Calorimetric Studies on the Binding of Substrates and the Inhibitor L-Alanine to Unadenylylated Glutamine Synthetase of *Escherichia coli*†

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ABSTRACT: Glutamine synthetase, a strictly regulated enzyme in *Escherichia coli*, is a dodecamer with 12 catalytic sites. Separate protein binding sites for the substrate L-glutamate and feedback inhibitor L-alanine have been demonstrated by measuring heats of binding of these effectors to the unadenylylated Mg-enzyme in the presence of saturating ADP at pH 7.2 at 30 °C: (a) The heat of binding L-glutamate to the enzyme is large and negative in Tris buffer ($Q'_{max} = -5.9$ kcal/mol of subunit), whereas that for binding L-alanine at 30 mM concentration (K_D' unknown) is approximately zero (−0.2 kcal/mol of subunit); addition of 30 mM L-alanine to the enzyme already saturated with L-glutamate (60 mM) produces a negative heat of -1.1 ± 0.5 kcal/mol of subunit. (b) The heat for simultaneously binding L-glutamate (60 mM) and L-alanine (30 mM) is more exothermic than the heat of binding either ligand alone. (c) In the presence of saturating ADP and 20 mM P_i (with ADP + P_i combined to simulate ATP), the binding of L-glutamate to the enzyme becomes weaker and the heat of binding L-alanine becomes positive; the observed heat of binding L-alanine to the enzyme in the presence of L-glutamate remains positive and is different from that predicted for direct competition between these two ligands for the same site. For binding L-glutamate to the ADP-Mg-enzyme complex, $\Delta G' = -3.81$ kcal/mol of subunit (computed from $K_A' = 560\ M^{-1}$ assuming a standard state for hydrogen ion activity of $10^{-7.2}$ M), $\Delta H' = -7.7$ kcal/mol of subunit, and $\Delta S' = -13$ cal/(deg mol of subunit) with a proton uptake of 0.2 equiv/subunit. These binding parameters are the same as those for L-glutamine binding to the ADP-Mn-enzyme complex, suggesting similar binding mechanisms. Thermodynamic parameters for the binding of L-glutamate and of L-alanine to the ADP-P_i-Mg-enzyme complex and for the binding of P_i to the ADP-Mg-enzyme complex also are given. The K_D' for the dissociation of L-alanine from the ADP-P_i-Mg-enzyme-Ala complex is 15 mM and $\Delta H'$ for the corresponding binding reaction is +1.8 kcal/mol of subunit. The calorimetric results are consistent with a strong synergistic effect between L-alanine and ADP + P_i and with an antagonistic effect between L-glutamate and ADP + P_i. The biosynthetic reaction products ADP + P_i mimic the action of ATP on L-alanine binding to the enzyme but differ from the effect of ATP on L-glutamate binding.

Glutamine synthetase catalyzes the reaction:

$$\text{L-glutamate} + \text{ATP} + NH_4^+ \overset{Me^{2+}}{\rightleftharpoons} \text{L-glutamine} + \text{ADP} + P_i \quad (1)$$

The biosynthetic product, L-glutamine, is an important intermediate in the assimilation of ammonia by *E. coli*. Besides being the source of nitrogen for the biosynthesis of a variety of chemically different end products, L-glutamine is important in an ATP-dependent synthesis of most amino acids (including L-alanine) through a coupling of the reactions catalyzed by glutamine synthetase, glutamate synthase, and various glutamate-α-ketoacid transaminases (Stadtman, 1973). The complex regulation of glutamine synthetase activity in *E. coli* has been extensively reviewed (Holzer, 1969; Shapiro & Stadtman, 1970; Stadtman et al., 1970; Stadtman & Ginsburg, 1974). Control of this enzyme activity in *E. coli* has been shown to involve repression of enzyme synthesis by high concentrations of ammonia, feedback inhibition by end products of glutamine metabolism, and covalent modification of glutamine synthetase by adenylylation and deadenylylation, which is modulated by a cascade system consisting of several metabolite-regulated enzymes and a small regulatory protein (Adler et al., 1975). Adenylylation of glutamine synthetase (Shapiro et al., 1967) dramatically affects catalytic activity and divalent cation (Me^{2+}) specificity of the enzyme in reaction 1 (Wulff et al., 1967; Kingdon et al., 1967). Adenylylation of glutamine synthetase also affects the susceptibility of the enzyme to feedback inhibition in biosynthetic catalysis (Kingdon et al., 1967).

Physical and chemical properties of glutamine synthetase from *E. coli* have been reviewed (Ginsburg, 1972). The enzyme is composed of 12 apparently identical subunits, each of 50 000 molecular weight (Woolfolk et al., 1966; Valentine et al., 1968; Shapiro & Ginsburg, 1968). Each subunit of the enzyme has an adenylylation site within the polypeptide chain (Shapiro & Stadtman, 1968; Heinrikson & Kingdon, 1971). The enzyme has been shown also to have 12 catalytic sites, which involve metal ion binding (Hunt et al., 1975). In view of the dodecameric structure of the enzyme, each subunit of glutamine synthetase most probably possesses in addition to the adenylylation site: a catalytic site; divalent cation sites (Denton & Ginsburg, 1969); and allosteric sites (Ginsburg, 1969).

Dahlquist & Purich (1975) have examined the interaction of unadenylylated glutamine synthetase with several substrates and effectors using magnetic resonance techniques. They concluded that the substrate L-glutamate displaces the inhibitor L-alanine from the enzyme but we question this interpretation of these data. Although previous steady-state kinetic studies of the Mg^{2+}-supported biosynthetic reaction 1 were equivocal on this point, more recent kinetic results of Rhee & Chock (1976a) indicate that L-glutamate and L-alanine bind

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at separate sites. We have used calorimetry in the present study to determine whether or not L-alanine and L-glutamate have separate binding sites or a common binding site for which these ligands compete on the enzyme. The unadenylylated Mg^{2+}-enzyme was used because this enzyme form is most susceptible to inhibition by L-alanine (Kingdon et al., 1967). A calorimetric approach for determining the existence of separate sites was explored previously (Ross & Ginsburg, 1969; Shrake et al., 1976, 1977). Chock et al. (1977) have undertaken a complementary study of the binding of L-glutamate and L-alanine and other ligands to unadenylylated glutamine synthetase, again using magnetic resonance techniques.

