Catalytic Mechanisms of Glutamine Synthetase Enzymes

STUDIES WITH ANALOGS OF POSSIBLE INTERMEDIATES AND TRANSITION STATES*

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Glutamine synthetase enzymes isolated from pea seeds and from Escherichia coli are observed to behave differently in experiments designed to probe reaction mechanism. Although both enzymes were found to bind and release substrates in random order mechanisms (Wedler, F. C. (1974) J. Biol. Chem. 247, 5080-5087), isotopic exchanges with partial reaction systems indicative of a γ-glutamyl-phosphate intermediate are catalyzed only by the pea seed enzyme. The E. coli system fails to catalyze any exchanges at appreciable rates unless all substrates are present. This negative result implies either an absolute conformational requirement for bound substrates or that the putative complex (E · Glu-P · MgADP) is exceedingly tight. To test the latter, a nonreactive structural analog of γ-glutamyl-phosphate, namely 3-(phosphonoacetylamido)-L-alanine (PA,LA), has been synthesized. With the E. coli enzyme PA,LA was found to bind no more tightly than L-glutamate and is strictly competitive versus L-glutamate (Kᵢ = 3 mm). Thus, failure to catalyze partial exchange reactions indicative of γ-Glu-P is probably not attributable to tight complex formation. The binding of PA,LA with the pea seed enzyme apparently involves a two-step process: a rapid, reversible step in which PA,LA binds 10-fold more tightly than L-glutamate, followed by a slow (but reversible) process involving very tight PA,LA binding, apparently with enzyme isomerization promoted by nucleotide.

The specificity of the two enzymes toward L-methionine-SR-sulfoximine, Met(O)(NH), was also different. Competitive Kᵢ values, measured by steady state kinetics versus L-glutamate, are lower than I₅₀ values, measured by the [Met(O)(NH)] required for the half-maximal rate of enzyme inactivation in the presence of MgATP. For the E. coli enzyme Kᵢ = 1.5 μM, I₅₀ = 105 μM, and I₅₀/Kᵢ = 70; but for the pea seed enzyme, Kᵢ = 200 μM, I₅₀ = 1000 μM, and I₅₀/Kᵢ = 5.0. These and other data suggest that Met(O)(NH) in the E. coli enzyme active site may be an analog of a stabilized transition state or intermediate, with the imino group bound in the ammonia site but improperly situated for reaction with ATP. The pea seed enzyme exhibits much lower specificity for Met(O)(NH), but reaction with ATP occurs readily. Planar anions such as nitrate do not inhibit either enzyme as analogs of the transition state at the γ-phosphoryl of ATP.

It is proposed that the E. coli enzyme may form γ-Glu-P as a highly transient species, but does not stabilize or tightly bind this moiety. Rather, once ammonia is present, a stable tetrahedral intermediate at the γ-carboxylate of L-glutamate may be formed. In contrast, the pea seed enzyme appears to form γ-Glu-P preferentially, binding it tightly in the active site so as to exclude water, but binds the tetrahedral intermediate mimicked by Met(O)(NH) much less specifically. Differences in active site architecture may dictate such mechanistic differences, in particular the "tight" (nₜ) metal ion site recently discovered to be within bonding distance of the γ-carboxylate of L-glutamate in the E. coli enzyme.

Glutamine synthetase, a key enzyme in nitrogen metabolism (1), has been of interest to enzymologists in terms of its mechanisms of regulation and catalysis. At present there is considerable evidence (1–7) both in support of and against the possible existence of covalent intermediates in the pathway of the reactions catalyzed by these enzymes, especially γ-glutamyl-phosphate (γ-Glu-P). The majority of the attention has been focused on the enzymes from ovine brain, pea seeds, and Escherichia coli.

Considerable controversy has been generated by the earlier proposal of a "concerted," as opposed to a "stepwise" mechanism (via γ-glutamyl-P) for the E. coli enzyme (8), based on lack of observable independent exchanges without all substrates present. Since then, it has been shown that this en-
zyme can very slowly produce 5-oxoproline from L-glutamate and M"gATP and can also phosphorylate Met(OH)(NH)3 with M"g-ATP present (4). Evidence for an activated γ-glutamyl moiety (6) was further supported by chemical studies in which the γ-aldehyde can be isolated from quenched reactions after addition of hydride ions (7) and by fluorescent changes suggesting special conformations of the site related to the formation of an intermediate from L-glutamate and ATP (5). Although these results are interesting and suggestive, several key questions about the chemical nature of the intermediates remain unanswered. Since net turnover is rate-limited by product release rather than by covalent interconversion steps (2, 3, 5), it is difficult to define the chemistry of the reaction by the usual steady-state kinetic methods. Rapid quench experiments may perturb the conformation of the active site so as to alter kinetic responses under equilibrium exchange conditions. The results described here involve several new approaches to these problems, notably observations of the interaction of structural analogs of possible intermediates and transition states which may lie along the main reaction pathway. These studies have allowed new insights to differences in the chemistry dictated by enzyme active sites from different sources. It was very recently found that the rat brain possesses a partially inhibited kinetic responses under equilibrium exchange conditions indicative of random mechanisms. Thus, present attention is focused on these latter two systems with regard to possible differences in their mechanism of action.

