibly up to 2 equiv/subunit of Mn^{2+} or Mg^{2+} which is consistent with our previous finding of 2 equiv of Mn^{2+} bound per subunit in the presence of ADP and the transition-state analogue L-Met-*S*-sulfoximine phosphate (Maurizi et al., 1986). Mn^{2+} binds first to one site per subunit with $K_A' \simeq 1.5 \times 10^5$ M^{-1} and then to a second site per subunit as ADP·Mn with $K_A' \approx 1 \times 10^6$ M^{-1}. Mn^{2+} affinity for the Mn–ADP site appears to be weak ($K_A' < 10^4$ M^{-1}). Fluorescence and spectrophotometric titrations indicate that Mg^{2+} binds to these two sites with $K_A' \simeq 10^3$ M^{-1}. In the presence of Mn^{2+}, ADP binding ($K_A' \sim 10^6$ M^{-1}) reached saturation at ~ 1 equiv of ADP/subunit. The Job analysis of Figure 2 also shows that one ADP site per subunit must be occupied for activity expression. Thus, each subunit has two sites for metal ions and one site for ADP, and there was no evidence for cooperativity in binding. The site at which Mn^{2+} or Mg^{2+} alone binds is formally similar to the n_1 structural metal ion site on the *E. coli* enzyme subunit (Shapiro & Ginsburg, 1968; Hunt & Ginsburg, 1972, 1981). Arsenate or phosphate increases the affinity of the brain enzyme ~ 100-fold for ADP–Mn, as has been observed for *E. coli* glutamine synthetase (Hunt et al., 1975; Rhee et al., 1976). The high degree of similarity between the catalytic properties, substrate specificity, metal ion binding, and ligand affinities of the bacterial and brain glutamine synthetases suggests that the active sites are structurally similar as has been proposed (Meister, 1974).

The finding of two Mn^{2+} sites and one active site per subunit for the bovine brain enzyme is intriguing in light of the recent X-ray crystallographic structure published for the *Salmonella typhimurium* glutamine synthetase, which has a structure homologous to that of the *E. coli* enzyme (Almassy et al., 1986). The metal ion sites in the active sites of the bacterial enzyme are located near the intra-ring subunit bonding contacts in the carboxy-terminal domain (Almassy et al., 1986), whereas the nucleotide site is in the N-terminal domain of an adjacent subunit (Pinkofsky et al., 1984). Since the *E. coli* and *S. typhimurium* enzyme subunits are arranged as superimposed isologously bonded hexagonal rings, the six heterologous subunit bonding domains within a ring contribute six active sites (Almassy et al., 1986). The brain glutamine synthetase appears to be composed of two isologously bonded tetramers with D_4 symmetry (Haschemeyer, 1970; Wilk et al., 1969; Stahl & Jaenicke, 1972; Meister, 1974). Our data indicate that there should be four active sites per tetramer. It is highly likely, considering the similarity in the properties of the active sites of the bacterial and brain enzyme, that the active sites of the brain enzyme also are located between heterologously bonded subunits and are composed of parts of both subunits. In this case, active-site ligands might be expected to stabilize heterologous subunit contacts as observed with the bacterial enzyme (Maurizi & Ginsburg, 1982b, 1985). However, our attempts to form submolecular oligomers of the bovine brain enzyme or even to reconstitute stable enzyme activity after inactivation of the Mn^{2+}- or Mg^{2+}–enzyme forms with L-Met-*S*-sulfoximine plus ATP (Maurizi & Ginsburg, 1982a,b) were unsuccessful (unpublished data). It is possible that isologous contacts in the brain enzyme are not as strong as they are in the dodecameric structure of bacterial enzyme, so that cleavage of heterologous contacts might not result in stable oligomers containing two, four, or six subunits.