Materials and Methods

Glutamine Synthetase Preparations and Assay. Glutamine synthetase was purified by the procedure of Woolfolk et al. (1966) and stored as described previously (Shrake et al., 1977). Several enzyme preparations were used. All preparations were unadenylylated enzyme (i.e., those containing an average of 1 mol of 5′-adenylyl groups per mol of dodecamer) and all gave full activity in the saturating γ-glutamyltransferase assay at pH 7.57 at 37 °C (Hunt et al., 1975) and in the Mg^{2+}-dependent biosynthetic assay at 30 °C (Ginsburg et al., 1970). Protein stock solutions were prepared as before (Shrake et al., 1977), dialyzing the enzyme at 4 °C against Tris-HCl[1] or Hepes-KOH buffers, which contained 0.10 M KCl and $MgCl_2$.

Reagents. For removal of any divalent and trivalent metal ions, L-glutamate (Sigma Chemical Co., free acid) and L-alanine (Sigma Chemical Co.) were treated with Chelex 100 (Bio-Rad Laboratories) as described by Shrake et al. (1977). The ADP was obtained from Sigma Chemical Co. (grade III, sodium salt), assayed for concentration, and stored as before (Hunt et al., 1975). All other reagents were as described by Hunt et al. (1975).

Calorimetric Measurements. All heats of binding were measured at 30 ± 0.1 °C using an LKB Batch Microcalorimeter 10700 equipped with gold cells. Recording and measuring the heat of reaction, calibrating the calorimeter, loading the calorimeter cells, and running separate experiments to determine heats of dilution were all as described previously (Shrake et al., 1977).

All reactant solutions (pH 7.2 at 30 °C) were prepared on the day of the experiment using dialyzed protein stock solution (pH 7.2), 0.45 M L-glutamate (pH 7.5), 0.30 M L-alanine (pH 7.5), 0.12 M ADP (pH 6.9), 1.0 M KPO_4 (pH 7.8), final protein dialysate, and concentrated (twice concentration of final dialysates) buffer solutions when required. The L-glutamate, L-alanine, and ADP stock solutions were in water and were stored frozen. Reactant solutions containing adp and ADP + P_i were prepared by at most a 2% dilution with each effector. The pH adjustments and measurements were carried out as before (Shrake et al., 1977).

The sequential and simultaneous heats of binding (directly observed heats of reaction and substrate dilution corrections) of L-glutamate and L-alanine to unadenylylated enzyme in the presence of ADP (Figure 4) and of ADP + P_i (Figure 5) were measured at least twice, as were the heats of binding P_i[1](Table II). All heats of dilution of protein were measured once. The remaining heats that were used to determine the saturation curves for L-glutamate and for L-alanine binding to enzyme in the presence of ADP and of ADP + P_i, respectively (Figures 1 and 3), were measured once; they were repeated if an inconsistency occurred within either set of saturation data. The remaining heats that were used to determine the saturation curve for L-glutamate binding to enzyme in the presence of ADP + P_i (Figure 2) are averages of three determinations; the heats of dilution of L-glutamate for final concentrations of 5 and 7 mM were extrapolated from a linear heat of dilution curve, which had been determined over a concentration range of 56–10 mM L-glutamate.

TABLE I: Heats of Protonation of Buffers.

Buffers[a]	Heats of protonation[b] (kcal/mol H^+) Tris buffer	Hepes buffer
0.050 M Tris-HCl or 0.050 M Hepes-KOH, 0.050 M $MgCl_2$, and 0.10 M KCl	−11.1[c]	−4.8[d]
0.10 M Tris-HCl or 0.050 M Hepes-KOH, 0.020 M KPO_4, 0.050 M $MgCl_2$, and 0.10 M KCl	−10.0 ± 0.4	−3.8 ± 0.2[e]
0.10 M Tris-HCl or 0.050 M Hepes-KOH, 0.020 M KPO_4, 0.020 M $MgCl_2$, and 0.10 M KCl	−9.3 ± 0.7	−3.2 ± 0.3

[a] All buffers were of pH 7.2 at 30 °C. [b] Errors were calculated assuming an error of ±0.2 mcal in each measured heat of reaction. [c] Wadsö, 1968; at 25 °C. [d] Hunt et al., 1972; at pH 7.1 at 37 °C. [e] In order to prevent precipitation, which occurred in the absence of enzyme and/or effectors, the buffer was diluted by a factor of two with deionized water.

The initial protein concentration was 5–8 mg/mL. The amount of protein used varied from approximately 11–31 mg; the smaller amounts of enzyme were used in order to minimize large heats of dilution of substrate by decreasing initial protein volume (2–4 mL) and increasing initial substrate volume (2–5 mL). After each experiment the pH was measured to ensure that no shift in pH occurred during the experiment. All reaction mixtures containing enzyme were assayed by the pH 7.57 γ-glutamyltransferase reaction or Mg^{2+}-dependent biosynthetic reaction to ensure that fully active enzyme had been used.

In order to calculate proton uptake and enthalpies of binding (correcting for proton uptake), heats of protonation of the mixed buffers were measured (Table I). Because P_i buffers at pH 7.2, the presence of 20 mM P_i changes the heat of protonation of the buffers containing Tris and Hepes. Furthermore, Mg^{2+} reversibly binds to P_i thereby decreasing the buffering capacity of the P_i. The buffering effect of 2 mM Mg-ADP is negligible as are the relative buffering capacities of L-glutamate and L-alanine at pH 7.2. The presence of P_i only slightly changes calculation of proton uptake but does affect correction of observed enthalpy values for proton uptake.