EXPERIMENTAL PROCEDURES

Materials—Glutamine synthetase from green pea (dwarf Blue Bantam, untreated) was prepared according to Elliott (8) with the last step repeated twice, followed by chromatography on Bio-Gel A-0.5m. This resulted in an enzyme exhibiting a single band on polyacrylamide gel electrophoresis at pH 8.3 or in the presence of 0.1% sodium dodecyl sulfate (9) and which exhibited a specific activity of 485 units/mg. Unadenylated enzyme, prepared from Bio-Rad Laboratories. DEAE-cellulose (formate) used for separation of reaction components in exchange experiments was prepared from Whatman DE52 by batchwise treatment with 0.5 M formic acid, then washing to neutrality with double-distilled water. All inorganic salts and buffers were of ACS analytical grade. Enzymes were obtained from Sigma Chemical Co. The inhibitors L-methionine sulfone and L-methionine-SR-sulfoximine were also Sigma products, while 5'-adenylylimidodiphosphate was from P-L Biochemicals. Radioactive labeled substrates were from New England Nuclear, and [15N]ammonium chloride (99%) was from Bio-Rad Laboratories. DEAE-cellulose (formate) used for separation of reaction components in exchange experiments was prepared from Whatman DE52 by batchwise treatment with 0.5 M formic acid, then washing to neutrality with double-distilled water. All inorganic salts and buffers were of ACS analytical grade. Fluorescamine was a Roche product.

Synthesis and Analysis of PA,LA—3-(Phosphonoacetylamido)zingole (PA,LA) was synthesized in a manner somewhat analogous to that employed by Collins and Stark (11) for a similar compound. Preparation of phosphonoacetylized hydrazide was described earlier (11). The diazotization reaction of this intermediate compound (300 mg) was carried out in 10 ml of dimethylformamide (DMF) plus 0.5 ml of concentrated HCl and a stoichiometric amount of sodium nitrite (predissolved in 2.5 ml of dimethylformamide plus 0.5 ml of H2O, with stirring for 10 to 15 min). To this was then added 1:1 stoichiometric amounts of L-glutamine and ADP. At pH 6.5 to 6.8, a doublet at 83.6 for the β protons, and a doublet (J = 20) at 83.0 for the methane bridge protons. Splitting by protons on adjacent nitrogens was not observed, apparently due to rapid exchange with D2O, possibly catalyzed by the acidic phosphate or carboxylate groups. The peak positions and integrated areas (1:2:2, respectively) were compared to those of starting materials and were consistent with the structural assignment for PA,LA.

Mass spectral analysis was carried out with a Finnigan model 3500 instrument interfaced to a model 6000 data system. Direct chemical ionization analysis with methane carrier gas showed mass peaks for the parent ion plus H+, plus CH3+, and plus C2H5O+, as well as the key fragments for (HO,C(H,N)CH- and (-CH,NHCOOH,PO2H2) and derivatives thereof. Taken together, these results are consistent with the assigned structure for the product.

Enzyme Assays—Enzyme activity was routinely assayed by either the biosynthetic (P) releasing transferase (γ-glutamyl hydroxamate formation) techniques. These procedures used for PA,LA were as described by Shapiro and Stadtman (10). Procedures for the pea enzyme were as described by Elliott (8): 50 mM MgCl2 at pH 7.4, 30" for either the biosynthetic or forward transferase, using 25 mM NH4Cl or NH4OH, respectively. The pH-stat continuous assay technique (14) was used as a check on the fixed time incubation results. In the reverse transferase assay procedure, used with both enzymes at pH 7.4, the mixtures contained substrates at the following levels, in micromoles/ml: 50 imidazole, 50 Mgl2, 25 mM NH4Cl or NH4OH, respectively. The PH-star continuous assay technique (14) was used as a check on the fixed time incubation results. In the reverse transferase assay procedure, used with both enzymes at pH 7.4, the mixtures contained substrates at the following levels, in micromoles/ml: 50 imidazole, 50 MgCl2, 25 mM NH4Cl or NH4OH, respectively. The PH-star continuous assay technique (14) was used as a check on the fixed time incubation results. In the reverse transferase assay procedure, used with both enzymes at pH 7.4, the mixtures contained substrates at the following levels, in micromoles/ml: 50 imidazole, 50 MgCl2, 25 mM NH4Cl or NH4OH, respectively. The PH-star continuous assay technique (14) was used as a check on the fixed time incubation results. In the reverse transferase assay procedure, used with both enzymes at pH 7.4, the mixtures contained substrates at the following levels, in micromoles/ml: 50 imidazole, 50 MgCl2, 25 mM NH4Cl or NH4OH, respectively. The PH-star continuous assay technique (14) was used as a check on the fixed time incubation results. In the reverse transferase assay procedure, used with both enzymes at pH 7.4, the mixtures contained substrates at the following levels, in micromoles/ml: 50 imidazole, 50 MgCl2, 25 mM NH4Cl or NH4OH, respectively. The PH-star continuous assay technique (14) was used as a check on the fixed time incubation results. In the reverse transferase assay procedure, used with both enzymes at pH 7.4, the mixtures contained substrates at the following levels, in micromoles/ml: 50 imidazole, 50 MgCl2, 25 mM NH4Cl or NH4OH, respectively. The PH-star continuous assay technique (14) was used as a check on the fixed time incubation results. In the reverse transferase assay procedure, used with both enzymes at pH 7.4, the mixtures contained substrates at the following levels, in micromoles/ml: 50 imidazole, 50 MgCl2, 25 mM NH4Cl or NH4OH, respectively. The PH-star continuous assay technique (14) was used as a check on the fixed time incubation results. In the reverse transferase assay procedure, used with both enzymes at pH 7.4, the mixtures contained substrates at the following levels, in micromoles/ml: 50 imidazole, 50 MgCl2, 25 mM NH4Cl or NH4OH, respectively. The PH-star continuous assay technique (14) was used as a check on the fixed time incubation results. In the reverse transferase assay procedure, used with both enzymes at pH 7.4, the mixtures contained substrates at the following levels, in micromoles/ml: 50 imidazole, 50 MgCl2, 25 mM NH4Cl or NH4OH, respectively. The PH-star continuous assay technique (14) was used as a check on the fixed time incubation results. In the reverse transferase assay procedure, used with both enzymes at pH 7.4, the mixtures contained substrates at the following levels, in micromoles/ml: 50 imidazole, 50 MgCl2, 25 mM NH4Cl or NH4OH, respectively. The PH-star continuous assay technique (14) was used as a check on the fixed time incubation results. In the reverse transferase assay procedure, used with both enzymes at pH 7.4, the mixtures contained substrates at the following levels, in micromoles/ml: 50 imidazole, 50 MgCl2, 25 mM NH4Cl or NH4OH, respectively. The PH-star continuous assay technique (14) was used as a check on the fixed time incubation results. In the reverse transferase assay procedure, used with both enzymes at pH 7.4, the mixtures contained substrates at the following levels, in micromoles/ml: 50 imidazole, 50 MgCl2, 25 mM NH4Cl or NH4OH, respectively. The PH-star continuous assay technique (14) was used as a check on the fixed time incubation results.
with either a Haake water bath or Tecam Dri-Block devices, each thermostat to ±0.2° of the desired temperature. The standard biosynthetic assay method was used, and multiple (>3) determinations were carried out for each enzyme at each temperature, so that individual rates values had <5% error. The reaction velocities (micromoles/min/mg of protein) were converted to first-order rate constants \( k \) using the molecular weight of 50,000 for enzyme subunits, assuming homogeneous, 100% active enzyme. These values were plotted as \( \log k \) versus \( 1/T \) (°K).

