Thermodynamics of Active-Site Ligand Binding to *Escherichia coli* Glutamine Synthetase†

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ABSTRACT: Active-site ligand interactions with dodecameric glutamine synthetase from *Escherichia coli* have been studied by calorimetry and fluorometry using the nonhydrolyzable ATP analogue 5′-adenylyl imidodiphosphate (AMP-PNP), L-glutamate, L-Met-(*S*)-sulfoximine, and the transition-state analogue L-Met-(*S*)-sulfoximine phosphate. Measurements were made with the unadenylylated enzyme at pH 7.1 in the presence of 100 mM KCl and 1.0 mM $MnCl_2$, under which conditions the two catalytically essential metal ion sites per subunit are occupied and the stoichiometry of active-site ligand binding is equal to 1.0 equiv/subunit. Thermodynamic linkage functions indicate that there is strong synergism between the binding of AMP-PNP and L-Met-(*S*)-sulfoximine ($\delta\Delta G' = -6.4$ kJ/mol). In contrast, there is a small antagonistic effect between the binding of AMP-PNP and L-glutamate ($\delta\Delta G' = +1.4$ kJ/mol). Proton effects were negligible (≤ 0.2 equiv of H^+ release or uptake/mol) for the different binding reactions. The binding of AMP-PNP (or ATP) to the enzyme is entropically controlled at 303 K with $\Delta H = +5.4$ kJ/mol and $\Delta S = +150$ J/(K·mol). At 303 K, the binding of L-glutamate ($\Delta H = -22.2$ kJ/mol) or L-Met-(*S*)-sulfoximine [$\Delta H = -45.6$ kJ/mol with $\Delta C_p \simeq -670 \pm 420$ J/(K·mol)] to the AMP-PNP·Mn·enzyme complex is enthalpically controlled with opposing ΔS values of −29 or −46 J/(K·mol), respectively. The overall enthalpy change is *negative* and the overall entropy change is *positive* for the simultaneous binding of AMP-PNP and L-glutamate or of AMP-PNP and L-Met-(*S*)-sulfoximine to the enzyme. For the binding of the transition-state analogue L-Met-(*S*)-sulfoximine phosphate (which inactivates the enzyme by blocking active sites), both enthalpic and entropic contributions also are favorable at 303 K [$\Delta G' \simeq -109$ and $\Delta H = -54.8$ kJ/mol of subunit and $\Delta S \simeq +180$ J/(K·mol)].

Microcalorimetry has been used to obtain reliable thermodynamic information on protein–ligand interactions (Barisas & Gill, 1978; Biltonen & Langerman, 1979; Ross & Subramanian, 1981; Hinz, 1983; Chen & Wadsö, 1982). For *Escherichia coli* glutamine synthetase, calorimetry has been used to assess proton effects in metal ion and substrate binding and to establish separateness of binding sites for feedback inhibitors and substrates (Hunt et al., 1972; Shrake et al., 1977, 1978; Ross & Ginsburg, 1969; Gorman & Ginsburg, 1982). This paper presents thermodynamic parameters for the interactions of glutamine synthetase with active-site ligands obtained by using the nonhydrolyzable ATP analogue AMP-PNP,[1] L-glutamate, L-Met-(*S*)-sulfoximine (an analogue of L-glutamate), and the transition-state analogue L-Met-(*S*)-sulfoximine phosphate. The energetics of active-site ligand binding to this enzyme involve contributions from both ligand–protein and protein–protein interactions.

Glutamine synthetase forms an extremely tight, inactive complex with L-Met-(*S*)-sulfoximine phosphate, ADP, and 2 Mn^{2+} bound per subunit (Weishrod & Meister, 1973; Maurizi & Ginsburg, 1982a). Formation of this transition-state complex occurs by the enzymatic transfer of the γ-phosphoryl group of ATP to the imino nitrogen of L-Met-(*S*)-sulfoximine (Meister, 1974; Manning et al., 1969). Disruption of this inactive complex with recovery of active enzyme requires protonation of protein carboxylate groups and structural perturbations produced by high KCl concentrations and increased temperature (Maurizi & Ginsburg, 1982a).

An atomic model at 3.5-Å resolution of dodecameric glutamine synthetase from *Salmonella typhimurium* (which has a structure closely homologous to that of the *E. coli* enzyme) is now available from sophisticated X-ray crystallographic analysis in Eisenberg's laboratory (Almassy et al., 1986). An unusual feature of the enzyme structure is that the 12 active sites are formed at heterologous interfaces between subunits arranged within a hexagonal ring of two face-to-face eclipsed rings (Almassy et al., 1986). The 2 Mn^{2+} at each active site (Hunt et al., 1975; Hunt & Ginsburg, 1980) are in the subunit C-terminal domain (Almassy et al., 1986), whereas the nucleotide binding site is in the N-terminal region (Pinkofsky et al., 1984) of an adjacent subunit. It is possible that this type of highly cooperative protein–protein interaction to form active sites evolved because the regulation of glutamine synthetase activity, which has a central role in bacterial nitrogen metabolism (Stadtman & Ginsburg, 1974), is of paramount importance.

Previous results obtained in this laboratory are entirely consistent with the molecular model for glutamine synthetase proposed by Almassy et al. (1986). Binding of ligands to active

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[1] Abbreviations: AMP-PNP, 5′-adenylyl imidodiphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; GS, glutamine synthetase; MetSox, L-Met-(*S*)-sulfoximine (L-*S* diastereoisomer of L-methionine *S*-oxide *S*-imide); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; P_i, inorganic phosphate.

sites of the enzyme was found to enormously stabilize quaternary structures of glutamine synthetase (Maurizi & Ginsburg, 1982b, 1985). After partial autoinactivation of the enzyme with Mn^{2+}, L-Met-(*S*)-sulfoximine, and ATP (Maurizi & Ginsburg, 1982a), stable oligomers containing 4, 6, 8, and 10 subunits could be formed by cleavage of intra-ring noncovalent contacts across both hexagonal rings (Haschemeyer et al., 1982). Intra-ring heterologous subunit interactions were found to be stabilized by active-site ligand binding, and heterologous bonding between subunits within the same ring appeared to require the integrity of the isologous bonding domains with opposing subunits since no oligomeric structures with an odd number of subunits were detected. The strong linkage between active-site ligand binding and intersubunit bonding strength can be more readily understood on the basis of the location of active sites at heterologous contacts, since active sites of glutamine synthetase are widely separated. Almassy et al. (1986) give ~45 Å for the distance between Mn^{2+} ions at neighboring active sites within the same ring of 6 subunits and ~52 Å for the smallest separation of active-site metal ions in opposite rings of the dodecamer. These distances are shorter than those estimated by fluorescence energy transfer: nucleotide probes at active sites within the same hexagonal ring were 56–60 Å apart, whereas the nearest ATP probes in opposite rings were 60–78 Å (Maurizi et al., 1986a). Apparently, the adenosine moiety of each ATP molecule binds more toward the exterior surface of the enzyme than do the Mn^{2+} ions.