Prior to calorimetric measurements, solubility studies were performed on reactant solutions in order to avoid precipitation. Occasionally magnesium phosphate still precipitated during a calorimeter experiment causing a large drift in baseline that made any measurement of heat of reaction impossible.

Results

All experiments were performed in the presence of saturating (2.0 mM free) ADP ($K_A \simeq 10^4\ M^{-1}$; Hunt et al., 1975; Rhee & Chock, 1976b). The stoichiometry of ADP binding is 12 equiv/dodecamer. To maintain saturation of the unadenylylated glutamine synthetase with Mg^{2+}, calorimetric measurements involving L-glutamate were made in the pres-

[1] Abbreviations used: Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; GS, glutamine synthetase subunit.

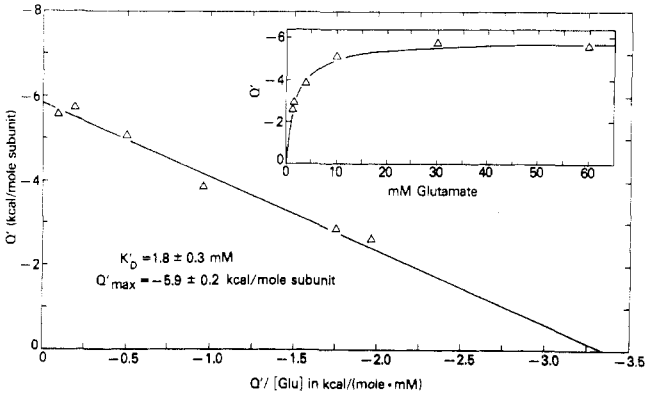

FIGURE 1: Thermal saturation data for binding L-glutamate to unadenylylated glutamine synthetase in the presence of 2.0 mM free ADP in 0.050 M Tris-HCl, 0.10 M KCl, and 0.050 M $MgCl_2$ of pH 7.2 at 30 °C (Δ). The fitted Scatchard equation and corresponding saturation curve (inset) are plotted using K_D' = 1.8 mM and Q'_{max} = −5.9 kcal/mol of subunit.

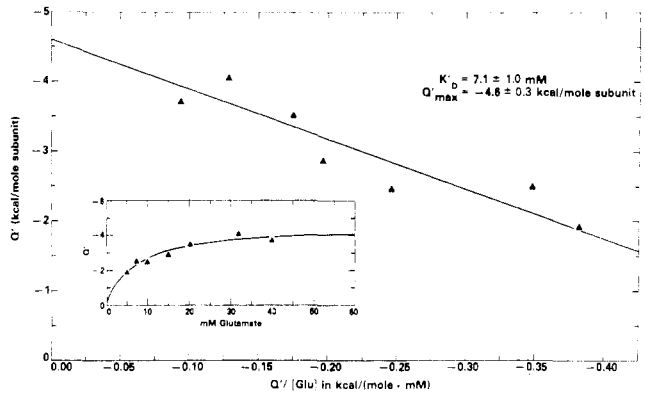

FIGURE 2: Thermal saturation data for binding L-glutamate to unadenylylated glutamine synthetase in the presence of 2.0 mM free ADP and 20 mM KPO_4 (ADP + P_i) in 0.10 M Tris-HCl, 0.10 M KCl, and 0.050 M $MgCl_2$ of pH 7.2 at 30 °C (▲). The fitted Scatchard equation and corresponding saturation curve (inset) are plotted using K_D' = 7.1 mM and Q'_{max} = −4.6 kcal/mol of subunit.

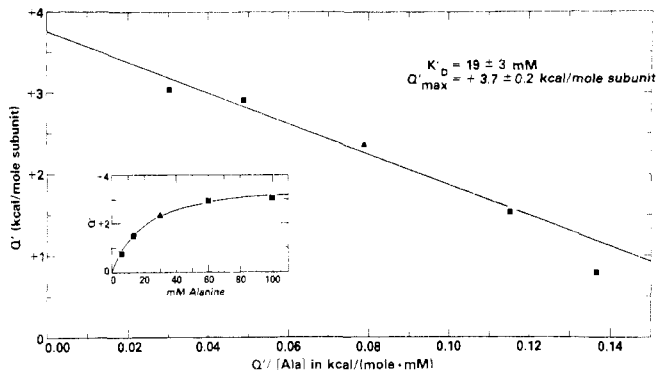

FIGURE 3: Thermal saturation data for binding L-alanine to unadenylylated glutamine synthetase in the presence of 2.0 mM free ADP and 20 mM KPO_4 (ADP + P_i) in 0.10 M Tris-HCl, 0.10 M KCl, and 0.020 M $MgCl_2$ (■) or 0.050 M $MgCl_2$ (▲) of pH 7.2 at 30 °C. The fitted Scatchard equation and corresponding saturation curve (inset) are plotted using K_D' = 19 mM and Q'_{max} = +3.7 kcal/mol of subunit.