Isotopic Exchanges—Procedures for observing exchange kinetics between reactant and product substrate pools have been described elsewhere (2, 3), including procedures for separation of substrate pairs of interest and special techniques involving \(^{15}\text{N}-\)labeled compounds.

Solutions were rendered free of ammonia or nucleotide by rapid exposure to Dowex 50-K+ or Dowex 1-Cl—, respectively, immediately prior to use. As an extra precaution, enzyme solutions were first greatly diminished in ammonia by exhaustive reaction with excess MgATP and L-glutamate (4, 6). Enzyme was similarly rendered free of ATP with 10 mM NH\(_4\)Cl, 10 mM L-glutamate, and 50 mM MgCl\(_2\). Analysis for ammonia before and after such treatment was by the Nessler microdetermination of San Pietro (18) that detected NH\(_3\) at >10\(^{-4}\) M. Nucleotide was detected as ribose in the orcinol-H\(_2\)SO\(_4\) colorimetric procedure of Brueckner (19), used at micro scale and sensitive to ribose at >10\(^{-6}\) M. Dowex treatment after enzymic reaction removed NH\(_3\) or nucleotide to below the levels of detection in all cases, and that corresponds to <0.1 \( K_m \) for either substrate with either enzyme (8, 10).

RESULTS

Partial Exchange Reactions—Observation of independent partial exchange reactions in a multisubstrate enzyme system can provide insights to possible chemical intermediates. If \( \gamma\)-glutamyl-phosphate is readily formed by glutamine synthetase in a reaction such as

\[
\text{E} + \text{Glu} \rightleftharpoons (\text{E} - \text{Glu-ATP}) \rightleftharpoons (\text{E} - \text{Glu-P}) \rightleftharpoons (\text{E} - \text{Gln-P}) + \text{NH}_3 + \text{ADP} + \text{P}_i
\]

one should be able to observe both an ammonia- and glutamate-independent, glutamate-dependent ATP-ATP exchange, and a nucleotide-independent, \( \text{P}_i \)-dependent \( \text{NH}_3\)-Gln exchange.

Table I presents the results of attempts to observe four different isotopic exchange reactions with all substrates present and with various partial reaction systems. With each enzyme these were Glu-Gln, \( \text{NH}_3\)-Gln, ADP-ATP, and \( \text{P}_i\)-ATP. Very striking differences between the bacterial and plant enzymes are immediately apparent. The unadenylylated \( E. \text{coli} \) enzyme used here, as with the adenylylated enzyme, catalyzes only those partial reactions indicative of \( \gamma\)-glutamyl-phosphate formation, at approximately 10 to 20% the rate with the complete system. This is the first affirmative report of any such exchanges for a purified \( \gamma\)-glutamyl synthetase that occur at appreciable rates (3, 6). The formation of \( \gamma\)-glutamyl-phosphate by the \( E. \text{coli} \) enzyme was inferred by Weisbrod and Meister (45) for the pea seed enzyme, who observed release of 5-oxoproline from the \( E. \text{coli} \) enzyme incubated with \( \gamma\)-glutamyl-phosphate and M\(^{2+}\)ATP, but these rates were slower by approximately 10\(^3\) than those for net turnover with ammonia present.

For either enzyme with the complete reaction systems present, the relative rates of the individual exchanges are unequal. This could not be true if the rate of the catalytic process was the slowest step in net turnover. Therefore, the individual substrate release steps appear to be the main contributors to rate limitation in overall catalysis.