Materials and Methods

The preparations of *E. coli* glutamine synthetase that were used in these studies were unadenylylated with an average of 0.8 equiv of covalently bound 5′-adenylate groups per dodecamer. The enzyme had a specific activity of 135 ± 10 units/mg (Maurizi & Ginsburg, 1986) in the γ-glutamyl transfer assay at pH 7.57 and 37 °C (Stadtman et al., 1979), which was routinely used to measure GS activity before and after calorimetric experiments. The enzyme was stored at 4 °C as a suspension in 50% $(NH_4)_2SO_4$ containing 5 mM $MnCl_2$ and 5 mM imidazole; protein was collected as needed by centrifugation and dialyzed vs. three changes over 36 h of 1000-fold volumes of buffer, pH 7.1 (at 25 or 30 °C), containing 100 mM KCl, 1.0 mM $MnCl_2$, and 20 mM Hepes/KOH, 20 mM Pipes/KOH, or 20 mM Tris-HCl. Protein concentrations were determined from published absorption coefficients (Ginsburg et al., 1970), and a subunit M_r of 50000 was used in calculating the equivalents of active sites.

L-Met-(*S*)-sulfoximine was a gift from F. C. Wedler and Y. Sugiyama from the Pennsylvania State University, and solutions of this compound were stored at 4 °C in the desired buffer. Concentrations of L-Met-(*S*)-sulfoximine were determined by the modified ninhydrin colorimetric analysis of Rosen (1957) and by glutamine synthetase inactivation as described by Shrake et al. (1982). L-Met-(*S*)-sulfoximine phosphate was prepared and stored frozen and then repurified, the pH was adjusted to pH 7.1 at 30 °C, and it was assayed as described by Maurizi et al. (1986b) just prior to calorimetric measurements. L-Glutamic acid (Sigma) was freed of di- and trivalent metal ions by passage through a column of Chelex 100 (100–200 mesh from Bio-Rad) in the K^+ form (Hunt & Ginsburg, 1980), recrystallized from ethanol–H_2O, and stored as frozen 1 M stock solutions in H_2O at pH 7; just prior to calorimetric experiments, pH adjustments of L-glutamate solutions were made with KOH so that dilutions (~22-fold) with final dialysates of the protein produced no pH change at 30 °C. AMP-PNP was obtained as the tetralithium salt from Boehringer-Mannheim GmB and was determined to be >95% pure by phosphate analysis (Rockstein & Herron, 1951) before and after hydrolysis in 0.9 N HCl at 100 °C for 10 min, by UV absorbance with $\epsilon_m = 15.4 \times 10^3$ M^{-1} cm^{-1} at 259 nm at pH 7, and by ascending thin-layer chromatography on poly(ethylenimin)–cellulose (PEI–cellulose) sheets (Polygram gel 300 PEI from Brinkman) with KH_2PO_4 at pH 3.4 as the solvent (Cashel et al., 1969). Solutions of AMP-PNP in H_2O (~12 mM) for calorimetry were stored frozen for <1 month after pH adjustment to pH 7.0 with HCl. There was no contamination of AMP-PNP with ATP since prolonged incubations of glutamine synthetase with AMP-PNP plus L-Met-(*S*)-sulfoximine produced no inactivation of the enzyme. Water was distilled and then deionized and filtered through a Millipore Q2 reagent grade system. All other chemicals were analytical reagent grade.

L-Met-(*S*)-sulfoximine binding determinations utilized the Amicon MPS-1 micropartiaion system with YMT membranes that had been thoroughly washed with water and then buffer. Protein solutions (4.4–8.8 μM subunit concentrations) were equilibrated with 0.1 mM free AMP-PNP and varying concentrations of L-Met-(*S*)-sulfoximine (12–71 μM) in 20 mM Hepes/KOH, 100 mM KCl, and 1.0 mM $MnCl_2$, pH 7.2, for ~2 h at 26 °C and then centrifuged in a swinging bucket Sorvall GLC centrifuge until ~60–70% of the solution had passed through the membrane. After ultrafiltration, filtrates were determined to be protein free and the L-Met-(*S*)-sulfoximine concentrations were measured by quantitative inactivation of ~50% of available GS catalytic sites in the presence of excess ATP (2 mM) in separate incubations (Shrake et al., 1982) for 5 h at 26 °C and 24 h at 4 °C to ensure that the autoinactivation reaction had gone to completion (Maurizi & Ginsburg, 1986). Measurements of L-Met-(*S*)-sulfoximine concentrations were ±10% of theoretical values in control filtrates which had been incubated under the same conditions without protein present.

Nucleotide binding was determined by measuring the enhancement of enzyme tryptophanyl fluorescence upon addition of AMP-PNP or ATP to solutions of GS with 0.69 or 2.29 μM subunit concentration, respectively. Measurements were made at constant temperature (20, 25, 30, 35, or 41 °C) with ~1.1-mL solutions of enzyme in standard 3-mL fluorescence cuvettes; excitation was at 300 nm while emission at 350 nm was monitored. Nucleotides were added in 1-μL aliquots (with a Hamilton syringe dispenser), and measurements were corrected for dilution effects. Hill plots were constructed assuming that the fractional change in protein fluorescence was proportional to nucleotide binding.

Instrumentation. Ultraviolet absorption measurements and spectra were recorded with a Perkin-Elmer 320 spectrophotometer equipped with a thermostable cell holder. Fluorescence measurements were made with a Perkin-Elmer 650-40 spectrofluorometer with water-jacketed cuvette holders. Temperature of solutions was monitored with a YSI (Yellow Spring Instrument Co., Inc.) thermistor probe attached to a YSI telethermometer.

Calorimetric Measurements. Unless otherwise indicated, heats of ligand binding were measured by using an LKB batch microcalorimeter, Model 10700, equipped with 18-carat gold cells[2] and a Keithley Model 147 nanovolt detector set on the 10-μV scale. Calibrations for the calorimeter and separate measurements of the heats of reaction and the heats of dilution of ligand or of protein were as described by Shrake et al.

[2] The presently installed gold cells are replacements for those that developed a reaction with Tris buffers (Gorman & Ginsburg, 1982).

(1977). The electrical calibration heaters of the instrument were determined to be within <±5% of the chemical heat generated by diluting sucrose (Gucker et al., 1939). The thermopiles of the batch calorimeter used were well matched, as determined by generating 2–8 mcal in both cells by dilution of sucrose and measuring at net heat of <0.1 mcal.[3]

The LKB Model 2107-350 microtitrator was later attached to the LKB batch microcalorimeter. A complete description of this microtitrator unit and detailed procedures for its use and calibration are given by Chen and Wadsö (1982). The LKB T-valves inserted between the syringes and flow lines (Teflon tubing) developed leaks with time and were replaced with Omnifit flangeless valves (Omnifit, Ltd.). After the syringes and flow lines were filled with solutions, the tapered ends of the tubing were placed in a sealed T of a short segment of silicon tubing on top of the block during temperature equilibration and also when applicable during an initial batch-type experiment. Just prior to the beginning of a titration experiment, 5-μL amounts were dispensed to make certain that flow lines were completely filled with solution, and then the Teflon tubes (in tightly fitting short sleeves of silicon tubing) were inserted into the large compartments of the sample and reference cells. Stoppers then were placed on the small sides of the cell compartments. The duration (approximately 18, 22, 27, 38, or 45 min) between additions (5–25 μL) from 0.50-mL Hamilton syringes and the volume injected per cycle were controlled by the LKB programmer of the microtitrator. The differential calorimetric signal was amplified by use of a Keithley Model 147 nanovolt null detector which was connected to a strip chart recorder for monitoring reactions and also interfaced to an IBM PC.