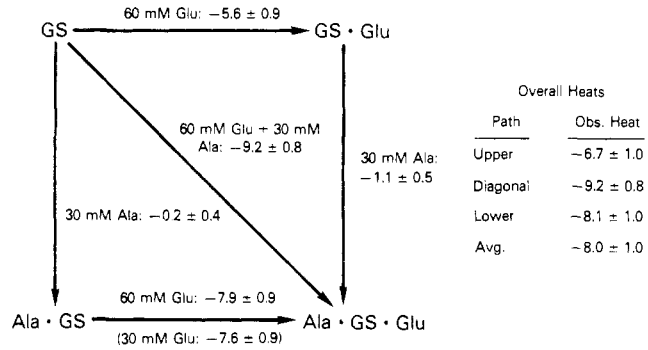

FIGURE 4: Case I (ADP alone). Observed heats in kcal/mol of subunit (uncorrected for level of enzyme saturation) for binding L-glutamate and L-alanine (sequentially and simultaneously) to unadenylylated glutamine synthetase in the presence of 2.0 mM free ADP in 0.10 M Tris-HCl, 0.10 M KCl, and 0.050 M $MgCl_2$ of pH 7.2 at 30 °C. Errors in constituent individual reactions were computed assuming an error of ±0.2 mcal in each observed heat. The overall heats (GS → Ala·GS·Glu) for the upper, diagonal, and lower paths (for the 60 mM L-glutamate-30 mM L-alanine data) are given together with the average heat for the three paths. The error in each path is the square root of the sum of the squares of errors for constituent reactions; the error with the average path is the standard deviation.

ence of 50 mM Mg^{2+} and those of L-alanine binding to the enzyme in the absence of L-glutamate were in 20 or 50 mM Mg^{2+} and those of P_i binding were in 20 mM Mg^{2+}.

Thermal saturation data (Figures 1-3) gave dissociation constants and Q'_{max} values, which were obtained by fitting the Scatchard equation to these data. An iterative least-squares fitting procedure was used as described previously (Shrake et al., 1977). A stoichiometry of 12 equiv/dodecamer was assumed for L-glutamate and for L-alanine binding. We assumed an error of ±0.2 mcal in the heats of reaction and no error in substrate concentration. Errors in the K_D' values were estimated by considering scatter in the experimental points. Errors in the Q'_{max} values derive from those in the corresponding K_D' values. These errors are given in Figures 1-3 and the ranges of enzyme saturation were: 49-98%, 42-88%, and 21-81%, respectively. All data points are within ±0.2 mcal of the lines corresponding to the fitted Scatchard equations. Thus, all Scatchard plots are linear within experimental error.

The sequential and simultaneous heats of binding L-glutamate and L-alanine to unadenylylated glutamine synthetase in the presence of ADP and of ADP + P_i are in Figures 4 and 5, respectively; these are directly measured heats (corrected for heats of dilution) in Tris buffers with no correction for the level of enzyme saturation. Some of the corresponding heats in Hepes buffers are given in Table II. The heats of binding P_i to the ADP-Mg-enzyme complex (uncorrected for saturation) in Tris and Hepes buffers are given in Table II. Measuring the heat of binding P_i was necessary in order to obtain enthalpies of binding for L-glutamate and L-alanine from observed heats. Errors in Figures 4 and 5 and in Table II were computed assuming an error of ±0.2 mcal in each directly measured heat.

Table III contains observed and estimated dissociation constants for the given reactions. Estimated dissociation constants were calculated assuming random binding of L-glutamate, P_i, and L-alanine to separate enzyme sites. The thermodynamic parameters for those binding reactions for which the data are available are presented in Table IV. The free energy changes were calculated from the association constants (expressed in units of M^{-1}) assuming a standard state for hydrogen ion activity of $10^{-7.2}$ M. The enthalpy changes were corrected for proton uptake.

Case I: In the Presence of ADP. Binding L-glutamate to unadenylylated glutamine synthetase in Tris buffer (Figure 1) is quite exothermic (Q'_{max} = −5.9 kcal/mol of subunit) and K_D' is 1.8 mM. The heat of binding L-alanine at 30 mM concentration to the enzyme (Figure 4) is slightly exothermic or

TABLE II: Observed Heats for Binding L-Glutamate, P_i, and L-Alanine to Unadenylylated Glutamine Synthetase at pH 7.2 and 30 °C.

Ligand added	Final free concn of ligand (mM)	Effector(s) present (final free concn)	Obsd heats of binding[a,b] In Tris-HCl	In Hepes-KOH
L-Glutamate	30	2.0 mM ADP	−5.8 ± 0.9[c,d]	−6.5 ± 0.8
L-Alanine	30	2.0 mM ADP	−0.2 ± 0.4[d]	−0.4 ± 0.5
P_i	10	2.0 mM ADP	+1.7 ± 0.5[e]	+0.3 ± 0.5[e]
L-Glutamate	20	2.0 mM ADP + 20 mM P_i	−3.5 ± 0.8[c]	−4.2 ± 0.6
L-Alanine	30	2.0 mM ADP + 20 mM P_i	+2.4 ± 0.4[c]	+1.5 ± 0.4[e]

[a] Errors were calculated assuming an experimental error of ±0.2 mcal in each directly measured heat of reaction. [b] All buffers contained: 0.10 M Tris-HCl or 0.050 M Hepes-KOH, 0.10 M KCl, and 0.050 M $MgCl_2$ of pH 7.2 unless otherwise noted. [c] These values are from the corresponding data sets used to determine the thermal saturation curves in Figures 1-3. [d] This buffer contained 0.050 M Tris-HCl. [e] This buffer contained 0.020 M $MgCl_2$.

zero; this heat is also very small in Hepes buffer (Table II). This may be due to either very weak binding of L-alanine (Ginsburg, 1969) or to a very small enthalpy of binding L-alanine under these conditions.

For showing separateness of binding sites for L-glutamate and L-alanine, consider the heat of binding L-alanine to the enzyme in the presence of 60 mM (saturating) L-glutamate; at 30 mM L-alanine (Figure 4), this value is −1.1 ± 0.5 kcal/mol of subunit (an average of three determinations). If L-alanine and L-glutamate were competing for the same site and if, in the extreme case, L-alanine (30 mM) completely displaced the saturating L-glutamate, the observed heat would be +5.4 kcal/mol of subunit. All intermediate cases of competition, in which only part of the glutamate is displaced by the alanine, predict smaller yet positive heats. Because 60 mM L-glutamate is saturating and its heat of binding is large and negative and that for L-alanine at 30 mM concentration is very small, *any negative* heat generated on binding L-alanine in the presence of saturating L-glutamate must derive from L-alanine binding to a site on the enzyme subunit that is separate from that for L-glutamate.

