Calorimetric and Equilibrium Binding Studies of the Interaction of Substrates with Glutamine Synthetase of *Escherichia coli*†

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ABSTRACT: Interactions of two substrates (L-glutamine and ADP) and Mn^{2+} ions with glutamine synthetase from *Escherichia coli* have been studied by calorimetry and equilibrium dialysis techniques. In addition, the use of calorimetry for establishing separateness of binding sites of different enzyme ligands is considered. The thermodynamic parameters for the sequential and simultaneous binding of L-glutamine and ADP to the unadenylylated Mn-enzyme have been determined. Thermal saturation curves for the binding of L-glutamine to the enzyme in the presence and absence of ADP were obtained at pH 7.1 and 30 °C. With saturating ADP and without ADP present, $\Delta G'$ values for L-glutamine binding are −3.83 and −2.96 kcal/mol of subunit, respectively. The corresponding $\Delta H'$ values are −7.4 and −9.7 kcal/mol of subunit. Without L-glutamine present, the $\Delta G'$ value for ADP binding is −7.60 kcal/mol of subunit and $\Delta H'$ is −5.7 kcal/mol of subunit. The synergistic effect between the binding of L-glutamine and ADP is reflected in the free-energy data. The overall entropy of binding both ADP and L-glutamine to the enzyme is negative, whereas the entropies of binding for ADP are positive and those for L-glutamine are negative. The proton uptake upon binding L-glutamine or ADP to the enzyme is very small; the total proton uptake on binding both substrates is ~0.2 equiv of H^+/subunit. Apparent association constants for L-glutamine and for Mn^{2+} binding to the fully adenylylated enzyme at pH 7.2 are essentially the same as those measured with the unadenylylated enzyme, even though these enzyme forms have quite different K_m values for L-glutamine and for Mn^{2+} in the γ-glutamyl transfer reaction. Kinetic analyses with adenylylated Mn-enzyme show a strongly antagonistic effect between L-glutamine and hydroxylamine and synergistic effects between L-glutamine and arsenate and between L-glutamine and ADP. Equilibrium measurements of Mn^{2+} binding to the fully adenylylated enzyme show that L-glutamine enhances the apparent affinity for Mn^{2+} at n_2 subunit sites (~30-fold) without affecting Mn^{2+} binding to the high-affinity n_1 subunit sites; with a saturating concentration of L-glutamine, $K_2' \simeq K_1' = 1.9 \times 10^6\ M^{-1}$ for Mn^{2+} binding to n_1 and n_2 subunit sites of the dodecamer. In hybrid enzyme molecules (those containing both adenylylated and unadenylylated subunits), heterologous interactions between the two types of subunits seem to decrease the apparent affinity for Mn^{2+} at n_1 but not at n_2 subunit sites.

Glutamine synthetase from *E. coli* is a dodecamer composed of 12 apparently identical subunits (Woolfolk et al., 1966; Valentine et al., 1968). The enzyme is isolated as a homogeneous protein of 600 000 molecular weight (Shapiro and Ginsburg, 1968); some physical-chemical properties of this protein have been reviewed (Ginsburg, 1972). A dominant control of glutamine synthetase activity in *E. coli* occurs through enzyme-catalyzed adenylylation and deadenylylation reactions (*see reviews*: Stadtman et al., 1968; Holzer, 1969; Stadtman and Ginsburg, 1974). The site of adenylylation in glutamine synthetase is a specific tyrosyl residue within the subunit polypeptide chain of 50 000 molecular weight. Upon adenylylation, a stable 5′-adenylyl-*O*-tyrosyl derivative is formed (Shapiro and Stadtman, 1968; Heinrikson and Kingdon, 1971). Adenylylation, which can occur to the extent of 12 equiv of AMP groups covalently attached per dodecamer (Kingdon et al., 1967), markedly affects various catalytic parameters of glutamine synthetase (Shapiro and Stadtman, 1970a). One of the more striking effects of adenylylation is the change from a Mg^{2+}- to a Mn^{2+}-dependent enzyme form in biosynthetic catalysis (Kingdon et al., 1967). Rhee et al. (1976) recently have proposed that the inability of Mn^{2+} to support the biosynthetic activity of the unadenylylated enzyme is due to product inhibition by ADP and orthophosphate; the unadenylylated enzyme has a much higher affinity for ADP-Mn in the presence of L-glutamine and orthophosphate or L-glutamine and arsenate than has adenylylated glutamine synthetase (Rhee and Chock, 1976; Hunt et al., 1975). In fact, Mn^{2+} supports the activities of both adenylylated and unadenylylated enzymes in the γ-glutamyl transfer reaction (Kingdon et al., 1967; Wulff et al., 1967):

$$\text{L-glutamine} + NH_2OH \xrightarrow[\text{arsenate}]{\text{ADP, } Mn^{2+}} \gamma\text{-glutamylhydroxamate} + NH_4^+ \quad (1)$$

in which ADP and arsenate are nonconsumable substrates in this uncoupling of the reverse biosynthetic reaction.

Divalent cation-free glutamine synthetase is activated (*tightened*) by Mn^{2+} or Mg^{2+} binding to the extent of 12 equiv of metal ion per dodecamer (Kingdon et al., 1968; Denton and Ginsburg, 1969; Hunt and Ginsburg, 1972). The tightening process involves a conformational change in the protein structure (Shapiro and Ginsburg, 1968; Ginsburg, 1972). Denton and Ginsburg (1969) observed that adenylylation decreased the apparent affinity for Mn^{2+} at the tightening or activating site (n_1) of each subunit while not affecting Mn^{2+} binding to a second subunit site (n_2). However, these earlier studies were made with glutamine synthetase preparations containing an average of 2 and 9 equiv of covalently bound

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AMP per dodecamer ($GS_{\overline{2}}$ and $GS_{\overline{9}}$)[1] and thus were possibly representative of hybrid enzyme forms containing both types of subunits (Stadtman et al., 1970; Ciardi et al., 1973). Consequently, the binding of Mn^{2+} to fully adenylylated and to partially adenylylated enzymes is reexamined here.

In more recent studies, Hunt et al. (1975) further showed that two Mn^{2+} sites (n_1 and n_2) per enzyme subunit must be saturated for activity expression in the γ-glutamyl transfer reaction. The binding of Mn^{2+} to the enzyme is random. An active enzyme complex can be formed by sequential binding of Mn^{2+} and ADP or by the direct binding of ADP-Mn complex to the second subunit metal-ion site with Mn^{2+} already bound at the n_1 site. A 1:1 interaction between ADP-Mn and each subunit of the unadenylylated dodecamer was demonstrated. Glutamine considerably enhances the affinity of the unadenylylated enzyme for Mn^{2+} at the second subunit site without affecting the affinity for Mn^{2+} at the first subunit site (Hunt et al., 1975) and this was confirmed by the nuclear magnetic resonance experiments of Villafranca et al. (1976).

Calorimetry was used previously to study the interactions of effectors with glutamine synthetase from *E. coli* (Ross and Ginsburg, 1969; Hunt et al., 1972). Two substrates, for which the enzyme has separate sites, have been used in the present calorimetric study to establish conditions for demonstrating separateness of ligand-binding sites. In a previous calorimetric study of the interaction of Mn^{2+} with the enzyme at pH 7.2, proton release during the binding reaction was quantitated by using Tris and Hepes buffers, which have quite different heats of protonation (Hunt et al., 1972). We have used these same buffers in this study to measure proton uptake or release during the binding of substrates to glutamine synthetase.

Materials and Methods

Glutamine Synthetase Preparations and Assay. Glutamine synthetase was isolated by the procedure of Woolfolk et al. (1966) from *E. coli* grown in 350 L of medium containing limiting ammonia or 35 mM L-glutamate and 0.67 M glycerol (Phares, 1971; Senior, 1975). The enzyme was purified to homogeneity by five to six acid–ammonium sulfate precipitations after the acetone step (Woolfolk et al., 1966). The state of adenylylation of purified enzyme preparations was determined both by the UV absorption spectrum (Ginsburg et al., 1970) and by assay (Stadtman et al., 1970). Three enzyme preparations were used: unadenylylated glutamine synthetase ($GS_{\overline{1}}$), partially adenylylated glutamine synthetase ($GS_{\overline{4.7}}$), and fully adenylylated glutamine synthetase ($GS_{\overline{12}}$), which was prepared by adenylylating $GS_{\overline{4.7}}$ in vitro (Ginsburg, 1970). The adenylylation procedure was as described except that the adenylylation reaction at pH 8.0 contained 5 mM ATP, 30 mM $MgCl_2$, 10 mM L-glutamine, 10 mM imidazole hydrochloride, 360 mg of $GS_{\overline{4.7}}$, and 212 units of glutamine synthetase adenylyltransferase (Caban and Ginsburg, 1976). After 50 min at 37 °C, the Mg^{2+}-dependent biosynthetic activity was 1% of that of $GS_{\overline{4.7}}$ and the reaction was terminated by adding 30% (v/v) of saturated ammonium sulfate (4 °C) and 1% (v/v) of 1 M $MnCl_2$; the adenylylated glutamine synthetase ($GS_{\overline{12}}$) was purified as described (Ginsburg, 1970).