Software was written by M.B.B. to acquire output voltage–time data at the rate of 3 readings/s which could be later integrated numerically by the trapezoidal method. At the end of a set of injections (or of a batch-type mixing experiment), two mixes were made at 10-min intervals to correct for frictional heat (≤0.03 mcal). Integration limits were minimized, and in cases in which a base-line shift occurred after mixing, the following base line was used. Electrical calibrations were run separately (with and without solutions in the calorimeter cells). By use of the microtitrator and the procedure of Chen and Wadsö (1982), the heat of dilution of sucrose was within 3% of the calculated value of Gucker et al. (1939) and the heat of protonation of Hepes buffer at 30 °C was −5.1 ± 0.1 kcal/mol, which is in good agreement with the value of Beres and Sturtevant (1971) after correction for the temperature difference between measurements (Roy et al., 1980), −5.0 kcal/mol.

A calorimetric titration experiment also was performed by E.G.G. in the laboratory of Dr. Neal Langerman using his modified Tronac Model 450 isoperibol calorimeter (Beaudette & Langerman, 1978).

Results

Active-Site Ligand Binding. In order to know the fractional saturation of the enzyme at a given ligand concenration for calorimetric measurements and to calculate $\Delta G'$ values, the necessary binding constants were determined (Table I). Throughout these studies, buffers at pH 7.1–7.2 contained 1.0 mM $MnCl_2$ under which conditions the two essential divalent cation sites at each subunit active site are occupied by Mn^{2+} (Hunt et al., 1975).

[3] In order to more easily compare the thermodynamic parameters of this paper with those previously measured, units of calories are used; for conversion to the International System of units (SI), 1 cal = 4.184 J.

Table I: Affinity Constants at 303 K for Glutamine Synthetase Binding Active-Site Ligands in the Presence of 1.0 mM $MnCl_2$ and 100 mM KCl, pH 7.1

binding reaction (Mn·GS ≡ enzyme subunit)	K_A' (M^{-1})
Mn·GS + AMP-PNP	$(8.70 \pm 2) \times 10^{6}$ [a]
Mn·GS·AMP-PNP + MetSox	$(3.0_3 \bullet 1) \times 10^{5}$ [b]
Mn·GS + MetSox	$2.3_2 \times 10^{4}$ [c]
Mn·GS·MetSox + AMP-PNP	$1.1_3 \times 10^{8}$ [d]
Mn·GS + ATP	$(1.0_7 \bullet 0.2) \times 10^{6}$ [a]
Mn·GS·AMP-PNP + Glu	$1.9_1 \times 10^{2}$ [d]
Mn·GS + Glu	$3.3_3 \times 10^{2}$ [e]
Mn·GS·Glu + AMP-PNP	5.0×10^{6} [f]

[a] At least two sets of fluorescence titration data at 30 °C (see Figure 2, for example) were used to calculate K_A' values, and in addition, linear fits of ln K vs. $1/T$ plots of titration data obtained at 20, 25, 30, 35, and 41 °C were used for error estimates. [b] The average of nine direct binding determinations at 26 °C with 3.7–10 μM = [free L-Met-(*S*)-sulfoximine] in the presence of saturating (0.1 mM free) AMP-PNP gave $(3.86 \pm 0.95) \times 10^5$ M^{-1} for K_A'; this K_A' value then was corrected to 30 °C by using the ΔH value obtained at 303 K (Table II) and eq 1. [c] From Gorman and Ginsburg (1982). [d] The value of K_A' was calculated from the reciprocal relationship $K_1K_2 = K_3K_4$ (Wyman, 1970) for the sequential binding of two ligands by two pathways. [e] From Villafranca et al. (1975). [f] From Figure 2.

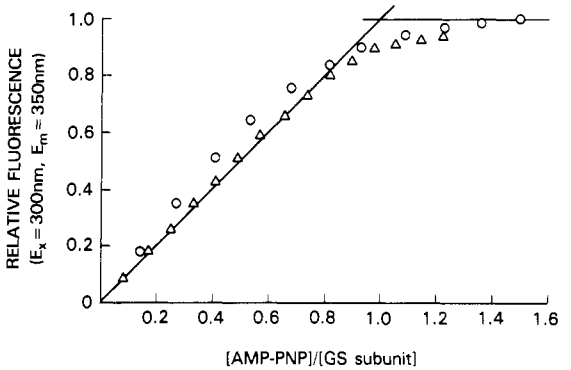

FIGURE 1: Fluorescence titrations of glutamine synthetase with AMP-PNP at 25 °C in 20 mM Hepes/KOH, 100 mM KCl, and 1.0 mM $MnCl_2$, pH 7.2, buffer. Glutamine synthetase subunit concentrations were 13.0 (△) and 2.3 μM (○) in the absence and presence of 2.3 μM L-Met-(*S*)-sulfoximine, respectively. Enzyme solutions (1.1 mL) were titrated with 0–15 μL of AMP-PNP, and the fluorescence enhancement at 350-nm emission (with excitation at 300 nm) was measured as a function of the molar ratio of AMP-PNP to enzyme subunit.

Figure 1 shows fluorescence titrations of the Mn·enzyme complex (at relatively high concentration) with the nonhydrolyzable ATP analogue AMP-PNP in the absence and presence of L-Met-(*S*)-sulfoximine. The binding of AMP-PNP to manganese glutamine synthetase is quite tight, and a stoichiometry of 1 equiv of AMP-PNP per enzyme subunit is indicated. A 2.5-fold enhancement of the protein tryptophanyl residue fluorescence occurs on binding AMP-PNP or ATP to the Mn·enzyme. The fluorescence enhancement produced by saturating L-Met-(*S*)-sulfoximine is ~65% with either Mg·enzyme (Rhee et al., 1981) or Mn·enzyme but ≤3% when added to the Mn·AMP-PNP·enzyme complex. When the enzyme was titrated with AMP-PNP in the presence of L-Met-(*S*)-sulfoximine (equimolar to the subunit concentration), the fluorescence changes indicated cooperative interactions between these two ligands. Since the affinity of the enzyme for L-Met-(*S*)-sulfoximine in the absence of AMP-PNP is 2.8×10^4 M^{-1} at 24 °C (Shrake et al., 1982), only ~6% of the sites will be filled with this ligand in the absence of AMP-PNP under the conditions of Figure 1. The fluorescence changes produced by AMP-PNP added to 2.3 μM enzyme subunit in the presence of 2.3 μM L-Met-(*S*)-