The overall heats (GS → Ala·GS·Glu) of the three paths (Figure 4) must be equal regardless of whether L-glutamate and L-alanine have separate sites or a common binding site on the enzyme. Therefore, the most accurate value for the overall heat is the average value (−8.0 kcal/mol of subunit; Figure 4). At most, the deviations of the three overall heats from the average value are only slightly greater than the computed errors in each of the overall heats. Thus, the estimated errors in the heats of the constituent reactions of Figure 4, which were used to calculate the errors in the overall heats, are reasonable. Furthermore, the average value of the three overall heats indicates that the heat for the upper path is probably more negative than −6.7 kcal/mol of subunit; this suggests that the heat of binding L-alanine (30 mM) in the presence of 60 mM L-glutamate may be even more negative than −1.1 kcal/mol of subunit.

Separate binding sites for L-glutamate and L-alanine are also demonstrated by the heat measured for the simultaneous binding of L-glutamate (60 mM) and L-alanine (30 mM) to the enzyme (Figure 4); this value is −9.2 ± 0.8 kcal/mol of subunit. If only L-glutamate were bound during the simultaneous addition of both ligands (an extreme case), the heat evolved would be −5.6 ± 0.9 kcal/mol of subunit. If L-glutamate and L-alanine were competing for the same site, heats less negative than −5.6 kcal/mole subunit would result. Because all three heats of binding are negative or zero and because the simultaneous heat of binding both ligands is *more negative* than either individual heat of binding, L-glutamate and L-alanine must bind at separate sites on the enzyme.

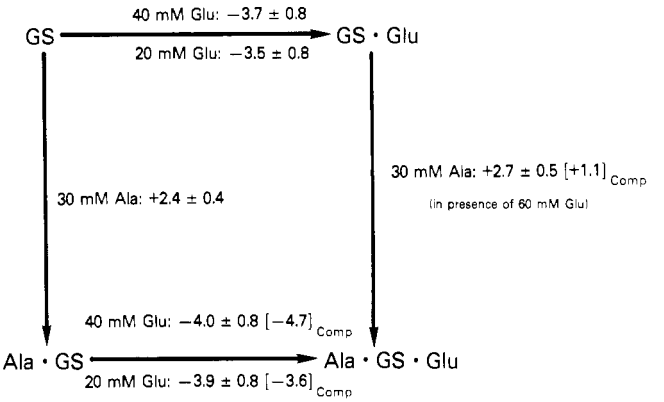

FIGURE 5: Case II (ADP + P_i). Observed heats (uncorrected for level of enzyme saturation) for sequentially binding L-glutamate and L-alanine to unadenylylated glutamine synthetase and the corresponding theoretical heats of binding for competition between these ligands for a mutually exclusive site (values in brackets; see Results). All values are in kcal/mol of subunit. Observed heats were measured in the presence of 2.0 mM free ADP and 20 mM KPO_4 (ADP + P_i) in 0.10 M Tris-HCl, 0.10 M KCl, and 0.050 M $MgCl_2$ of pH 7.2 at 30 °C. Errors in individual reactions were calculated assuming an error of ±0.2 mcal in observed heats.

In the presence of 30 mM L-alanine, the heat for binding L-glutamate at 30 mM concentration agrees quite well with that at 60 mM concentration (Figure 4). This is anticipated for separate ligand binding sites with no antagonism between the binding of ligands since in the absence of L-alanine the saturation levels at 30 and 60 mM L-glutamate are 94 and 97%, respectively (Figure 1). The heat of binding L-alanine (30 mM) to the enzyme in the presence of 60 mM L-glutamate is more negative than the very small heat observed in the absence of L-glutamate (Figure 4); this indicates either a synergistic effect between the binding of L-alanine and L-glutamate or a more negative enthalpy of binding L-alanine to the ADP-MgGS-Glu complex than to the ADP-MgGS complex. In fact, synergism between L-alanine and L-glutamine binding in the absence of ADP was suggested previously by equilibrium dialysis measurements (Ginsburg, 1969).

Case II: In the Presence of ADP + P_i. The scheme for the sequential binding of L-glutamate and L-alanine to the enzyme in the presence of saturating ADP and 20 mM KPO_4 is given in Figure 5. Measuring the heat of binding L-alanine (30 mM) in the presence of 60 mM L-glutamate was feasible. However, for the L-glutamate binding reaction, the maximum final concentration of L-glutamate was 40 mM because initial concentrations in excess of 60 mM resulted in precipitation (see Materials and Methods).

In order to show the existence of separate binding sites for L-glutamate and L-alanine in this case, the predicted heats of

binding one ligand in the presence of the other assuming direct competition between them for the same site were computed[2] for comparison with the observed values. This was necessary because the heats of binding each ligand were of different signs and because the level of enzyme saturation with P_i changed on the addition of each ligand (see Discussion). The observed heat of binding L-alanine (30 mM) in the presence of 60 mM L-glutamate, +2.7 ± 0.5 kcal/mol of subunit (Figure 5) does not agree with that predicted for direct competition between L-glutamate and L-alanine for the same site, +1.1 kcal/mol of subunit (Figure 5). This result is consistent with the existence of separate binding sites for L-glutamate and L-alanine on the ADP-P_i-Mg-enzyme complex.

Discussion

In their NMR studies with unadenylylated glutamine synthetase, Dahlquist & Purich (1975) observed in the presence of the Mn-enzyme a slight sharpening of the methyl resonance of L-alanine by L-glutamate.[3] They interpreted this as displacement of L-alanine by L-glutamate from a common site (or mutually exclusive sites) on the enzyme. This conclusion is not supported by our results.