Various explanations are possible for failure of an enzyme to catalyze partial exchange reactions, even if a covalent intermediate is formed. These may include (a) a particular ordered mechanism of substrate binding or release, (b) formation of an exceptionally tight enzyme-intermediate complex, or (c) an active site conformational requirement for all substrates to be present. One may not invoke a as the basis for the lack of exchanges by the \( E. \text{coli} \) enzyme, since random order substrate binding mechanisms were found for both the adenylated and unadenylated forms (2, 3). To test hypothesis b, the binding of a nonreactive structural analog of \( \gamma\)-Glu-P to the plant and bacterial enzymes was investigated (see below).

As a probe for explanation (c), various K\(^+\) ion concentrations from 0.02 to 0.50 M were used to stimulate the exchanges catalyzed by the \( E. \text{coli} \) enzyme with the complete reaction system or to produce stimulation of the exchanges absent with partial reaction systems. All results were negative. This implies either that hypothesis c is incorrect with regard to glutamate promoted ADP-ATP exchange, or that this enzyme does not recognize K\(^+\) as an analog of NH\(_3\). The active site may be specific for neutral NH\(_3\) rather than NH\(_{3}^+\) ion.

Binding of \( \text{PA}_{LA} \)—The synthesis of a nonreactive analog of \( \gamma\)-Glu-P (I), namely 3-(phosphonoacetylamido)-L-alanine, abbreviated \( \text{PA}_{LA} \) (II), is described under “Experimental Procedures.”

Enzyme binding of \( \text{PA}_{LA} \) was first studied by steady-state
kinetics with PA₅LA versus L-glutamate as the varied substrate. PA₅LA was allowed to interact with each enzyme in several different ways: first, by addition of enzyme to assay mixtures containing PA₅LA and substrates, second, by preincubation of enzyme with PA₅LA at the assay temperature for 15 min prior to addition of this enzyme to assay mixture containing substrates and an identical concentration of PA₅LA. Each of these procedures was also carried out in the presence and absence of ADP and MgCl₂. The data of Table II, part A, indicate markedly different behavior of the E. coli and pea seed enzymes.

With the E. coli enzyme, the Kᵢ of PA₅LA (3 mM) is comparable to the Kᵢₑ of L-glutamate (2.5 mM). Inhibition patterns and slope replots showed that PA₅LA is competitive versus L-glutamate, and preincubation of PA₅LA with enzyme does not alter the Kᵢ value. Added MgADP enhances the affinity of enzyme for PA₅LA, just as it is known to do by synergism of binding (2) with L-glutamate.

Two nonkinetic spectroscopic observations are also in accord with the weak interaction of PA₅LA with E. coli glutamin synthetase. The perturbation of the ESR spectrum of bound Mn(II) in the tight (n) sites of the enzyme by PA₅LA is almost indistinguishable from that observed with L-glutamate. Second, fluorescence changes induced in the enzyme by bound PA₅LA alone are not distinguishable from those induced by L-glutamate alone.

PA₅LA interacts with the pea seed enzyme very differently. With no preincubation in the absence of MgADP, PA₅LA binds approximately 10-fold more tightly than L-glutamate in a manner competitive with L-glutamate. Upon preincubation or addition of MgADP, the type of inhibition changes from competitive to noncompetitive, and the apparent (noncompetitive) binding constants decrease markedly to a value of 18 μM upon preincubation with PA₅LA and MgADP. The competitive constants, Kᵢₑ extracted from the noncompetitive inhibition patterns (see Table II and "Experimental Procedures") show a less drastic decrease in the presence of MgADP or with preincubation.

Incubation of the pea seed enzyme with PA₅LA showed the basis for the conversion from competitive to noncompetitive inhibition to be a time-dependent loss of activity in a first-order process, induced by PA₅LA and enhanced by MgADP as shown in Fig. 1.

An alternative method of determining binding constants for PA₅LA with the pea seed enzyme was to observe the rate of the PA₅LA-induced inhibition as a function of PA₅LA concentration, in the presence and absence of ADP and MgCl₂. These data, shown in Table II, part B, indicate that the concentrations of PA₅LA required for the half-maximal rate of activity inhibition, called Iₐ₅₀ agree reasonably well with the Kᵢₑ values calculated from steady state kinetics (part A). These activation rate studies indicate that MgADP lowers the Iₐ₅₀ value rather than increasing the maximal rate of inactivation by PA₅LA, hᵢ (max).

Nature of PA₅LA Inhibition of Pea Seed Enzyme—PA₅LA does contain a potentially labile amide bond, and thus the possibility exists that inactivation of the pea seed enzyme could result from a functional group in the pea seed enzyme active site covalently reacting with the δ-carbonyl group of PA₅LA. The data of Table III indicate that the PA₅LA-induced inhibition is completely reversible. Enzyme that was first inhibited by PA₅LA in the presence or absence of MgADP, then dialyzed against 0.1 M L-glutamate and 0.2 M KCl, was completely reactivated. Thus, inhibition by PA₅LA is tight but reversible.