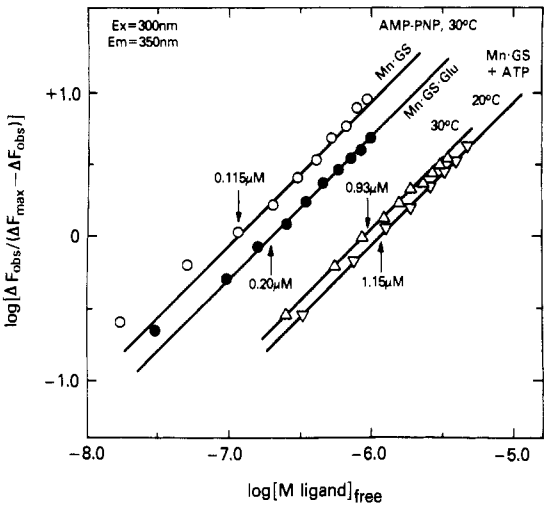

FIGURE 2: Hill plots of fluorescence titrations of glutamine synthetase with AMP-PNP and with ATP in 20 mM Hepes/KOH, 100 mM KCl, and 1.0 mM $MnCl_2$, pH 7.1. The experimental data are plotted assuming that the fractional saturation is $\bar{Y} = \Delta F_{obsd}/\Delta F_{max}$ and c_{free} = total molar ATP (or analogue) − ($\bar{Y}$ × total molar enzyme subunit), where ΔF_{obsd} and ΔF_{max} are the observed and maximum fluorescence changes, respectively, at a given nucleotide concentration and at a saturating nucleotide concentration at which no further fluorescence change occurs. Slopes were unity within experimental error, and the arrows indicate the values of $[S]_{0.5} = c_{free}$ at log $\bar{Y}/(1 - \bar{Y}) = 0$. Shown are titrations with AMP-PNP at 30 °C (○) or with ATP at 20 (▽) and 30 °C (△) at subunit concentrations of 0.69 and 2.29 μM, respectively. When L-glutamate (87 mM) was present (●), the enzyme was 97% saturated with L-Glu (Mn·GS·Glu).

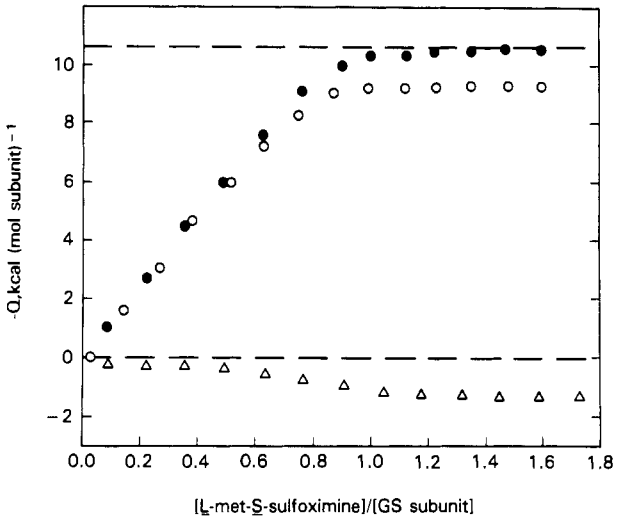

FIGURE 3: Titration isotherm for binding of L-Met-(*S*)-sulfoximine to glutamine synthetase (GS) at pH 7.2 and 25 °C in the presence of 1 mM AMP-PNP in 20 mM Pipes/KOH, 100 mM KCl, and 1.0 mM $MnCl_2$. Buffer (△) or protein (36.4 mg) solution (○), 2 mL, was placed in the microreaction vessel at 25 °C of the Tronac Model 450 calorimeter, and 15 μL of 188 mM AMP-PNP in buffer was added before titration with 19 mM MetSox. The data are plotted as observed heat (Q) in kcal (mol of subunit)$^{-1}$ vs. the ratio of MetSox added per enzyme subunit, and the net isotherm is shown by the solid symbols. This experiment was performed in the laboratory of Neal Langerman (Utah State University) by E.G.G. in July 1981, and initial calculations for the titration isotherm data were as described by Beaudette and Langerman (1978).

sulfoximine indicate that the binding of the two ligands to a subunit site (see below) increases the affinity of an adjacent subunit for either of the two ligands which by themselves produce a fluorescence enhancement.

Figure 2 shows Hill plots of fluorescence titration data using the enhancement of protein tryptophanyl residue fluorescence as a measure of the binding of AMP-PNP or ATP to the manganese enzyme (Mn·GS) subunit. The enzyme has an ~8-fold higher affinity for AMP-PNP than for ATP, and the presence of saturating L-glutamate (Mn·GS·Glu) is antagonistic to the binding of AMP-PNP. The slopes of each set of data in Figure 2 were unity within experimental error, indicating that the binding of AMP-PNP or ATP is noncooperative, and the value of $[S]_{0.5}$ is equal to K_D'. Titrations of the enzyme with ATP and AMP-PNP were performed at several temperatures in order to estimate the van't Hoff enthalpy (ΔH_{vH}) for binding ATP or AMP-PNP from

$$\ln (K_2/K_1) = (\Delta H_{vH}/R)(1/T_1 - 1/T_2) \qquad (1)$$

which assumes that ΔH_{vH} is temperature invariant, and the subscripts of the apparent affinity constants ($1/K_D'$ values) refer to the corresponding absolute temperatures in K, T_1 and T_2. In the temperature range of 20–41 °C, ΔH_{vH} values of +3 ± 2 kcal/mol were estimated for the binding of ATP or AMP-PNP. The values of ΔH_{vH} were disappointing in their lack of precision but the estimated ΔH_{vH} value for binding AMP-PNP is in the range of the measured enthalpy change of +1.3 kcal/mol (see below). The results do suggest that the enthalpy change for binding ATP to the manganese enzyme is similar to that for AMP-PNP binding.

Once the affinity constants for binding L-Met-(*S*)-sulfoximine to the AMP-PNP·Mn-enzyme complex were determined, this value and the K_A' values for binding AMP-PNP and L-Met-(*S*)-sulfoximine to the Mn-enzyme complex could be used to calculate the unknown K_A' value in the thermodynamic cycle for binding two ligands to glutamine synthetase (Table I). Likewise, measurement of the K_A' value for binding AMP-PNP to the L-Glu·Mn-enzyme complex allowed calculation of the K_A' value for binding L-glutamate to the AMP-PNP·Mn-enzyme complex since K_A' values for binding L-glutamate and AMP-PNP to the Mn-enzyme complex were known (Table I). Thermodynamic linkage functions (Wyman, 1970) show that there is a strong synergism between the binding of AMP-PNP and L-Met-(*S*)-sulfoximine. In contrast, there is a slight antagonistic effect between the binding of the ATP analogue and L-glutamate.