Case I: In the Presence of ADP. In the absence of ADP, K_D' values of 3–5 mM have been estimated for the dissociation of L-glutamate from both unadenylylated and adenylylated Mn-enzyme-glutamate complexes (Dahlquist & Purich, 1975; Villafranca & Wedler, 1974). K_D' values of 5–7 mM for the dissociation of L-glutamine from the Mn-enzyme-glutamine complexes were reported previously (Shrake et al., 1977). ADP is synergistic to the binding of L-glutamine to unadenylylated Mn-enzyme (Shrake et al., 1977). Thus, we chose to work in the presence of saturating ADP in an effort to saturate the enzyme with L-glutamate.

The thermodynamic parameters of Table IV for L-glutamate binding to the unadenylylated ADP-Mg-enzyme complex are identical with the corresponding $\Delta G'$, $\Delta H'$, and $\Delta S'$ values for L-glutamine binding to the ADP-Mn-enzyme complex (Shrake et al., 1977). Furthermore, the small proton uptake is the same for binding either substrate. These observations suggest that the mechanisms for the binding of the substrates L-glutamate and L-glutamine to the ADP-Me-GS complex are the same. The γ-carboxyl group of L-glutamate therefore does not appear to be directly involved in the binding mechanism.

TABLE III: Some Observed and Estimated Dissociation Constants of Unadenylylated Glutamine Synthetase.

Reaction[a]	K_D' (mM)[b]	Symbol
ADP-MgGS + Glu	1.8 (obsd)[c]	K_{Glu}°
ADP-MgGS + P_i	5.0 (obsd)[d]	K_P°
ADP-P_i-MgGS + Glu	27 (est)	K_{Glu}^{P}
ADP-MgGS-Glu + P_i	75 (est)	K_P^{Glu}
ADP-P_i-MgGS + Ala	15 (est)	K_{Ala}^{P}
ADP-MgGS-Ala + P_i	0.26 (est)	K_P^{Ala}

[a] MgGS represents the unadenylylated subunits of the Mg-enzyme. All unadenylylated Mg-enzyme subunits are in the form of the complex represented in the table. [b] The *estimated* dissociation constants are determined in the following way. Assuming separate binding sites and random binding for L-glutamate and P_i, the *apparent* dissociation constant for L-glutamate in the presence of a concentration $[P_i]$ of orthophosphate is given by: $K_{Glu}^{app} = K_{Glu}^{\circ}(1 + [P_i]/K_P^{\circ})/(1 + [P_i]/K_P^{Glu})$. K_{Glu}^{app} is 7.1 mM and $[P_i]$ is 20 mM (Figure 2). Solving the equation above gives K_P^{Glu}. Assuming the required reciprocal thermodynamic relationship, the dissociation constant for L-glutamate in the presence of saturating P_i is given by: $K_{Glu}^{P} = K_{Glu}^{\circ}K_P^{Glu}/K_P^{\circ}$. The estimated K_D' values for L-alanine are calculated in a similar manner assuming $K_{Ala}^{\circ} \simeq 300$ mM. Dahlquist & Purich (1975) estimated 300 mM for the K_D' for the dissociation of L-alanine from the MnGS-Ala complex and observed little effect of ADP on this constant. Furthermore, the value of K_{Ala}^{P} is not strongly dependent on the value used for K_{Ala}° when the latter is large (>100 mM). [c] This work; pH 7.2 at 30 °C. [d] Timmons et al. (1974); pH 7.2 at 25 °C.

For unadenylylated glutamine synthetase, Timmons et al. (1974) have estimated a K_D' value of 20 mM for the dissociation of L-glutamate from the MgGS-Glu complex and have measured a K_D' of 3 mM for the dissociation of L-glutamate from the ATP-MgGS-Glu complex. The latter value agrees well with the K_m for L-glutamate in the Mg^{2+}-supported biosynthetic reaction catalyzed by the unadenylylated enzyme (Denton & Ginsburg, 1970). The K_D' for the dissociation of L-glutamate from the ADP-MgGS-Glu complex is 1.8 mM (Figure 1). These values reflect a synergistic effect between the binding of L-glutamate and nucleotide (ADP or ATP) to the Mg-enzyme.

The results from the calorimeter experiments in the presence of saturating ADP (case I) are unequivocal in showing separateness of binding sites for L-glutamate and L-alanine on the unadenylylated Mg-enzyme: (a) Since the heat of binding L-glutamate (60 mM) is large and negative and that for L-alanine binding at 30 mM concentration is approximately zero (Figure 4), the *negative* heat evolved upon binding L-alanine (30 mM) in the presence of saturating (60 mM) L-glutamate (Figure 1) indicates separate binding sites for the substrate and inhibitor on the enzyme subunit. (b) Furthermore, because the absolute value of the heat for the simultaneous binding of L-glutamate (60 mM) and L-alanine (30 mM) to the enzyme is *greater than* the absolute value of either individual heat of binding ligand and because all three heats of binding are the same sign or zero, L-glutamate and L-alanine must bind at separate sites on glutamine synthetase. This attains for any level of enzyme saturation with either ligand.

Case II: In the Presence of ADP + P_i. Fluorescence studies of the L-alanine concentration dependence of the apparent dissociation constant for ATP gave dissociation constants for L-alanine in the absence and presence of saturating ATP of 300 and 7 mM, respectively (Dahlquist & Purich, 1975). This in-

[2] The predicted heats of binding one ligand in the presence of the other for direct competition between them for the same site were calculated by deriving expressions for the equilibrium concentrations of the allowed enzyme species (ADP-MgGS, ADP-P_i-MgGS, ADP-P_i-MgGS-Glu, ADP-P_i-MgGS-Ala, ADP-MgGS-Glu, and ADP-MgGS-Ala) in terms of total enzyme concentration, [Glu], [Ala], and the appropriate dissociation constants of Table III. Thus, the changes in saturation for each enzyme species for the desired reaction in Figure 5 were determined. The heats of formation of each species in Tris buffer from ADP-MgGS, P_i, Glu, and/or Ala were calculated from the $\Delta H'$ and proton uptake values for binding Glu and P_i to ADP-MgGS and from those for binding Glu and Ala to ADP-P_i-MgGS (see Table IV). The predicted heat for a particular competition reaction (Figure 5) is the sum of the products of the change in saturation for each species multiplied by the corresponding heat of formation. Because of the small change in saturation of ADP-MgGS-Ala, the contribution from this species was assumed negligible.