The possibility of a covalent reaction between the pea seed enzyme and PA₅LA was probed by a series of experiments with detection at the nanomolar level. A high concentration of pea seed enzyme, 100 nmol of active sites, was incubated with a 2-fold excess of PA₅LA and MgADP at pH 7.5, 30° for 30 min, in a total volume of 1.0 ml after which the enzyme was found by assay to be completely (>95%) inactivated. This mixture was then cooled to 4° and rapidly chromatographed on a Bio-Gel P-6 column (2 × 16 cm) equilibrated with 50 mM imidazole buffer, pH 7.5. The enzyme and the small molecules peaks were then collected separately and the latter concentrated in vacuo to a volume of 1 ml. The enzyme, freed of nonbound components, was then divided into two portions. One of these was heated to 100° for 5 min and the precipitated protein removed by centrifugation; the other portion was incubated with 0.2 M KCl overnight at 4°, then reduced in volume 10-fold by filtration on a Sartorius collodion bag device. The filtrate was collected and concentrated in vacuo to 1.0 ml. Enzyme in the concentrated solution was found to contain 80% of the original units of activity. Chromatographic separations were then carried out on each of the three small molecule fractions: (a) supernatant from the heat-killed PA₅LA-inhibited enzyme, (b) nonbound small molecules from the PA₅LA-inhibited enzyme from the Bio-Gel P-6 column, and (c) small molecules released from PA₅LA-inhibited enzyme by incubation with no excess PA₅LA and 0.2 M KCl, as well as on authentic PA₅LA and L-α,β-diaminopropionic acid (DAP). These fractions were reacted with fluorescamine and chromatographed on thin layer cellulose plates, using solvent system A according to Imai et al. (13).

Fig. 2 shows the results of these thin layer chromatography separations. PA₅LA and L-α,β-diaminopropionic acid clearly migrate with distinctly different Rᵢ values. In the initial incubation mixture among the nonbound small molecules (A), there is not detectable L-cytosine, nor does the heat-killed enzyme release any moiety with an RI different from PA₅LA. Finally, the dialysis reactivation of enzyme released only a component with Rᵢ different from PA₅LA. This is one of the cases where the dialysis reactivated enzyme released only a component with Rᵢ different from PA₅LA.

Interaction of Met(O) (NH) with Glutamine Synthetases—The binding of L-methionine-SR-sulfoxamine with the E. coli and pea seed glutamine synthetases was studied by several kinetic approaches. First, fixed levels of Met(O)(NH) were used in steady state experiments in competition with each of the substrates of the reaction. The results of two such experiments, carried out with L-glutamate as the varied substrate, are shown in Fig. 3. The v versus [S] plots indicate that the pea seed enzyme exhibits noncompetitive behavior, whereas the E. coli enzyme undergoes a large change in Kᵢₑ(Glu) at 5 to 10 μM Met(O)(NH), at which level of inhibitor the pattern is competitive, but changes to noncompetitive at 25 μM and higher Met(O)(NH). Values of Kᵢ and Kᵢₑ calculated from such plots are shown in Table IV, part A.

The basis for the noncompetitive inhibition patterns was explored further by incubation of Met(O)(NH) and also L-Met(O₂), with both enzymes in the presence of ATP and MgCl₂, with removal of samples at various times for assay. Related experiments with ovine brain and E. coli enzyme were reported previously (4, 20–22). Inhibition by L-Met(O₂) was completely and rapidly reversible with both enzymes, but inhibition by Met(O)(NH) was apparently irreversible and,
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TABLE II

Binding of glutamine synthetases with PA,LA

A, by steady state kinetics for PA,LA versus L-glutamate, pH 7.3, 50 mM MgCl₂ (where used). See text and "Experimental Procedures" for procedures and conditions. B, by rate of inhibition of pea seed glutamine synthetase, pH 7.5, 30°C (cf. Fig. 1).

<table>
<thead>
<tr>
<th>Additions (mm)³</th>
<th>Type inhibition pattern</th>
<th>Constant⁴</th>
<th>Additions (mm)³</th>
<th>Type inhibition pattern</th>
<th>Constant⁴</th>
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<td></td>
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<tr>
<td>E. coli (37°C)</td>
<td>PA,LA (1.31, 2.62) C</td>
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<td>Kᵢ = 3000</td>
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<td></td>
<td>PA,LA (0.55) + MgADP (0.02) C</td>
<td>Kᵢ = 910</td>
<td>PA,LA (0.55) + MgADP (0.02) C</td>
<td>Kᵢ = 910</td>
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</tr>
<tr>
<td>Pea seed (30°C)</td>
<td>PA,LA (1.31, 2.62) C</td>
<td>Kᵢ = 700</td>
<td>PA,LA (0.55) C</td>
<td>Kᵢ = 330</td>
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<tr>
<td></td>
<td>PA,LA (0.55) + MgADP (0.02) NC</td>
<td>Kᵢ = 500</td>
<td>PA,LA (0.55) + MgADP (0.02) NC</td>
<td>Kᵢ = 310</td>
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B. Additions

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<tr>
<td>PA,LA</td>
<td>500</td>
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<tr>
<td>PA,LA + ADP (0.1) + MgCl₂ (50)</td>
<td>150</td>
<td>7.7</td>
</tr>
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</table>

* No preincubation had taken place. ³ C = competitive; NC = noncompetitive. ² Kᵢ = competitive component of noncompetitive inhibition (see text). ⁴ Preincubation had taken place. ⁵ I₅₀ = concentration of inhibitor giving half-maximal rate of inactivation.

Fig. 1. Time dependence of inhibition of pea seed glutamine synthetase by PA,LA (0.1 mM) with and without added ADP (0.02 mM) and MgCl₂ (50 mM), pH 7.5, 30°C, as assayed by biosynthetic activity.

Reversibility of PA,LA inhibition of pea seed glutamine synthetase

Enzyme (100 units) was incubated with 0.1 mM PA,LA at pH 7.3, 30°C, in the presence or absence of 0.02 mM ADP and 50 mM MgCl₂ for 30 min, then dialyzed against two changes of 1000 volumes of 50 mM imidazole buffer, pH 7.3, containing 0.1 M L-glutamate and 0.2 M KCl, at 4°C for 24 h. Controls included enzyme with PA,LA omitted. Per cent activity was calculated from measured specific activity (units/mg).