Calorimetric Measurements. Figure 3 shows a titration isotherm for binding L-Met-(*S*)-sulfoximine to the AMP-PNP·Mn-enzyme complex at 25 °C. These data were obtained in a preliminary experiment using the Tronac Model 450 isoperibol calorimeter in N. Langerman's laboratory (Biltonen & Langerman, 1979) which required quite concentrated protein solutions; ~1 mM free AMP-PNP was used since the affinity of glutamine synthetase for this ATP analogue had not as yet been determined. Nevertheless, the plateau of the net isotherm was in excellent agreement with later experiments (Table II) and reached a maximum value of −10.5 ± 0.1 kcal (mol of subunit)$^{-1}$ when 1.0 equiv of L-Met-(*S*)-sulfoximine per subunit had been added. However, there appeared to be some cooperative effect in the heat evolved at subsaturating L-Met-(*S*)-sulfoximine that was not observed in later experiments (Figure 4; Table II). Possibly, the high concentrations of AMP-PNP and/or of protein employed in the experiment of Figure 3 led to higher heats at subsaturating L-Met-(*S*)-sulfoximine. In separate experiments in the batch calorimeter using Pipes, Hepes, and Tris buffers at pH 7.1 (30 °C), no significant proton release or uptake was detected for this binding reaction.

Figure 4 shows representative thermal titration data for the binding of L-Met-(*S*)-sulfoximine to the AMP-PNP·Mn-enzyme complex obtained at 25 and 30 °C with the LKB Model 2107-350 microtitrator accessory to the batch calo-

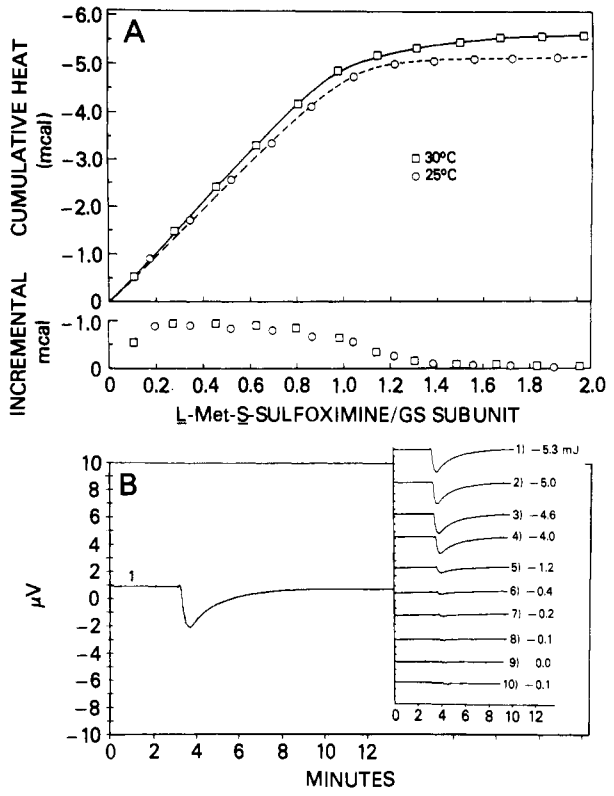

FIGURE 4: Thermal titrations of the AMP-PNP·Mn·enzyme complex with L-Met-(*S*)-sulfoximine at 25 or 30 °C, pH 7.1, performed with the LKB Model 10700 batch calorimeter equipped with a LKB 2107-350 microtitrator. The buffer was 20 mM Hepes/KOH, 100 mM KCl, and 1.0 mM $MnCl_2$, pH 7.1, and contained 0.1 mM free AMP-PNP; in (A), the reference and reaction cells each contained 4.0 mL of this mixture without and with 0.487 μmol of enzyme subunit, respectively. Injections of 10.5 μL of 10.8 mM MetSox in buffer with 0.1 mM AMP-PNP were made simultaneously into both cells for each cycle. In (A), cumulative heats and the incremental heats in millicalories vs. the equivalents of ligand added per enzyme subunit are shown. In (B), truncated voltage–time traces are shown for 10 consecutive additions of MetSox at ~25-min intervals to 0.464 μmol of enzyme subunit at 30 °C. The integrated areas were converted to the heat evolved in millijoules by means of electrical calibrations.

rimeter. A summary of data obtained by microtitration and batch calorimetry is given in Table II. Since the binding of the ligand is very tight (Table I) and there were no apparent cooperative effects, the heat released on binding subsaturating L-Met-(*S*)-sulfoximine in microtitration experiments gave additional measurements of the total enthalpy change for the binding reaction. Averaging all values gave $\Delta H = -10.9 \pm 0.5$ and -10.1 ± 0.4 kcal/mol at 30 and 25 °C, respectively, for the binding of L-Met-(*S*)-sulfoximine to the AMP-PNP·Mn·enzyme complex. The temperature dependence of the enthalpy change ($\delta\Delta H/\Delta T$) gives an estimate of $\Delta C_p \simeq -160 \pm 100$ cal/(K·mol).

The bottom panel of Figure 4A shows the incremental heat in millicalories vs. L-Met-(*S*)-sulfoximine added per subunit. A low first value can occur if there is evaporation in the Teflon tubing after lines are primed with ligand and inserted into calorimeter vessels. Figure 4B illustrates truncated voltage–time signals recorded during a titration of the AMP-PNP·Mn·enzyme complex at 30 °C (~25 min was chosen as the interval between injections so that thermal reequilibration before the next injection of ligand was assured). Integration of voltage–time outputs for 10 injections of subsaturating L-Met-(*S*)-sulfoximine (0.24 equiv/subunit) gave the heats in millijoules shown, using electrical calibrations for calculations of the heat released in each case. After five and six injections, 1.16 and 1.4 equiv of L-Met-(*S*)-sulfoximine per

Table II: Heats of Binding L-Methionine (*S*)-Sulfoximine to the AMP-PNP·Mn·Enzyme Complex at pH 7.1 Measured by Microtitration and Batch Calorimetry[a]

	$[\text{enzyme}]_{\text{total}}$ (mg)	$-\Delta H$ (kcal/mol)[b]
30 °C values		
microtitration plateau	24.4	11.3_5
	23.2	10.6
microtitration initial values	24.4	11.2, 11.0, 10.7
	23.2	11.2, 10.5, 9.7
batch	8.7	11.4[c]
		av $10.8_5 \pm 0.5$
25 °C values		
microtitration plateau	24.4	10.4
microtitrator initial values	24.4	10.5, 9.6, 9.9, 9.7
batch	8.7	9.8[c]
batch	4.6	10.5[c]
		av $10.0_6 \pm 0.4$

[a] The reaction cell contained glutamine synthetase in 2.4–4.0 mL in 20 mM Hepes/KOH, 100 mM KCl, and 1.0 mM $MnCl_2$, pH 7.1, with 0.1 mM free AMP-PNP present. The reference cell contained the same volume of buffer with 0.1 mM AMP-PNP. For microtitrator experiments, two 0.5-mL syringes (one per cell) containing 10.8 mM MetSox with 0.1 mM AMP-PNP in buffer at pH 7.1 were connected by Teflon tubing to each large cell compartment (Chen & Wadsö, 1982) and injections of 10.5 μL per cycle were made. For batch experiments, 2.0 mL containing 1.08 mM of MetSox in pH 7.1 buffer with 0.1 mM AMP-PNP was placed in the small compartment of the reaction cell and 2.0 mL of an identical solution without MetSox was placed in the opposing reference cell; reactions were initiated by mixing cell contents. [b] Since the binding of MetSox is very tight (Table I), the units of kcal/mol can be expressed in terms of the moles of added ligand at subsaturating concentrations. At saturating concentrations of MetSox, the moles of bound ligand was assumed to equal the moles of enzyme subunit present. [c] Corrected for heats of protein dilution (~4% of reaction heats) which were determined in separate experiments. The heat of dilution of MetSox (from 1.08 to 0.72 mM) in batch experiments was negligible.

enzyme subunit had been added and further additions of this ligand gave heats approximately equal to those of frictional mixing heats (not shown).