Glutamine synthetase preparations were stored at 4 °C in the presence of 5 mM $MnCl_2$ and 5 mM imidazole buffer as a precipitate in 50% saturated ammonium sulfate, from which the enzyme was collected by centrifugation as needed. Prior to all calorimetric studies, the protein was extensively dialyzed by multiple changes in buffers containing 0.050 M Tris-HCl (or 0.050 M Hepes-KOH), 0.10 M KCl, and 0.001 M $MnCl_2$ at pH 7.1 (30 °C). Protein concentrations were determined spectrophotometrically at 290 nm ($A_{290}^{0.1\%} = 0.385$; Shapiro and Stadtman, 1970b) and at 280 nm as described previously (Ginsburg et al., 1970). All glutamine synthetase preparations were 120–130 units/mg in the saturating γ-glutamyltransferase assay at pH 7.57, which is independent of the state of adenylylation (Hunt et al., 1975); this assay system was used also during enzyme purifications, for assaying enzyme samples before and after equilibrium dialysis for Mn^{2+} binding studies, and for assaying $GS_{\overline{12}}$ samples for calorimetric studies. Before and after calorimetric studies with unadenylylated glutamine synthetase ($GS_{\overline{1}}$), the Mg^{2+}-dependent biosynthetic activity was measured at 25 (38–40 units/mg) or at 30 °C (55 units/mg) as described by Ginsburg et al. (1970).

The K_m values for L-glutamine in the γ-glutamyl transfer reaction (Shapiro and Stadtman, 1970b) were determined with the conditions specified in Table I. With all other substrates at saturating concentrations, two assay mixtures containing 0 and 150 mM L-glutamine were prepared and mixed in varying proportions to give the final concentrations of L-glutamine required. When a second substrate (arsenate, ADP, or NH_2OH) was varied, assay mixtures with 60 mM L-glutamine and without L-glutamine were prepared at each subsaturating concentration of the second substrate and then mixed in varying proportions. Generally, L-glutamine saturation curves for four concentrations of the second substrate varied were determined and double-reciprocal plots were constructed as described by Dixon and Webb (1964). Reactions were initiated by adding 20 μL of glutamine synthetase. The linearity of the reactions with time was checked by incubating the reactions for different times. At low substrate concentrations, the enzyme was diluted by increasing the reaction volume two- to fourfold. In these cases, a two- to fourfold more concentrated $FeCl_3$–trichloroacetic acid solution (Shapiro and Stadtman, 1970b) was used to stop the reactions. Reaction mixtures containing no glutamine and those containing no enzyme were used for blank corrections. A Gilford 300 N spectrophotometer was used to measure the developed color at 540 nm.

Reagents. Standardized $MnCl_2$ and ADP stock solutions and all other reagents were as described by Hunt et al. (1975). Deionized, ultrafiltered water was obtained by passing distilled water through a Milli-Q2 reagent grade water system of the Millipore Corp. Divalent and trivalent metal ions were removed from 200 g of L-glutamine (grade III; Sigma Chemical Co.) using a 5 × 26 cm column of Chelex 100, 200–400 mesh (Bio-Rad Laboratories). Glutamine in the effluent (pH 7.6) was adjusted to its isoelectric pH (pH 4.9) with 37% HCl, precipitated with absolute ethanol, and suction dried using absolute ethanol washes. Solutions of L-glutamine purified in this manner were free of manganese and zinc at levels detectable by atomic absorption spectroscopy ($<10^{-7}$ M).

pH Meter Measurements of Proton Uptake or Release. The techniques and instrumentation for measuring proton uptake or release from glutamine synthetase during binding reactions were as described previously by Hunt and Ginsburg (1972). For measuring proton uptake upon L-glutamine binding to the native unadenylylated enzyme, three jacketed cells (thermostated at 37 °C) in tandem containing dialyzed glutamine synthetase (~5 mg in 0.8 mL of 3 mM Tris-HCl, 100 mM KCl, and 1 mM $MnCl_2$, pH 7.1–7.2) and 150 and 30 mM L-glutamine in the same buffer were used. For measurements in

[1] Abbreviations used are: $GS_{\overline{n}}$, a glutamine synthetase preparation with an average state of adenylylation, $\overline{n}$ (where the number of adenylylation sites for covalent attachment of 5′-adenylic acid residues per enzyme dodecamer equals 12); Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

the presence of ADP, the protein solution and buffers contained 0.1 mM free ADP. All solutions were transferred so as to minimize CO_2 absorption and were matched in pH (±0.002 pH unit) by adding either HCl or KOH initially and prior to calibrations. For each experiment, the observed changes in pH (ΔpH) for three reactions (with calibrations) were measured: (1) the binding of L-glutamine (200 μL of 150 mM Gln) to the enzyme (800 μL); (2) the dilution of 800 μL of enzyme–glutamine complex with 200 μL of 30 mM L-glutamine; (3) the dilution of 200 μL of 150 mM L-glutamine into 800 μL of dialysate. For conversion of the observed ΔpH to equivalents of H^+ released (ΔH^+), several 2-μL aliquots of 0.020 M HCl were added. Reactions 2 and 3 are corrections for 1. Usually, the ΔH^+ for reaction 2 was negligible, but that for reaction 3 was substantial. The corrected ΔH^+ for glutamine binding to GS(±ADP) is: (ΔH^+ for GS + Gln) − (ΔH^+ for GS–Gln complex dilution with Gln)/0.8 − (ΔH^+ for dialysate + Gln), where negative and positive signs are for apparent proton release and uptake, respectively.

For studies with *relaxed* GS_T, divalent cations were removed from the enzyme as described by Hunt et al. (1972). The proton release upon binding Mn^{2+} with and without L-glutamine present was measured as before (Hunt et al., 1975) and the proton uptake upon L-glutamine binding to the freshly tightened enzyme (Hunt and Ginsburg, 1972) was measured as described above.

Calorimetric Measurements. Heats of binding of glutamine and ADP to glutamine synthetase were measured at 30 ± 0.1 °C using an LKB Batch Microcalorimeter 10700 equipped with gold cells. A Keithley 147 Nanovolt Null detector coupled with a Honeywell Electronik 194 recorder equipped with a Disc ball and disk integrator and Disc 610 automatic printer were used to amplify and record output from the calorimeter. The electrical calibration heaters were checked by measuring the precision of the heat of dilution of sucrose (Gucker et al., 1939) and of the heat of protonation of Tris buffer (Wadsö, 1968). All binding experiments were performed using the 10-μV output of the nanovolt null detector. The same cell was used as the reaction cell for all binding experiments and matching volumes of final dialysate were used in the reference cell. All heats of reaction, of dilution of substrate, and of dilution of protein were determined in separate experiments in order to avoid errors due to differences in response of the thermopiles in each cell. Air-dried cells were found to give more reproducible heats than wet cells; thus, dry cells were used for all experimental work. Occasionally, small shifts in baseline occurred during the mixing cycle of the reaction; the most reproducible results were obtained by using the following baseline for the reaction and averages of the leading and following baselines for the electrical calibrations and for the frictional heats. To ensure complete mixing, double-mixing cycles were used. For each experiment, two electrical calibrations and two friction runs were carried out. A reaction (binding or dilution) was assumed complete 20 min after initiation of the double-mixing cycle. Initial protein concentrations were ~6 mg/mL and initial volumes of protein (2–4 mL) and substrate (2–4 mL) reactant solutions were varied in order to minimize corrections for heats of dilution of substrates (*e.g., see Table II*).

All reactant solutions were prepared on the day of the experiment using a dialyzed protein stock solution, purified L-glutamine (dry powder), and 0.1 M ADP in water at pH 6.9 (stored frozen). The glutamine solutions were prepared in buffer at a pH slightly above that of the other reactant solution. Reactant solutions containing ADP were prepared by a 1% dilution of final dialysate, L-glutamine solution, or protein solution. When preparing enzyme reactant solutions containing ADP or L-glutamine, the pH of the substrate solution added to the enzyme was such that minimal changes in the pH of the protein solution occurred.