[3] Interaction of the paramagnetic Mn^{2+} ion bound to glutamine synthetase with the nuclear spins of L-alanine bound to the enzyme results in a broadening of the NMR resonance of the methyl doublet of L-alanine relative to that of free L-alanine. Such an interaction occurs only if the ligand whose nuclear spins are affected and the paramagnetic species are both bound to the protein. The sharpening of the methyl resonance of L-alanine by the addition of L-glutamate may arise for one or more of several reasons (Dahlquist & Purich, 1975): (1) a decrease in the affinity of L-alanine for the Mn-enzyme-glutamate complex; (2) an increase in the bound lifetime of L-alanine, thereby possibly increasing its affinity for the complex; (3) a conformational change that increases the distance from the bound Mn^{2+} ion to the bound L-alanine; (4) the displacement of Mn^{2+} by L-glutamate.

TABLE IV: Thermodynamic Parameters for Binding L-Glutamate, P_i, and L-Alanine to Unadenylylated Glutamine Synthetase at 30 °C and pH 7.2.[a]

Reaction[b]	$\Delta G'$[c] (kcal/mol of subunit)	$\Delta H'$[d] (kcal/mol of subunit)	$\Delta S'$[e] (cal/(deg mol of subunit))	Proton uptake[f] (equiv/subunit)
ADP-MgGS + Glu	−3.81	−7.7	−13	0.2
ADP-MgGS + P_i	−3.17[g]	−1.1	+7	0.3
ADP-P_i-MgGS + Glu	−2.18	−4.1	−6	1.1
ADP-P_i-MgGS + Ala	−2.53	+1.8	+14	0.2
ADP-MgGS-Glu + P_i[h]	−1.54	+2.5	+13	1.2

[a] Measurements were made in buffers containing 0.10 M KCl and saturating $MgCl_2$ (0.050 or 0.020 M). [b] Stoichiometry of L-glutamate binding is assumed to be 1 mol per mol of subunit and that of P_i and that of L-alanine are assumed to be the same. [c] Free energy changes were calculated from the association constants expressed in units of M^{-1} (corresponding K_D' values are given in Table III), assuming a standard state for hydrogen ion activity of $10^{-7.2}$ M. [d] Enthalpy changes were corrected for proton uptake using the appropriate $\Delta H'_{Tris}$ and $\Delta H'_{Hepes}$ values and heats of protonation (Table I). For the first reaction, $\Delta H'_{Tris}$ is the Q'_{max} value of Figure 1 and $\Delta H'_{Hepes}$ derives from Table II and the K_D' value of Figure 1. For the second reaction, $\Delta H'_{Tris}$ and $\Delta H'_{Hepes}$ values derive from Table II and the appropriate K_D' value of Table III. $\Delta H'_{Tris}$ for the third reaction was calculated from that for the first reaction, that for the second reaction, Q'_{max} of Figure 2, and changes in saturation of the relevant enzyme species on adding saturating L-glutamate (ADP-MgGS-Glu, P_i-ADP-MgGS, and P_i-ADP-MgGS-Glu). The saturation changes were computed from appropriate K_D' values of Table III. The $\Delta H'_{Hepes}$ value was similarly calculated. $\Delta H'_{Tris}$ for the fourth reaction was calculated from that for the second reaction, Q'_{max} of Figure 3, and changes in saturation of the relevant enzyme species on adding saturating L-alanine (P_i-ADP-MgGS and P_i-ADP-MgGS-Ala; because of the small change in saturation of ADP-MgGS-Ala, the contribution from this species was assumed negligible). The saturation changes were computed from appropriate K_D' values of Table III. The $\Delta H'_{Hepes}$ value was similarly calculated. [e] Entropy changes were computed from the relationship $\Delta S' = (\Delta H' - \Delta G')/T$, where T is the absolute temperature (303 K). [f] Proton uptake was calculated using the appropriate $\Delta H'_{Tris}$ and $\Delta H'_{Hepes}$ values and the heats of protonation of appropriate buffers (Table I). [g] The binding measurement was made at 25 °C (Timmons et al., 1974). The $\Delta G'$ value was corrected to 30 °C using $\Delta H'$. [h] All thermodynamic parameters for this reaction were computed assuming separate binding sites and random binding for L-glutamate and P_i and using the required reciprocal thermodynamic binding relationship.

dicates an extremely strong synergistic effect between the binding of L-alanine and ATP to the enzyme.

ATP also caused a sharpening of the methyl resonance in the NMR spectrum of L-alanine in the presence of unadenylylated Mn-enzyme (Dahlquist & Purich, 1975); ADP + P_i showed the same dramatic sharpening of the L-alanine line width, whereas ADP or P_i alone had little effect. The foregoing suggest that ADP + P_i, like ATP, has a synergistic effect on the binding of L-alanine and that separately neither ADP nor P_i perturbs the binding of L-alanine to the enzyme.

In an attempt to mimic the action of ATP, the heats of binding L-alanine were measured in the presence of saturating ADP and 20 mM KPO_4 (Figure 3). In the presence of ADP + P_i, the apparent enthalpy of binding L-alanine in Tris buffer becomes positive (see Table II) and large enough to obtain a thermal saturation curve for L-alanine binding (Figure 3). The calorimetric results are completely compatible with the fluorescence and NMR observations of Dahlquist & Purich (1975) on L-alanine binding to the enzyme without effectors and in the presence of ATP, ADP, P_i, or ADP + P_i. The small heat on binding L-alanine in the presence of ADP alone (case I) may well reflect very weak binding, whereas in the presence of ADP + P_i (case II), the apparent dissociation constant is 19 mM (Figure 3).