Figure 5 shows enthalpies of binding L-glutamate to manganese glutamine synthetase in the absence and presence of saturating AMP-PNP in Pipes, Hepes, and Tris buffers at pH 7.1 and 303 K. Enthalpies for binding AMP-PNP to the Mn·enzyme·L-Glu complex were measured also in the different buffers. Values of ΔH for each reaction are plotted as a function of the enthalpy change for protonation of the buffer at 30 °C. Details of the batch calorimetry experiments for determination of the values in Figure 5 are given in the legend. Large amounts of protein were required since measured heats of binding were small. Also, corrections for endothermic heats of dilution of L-glutamate solutions were substantial (~20%). The slopes of the fitted lines in Figure 5 give ≤0.14 equiv of H^+ taken up by the enzyme subunit in the binding reactions shown. The intercepts of Figure 5 give ΔH values in kilocalories per mole corrected for buffer deprotonation.

Binding L-Met-(*S*)-sulfoximine to the unadenylylated, manganese enzyme produces a release of ~0.1 equiv of H^+/subunit (Gorman & Ginsburg, 1982). In the present studies, proton effects also were negligible (≤0.2 equiv of H^+/subunit) for binding AMP-PNP to the Mn·enzyme or to the Mn·enzyme·MetSox complex as well as for binding L-Met-(*S*)-sulfoximine to the AMP-PNP·Mn·enzyme complex (as judged by calorimetric measurements in Pipes, Hepes, and Tris buffers at pH 7.1).

The heat of binding the transition-state analogue L-Met-(*S*)-sulfoximine phosphate to glutamine synthetase also was measured by batch calorimetry at 30 °C (Table III). This analogue of the postulated glutamyl phosphate–ammonia

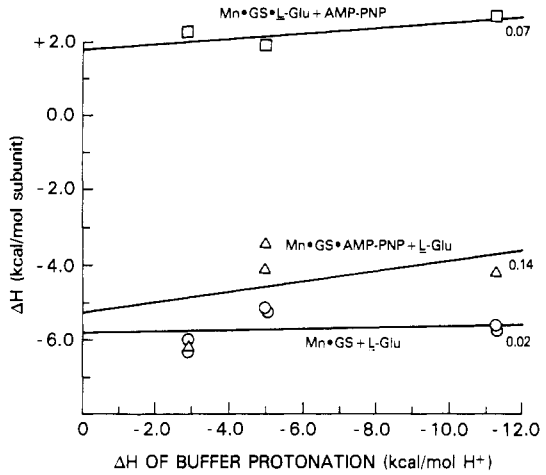

FIGURE 5: Enthalpies of binding and proton effects for L-glutamate and AMP-PNP binding to glutamine synthetase at pH 7.1 and 303 K. Batch calorimetry experiments were performed with 2.0 mL of the dialyzed enzyme (26–29 mg) and 4.0 mL of ligand prepared in dialysis buffer for a final concentration of 0.1 mM free AMP-PNP and/or 30 mM L-glutamate. Buffers were 20 mM Pipes, Hepes, or Tris with 100 mM KCl and 1.0 mM $MnCl_2$ at pH 7.1 (30 °C). The heats of protein and ligand dilutions were determined in separate experiments for corrections of reaction heats; corrections were ~4% for protein dilutions, ~20% for dilution of L-glutamate from 45 to 30 mM (~+0.65 ± 10% in three determinations in each buffer), and negligible for AMP-PNP dilutions. For L-glutamate binding, corrected reaction heats (−2.6 to −3.2 ± <0.05 mcal) then were divided by 0.909 to correct to full enzyme saturation with L-glutamate. The enthalpy change for each binding reaction (where Mn•GS refers to the enzyme subunit saturated with Mn^{2+}) in kcal (mol of subunit)$^{-1}$ is plotted as a function of ΔH values for buffer protonation, which are −2.9 and −5.0 kcal (mol of H^+)$^{-1}$ for Pipes and Hepes, respectively (Beres & Sturtevant, 1971; Roy et al., 1980), and −11.3 kcal (mol of H^+)$^{-1}$ for Tris (Chen & Wadsö, 1982). The slopes of linear fits to each data set are given and correspond to the equivalents of H^+ taken up by the enzyme subunit, and the intercepts give the ΔH values corrected for buffer deprotonation for the different reactions.

Table III: Heat of Binding the Transition-State Analogue L-Methionine (*S*)-Sulfoximine Phosphate to Manganese Glutamine Synthetase at pH 7.1 and 303 K[a]

enzyme (μmol of subunit)	observed heat (mcal)	corrected heat[b] (mcal)	ΔH (kcal/mol)
0.139	−1.54	−1.81	−13.0
0.174	−1.94	−2.21	−12.7
0.174	−2.08	−2.35	−13.5
			av −13.1 ± 0.4

[a] Determined by batch calorimetry at 30 °C using 2.0 mL of enzyme solution and 4.0 mL of a 1.2–1.5-fold excess of L-methionine (*S*)-sulfoximine phosphate to enzyme subunit concentration in 20 mM Hepes/KOH, 100 mM KCl, and 1.0 mM $MnCl_2$ at pH 7.1. At the end of each experiment, the enzyme was >98% inactivated. [b] Observed heats were corrected for the heat of protein dilution (−0.07 mcal) and the heat of ligand dilution (+0.34 ± 0.28 mcal in six separate experiments).

adduct intermediate in biosynthetic catalysis (Meister, 1974) binds noncovalently with a very high affinity ($K_A' \simeq 10^{19}$ M^{-1}) and thereby inactivates the enzyme by blocking active sites (Maurizi & Ginsburg, 1982a). The transition-state analogue is very stable when bound to the enzyme (Maurizi & Ginsburg, 1982b, 1986) but was found to be only marginally stable in protein-free solutions at pH 7.1. For the experiments of Table III, L-Met-(*S*)-sulfoximine phosphate was repurified and used shortly thereafter (see Materials and Methods). The slow breakdown of this analogue in the pH 7.1 buffer used for the experiments of Table III led to a wider variation in the six measured heats of dilution than normally observed. Taking into account the error in the correction for ligand dilution, the