Before loading the calorimeter cell, the pH of the substrate reactant solution was decreased with dilute HCl to match within 0.01 pH unit the pH of the protein reactant (or diluting) solution. Closely matching the pH levels of solutions before mixing in the calorimeter is necessary for reproducible heats; pH adjustments were downward to avoid manganese oxide formation. The pH of the reaction solution was measured at the end of each experiment to ensure that no pH shifts occurred during the experiment. For pH measurements, a Radiometer pH Meter 26 equipped with a Radiometer combined glass electrode (Type GK 2322C; suitable for use with Tris buffers) was used. The pH meter was standardized with pH 7 and 4 reference buffers before each measurement. The solutions were loaded volumetrically (checked by weight) into the calorimeter cell by means of calibrated syringes fitted with Teflon needles.

In general, the heats of binding L-glutamine to the enzyme (observed heats of reaction and dilution corrections) that were used to determine thermal saturation functions were measured once. These measurements were repeated, however, when an inconsistency within a particular set of saturation data occurred. All measurements involving the heat of binding ADP (Table III) were made at least twice (i.e., duplicate measurements of the heat of reaction with GS_T and of the heat of dilution of substrate(s)).

To detect and measure potential proton uptake or release attending binding reactions, the thermal-binding reactions were performed in both Tris and Hepes buffers. These buffers have quite different heats of protonation: −11.1 kcal/mol for Tris (Wadsö, 1968) and −4.8 kcal/mol for Hepes (Hunt et al., 1972). Any difference between observed heats of binding in these two buffers is due to proton release or uptake for the reaction under consideration.

Equilibrium Dialysis Measurements of Mn^{2+} Binding. Mn^{2+} binding to $GS_{\overline{12}}$ and to $GS_{\overline{4.7}}$ was measured using an Instrumentation Laboratories Model 153 atomic absorption spectrophotometer as described by Hunt et al. (1975). A stock protein solution (~16 mg/mL) was first dialyzed against 20 mM Tris-HCl, 100 mM KCl, and 1 mM $MnCl_2$ buffers at pH 7.2. For Mn^{2+}-binding measurements, dialysates were prepared from 0.020 M Tris-HCl–0.10 M KCl buffer (pH 7.17) to which standardized $MnCl_2$ (<0.5%, v/v) was added only if the final free concentration of Mn^{2+} was to be greater than 2×10^{-6} M. When L-glutamine was present, this compound was weighed and added to the Tris-KCl buffer with adjustment of the pH to 7.17 before adding $MnCl_2$. For each binding experiment an aliquot of the stock protein (2–6 mg) was placed inside the dialysis tubing, which had been treated as described previously by Hunt et al. (1975), and the protein solution was diluted to 1.0 mL with dialysate before tying the tubing. Generally, the protein was dialyzed against 100 mL of dialysate with magnetic stirring; for low free Mn^{2+} concentrations ($<2 \times 10^{-6}$ M), the volume of dialysate was increased (200–500 mL). After 12–19 h at 4 °C, dialysis was continued for 22–28 h at room temperature. Equilibration was complete in this time, since identical results were obtained after 24 and 48 h at room temperature. In these studies, bacterial growth in dialysates containing glutamine was not a problem (Hunt et al., 1975); surgical gloves for handling the dialysis bags and ultrafiltered water were used.

After equilibration, a 20-μL aliquot of the dialyzed protein solution was removed for enzymatic assay and the contents of

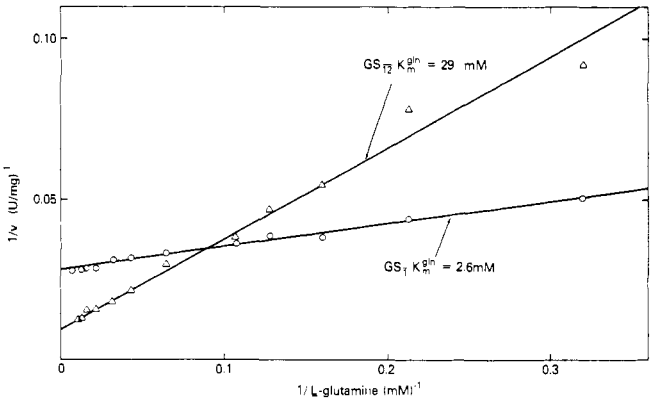

FIGURE 1: Double-reciprocal plots of velocity vs. L-glutamine concentration for adenylylated (Δ) and unadenylylated (O) glutamine synthetase in the Mn^{2+}-supported γ-glutamyl transfer reaction at 30 °C (pH 7.2). Assay conditions were as given in Table I (without variation of a second substrate) with either 0.49 μg of $GS_{\overline{12}}$ (V_{max} = 103 units/mg) or 1.30 μg of $GS_{\overline{1}}$ (V_{max} = 36 units/mg).

each bag were diluted to 10 mL with deionized water after rinsing the dialysis bag with this water. Quantitative recovery of the protein in each case was confirmed spectrophotometrically. The Mn^{2+} concentrations of the freshly diluted protein and of the dialysate were measured by atomic absorption (Hunt et al., 1975).

The saturation of glutamine synthetase with Mn^{2+} (Denton and Ginsburg, 1969; Hunt et al., 1975) is expressed by:

$$\bar{\nu} = (n_1K_1'c)/(1 + K_1'c) + (n_2K_2'c)/(1 + K_2'c) \quad (2)$$

where $\bar{\nu}$ is the experimentally determined number of moles of Mn^{2+} bound per mole of enzyme subunit (50 000g) at a free concentration c of Mn^{2+}, and K_1' and K_2' are the apparent association constants at pH 7.17 (25 °C) for Mn^{2+} binding to the n_1 and n_2 independent subunit sites, respectively. The values of K_1' and K_2' were determined by fitting the experimental values of $\bar{\nu}$ and [Mn^{2+}] with eq 2, setting n_1 and n_2 each at unity and performing an iterative adjustment of K_1' and K_2'.

Results

Kinetics. Double-reciprocal plots for L-glutamine saturation functions in the Mn^{2+}-supported γ-glutamyl transfer reaction are presented in Figure 1 for adenylylated and unadenylylated glutamine synthetase. The assay conditions were chosen to approximate those of the calorimetric studies. The enzyme forms $GS_{\overline{1}}$ and $GS_{\overline{12}}$ have quite different K_m values for L-glutamine; $GS_{\overline{12}}$ has an 11-fold lower apparent affinity for this substrate than has $GS_{\overline{1}}$.

Further kinetic analyses (Table I) were performed because the calorimetric studies presented below show that $GS_{\overline{1}}$ and $GS_{\overline{12}}$ have essentially the same intrinsic binding constant for L-glutamine in the absence of other substrates. Since the mechanism of glutamine synthetase catalysis involves a random binding of substrates and rapid equilibrium (Stadtman and Ginsburg, 1974; Rhee and Chock, 1976; Rhee et al., 1976), K_m values for substrates of this enzyme generally reflect corresponding K_D' values. In order to assess which substrates influence the interaction of the adenylylated enzyme with glutamine, L-glutamine and a second substrate were varied simultaneously in the γ-glutamyl transfer reaction (Dixon and Webb, 1964). The results from these studies are given in Table I. When a second substrate was varied, the K_m value for L-glutamine in Table I is that value extrapolated to zero concentration of the substrate being varied. The extrapolated

TABLE I: Apparent K_m Values for L-Glutamine with Adenylylated Glutamine Synthetase.