<table>
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<th>Additions</th>
<th>% activity</th>
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<td>None</td>
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</tr>
<tr>
<td>PA,LA</td>
<td>100</td>
</tr>
<tr>
<td>PA,LA + MgADP</td>
<td>100</td>
</tr>
</tbody>
</table>

Initially 100% activity. After preincubation, 100% activity. After dialysis, 12.9% activity. After dialysis, 12.9% activity.

Met(O)(NH) was also observed to be competitive versus NH₃ with the E. coli enzyme but showed noncompetitive kinetic patterns versus NH₃ with the pea seed enzyme (Table IV, part A). This may indicate a specific interaction of Met(O)(NH) with the ammonia site of the E. coli enzyme that does not occur in the pea seed system. The noncompetitive behavior of NH₃ versus Met(O)(NH) with the pea enzyme is not in agreement with the earlier suggestion by Gass and Meister (23) that the methyl group of those inhibitors may occupy the NH₃ site of the ovine brain enzyme, although this system may behave differently from the pea seed enzyme.

Binding constants for Met(O)(NH) to the two enzymes were also obtained by observing the rate of inhibition by Met(O)(NH) plus MgATP as a function of Met(O)(NH) concentration. One may then plot the first order rates, kᵢ (s⁻¹), versus [Met(O)(NH)] or as double-reciprocal plots. From these data for each enzyme, one may calculate I₅₀, the [Met(O)(NH)] required to achieve the half-maximal inhibition rate, kᵢ (max). One may then compare kᵢ (max) with the rate constants for net turnover and I₅₀ with the Kᵢ and Kᵢ (max) values from steady state experiments. Such comparisons (Table IV) show that the I₅₀ values are significantly higher than the Kᵢ or Kᵢ (max) values, by approximately 70-fold for the E. coli enzyme but only 5.0-fold for the pea seed enzyme. This implies that a higher level of Met(O)(NH) is required to irreversibly inhibit the enzyme in the presence of MgATP alone than is required to compete with L-glutamate for a binding site.² kᵢ (max) values are approximately 70-fold higher than the kᵢ (max) values for the E. coli enzyme.

Weisbrod and Meister (4) found a higher rate of irreversible inhibition of the Escherichia coli Me₂⁺ATP and the S isomer of L-
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Fig. 3. Inhibition of (A) pea seed and (B) Escherichia coli glutamine synthetase (GS) by Met(O)(NH) in competition versus L-glutamate, presented as V versus V/S plots. The concentrations of inhibitor (micromolar) in each experiment are shown adjacent to each line; 0 represents zero. Assay incubation conditions were (A) 10 min, 30°C, and (B) 15 min, 37°C. V is ΔA0/min/unit time (see "Experimental Procedures").

naturally 10³ slower than turnover rates (see Fig. 6).

Of particular interest is the strikingly low value of Kᵵ for Met(O)(NH) with the E. coli enzyme, fully 3 orders of magnitude below the Kᵵ of L-glutamate (10), and more than 2 orders of magnitude below the Kᵵ values for Met(O)(NH) with the pea seed enzyme. This value has been confirmed independently by titration of unadenylylated enzyme with Met(O)(NH) in the presence of ATP by pulsed NMR, observing changes in water proton relaxation rates induced by Mn(II) at the n₁ sites (Table IV, part C, and Ref. 24). This verifies that Met(O)(NH) alone binds to the site with unusually high affinity. Such specific recognition of tetrahedral geometry at the 5-atom of an analog of glutamate thereof is consistent with Met(O)(NH) being a transition state analog (24, 25).

With the pea seed enzyme, if the assay incubation time in the experiments as in Fig. 3 (Met(O)(NH) versus L-glutamate as the varied substrate) was varied, the kinetic patterns showed a trend from noncompetitive toward competitive at shorter times. This suggests that the noncompetitive behavior is due to irreversible time-dependent inhibition when Met(O)(NH) and ATP bind to the active site rather than to Met(O)(NH) binding to or acting at an allosteric (nonsubstrate) site. When either enzyme, inhibited by Met(O)(NH) and MgATP, was dialyzed versus 1000 volumes of buffer with 100 mM L-glutamate added, no reactivation occurred.

It was found, however, that the competitive Kᵵ values (Kᵵ) for Met(O)(NH) with the pea seed enzyme, obtained in steady state competition versus L-glutamate (Table IV, part A), were dependent on assay incubation time. As shown in Fig. 5, the value diminished from 200 μM at zero incubation time to a value of 25 μM at 20 min. Since Kᵵ is the competitive component of a noncompetitive or mixed inhibition, this implies a time-dependent tightening of the reversible binding of Met(O)(NH) to the pea seed enzyme.

Binding of L-Met(O₂)₅—When steady state kinetic studies with L-glutamate as a competitor of L-methionine sulfone were carried out, as was done for Met(O)(NH) in Fig. 3, Kᵵ values were obtained. The Kᵵ for L-Met(O₂)₅ for the pea seed enzyme was 620 μM; Kᵵ for the E. coli enzyme was 240 μM. These values are 100-fold higher than Kᵵ for the E. coli enzyme with Met(O)(NH) versus L-glutamate was also observed by Ronzio et al. (22).
Met(O)(NH), but only 1.6-fold higher than the $K_i$ for Met(O)(NH) with the pea enzyme. This implies that E. coli enzyme shows much greater specificity for Met(O)(NH) over L-Met than does the pea seed enzyme. This indicates a special interaction for the imino group of Met(O)(NH) in the active site, probably via polar or hydrogen binding, as discussed below.