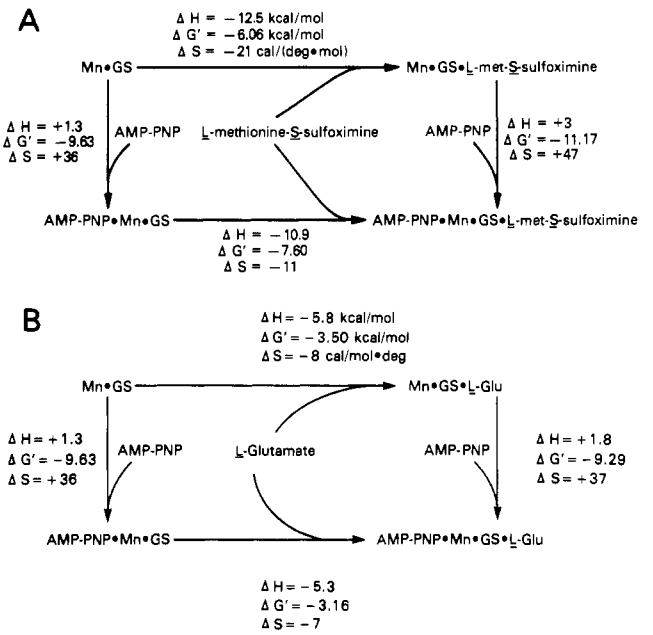

FIGURE 6: Thermodynamic cycles for binding two active-site ligands to each subunit of manganese glutamine synthetase at pH 7.1 and 303 K. In (A), the cycle for binding L-Met-(*S*)-sulfoximine and AMP-PNP by two pathways to give the AMP-PNP•Mn•GS•MetSox subunit complex is shown with thermodynamic parameters for each binding reaction. In (B), the cycle for binding L-glutamate and AMP-PNP by two pathways to give the AMP-PNP•Mn•GS•Glu complex is shown with $\Delta G'$, ΔH, and ΔS values for each binding reaction. All ΔH values were measured by calorimetry, and $\Delta G'$ values in each cycle were calculated from K_A' values (Table I). The units of ΔH and $\Delta G'$ are kcal (mol of subunit)$^{-1}$, and for $\Delta G'$ values, the standard state for H^+ activity is $10^{-7.1}$ M. Values of ΔS were calculated from corresponding $\Delta G'$ and ΔH values and are given in cal (deg•mol of subunit)$^{-1}$.

corrected enthalpy change for binding L-Met-(*S*)-sulfoximine phosphate is −13.1 ± 1.6 kcal/mol of enzyme subunit. At the end of each experiment in Table III, the enzyme was >98% inactivated, indicating that all subunits of the dodecamer were complexed to the transition-state analogue.

Thermodynamic cycles for binding L-Met-(*S*)-sulfoximine and AMP-PNP and for binding L-glutamate and AMP-PNP are shown in panels A and B, respectively, of Figure 6. Thermodynamic parameters are given for each binding reaction. All ΔH values were directly measured at 303 K with a standard deviation of ~±10%. The calculated overall enthalpy change for forming the fully liganded enzyme subunit from the unliganded Mn•enzyme subunit in each cycle was the same (±0.1 kcal/mol) by the two binding pathways. For each cycle, $\Delta G'$ values were calculated from apparent affinity constants from the relationship $\Delta G' = -RT \ln K_A'$, where three of the four K_A' values for each cycle were known and the fourth was calculated (Table I). The entropy change for each binding reaction in Figure 6 was computed from $(\Delta H - \Delta G')/T$, where T is the absolute temperature (303 K).

Discussion

The data of Figure 6 illustrate thermodynamic linkage relationships for binding two ligands by two pathways. A strong synergism between the binding of L-Met-(*S*)-sulfoximine and the ATP analogue AMP-PNP is apparent from the $\Delta G'$ values given in Figure 6A, which show that the binding reaction for a given ligand is considerably more exergonic in the presence than in the absence of other ligand ($\delta\Delta G' = -1.54$ kcal/mol). In contrast, there is some antagonism between the binding of L-glutamate and AMP-PNP to the subunit active site (Figure 6B; $\delta\Delta G' = +0.34$ kcal/mol).[4]

Table IV: Thermodynamic Parameters at 303 K for Active-Site Ligand Binding to Glutamine Synthetase at pH 7.1 in the Presence of 1.0 mM $MnCl_2$ and 100 mM KCl[a]

binding reaction (Mn·GS ≡ enzyme subunit)	$\Delta G'$ (kcal/mol)	ΔH (kcal/mol)	ΔS [cal/(K·mol)]	$-T\Delta S$ (kcal/mol)
Mn·GS + ADP[b]	−7.60	−5.7	+6	−1.9
Mn·GS + ATP	−8.36	+3 ± 2_{vH}	+37	−11.2
Mn·GS + AMP-PNP	−9.63	+1.3	+36	−10.9
Mn·GS + L-glutamine[b]	−2.96	−9.7	−22	+6.7
Mn·GS + L-glutamate	−3.50[c]	−5.8	−8	+2.3
Mn·GS + L-Met-(*S*)-sulfoximine[d]	−6.06	−12.5	−21	+6.4
Mn·GS + L-Met-(*S*)-sulfoximine phosphate	−26[e]	−13.1	+43	−12.9

[a] For free energy values ($\Delta G'$), the standard state for hydrogen ion activity is $10^{-7.1}$ M. Enthalpy changes were determined by calorimetry with a standard deviation of ~±10% except in the case of ATP binding for which a van't Hoff ΔH_{vH} value was estimated. The entropy of binding for each reaction is computed from $(\Delta H - \Delta G')/T$, where T is the absolute temperature (303 K). [b] Shrake et al. (1977). [c] Villafranca et al. (1975). [d] Gorman and Ginsburg (1982). [e] Estimated from a second-order rate of ~10^6 M^{-1} s^{-1} for inactivation by L-Met-(*S*)-sulfoximine phosphate at 25 °C, pH 7, and an extrapolated rate of dissociation of 10^{-13} s^{-1} for reactivation at pH 7 and 25 °C (Maurizi & Ginsburg, 1982a).

Overall enthalpy changes for forming the fully liganded enzyme subunit from the unliganded Mn·enzyme subunit by two pathways in each cycle of Figure 6 can be computed from the calorimetric measurements of ΔH for each reaction and are the same within experimental error. The binding of either L-Met-(*S*)-sulfoximine or L-glutamate to the Mn·enzyme complex was less exothermic in the presence than in the absence of AMP-PNP (Figure 6). Previously, ADP was found to make the binding of L-glutamine ~24% less exothermic while increasing 4.3-fold the affinity of the Mn·enzyme for L-glutamine (Shrake et al., 1977). Also, enthalpy changes for binding L-glutamate to the ADP·Mg·enzyme and ADP·P_i·Mg·enzyme complexes were −7.7 and −4.1 kcal/mol, respectively, at pH 7.2 and 303 K, although in this case the binding of ADP + P_i to active sites substantially decreased the affinity for L-glutamate (Shrake et al., 1978). The value of ΔH = −5.3 kcal/mol for the binding of L-glutamate to the AMP-PNP·Mn·enzyme complex (Figure 6B) is intermediate to the previously measured ΔH values for binding L-glutamate to the ADP·Mg·enzyme ± P_i complexes.

Table IV gives thermodynamic parameters at pH 7.1 and 303 K for binding various active-site ligands to glutamine synthetase saturated with Mn^{2+}. In contrast to the exothermic enthalpy for binding ADP, the tighter binding of ATP or AMP-PNP is slightly endothermic. The entropy changes on binding ADP, ATP, or AMP-PNP are positive, consistent with disruption of bound water as the nucleotide coordinates to the metal ion at the subunit n_2 site. The enthalpy and entropy changes on binding ATP or AMP-PNP to the enzyme are indicative of hydrophobic and/or ionic (charge neutralization) interactions (Ross & Subramanian, 1981).