Second substrate varied[a]	K_m^{gln}[b] (mM)	Intersect pt in ref to abscissa[b]
None	29	
Arsenate	100	Above
ADP	25[c]	Above
NH_2OH	2.1	Below

[a] Unless a second substrate was varied, γ-glutamyl transfer assays at 30 °C and pH 7.2 (see Methods) contained: 1.0 mM $MnCl_2$, 40 mM NH_2OH, 0.40 mM ADP, 100 mM KCl, 50 mM Tris-HCl, 20 mM potassium arsenate, varying L-glutamine (0–150 mM), and 0.62 μg of $GS_{\overline{12}}$. When a second substrate was varied, saturation curves for L-glutamine (0–60 mM) were determined at 0.5, 1.0, 5.0, and 20.0 mM arsenate or 0.033, 0.046, 0.071, and 0.125 mM ADP or 1.0, 2.0, 5.0, and 10.0 mM NH_2OH. The concentrations of those components not varied were as given above. [b] K_m^{gln} values were obtained from double-reciprocal plots of velocity vs. glutamine concentration. When a second substrate was varied, the K_m^{gln} value was obtained from the intersection point of such double-reciprocal plots at the different arsenate, ADP, or NH_2OH concentrations (Dixon and Webb, 1964). The intersection point occurs at an abscissa value of $-1/K_D'$ for glutamine (i.e., $-1/K_m^{gln}$ extrapolated to 0 concentration of the second substrate varied). Similarly, extrapolating to 0 glutamine concentration, K_m values for arsenate, ADP, and NH_2OH were 2.6, 0.069, and 0.41 mM, respectively. [c] This value should be compared with that obtained from a secondary double-reciprocal plot of V_{max} vs. [glutamine] of these data, which gives K_m^{gln} = 15 mM for a saturating concentration of ADP, rather than with the 29 mM value.

K_m^{gln} values therefore reflect the binding of L-glutamine to the enzyme in the presence of all other substrates except that being varied. In order to express activity, the enzyme subunit must bind two Mn^{2+} ions, ADP, arsenate, NH_2OH, and L-glutamine (Hunt et al., 1975).

In double-reciprocal plots of v vs. [L-glutamine] at different concentrations of a second substrate, the lines either intersected above or below the abscissa (Table I), which indicates synergism or antagonism, respectively, between glutamine and the second substrate being varied. Synergistic effects of ADP and arsenate on the affinity of $GS_{\overline{12}}$ for L-glutamine are indicated. Arsenate has the greater effect on the K_m^{gln} value. Hydroxylamine exerts a marked antagonistic effect on the affinity of $GS_{\overline{12}}$ for L-glutamine. The K_m^{gln} value is 12-fold lower in the absence of hydroxylamine than in its presence. Extrapolating to zero hydroxylamine, the K_m^{gln} value of $GS_{\overline{12}}$ is nearly the same as the K_D^{gln} value measured for $GS_{\overline{1}}$ by calorimetry in the presence of ADP. The analyses of Table I could not be made with $GS_{\overline{1}}$ because the affinity of the unadenylylated enzyme for ADP-Mn and for hydroxylamine under assay conditions is too great.

Calorimetry. Thermal saturation data for the binding of L-glutamine to $GS_{\overline{1}}$ in Tris and in Hepes buffers and for the binding of L-glutamine to $GS_{\overline{12}}$ in Tris buffer are presented in Figure 2. Analogous thermal saturation data for the binding of L-glutamine to $GS_{\overline{1}}$ in the presence of saturating ADP are given in Figure 3.

All calorimetric studies were made in the presence of 1 mM free Mn^{2+} under which conditions two Mn^{2+} binding sites of each enzyme subunit are saturated (Denton and Ginsburg, 1969; Hunt et al., 1975). All experiments involving ADP were performed at ≥97% saturation of the enzyme with ADP (Table III). The stoichiometry of ADP binding is 12 equiv per glutamine synthetase dodecamer (Hunt et al., 1975) and that for L-glutamine is assumed to be the same. The thermal saturation

TABLE II: Thermal Saturation Data for L-Glutamine Binding to Adenylylated Glutamine Synthetase.[a]

Initial vol of protein[b] (mL)	Initial vol of substrate[b] (mL)	Final substrate concn (mM)	Heat obsd (mcal)	Heat of dilution of substrate (mcal)	Heat of reaction[c] (mcal)	Q' (kcal/mol of subunit)
2.00	4.00	30.8	−1.48	+0.41	−1.78	−7.75
4.00	2.00	15.8	−2.67	+0.54	−3.10	−6.75
4.00	2.00	7.9	−2.54	Negl	−2.43	−5.29
4.00	2.00	4.2	−2.06	Negl	−1.95	−4.25
4.00	2.00	1.5	−0.85	Assumed negl	−0.74	−1.6

[a] Microcalorimetric measurements at 30 °C with a buffer of 0.050 M Tris-HCl, 0.10 M KCl, and 0.001 M $MnCl_2$ at pH 7.1. [b] Volumes before mixing in the smaller and larger compartments of the reaction cell. The reference cell contained the same volumes of dialysate. Initial protein concentration was 5.74 mg/mL in each case. [c] Heat of reaction is the observed heat of reaction corrected for heat of dilution of substrate and of protein; the heat of dilution of the protein was −0.11 mcal. The estimated error in each heat of reaction is ±0.17 mcal.

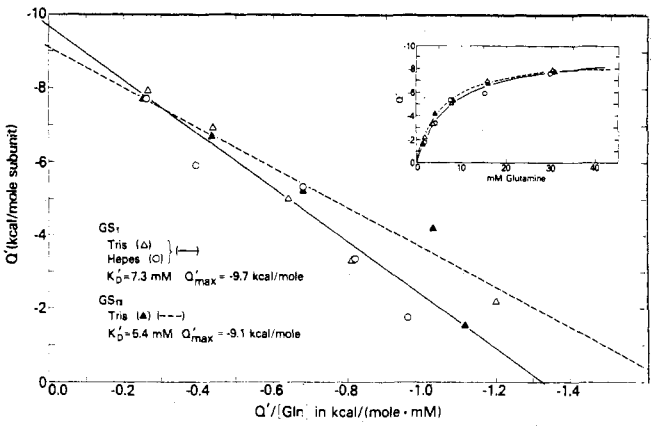

FIGURE 2: Thermal saturation data for the binding of L-glutamine to GS_T and $GS_{\overline{T}}$ in 0.050 M Tris-HCl or 0.050 M Hepes-KOH, 0.10 M KCl, and 0.001 M $MnCl_2$ of pH 7.1 at 30 °C. The data for GS_T in Tris (Δ) and Hepes (O) were fitted simultaneously to the Scatchard equation (—). The data for $GS_{\overline{T}}$ in Tris (▲) were fitted separately to the Scatchard equation (- - -). The inset shows the corresponding saturation curves constructed from the fitted Scatchard equations using the appropriate K_D' and Q_{max}' values.

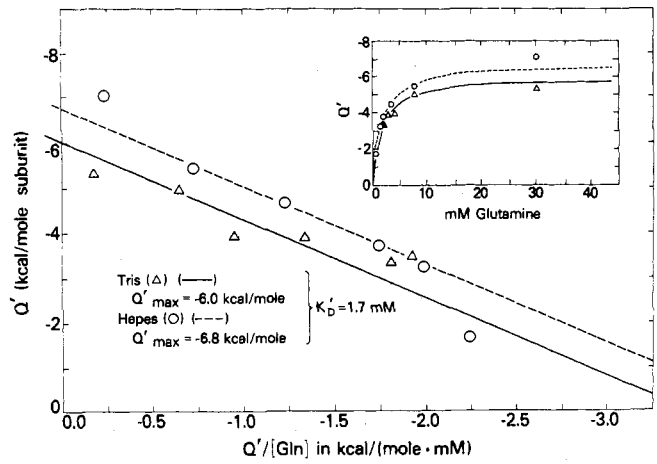

FIGURE 3: Thermal saturation data for the binding of L-glutamine to GS_T in the presence of saturating (0.1 mM free) ADP in 0.050 M Tris-HCl or 0.050 M Hepes-KOH, 0.10 M KCl, and 0.001 M $MnCl_2$ of pH 7.1 at 30 °C. Initially, the Tris (Δ) and Hepes (O) data were fitted separately to the Scatchard equation giving 1.2 and 2.3 mM for the K_D', respectively; the average value (1.7 mM) was used for the K_D'. The Tris and Hepes data were refitted separately to the Scatchard equation fixing K_D' at 1.7 mM and allowing only the Q_{max}' value to vary in each case. Plots of the fitted Scatchard equations and corresponding saturation curves (inset) in Tris (—) and Hepes (- - -) are presented.

data for L-glutamine binding to adenylylated and unadenylylated glutamine synthetase in the absence of ADP (Figure 2) spanned the range of 20–80% enzyme saturation; with saturating ADP present (Figure 3), the data covered the range of 30–90% enzyme saturation with L-glutamine. Representative thermal saturation data for L-glutamine binding to the enzyme are given in Table II; the data are for L-glutamine binding to $GS_{\overline{T}}$ in Tris buffer.