**pH Titration of Met(O)(NH)**—The possible state of protonation of the imino group at pH 7 was investigated by a pH titration with KOH of Met(O)(NH) from pH 1.0 to 12.0, then comparing the resultant curve to that obtained upon titration of an equimolar amount of L-methionine. In the region of pH 1.5 to 3.0, two titrable groups are observed for Met(O)(NH) but only one for L-Met. Both Met(O)(NH) and L-Met showed a single group of $pK_a 9.0$ to 9.5 ($\alpha$-NH$_2$).

**Studies with Other Inhibitors**—Several other inhibitors and combinations of those were studied as possible analogs of intermediates or transition states. First, as an analog of the trigonal bipyramidal structure of the $\gamma$-phosphoryl of ATP, the planar nitrate ion was tested. Potent inhibition of pyruvate kinase (26) by nitrate was reported previously. Here, nitrate varied from 1 PM to 206 mM. Similar negative results were obtained with other planar anions tested: bicarbonate, formate, and isothiocyanate.

Second, both enzymes were incubated with Met(O)(NH) and MgApp(NH)p, and activity was observed as a function of time (Table V). The E. coli enzyme exhibited no loss of activity. Although the pea seed enzyme showed a time-dependent loss of 50% of the original activity, 96% of this was recovered upon dialysis overnight against buffer (pH 7.4, 50 mM imidazole 100 mM KCl, 4°C). These results suggest that irreversible inactivation (Fig. 4) by Met(O)(NH) requires covalent phosphorylation by ATP, but conformational changes can yield tight inhibitory complexes.

**Energetics of Net Turnover**—A study of the temperature dependences of net turnover rate for the E. coli and pea seed enzyme also shows significant differences in behavior (Fig. 6). The activation energy, $E_a$, for the E. coli enzyme is approximately 3-fold greater than that for the pea seed enzyme. A value of $E_a = 5.4$ kcal/mol for the pea seed enzyme can also be calculated from the data of Pushkin et al. (27). Up to the point that thermal denaturation begins to occur within the reaction incubation time, both plots are linear. The sharp decrease in activity above 45°C in the 20-min assay was also observed by these researchers (27) and apparently due to a high cooperative thermal denaturation process. This process does not occur appreciably in 20 min or alter the slope of the Arrhenius plot below 40°C, however.

Calculated values of other activation parameters are presented in Table VI. Comparing these values, it is clear that $\Delta G^\ddagger$ and the rate-limiting step are dominated by different activation parameters: $\Delta H^\ddagger$ for the E. coli system but $\Delta S^\ddagger$ for the pea seed enzyme. Since the slow step in turnover is substrate release (Table I) this may suggest that this difference in dominant parameters could reflect functional differences between the two active sites, such as differences in conformational states prior to or after product release.

One may also calculate from these data that the magnitude of the first order turnover rate at 37°C is such that any covalent intermediate in the reaction pathway must be formed and broken down with a half-life ($t_{1/2}$) less than 7.5 ms.

**DISCUSSION**

Although recent reviews (28-31) have discussed synthetase type enzyme mechanisms in terms of covalent intermediates, with special attention paid to acyl phosphates (32), only in a few cases has it been demonstrated unambiguously that these compounds are stabilized (tightly bound) and kinetically viable in the main reaction pathway. Based largely on demonstrations that glutamine synthetase can form phosphorylated derivatives of Met(O)(NH), L-Met(O$_2$), and L-glutamate, it has been proposed that all glutamine synthetases utilize nearly identical reaction mechanisms (4, 28, 32). The present data provide several new lines of evidence that contradict this view, each of which has implications for reaction mechanism.

The E. coli enzyme fails to catalyze independent exchanges indicative of $\gamma$-Glu-P and binds PA$_2$LA no more tightly than l-glutamate, whereas the pea seed enzyme does catalyze these exchanges, but binds PA$_2$LA very tightly but reversibly. Thus, one must reject the earlier assertion that "the inability of glutamine synthetase (E. coli) to catalyze ADP-ATP exchange in the presence of glutamate is readily explained by the stability of the ADP-$\gamma$-glutamyl-phosphate-enzyme complex and its ability to yield S-oxoproline" (28). Indeed, unusual stability of
Experimental Procedures" for details. Substrate levels used were 
NH$_2$Cl; above 37$, the levels were twice these, except that MgCl$_2$ was 
held at 50 mM.

That Met(O)(NH) can displace L-glutamate from the 
Three data are not inconsistent with the transient exist-
ence of Met(O)(NH) as an unstable intermediate, however. 

The present data indicate that Met(O)(NH) for the pea 
seed enzyme increases with time, but remains reversible prior 
to phosphorylation, suggesting that two forms of the active 
site must be available for competitive binding of Met(O)(NH) versus L-glutamate. Finally, the dominance of the entropic 
term (Table VI) for net turnover with the pea seed enzyme 
may indicate active site isomerizations necessary for substrate 
binding or product release. Incubation of the pea seed enzyme 
with ATP prior to assay has been reported to be necessary to 
achieve optimal $V_{	ext{max}}$ values (33).

Hypotheses—From the present data, we propose that the 
minimum differences between geometries of the most stabi-
ized intermediates for each of the two enzymes studied are 
those for a trigonal versus a tetrahedral geometry at the 5-
atom. The respective bond angles between the ammonia nitro-
gen and the carbon-oxygen at C-5 (90° or $\pi 90^\circ$) are critical, 
and those for a trigonal versus a tetrahedral geometry at 
the 5-position of glutamate, and discrete stepwise phosphorylation 
may indicate active site isomerizations necessary for substrate 
binding or product release. Incubation of the pea seed enzyme 
with ATP prior to assay has been reported to be necessary to 
achieve optimal $V_{	ext{max}}$ values (33).