The enzyme has a higher affinity for L-Met-(*S*)-sulfoximine than for L-glutamate or L-glutamine, and the binding of the analogue is considerably more exothermic than is L-glutamate binding (Table IV). A small, negative ΔC_p value of −160 ± 100 kcal/mol for L-Met-(*S*)-sulfoximine binding to the AMP-PNP·Mn·enzyme complex was estimated from thermal titrations at 25 and 30 °C, suggesting a minor role of water in this binding process. Entropy changes are negative for binding L-glutamate and are ~3-fold more negative for binding L-glutamine or L-Met-(*S*)-sulfoximine to glutamine synthetase (Table IV), suggesting that different ordering effects occur on binding these substrates. The binding of L-Met-(*S*)-sulfoximine to glutamine synthetase produces a burial of 1 tyrosyl residue/subunit (Shrake et al., 1980, 1982) and an enhancement of tryptophanyl residue fluorescence (Rhee et al., 1980). Also, the binding of L-Met-(*S*)-sulfoximine produces a total obstruction of water exchange with Mn^{2+} bound at the subunit n_1 site and dramatic changes in the EPR spectrum (Villafranca et al., 1975). Both local and gross conformational changes are promoted by L-Met-(*S*)-sulfoximine binding to the unadenylylated enzyme, since difference sedimentation velocity measurements showed that the liganded dodecamer has a more compact structure (Shrake et al., 1980).

The conformational changes observed on binding the transition-state analogue L-Met-(*S*)-sulfoximine phosphate to unadenylylated glutamine synthetase are even more pronounced than those observed with L-Met-(*S*)-sulfoximine (Maurizi & Ginsburg, 1986). Both intra- and intersubunit bonding domains are enormously strengthened by binding the inactivating transition-state analogue (Maurizi & Ginsburg, 1982b, 1985). The large negative $\Delta G'$ value for binding L-Met-(*S*)-sulfoximine phosphate in Table IV was estimated from the second-order rate constant of ~10^6 M^{-1} s^{-1} for inactivation of the enzyme at pH 7 and 25 °C and an extrapolated first-order rate of dissociation leading to reactivation at pH 7 of ~10^{-12} s^{-1} (Maurizi & Ginsburg, 1982a). If ADP is present with L-Met-(*S*)-sulfoximine phosphate, ADP also binds very tightly ($K_A' \simeq 10^{12}$ M^{-1}) to each enzyme subunit (Maurizi & Ginsburg, 1982a), as do the two Mn^{2+} ($K_A' > 10^9$ M^{-1}) in the inactive complex (Hunt & Ginsburg, 1980). Although ADP binding is not necessary for inactivation, ADP is synergistically bound to the enzyme subunit with Mn^{2+} and L-Met-(*S*)-sulfoximine phosphate (Maurizi & Ginsburg, 1982a).

The columm headed by $-T\Delta S$ in Table IV is included for comparison with ΔH and $\Delta G'$ values to assess whether binding reactions are enthalpically or entropically controlled at 303 K. ATP or AMP-PNP binding is entropically controlled since ΔH is positive and $-T\Delta S$ is large and negative. The binding of L-glutamate (or analogues) is enthalpically controlled since the $-T\Delta S$ value is positive. However, net reactions for binding both AMP-PNP (or ATP) and L-Met-(*S*)-sulfoximine and for binding both AMP-PNP (or ATP) and L-glutamate are enthalpically and entropically controlled, respectively (Figure 6). The binding of the transition-state analogue L-Met-(*S*)-sulfoximine phosphate involves both large favorable enthalpic and entropic contributions; when ADP also is synergistically bound to the subunit active site, the enthalpic term appears to dominate since the apparent constant for complex dissociation (reactivation) increases with increasing temperature (Maurizi & Ginsburg, 1982a).

Thermodynamic parameters for binding various substrates and substrate analogues to glutamine synthetase may be useful for mapping interactions at active sites. L-Glutamine and L-Met-(*S*)-sulfoximine interact with the L-glutamate and ammonia subunit sites, and both produce an ordering effect [$\Delta S \simeq -21$ cal/(K·mol)] with large negative ΔH values (without significant net proton uptake). In contrast, the

[4] The synergistic effect previously observed beween the binding of ATP and L-glutamate to the Mg·enzyme (Timmons et al., 1974) could be due to ATP hydrolysis (Meister, 1974) or subtle differences in binding modes of ATP and AMP-PNP.

binding of L-glutamate is less exothermic and the apparent ordering effect (as judged by the negative ΔS value) is less, possibly due to charge neutralization and changes in hydration waters. The binding of ADP is exothermic as is the binding of P_i to the ADP·Mg·enzyme complex (−1.1 kcal/mol; Shrake et al., 1977, 1978). In contrast, the binding of ATP or AMP-PNP is endothermic. Also, the binding of P_i to the ADP·Mg·enzyme·Glu complex is entropically dominated at 303 K (ΔH = +2.5 and $-T\Delta S$ = −4.0 kcal/mol) with an accompanying net proton uptake of 1.2 H^+ mol of subunit (Shrake et al., 1978). Similarly, the binding of AMP-PNP and L-glutamate to the Mn·enzyme at 303 K is entropically controlled (Figure 6B). The transition-state analogue L-Met-(*S*)-sulfoximine phosphate spans the active-site protein groups responsible for binding L-glutamate, ammonia, and the γ-phosphate of ATP. The free energy change for binding L-Met-(*S*)-sulfoximine phosphate is very exergonic, and both enthalpy and entropy changes are favorable and are similar to those observed for L-Met-(*S*)-sulfoximine and ATP binding, respectively. Thus, the thermodynamic parameters obtained for binding the transition-state analogue appear to reflect those obtained for ligand binding to the subunit sites responsible for binding L-glutamate, ammonia, and the γ-phosphate of ATP.

The thermodynamic parameters that have been obtained for active-site ligand binding to *E. coli* glutamine synthetase contain contributions from both protein–ligand and protein–protein interactions. The positive entropy change for interaction of the enzyme with a full complement of substrates could result from forcing the nucleotide into a more hydrophobic environment for interaction with the γ-carboxylate group of L-glutamate and concomitant changes in hydration. However, the ionic interactions of the γ-phosphate of ATP play a major role in contributing to the entropy changes observed. Also, interactions at the L-glutamate and ammonia sites produce ordering effects that are opposed by large exothermic heats of binding. Active-site ligand stabilization of intra- and intersubunit bonding domains to produce a more compact dodecameric structure would be expected to lead to negative values of ΔH and ΔS through the strengthening of hydrogen bonds in the protein interior and van der Waals interactions brought about by the partial withdrawal of nonpolar groups from water (Ross & Subramanian, 1981). These more remote stabilizing effects of active-site ligand binding appear to be offset by local charge neutralization effects at active sites.

Registry No. AMP-PNP, 25612-73-1; MetSox, 21752-32-9; ATP, 56-65-5; Glu, 56-86-0; GS, 9023-70-5; MetSox phosphate, 109528-34-9.

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