Dissociation constants and Q_{max}' values were obtained by fitting the thermal data (e.g., Table II) with the Scatchard equation:

$$Q' = -K_D'(Q'/[S]) + Q_{max}' \qquad (3)$$

where Q' is the measured heat of binding at a free concentration of substrate [S]. An iterative linear least-squares fitting procedure was used in which the experimental points were weighted according to the estimated error in both Q' and $Q'/[S]$ (Williamson, 1968; York, 1966). The negative slope and y intercept of the fitted equation are the K_D' and Q_{max}' values, respectively. We assumed an error of ±0.2 mcal in the heats of reaction and no error in substrate concentration.

In Figure 2, the thermal saturation data for the binding of L-glutamine to the unadenylylated enzyme (GS_T) in Tris and Hepes buffers were fitted together, since no systematic difference between the data sets was discernible; K_D' = 7.3 mM. The data set with the adenylylated enzyme ($GS_{\overline{T}}$) was fitted separately, yielding K_D' = 5.4 mM. An inspection of Figure 2, however, shows that the thermal saturation data for L-glutamine binding to GS_T and $GS_{\overline{T}}$ are essentially the same; this permits an error estimate of ±1 mM in the K_D' values and ±0.6 kcal/mol in the Q_{max}' values for the binding of L-glutamine to the enzyme. Thus, the equilibrium constants and the heats for binding L-glutamine to each enzyme subunit are essentially the same for the adenylylated and unadenylylated Mn-enzymes.

The calorimetric data for the binding of L-glutamine to the unadenylylated enzyme in Figure 2 were obtained in Tris and Hepes buffers, which differ by −6.3 kcal/mol in their heats of protonation. The heats of binding L-glutamine at the different concentrations of this substrate were the same within experimental error, indicating that no proton release or uptake occurs upon the binding of L-glutamine to the enzyme. This was corroborated in direct ΔpH studies presented below.

Thermal data for the binding of L-glutamine to unadenylylated glutamine synthetase in the presence of saturating ADP are shown in Figure 3. The data sets in Tris and in Hepes buffer were fitted separately due to a systematic trend for the heats measured in Hepes buffer to be slightly more negative than those measured in Tris buffer. This indicates a small

TABLE III: Observed Heats for the Binding of ADP and L-Glutamine to Unadenylylated Glutamine Synthetase at 30 °C.

Substrate added[a]	Final free concn of substrate(s) (mM)	Final enzyme saturation (%)	Obsd heats of binding[b] in different buffers[c] (kcal/mol of subunit) Q' in Tris-HCl	Q' in Hepes-KOH
ADP	0.1	97[d]	−4.9 ± 0.7	−5.3 ± 0.7
L-Glutamine	30	80[e]	−7.9 ± 0.7	−7.7 ± 0.7
ADP and L-glutamine	0.1; 30	99[f]; 95[g]	−10.7 ± 0.7	−12.4 ± 0.7
L-Glutamine	30 (in 0.1 mM ADP)	95[g] (99)[f]	−5.3 ± 0.7	−7.1 ± 0.7
ADP	0.1 (in 30 mM gln)	99[f] (95)[g]	−5.4 ± 0.4	−5.4 ± 0.4

[a] The stoichiometry of ADP binding is 1 mol per mol of subunit (Hunt et al., 1975) and that of glutamine binding is assumed to be the same. [b] The errors are calculated assuming an experimental error of ±0.17 mcal in each of the directly measured heats of reaction (e.g., see Table II). [c] Buffers were either 0.050 M Tris-HCl or 0.050 M Hepes-KOH at pH 7.1 containing 0.10 M KCl and 0.001 M $MnCl_2$. Throughout, 2 equiv of Mn^{2+} was bound per subunit. [d] $K_A' = 3.0 \times 10^5\ M^{-1}$ at 30 °C using $\Delta H'$ to correct the 25 °C value (Hunt et al., 1975). [e] $K_A' = 1.4 \times 10^2\ M^{-1}$ (Figure 2). [f] $K_A' = 1.2 \times 10^6\ M^{-1}$ (from $\Delta G'_{calcd}$ of Figure 6). [g] $K_A' = 5.9 \times 10^2\ M^{-1}$ (Figure 3).

proton uptake during L-glutamine binding. An independent fit of these data sets resulted in two values for K_D' (1.2 mM in Tris and 2.3 mM in Hepes buffer). Since K_D' is independent of buffers that do not interact with the protein, K_D' was assumed to be the average of these two values (1.7 ± 1 mM). The two data sets then were refitted keeping K_D' constant at 1.7 mM and allowing only the Q_{max}' values to vary. The Q_{max}' values were −6.0 ± 0.5 and −6.8 ± 0.6 kcal/mol of subunit in Tris and Hepes buffers, respectively; a comparison of *all* values permits an estimate of error. The difference in Q_{max}' values indicates that, at most, 0.2 equiv of proton uptake occurs upon the binding of L-glutamine to the unadenylylated Mn-enzyme subunit in the presence of saturating ADP.

The linearity in the thermal Scatchard plots of Figures 2 and 3 suggests that no cooperativity occurs in binding L-glutamine to the enzyme. Of the 27 data points in Figures 2 and 3, all except one were within ±0.2 mcal from the line corresponding to the fitted Scatchard equation. The K_D' values of Figures 2 and 3 show that ADP produces a fourfold enhancement in the affinity of the unadenylylated enzyme for L-glutamine. The heats of binding L-glutamine to the enzyme subunit and to the subunit-ADP complex also differ.

The sequential and simultaneous heats of binding of ADP and L-glutamine to unadenylylated glutamine synthetase at constant final free substrate concentrations are in Table III; these are directly measured heats (corrected for heats of dilution) in Tris and Hepes buffers with no correction for the level of enzyme saturation. Where the Q' value in Hepes is more negative than in Tris buffer, a small proton uptake for the binding reaction is indicated.

The data of Table III in Hepes buffer are presented schematically in Figure 4. The overall heats generated upon the addition of substrates sequentially or simultaneously (upper and lower or diagonal path, respectively) to free enzyme forming the Gln·GS·ADP complex are equal within experimental error (see Figure 4); this is also true for the data in Tris buffer of Table III, for which the average overall heat was −11.4 ± 1.7 kcal/mol of subunit. The equality of overall heats illustrated in Figure 4 is a measure of the internal consistency of the data (*see below*). The thermodynamic binding parameters in the presence of 0.10 M KCl and 0.001 M $MnCl_2$ at pH 7.1 and 30 °C for the scheme in Figure 4 are given in Figure 6 below.

ΔpH Studies. Previous titrations of divalent cation-free glutamine synthetase ($GS_{\bar{1}}$), in solutions of low-buffering capacity, with $MnCl_2$ have established that three protons are released upon Mn^{2+} binding to two subunit sites: 2 equiv of H^+

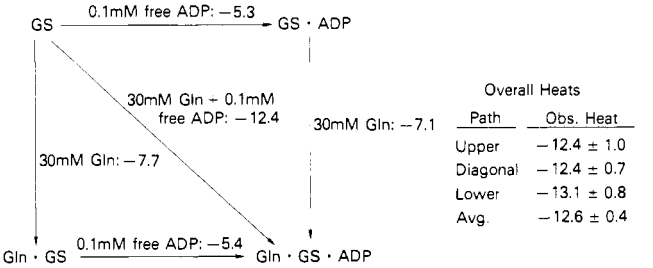

FIGURE 4: The observed heats in kcal/mol of subunit (uncorrected for the level of enzyme saturation) for binding ADP and L-glutamine to $GS_{\bar{1}}$ (sequentially and simultaneously) in 0.050 M Hepes-KOH, 0.10 M KCl, and 0.001 M $MnCl_2$ buffer of pH 7.1 at 30 °C. These data are taken from Table III where final saturation levels of substrates in each reaction are given. The overall heats (GS → Gln·GS·ADP) for the upper, diagonal, and lower paths are given together with the average value of the three paths. The error in each path is the square root of the sum of the squares of errors for the constitutent individual reactions presented in Table III; the error value with the average path is the standard deviation.

are released upon Mn^{2+} binding to a high-affinity (n_1) subunit site and 1 equiv of H^+ is released upon Mn^{2+} binding to a lower affinity (n_2) subunit site (Hunt and Ginsburg, 1972). Later it was found that L-glutamine markedly increased the affinity of n_2 subunit sites of $GS_{\bar{1}}$ for Mn^{2+} (Hunt et al., 1975; Villafranca et al., 1976; *see below*). In the studies of Hunt et al. (1975) the proton release appeared to be considerably decreased during titrations of $GS_{\bar{1}}$ with $MnCl_2$ in the presence of 30 mM L-glutamine (without and with ADP also present). These results suggested that there was a proton uptake upon L-glutamine binding to the enzyme. This conclusion is not corroborated by the calorimetric results presented in Figures 2 and 3. For this reason, some of the ΔpH experiments of Hunt and Ginsburg (1972) and Hunt et al. (1975) were repeated.