A scheme for the sequence and interrelationships of the 
most likely intermediates and transition states for glutamine 
synthetase-catalyzed reactions is proposed in Fig. 7. The pres-
ent data indicate that catalysis for both the pea seed and E. coli enzyme may proceed via intermediate 1. The negative 
results with planar anions as inhibitory analogs of metaphos-
phate indicate poor stabilization of the trigonal bipyramidal 
form of the y-phosphoryl of ATP in 1 and 2. Intermediate 2 is 
essentially the structure proposed previously for the "concerted" mechanism via a key transition state (3), although the degree of bond breaking or formation cannot be defined yet.

The pea seed enzyme probably catalyzes a reaction in which 
the most stable intermediate is y-Glu-P or some analog thereof, i.e. 3. In contrast, the E. coli enzyme active site 
 principally recognizes and stabilizes a tetrahedral geometry at 
position 5 of glutamate, and discrete stepwise phosphorylation 
may play a relatively minor role in this specificity, i.e. the 
most stable intermediate is probably 2 or 4.

Table VI

<table>
<thead>
<tr>
<th>Source</th>
<th>$E_a$ (e.u.)</th>
<th>$\Delta H$ (kal/mole)</th>
<th>$\Delta S$ (cal/mole K)</th>
<th>$T\Delta S$ (kal/mole K$^2$)</th>
<th>$\lambda$ (e.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>15.7</td>
<td>15.1</td>
<td>15.5</td>
<td>0.4</td>
<td>1.3</td>
</tr>
<tr>
<td>Pea seed</td>
<td>4.8</td>
<td>4.2</td>
<td>+15.5</td>
<td>-11.3</td>
<td>-36.5</td>
</tr>
</tbody>
</table>

This table shows the calculated activation parameters for glutamine synthetase-catalyzed reactions in E. coli and pea seed glutamine synthetase enzymes.

The exclusion of water from the site may be an essential 
feature of this class of enzymes, to prevent a futile glutamate-
ated ATPase activity from occurring at low levels of 
ammonia. An absolute requirement for bound NH$_2$ is merely 
substrate synergism in the extreme. The oxygen from glutama-
ate may not be readily transferred to the y-phosphate of ATP 
until and unless the tetrahedral intermediate is formed at 
least partially.

Specific interaction of the imino group of Met(O)(NH) with 
the ammonia site of the E. coli glutamine synthetase, but not 
with the ammonia site of the E. coli glutamine synthetase, is 
indicated by the high $K_a$/[$K_i$ ratio for 
Met(O)(NH) with the E. coli enzyme (Table IV), protection by 
NH$_3$ against Met(O)(NH) inhibition (Fig. 4), and the compe-
itive nature of Met(O)(NH) inhibition versus NH$_3$ in steady 
state experiments (Table IV). Also, the E. coli enzyme binds 
Met(O)(NH) 160-fold more tightly than it does L-Met(O$_2$). Ap-
parently, phosphorylation alone contributes much less to tight 
binding of L-glutamate or Met(O)(NH) with E. coli enzyme 
than with the pea seed system.

Especially with the pea seed enzyme, the data indicate 
conformational changes induced by substrates or inhibitors. 
PA$_2$LA binding appears to occur in two sequential stages, a 
rapidly reversible stage with $K_a$ or $K_i = 310$ to 700 $\mu$m (Table 
II), followed by a process in which binding becomes much 
tighter. Second, the time dependence of $K_i$ values for the 
interaction of Met(O)(NH) with the pea seed enzyme (Fig. 5) 
also tends to support an enzyme isomerization induced either 
by Met(O)(NH)$_2$, or nucleotide, or both. This plus the data of 
Table V indicate that the affinity of Met(O)(NH) for the pea 
seed enzyme increases with time, but remains reversible prior 
to phosphorylation, suggesting that two forms of the active 
site must be available for competitive binding of Met(O)(NH) versus L-glutamate. Finally, the dominance of the entropic 
term (Table VI) for net turnover with the pea seed enzyme 
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* B. Stokes and P. D. Boyer, personal communication.
Recent magnetic resonance studies with the E. coli enzymes suggest that metal ion in the n, site is very close to and interacts with the y-carboxylate of L-glutamate (or glutamine) so as to stabilize the tetrahedral intermediate (Fig. 7D). The formation of stable tetrahedral intermediates in the active sites of proteolytic enzyme has been observed by high resolution spectroscopic techniques (38, 39).

Stabilization of a chemical species or intermediate to very different degrees by the same type of enzyme from different sources is not a new concept. Familiar examples from the literature include the markedly different oxidation-reduction potentials seen for different cytochromes, and the Class I and Class II aldolases, in which activation of a carbonyl group is accomplished by different mechanisms. Among ligases, with succinyl-CoA synthetase, it has been shown that enzymes from different species stabilize the phospho-enzyme intermediate to different degrees (40), and there is increasing evidence that acyl adenylates may not be ubiquitous intermediates in several amino acid tRNA synthetase-catalyzed reactions (41, 42).

In conclusion, it seems reasonable that the microstructure of a given enzyme-active site may favor or disfavor the stabilization of a given intermediate or transition state. In reality, one mechanism or another ("concerted" or "stepwise") may never occur 100% under steady-state turnover conditions (43).

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