A novel finding in this study is the tight Cl^- binding site on each subunit ($K_A' = 1 \times 10^4$ M^{-1}) and the considerable structural perturbations (observed by both UV spectral changes and fluorescence quenching) caused by relatively low concentrations of this anion.[3] No other anions tested had the same effect at comparable concentrations (within a factor of 10). The effect of Cl^- was observed in the presence and absence of Mn^{2+} or Mg^{2+} and with or without ADP. In vivo, the enzyme is most likely to be in the chloride form at all times. Under these conditions, the affinity of the enzyme for Mg^{2+} or Mn^{2+} will be increased 2–4-fold (Table II), and the enzyme will be more sensitive to M^{2+}-promoted tryptophanyl residue exposure (Figure 6). Previously, we obtained evidence that glutamine synthetase in either bovine or ovine brain exists in vivo predominantly ($\geq 90\%$) as the Mg^{2+} form (Maurizi et al., 1986) so the effect of Cl^- will be mainly on Mg^{2+} binding. Of course, it is possible that Cl^- added in vitro is actually mimicking some anionic metabolic regulator that we have not tested. However, chloride has been reported to be a specific effector of various enzymes, and more recently Röhm (1985), for example, has examined the activating role of Cl^- on yeast aminopeptidase I. In this case, Cl^- was found to bind to an allosteric site and to improve the binding of Zn^{2+}, an essential component of aminopeptidase I. Asparagine synthetase from leukemia cells also is activated by low Cl^- concentrations ($K_A = 2$ mM), but the mechanism of the chloride effect is not known (Horowitz & Meister, 1972).

Binding different ligands to the active sites or to allosteric sites of the bovine brain enzyme promotes conformational changes as indicated by substantial quenching of tryptophanyl residue fluorescence. Contributions of individual ligands to

[3] A similar effect of Cl^- was observed with the glutamine synthetase purified from ovine brain (330 units/mg; Maurizi et al., 1986). Fluorescence titrations of the ovine brain enzyme with KCl gave $K_D' \approx 100 \pm 50$ μM, and the binding constant for Mn^{2+} (added as $MnSO_4$) was enhanced ~ 4-fold by the presence of 6.5 mM KCl in 40 mM Hepes/KOH, pH 7.0. A tryptophanyl residue quench on binding Mn^{2+} to the ovine brain enzyme previously had been observed by Wedler et al. (1982).

the overall quench, however, depend on the absence or presence of other effectors. For example, chloride added alone to the enzyme produced a smaller fluorescence quench (28%) than when added after Mn^{2+} or Mg^{2+} (33–35%) and caused an even smaller quench (15%) when added after Mn^{2+}, phosphate, and ADP. Active-site ligand binding therefore can influence the extent of the conformational change caused by Cl^- at the allosteric site. Arsenate, L-glutamate, and Cl^- produce similar protein fluorescence quenches, and no further quench is observed when Cl^- is added after saturating arsenate or L-glutamate. Both arsenate and L-glutamate appear to bind to allosteric sites as well as to substrate sites of the enzyme. Possibly, allosteric binding sites for arsenate, L-glutamate, and Cl^- overlap.

Both the trough at 299 nm in the UV difference spectrum and the quench in tryptophan fluorescence indicate that allosteric and active-site ligand binding cause at least one tryptophan per subunit to be exposed to solvent. Because there are eight tryptophanyl residues per subunit of the bovine brain enzyme, the magnitude of the fluorescence quench could indicate that these ligands produce a relaxation of the protein into a more open configuration. There was, however, a compensating burial of approximately two tyrosyl residues per subunit on binding M^{2+} and Cl^-. No change in quaternary structure of the enzyme occurred on binding ligands nor was there cooperativity in binding Cl^-, ADP, or Mn^{2+} to the brain enzyme. Thus, the ligand-promoted structural perturbations reported here appear to involve only subunit conformational changes.

In summary, bovine brain glutamine synthetase has been shown to have two essential M^{2+} sites per subunit: one occupied by Mg^{2+} or Mn^{2+} and the other occupied by M^{2+}–nucleotide for activity expression. Moreover, Cl^- bound to an allosteric site increases the affinity of the enzyme for M^{2+}.

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