In the present studies with divalent cation-free $GS_{\bar{1}}$, the presence of 30 mM L-glutamine during the titration of n_1 and n_2 subunit sites with Mn^{2+} was found to decrease the proton release by at most 0.2 H^+ equiv per subunit. Furthermore, pH measurements for ΔH^+ (*see Methods*) during the binding of L-glutamine (30 mM) to either the native enzyme or freshly tightened enzyme (with 0.1 or 1.0 mM free $MnCl_2$) resulted in 0.2 ± 0.2 equiv of H^+ uptake per subunit in the absence of ADP and 0.2 ± 0.1 equiv of H^+ uptake per subunit in the presence of 0.1 mM free ADP. These results are entirely consistent with those from the calorimetric studies. An explanation for the previous results of Hunt et al. (1975) is uncertain, but the divalent cation-free enzyme is unstable and a partial inactivation of the enzyme causes a decrease in the stoichiometry

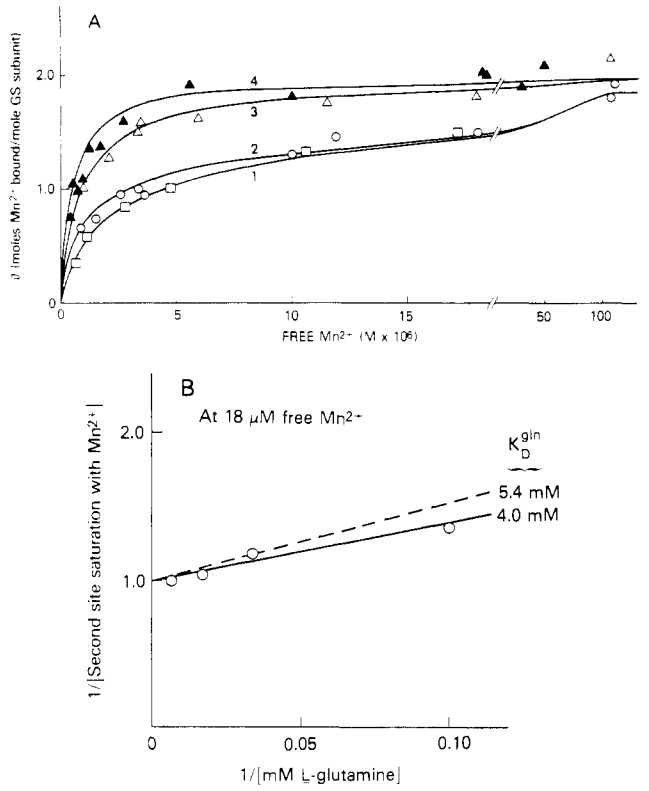

FIGURE 5: Equilibrium binding of Mn^{2+} to glutamine synthetase at pH 7.2 (~25 °C). In Figure 5A, equilibrium dialysis measurements in 0.020 M Tris-HCl and 0.10 M KCl buffer of Mn^{2+} binding to the fully adenylylated enzyme ($GS_{\overline{12}}$) were made without L-glutamine present (○) and with 30 (△) or 150 mM (▲) L-glutamine present. Measurements of Mn^{2+} binding to a partially adenylylated enzyme preparation ($GS_{\overline{4,7}}$) were made without glutamine present (□). The curves are theoretical for Mn^{2+} binding independently to n_1 and n_2 sites of each subunit with apparent association constants of K_1' and K_2', respectively (*see Methods*). *Curve 1* for Mn^{2+} binding to $GS_{\overline{4,7}}$ was constructed from $K_1' = 8.6 \times 10^5\ M^{-1}$ and $K_2' = 6 \times 10^4\ M^{-1}$; *curves 2–4* for Mn^{2+} binding to $GS_{\overline{12}}$ are theoretical for $K_1' = 1.9 \times 10^6\ M^{-1}$ and $K_2' = 6 \times 10^4\ M^{-1}$ (*curve 2*) or $K_2' = 5.9 \times 10^5\ M^{-1}$ (*curve 3*) or $K_2' = 1.9 \times 10^6\ M^{-1}$ (*curve 4*). In Figure 5B, a double-reciprocal plot of the n_2 site saturation of the $GS_{\overline{12}}$ subunit with Mn^{2+} (where $\bar{\nu}_2 = \bar{\nu}_{obsd} - 1.0$) is plotted vs. [L-glutamine] at a constant free Mn^{2+} concentration. Mn^{2+} binding data (○) at 18 μM free Mn^{2+} were obtained with $GS_{\overline{12}}$ at 10, 30, 60, and 150 mM L-glutamine and the K_D^{gln} value is the slope of the solid line fitting these points. The dashed line corresponds to the calorimetrically measured value.

of H^+ release during a titration with Mn^{2+} (Hunt et al., 1972).

Mn^{2+} Binding to Partially and Fully Adenylylated Glutamine Synthetase. Equilibrium measurements for Mn^{2+} binding to $GS_{\overline{4,7}}$ and to $GS_{\overline{12}}$ at pH 7.17 and ~25 °C are shown in Figure 5A; curves 1 and 2, respectively, of Figure 5A were fitted to these data and are theoretical for two noninteracting (independent) Mn^{2+}-binding sites (n_1 and n_2) per enzyme subunit with the K_1' and K_2' stability constants given in the figure legend. The lower affinity n_2 sites of both enzyme preparations have the same apparent association constant (K_2') for Mn^{2+}. However, the K_1' stability constant for the high-affinity n_1 subunit sites of $GS_{\overline{12}}$ was twofold greater than that for the $GS_{\overline{4,7}}$ preparation. This is a significant difference because equilibrium measurements of Mn^{2+} binding to these two enzyme preparations were made at the same time, both preparations were fully active catalytically during the study, and the fully adenylylated enzyme ($GS_{\overline{12}}$) was obtained by enzymatic adenylylation of the $GS_{\overline{4,7}}$ preparation.

Mn^{2+} binding to the fully adenylylated enzyme was measured also in the presence of L-glutamine. As was found previously for the unadenylylated enzyme (Table IV; Hunt et al., 1975; Villafranca et al., 1976), glutamine enhances the affinity of the n_2 sites of $GS_{\overline{12}}$ for Mn^{2+} without affecting the affinity of n_1 subunit sites for Mn^{2+}. In fact, the data for Mn^{2+} binding to $GS_{\overline{12}}$ in the presence of 30 mM L-glutamine could be fit (*curve 3* of Figure 5A) with the K_1' and K_2' stability constants determined by Hunt et al. (1975) for Mn^{2+} binding to $GS_{\overline{1}}$ in the presence of 30 mM L-glutamine. Increasing the glutamine concentration to 150 mM (*curve 4* of Figure 5A) further increased the K_2' stability constant to $K_2' \simeq K_1'$ for Mn^{2+} binding to $GS_{\overline{12}}$.

The binding of Mn^{2+} to the lower affinity n_2 sites of $GS_{\overline{12}}$ was measured as a function of L-glutamine concentration at a fixed level of free Mn^{2+} equal to 18 μM. Under these conditions, the n_1 sites were saturated with Mn^{2+} and $\bar{\nu}_2$ varied from 0.5 to 1.0. The results are shown in Figure 5B as a double-reciprocal plot for which the slope corresponds to $K_D^{gln} = 4 \pm 2$ mM assuming an error in $\bar{\nu}$ of ± 0.1 for Mn^{2+}-binding measurements. This value for the apparent dissociation constant of L-glutamine is in excellent agreement with the K_D' value of 5.4 ± 1 mM obtained by calorimetry (dashed line in Figure 5B).

Discussion

Mn^{2+} Binding. Table IV summarizes apparent association constants K_1' and K_2' for Mn^{2+} binding to different glutamine synthetase preparations at pH 7.0–7.2. From studies of Mn^{2+} binding to $GS_{\overline{2,3}}$ and $GS_{\overline{9}}$ preparations, Denton and Ginsburg (1969) reported that adenylylation decreased the apparent affinity of n_1 sites for Mn^{2+} without affecting the apparent affinity of n_2 subunit sites for Mn^{2+}. The n_1 and n_2 Mn^{2+} subunit binding sites were apparently independent (noninteracting); furthermore, with either enzyme preparation, the 12 high-affinity Mn^{2+}-binding sites per dodecamer were apparently equivalent and independent.