The K_D' for the dissociation of P_i from the ADP-P_i-MgGS complex is 5 mM (Timmons et al., 1974). Thus, 20 mM P_i is subsaturating (approximately 80% in the absence of L-glutamate and L-alanine) and the resulting dissociation constants and Q'_{max} values in the presence of 20 mM P_i given in Figures 2 and 3 are *apparent* thermodynamic parameters. The K_D' for the dissociation of L-alanine from the ADP-P_i-MgGS-Ala complex is estimated as 15 mM (Table III).

The binding of L-glutamate to glutamine synthetase is weaker in the presence of ADP + P_i than in the presence of ADP alone with apparent dissociation constants of 7.1 and 1.8 mM, respectively (Figures 1 and 2). This may be interpreted as antagonism between the binding of L-glutamate and P_i at separate sites or competition between them for mutually exclusive sites (or a common site). The calculated competition constant[4] for L-glutamate in the presence of 20 mM P_i is 9 mM, which is in accord with the observed value of 7.1 ± 1 mM (Figure 2). However, competition predicts a negative enthalpy of binding for P_i in Tris buffer (−1.6 kcal/mol of subunit),[5] whereas the observed value in Tris buffer is positive (+2.6 ± 0.8 kcal/mol of subunit; see Tables II and III). Thus, L-glutamate and P_i must bind at separate, nonmutually exclusive sites. The K_D' for the dissociation of L-glutamate from the ADP-P_i-MgGS-Glu complex is estimated as 27 mM and is 15-fold greater than that measured in the absence of P_i (Table III). These data quantitate the antagonistic effect between the binding of L-glutamate and P_i to unadenylylated Mg-enzyme in the presence of saturating ADP.

The synergistic effect between the binding of L-glutamate and ADP to unadenylylated Mg-enzyme is overcome by the antagonistic effect between L-glutamate and P_i in the presence of saturating ADP. The net effect is an antagonistic effect between L-glutamate and the combination ADP + P_i. However, a strong synergistic effect between L-glutamate and ATP has been observed (Timmons et al., 1974). Thus, in the case of L-glutamate, the combination ADP + P_i does *not* mimic the behavior of ATP.

The entropies of binding P_i to ADP-MgGS and to ADP-MgGS-Glu (Table IV) are both positive and the latter is more positive than the former. This suggests that binding P_i to the ADP-Mg-enzyme complex is disruptive and more disruptive in the presence of bound L-glutamate. The negative entropies

[4] $K_D^{CompA} = K_D^A(1 + ([B]/K_D^B))$, where K_D^{CompA} is the competition constant (or *apparent* dissociation constant) for ligand A in the presence of ligand B at a concentration [B], and K_D^A and K_D^B are dissociation constants for ligands A and B, respectively (each in the absence of the other). For example, if L-glutamate and P_i were competing for mutually exclusive sites (or a common site), the predicted value of the competition constant for L-glutamate ($K_D^{CompGlu}$) in the presence of 20 mM P_i would be 9 mM assuming K_D^P and K_D^{Glu} are 5 and 1.8 mM, respectively.

[5] The predicted enthalpy of binding P_i in Tris buffer assuming mutually exclusive sites (or a common site) for P_i and L-glutamate was calculated in the following way. The difference between the Q'_{max} values in Figures 2 and 1 is the heat of displacement of all the P_i by L-glutamate in Figure 2. In the absence of L-glutamate, 20 mM P_i is 80% saturating.

of binding L-glutamate (Table IV) reflect the above effect. This may arise from steric considerations since P_i has an additional oxygen atom in the ADP + P_i combination relative to ATP (Rhee et al., 1976). Furthermore, a large proton uptake occurs on binding L-glutamate to ADP-P_i-MgGS, whereas the proton uptake attending the binding of this ligand to ADP-MgGS is small (Table IV); the proton uptake data for the binding of P_i demonstrate the reciprocal effect (Table IV). The large proton uptake may involve protonation of the enzyme complex (ADP-P_i-MgGS-Glu) thereby reducing repulsive interactions between negatively charged groups. The steric effect may also explain the antagonism between L-glutamate and ADP + P_i binding in contrast to the synergism between L-glutamate and ATP binding.

The enthalpies of binding L-glutamate to ADP-MgGS and to ADP-P_i-MgGS are both negative, whereas that for L-alanine binding to ADP-P_i-MgGS is positive (Table IV). On binding to the ADP-P_i-MgGS complex, L-glutamate exhibits a large proton uptake whereas that attending the binding of L-alanine is small (Table IV). The combination ADP + P_i is antagonistic to the binding of L-glutamate but synergistic to that of L-alanine. These observations indicate very dissimilar modes of binding for these two ligands. In addition, the observed heat of binding L-alanine (30 mM) in the presence of ADP + P_i and 60 mM L-glutamate does not agree with that predicted for direct competition for a common site or mutually exclusive sites (Figure 5). Furthermore, the observed heat of binding one ligand is unchanged in the presence of the other. These results indicate the existence of separate binding sites for L-glutamate and L-alanine on the ADP-P_i-MgGS complex.

Conclusion

The calorimetric studies presented here demonstrate the existence of separate sites on unadenylylated glutamine synthetase for the substrate L-glutamate and the feedback inhibitor L-alanine, thereby establishing the latter as an allosteric effector of the Mg-enzyme. All calorimetric data are compatible with the experimental results of Dahlquist & Purich (1975). The single difference is a matter of interpretation of a portion of the NMR data; the calorimetric data suggest that binding L-glutamate to the enzyme induces a conformational change in the complex such that the distance between the bound L-alanine and Mn^{2+} ion increases and/or such that the bound lifetime of L-alanine increases.

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