The binding of Mn^{2+} to fully adenylylated glutamine synthetase ($GS_{\overline{12}}$) was measured here to see if adenylylation produced a monotonic decrease in the K_1' stability constant. The fully adenylylated enzyme requires a higher Mn^{2+} concentration for activity expression in the γ-glutamyl transfer reaction than does the unadenylylated enzyme (unpublished data of E. R. Stadtman and P. Z. Smyrniotis); however, this could reflect the affinity difference between unadenylylated and adenylylated subunits for ADP-Mn reacting at n_2 sites (Hunt et al., 1975) rather than an affinity difference for Mn^{2+} reacting at n_1 and n_2 sites.

The fully adenylylated enzyme ($GS_{\overline{12}}$) was found here to have the same K_1' stability constant as that measured previously for unadenylylated enzyme preparations ($GS_{\overline{1}}$ and $GS_{\overline{1,7}}$). Adenylylation of a dodecamer subunit therefore does not affect the affinity of that subunit for Mn^{2+}. In contrast, partially adenylylated enzymes ($GS_{\overline{4,7}}$ and $GS_{\overline{9}}$) have lower K_1' values (Table IV). This suggests that heterologous interactions between adenylylated and unadenylylated subunits in hybrid enzyme molecules (i.e., those containing both types of subunits) decrease the apparent affinity of n_1 subunit sites for Mn^{2+}. The studies of Ciardi et al. (1973) showed that enzymes isolated at intermediate adenylylation states contain hybrid forms. The more pronounced decrease in the K_1' stability constants of the higher, intermediate adenylylation states (Table IV) may reflect a greater proportion of hybrid molecules in these enzyme preparations. In fact, the $GS_{\overline{4,7}}$ preparation appeared to contain a high percentage of unadenylylated dodecamers and, conversely, a low percentage of hybrids in preliminary experiments of J. Davis and E. R. Stadtman (unpublished results).

TABLE IV: Summary of Equilibrium Measurements of Mn^{2+} Binding to Glutamine Synthetase in the Absence or Presence of L-Glutamine.[a]

Enzyme prep	L-Glutamine present (mM)	pH	Temp (°C)	$K_1' \times 10^{-6}$ (M^{-1})	$K_2' \times 10^{-4}$ (M^{-1})	Ref
$GS_{\overline{1}}$	0	7.2	~25	1.9		*b*
	0	7.2	37		2	*c*
	30	7.2	~25	1.9	59	*b*
$GS_{\overline{1.7}}$	0	7.0	25	2	2.2	*d*
	50	7.0	25	2	33	*d*
$GS_{\overline{4.7}}$	0	7.2	~25	0.9	6	*e*
$GS_{\overline{9}}$	0	7	4	0.1	1	*f*
$GS_{\overline{9}\pm\overline{2}}$[g]	0	7	4	0.2		*g*
$GS_{\overline{12}}$	0	7.2	~25	1.9	6	*e*
	30	7.2	~25	1.9	59	*e*
	150	7.2	~25	1.9	190	*e*

[a] All binding measurements were made in buffers containing 0.020 M Tris-HCl (or 0.010 or 0.020 M imidazole hydrochloride) and 0.10 M KCl (without or with L-glutamine) at the pH and temperature indicated; K_1' and K_2' are apparent association constants for Mn^{2+} binding to n_1 and n_2 independent enzyme subunit sites, respectively. [b] Hutn et al. (1975). [c] Hunt and Ginsburg (1972). [d] Villafranca et al. (1976). [e] This paper, Figure 5A. [f] Denton and Ginsburg (1969). [g] Villafranca and Wedler (1974). The state of adenylylation of the enzyme preparation used by Villafranca and Wedler is uncertain because the adenylylation state of glutamine synthetase isolated from *E. coli* W cells grown under the conditions of Woolfolk et al. (1966) varies. Such enzyme preparations usually contain 7–11 mol of covalently bound AMP per mol of enzyme. The fully adenylylated enzyme is obtained only by enzymatic adenylylation in vitro.

In the absence of L-glutamine, the K_2' stability constants for the binding of Mn^{2+} to n_2 subunit sites of the different enzyme preparations (Table IV) are about the same. However, the K_2' values for Mn^{2+} binding to n_2 sites of $GS_{\overline{4.7}}$ and $GS_{\overline{12}}$ are somewhat higher than K_2' values measured previously for $GS_{\overline{1}}$, $GS_{\overline{1.7}}$, and $GS_{\overline{9}}$. This could be due to temperature and pH differences between studies.

As shown also in Table IV for unadenylylated and adenylylated enzymes, L-glutamine has the capacity to increase the apparent association constant for Mn^{2+} binding to n_2 subunit sites (K_2') without changing the apparent affinity of n_1 sites for Mn^{2+}. With $GS_{\overline{12}}$, a saturating concentration of L-glutamine increases the K_2' value 32-fold so that $K_2' \simeq K_1'$. The influence of L-glutamine on enhancing the affinity of n_2 subunit sites for Mn^{2+} is proportional to the binding of L-glutamine to the enzyme subunits (Figure 5B).

Villafranca et al. (1976) have studied proton relaxation rates (PRR) of water with Mn^{2+} ions bound at the n_1 and n_2 subunit sites of $GS_{\overline{1.7}}$. From these data, they determined that two rapidly exchanging water molecules interact with each bound Mn^{2+}. With Mn^{2+} bound at both n_1 and n_2 subunit sites of $GS_{\overline{1.7}}$, L-glutamine (50 mM) or ADP (0.25 mM) decreased from 4 to ~2 the number of rapidly exchanging water molecules in the primary coordination shells of bound Mn^{2+}. As stated by Villafranca and Wedler (1974), a decrease in the number of rapidly exchanging water molecules from the primary coordination sphere of bound Mn^{2+} can arise either from displacement of a water ligand by a substrate or protein ligand or by restriction of a water ligand on the bound Mn^{2+} so that its exchange is slow compared with the other water molecules. Since Mn^{2+} can bind to n_2 subunit sites as the ADP-Mn complex (Hunt et al., 1975), the PRR results with ADP present are consistent with substrate substitution into the coordination sphere of Mn^{2+} bound at n_2 sites. The fact that L-glutamine does not influence K_1' while having the capacity to increase the magnitude of K_2' to that of K_1' suggests that the effect of L-glutamine in the PRR experiments of Villafranca et al. (1976) is to decrease the number of rapidly exchanging water molecules of Mn^{2+} bound at the n_2 sites.

The PRR studies of Villafranca and Wedler (1974) on glutamine synthetase at a high intermediate adenylylation state (Table IV) were performed with 0.1 mM free Mn^{2+} under which conditions 100% of n_1 sites and 50–67% of the n_2 sites were occupied by Mn^{2+}. Under these conditions, three rapidly exchanging water molecules were found for the protein-bound Mn^{2+}; L-glutamate decreased this number to two. If L-glutamate (like L-glutamine) enhances the affinity of n_2 sites for Mn^{2+}, the PRR results of Villafranca and Wedler (1974) with the partially adenylylated enzyme (correcting for saturation of the n_2 sites with Mn^{2+}) are the same as those with the unadenylylated enzyme (Villafranca et al., 1976). These enzyme preparations had quite different K_1' stability constants for Mn^{2+} (Table IV), but apparently the same number of rapidly exchanging water molecules at the n_1 Mn^{2+} sites. Thus, the free energies of binding Mn^{2+} do not correlate with the number of rapidly exchanging water molecules because the K_1' stability constant derives in part from the free energy of protein ligand substitution into the coordination sphere of Mn^{2+} and also in part from the free energy of the conformational change induced by Mn^{2+} binding to n_1 sites (Denton and Ginsburg, 1969; Ginsburg, 1972).

Calorimetry. Calorimetric studies (Figures 2 and 3) of the binding of L-glutamine to unadenylylated and to adenylylated glutamine synthetase demonstrate that accurate binding constants can be obtained from thermal saturation data for the binding of low-affinity ligands to proteins. Any concomitant proton uptake or release on binding either L-glutamine or ADP to the unadenylylated enzyme was measured using two *noninteracting* buffers with different heats of protonation. Essentially no proton uptake or release attends the binding of L-glutamine to $GS_{\overline{1}}$ in the absence of ADP and at most an uptake of 0.2 H^+ equiv per subunit occurs in the presence of saturating ADP. This result negates a previous prediction of Hunt et al. (1975) that a substantial proton uptake occurs upon binding L-glutamine to the enzyme. Saturation of the unadenylylated enzyme with ADP enhances the affinity of each subunit for L-glutamine fourfold. Figures 2 and 3 also show that the heats for binding L-glutamine to the free enzyme and to the enzyme-ADP complex differ. These calorimetric data indicate that ADP induces a conformational change in the enzyme subunit structure that affects the affinity for L-glutamine, slightly perturbs an ionizable group, and alters the

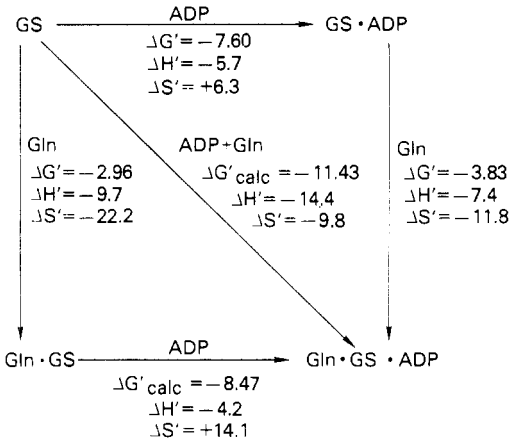

FIGURE 6: Thermodynamic parameters for the binding of ADP and L-glutamine (sequentially or simultaneously) to unadenylylated Mn-enzyme at pH 7.1 and 30 °C. The $\Delta G'$ and $\Delta H'$ values are expressed in kcal/mol of subunit and the $\Delta S'$ values are given in cal/(deg·mol of subunit). For free-energy values ($\Delta G'$), the standard state for hydrogen-ion activity is $10^{-7.1}$ M. The $\Delta G'$ values for glutamine binding in the absence and presence of saturating ADP are calculated from the corresponding K_D' values derived from fitting the Scatchard equation (see Figures 2 and 3). The value for $\Delta G'$ for ADP binding to $GS_{\overline{1}}$ at 30 °C is determined from $K_A' = 3.5 \times 10^5\ M^{-1}$ at 25 °C (Hunt et al., 1975) and the measured $\Delta H'$. The calculated free energies of binding ($\Delta G'_{calcd}$) are computed from the measured $\Delta G'$ values in the figure. The enthalpy values ($\Delta H'$) are corrected for any heat due to proton uptake occurring upon substrate binding (i.e., $\Delta H'$ is the enthalpy of binding in a buffer with zero heat of protonation); these values are computed from the enthalpies in Tris and Hepes ($\Delta H'_{Tris}$ and $\Delta H'_{Hepes}$, respectively). The enthalpies of binding glutamine in the absence and presence of saturating ADP in the two buffers are the corresponding Q_{max}' values in Figures 2 and 3, respectively. The enthalpy of binding ($\Delta H'_{Tris}$ or $\Delta H'_{Hepes}$) of ADP in the absence of glutamine is the corresponding observed heat of binding (Table III) corrected for enzyme saturation. The overall heats of binding (GS → Gln·GS·ADP) in Tris or in Hepes (at 30 mM glutamine and 0.1 mM free ADP) for the diagonal and lower paths are corrected for saturation by multiplying by the ratio of the overall enthalpy ($\Delta H'_{Tris}$ or $\Delta H'_{Hepes}$) for the upper path to the overall heat of binding for the upper path (at 30 mM glutamine and 0.1 mM free ADP) in Tris or Hepes. The enthalpy of binding ($\Delta H'_{Tris}$ or $\Delta H'_{Hepes}$) of ADP in the presence of saturating glutamine is the corresponding overall enthalpy for the lower path minus the corresponding enthalpy of binding glutamine in the absence of ADP. The entropy of binding for each reaction is computed from the relationship: $\Delta S' = (\Delta H' - \Delta G')/T$, where T is the absolute temperature (303 K).

enthalpy of binding L-glutamine (see Figure 6). Calorimetry therefore has proven a useful technique for obtaining thermodynamic parameters for weak enzyme–ligand interactions.

In addition, thermal saturation data for the binding of L-glutamine to the fully adenylylated enzyme ($GS_{\overline{12}}$) were obtained because $GS_{\overline{12}}$ has an 11-fold higher K_m^{gln} in the γ-glutamyl transfer reaction than has $GS_{\overline{1}}$ (Figure 1). From calorimetric measurements in the absence of other substrates, the dissociation constants for L-glutamine binding to $GS_{\overline{1}}$ and to $GS_{\overline{12}}$ were essentially the same; $K_D^{gln} = 7.3 \pm 1$ and 5.4 ± 1 mM, respectively. Kinetic analyses using a two-substrate variation technique (Dixon and Webb, 1964) then were performed in order to establish which substrates influence the affinity of $GS_{\overline{12}}$ for L-glutamine under assay conditions. Synergistic effects between L-glutamine and arsenate and between L-glutamine and ADP and a strongly antagonistic effect between L-glutamine and hydroxylamine were found (Table I). In the case of the unadenylylated enzyme, the apparent agreement between the K_D^{gln} value in the presence of ADP determined calorimetrically and the K_m^{gln} value is probably coincidental.

Heat of reaction was investigated as a potential probe for studying separateness of binding sites for various effectors. We chose the L-glutamine–ADP case as a model because these two substrates have separate binding sites and other pertinent data are available. Equilibrium dialysis studies of the binding of ADP to unadenylylated glutamine synthetase in the absence and presence of 30 mM L-glutamine (Hunt et al., 1975) showed that 0.1 mM free ADP is saturating. Any heat produced upon the addition of 30 mM L-glutamine to the enzyme already saturated with ADP must derive from the binding of L-glutamine to a site separate from that for ADP. This experiment performed in Tris or Hepes buffer liberated heat (Table III). Furthermore, simultaneous or sequential (either order) addition of ligands should result in the liberation of the same overall observed heat (i.e., uncorrected for enzyme saturation) in forming the ternary complex from the free, unliganded enzyme (see Figure 4), assuming that final concentrations of ligands are kept constant and that the kinetics of binding are rapid relative to the time scale of the calorimeter experiment and that binding is either random or ordered. However, equality of overall heats via the three possible paths shown in Figure 4 is not sufficient for showing separateness of sites, since ligands competing for the same site also should evolve equal overall heats under the same conditions; a mixed case also predicts equal overall heats. Nevertheless, demonstrating equality of overall heats is important because it is a measure of the internal consistency of the data.

In a previous study establishing separateness of sites, Ross and Ginsburg (1969) measured the individual and simultaneous heats of binding of two feedback inhibitors at saturating concentrations to glutamine synthetase. This approach can be used when all heats of binding are the same sign; for separate sites, the absolute value of the heat for the simultaneous binding reaction will exceed the absolute value of the enthalpy of binding each ligand alone.

All the thermodynamic parameters for the binding reactions leading to the formation of the Gln·GS·ADP complex are given in Figure 6. The free-energy change for the diagonal path and that for the second reaction in the lower path are calculated assuming that the overall free-energy change is the same via all three paths. The overall free-energy change is highly exergonic and those for the constituent reactions are exergonic. The two experimental $\Delta G'$ values for the binding of L-glutamine in the absence and presence of ADP reflect the synergistic effect. The equilibrium dialysis data for ADP binding to unadenylylated glutamine synthetase in the absence and presence of 30 mM L-glutamine were approximately the same (Hunt et al., 1975); however, the error of these measurements is sufficiently large to accommodate the calculated free energy of binding ADP in the presence of L-glutamine (assuming the thermodynamically required reciprocal synergistic effect). The enthalpies of Figure 6 are all from measurements made in this study so that overall values, although equal within experimental error, are slightly different; this leads to slightly different overall values for the entropy also. The enthalpies of binding have been corrected for any small proton uptake in the different reactions. All the binding reactions of Figure 6 are considerably exothermic. For both substrates, the enthalpy of binding one becomes less negative in the presence of the other; this further attests to interactions between the ADP and L-glutamine binding sites. The overall entropy of binding is negative, whereas the entropies of binding for ADP are positive and those for L-glutamine are negative; the following species are listed in sequence from the most ordered to the most disordered state: Gln·GS, Gln·GS·ADP, GS, and GS·ADP. Thus, the ternary complex is more ordered than the free, unliganded enzyme, suggesting that a disruption in solvent or ligand

structure (protein and/or water ligands) on binding ADP is overcome by the ordering effects of L-glutamine binding. With both substrates, the entropy of binding one becomes more positive in the presence of the other. In each case, the increased entropy not only compensates for the corresponding less negative enthalpy but dominates, giving rise to a more negative free energy (synergistic effect).

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