Catalytic Mechanisms of Glutamine Synthetase Enzymes

STUDIES WITH ANALOGS OF POSSIBLE INTERMEDIATES AND TRANSITION STATES*

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FREDERICK C. WEDLER AND BRADLEY R. HORN

From the Department of Chemistry, Biochemistry Program, Rensselaer Polytechnic Institute, Troy, New York 12181

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 Iₐ 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, Iₐ = 105 μM, and Iₐ/Kᵢ = 70; but for the pea seed enzyme, Kᵢ = 200 μM, Iₐ = 1000 μM, and Iₐ/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" (n₉) metal ion site recently discovered to be within bonding distance of the γ-carboxylate of L-glutamate in the E. coli enzyme.
Glutamine Synthetase Reaction Mechanisms

Enzyme can very slowly produce Soxoproline from Gglutamate (optical) techniques used. One is also limited in rapid kinetic approaches by the specificity of information about the actual chemistry that one can obtain from the spectroscopic (usually optical) techniques used.

The results described here involve several new approaches to these problems, notably observations of the interaction of structural analogs at 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 found by NMR that the active site possesses a partially ordered mechanism of substrate binding and release that prevents observation of partial exchange reactions indicative of γ-Glu-P (2). However, the pea seed and E. coli enzymes exhibited 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 3.5 or in the presence of 0.1% sodium dodecyl sulfate (9) and which exhibited a specific activity of 465 units/mg. Unadenylylated enzyme, prepared from Biocherichia coli and purified to homogeneity according to Shapiro and Studtmann (10), was found by assay and spectrophotometric techniques to contain an average of 1.7 or 3.0 AMP groups/dodecamer (separate preparations).

The reactants L-glutamate, L-glutamine, ADP, and ATP were of highest purity obtainable, supplied by Sigma Chemical Co. The inhibitors L-methionine sulfoxide and L-methionine-SR-sulfloxime were also Sigma products, while 5'-adenylylimidodiphosphate was from P-L Biochemicals. Radioactive labeled substrates were from New England Nuclear, and [14N]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 grade. Fluorescamine was a Roche product.

Synthesis and Analysis of PA,LA—3-(Phosphonoacetylamido)-L-alanine (P A, LA) was synthesized in a manner somewhat analogous to that employed by Collins and Stark (11) for a similar compound. Preparation of phosphonoacetylamido 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-glutamate and ATP (50 mM). After reaction for 1 h at 37°C, a 10% (w/v) solution of sodium metaphosphoric acid (158 mg, 2.10 mg in 3.0 ml of dimethylformamide) and tributylamine (Eastman, redistilled, 2.4 ml), then holding the temperature at 20°C for 2 h, then 4°C for 2 h. The solvent was then removed in vacuo and the residue dissolved in a minimal volume of water, stored overnight at 4°C, then passed over a Dowex 50-X8 column (1.0 x 30 cm) at pH 4.5 to 6.5 to remove unreacted diamine. The unabsorbed material was detected by ninhydrin and the latter two-thirds of the peak concentrated to dryness in vacuo. The product was then recrystallized twice from water/acetone and three times from water/ethylacetate. Being iodine-free, decomposed rapidly and giving a cut-clear melting point.

Total phosphorus analysis (19) of PA,LA indicated a purity of >95%. Thin layer chromatography as the fluorescamine derivative on silica gel and on cellulose, according to Imai et al. (13), with their solvent system A, showed a single spot, with no contamination of diaminopropionate. Elemental analysis for C13,H16,O4P gave:

\[
\begin{align*}
\text{C}_13\text{H}_16\text{O}_4\text{P} & \\
\text{Calculated:} & \text{C} 26.5, \text{H} 4.9, \text{N} 12.4, \text{P} 13.7 \\
\text{Found:} & \text{C} 26.2, \text{H} 5.2, \text{N} 12.2, \text{P} 13.5
\end{align*}
\]

The infrared spectrum of the product, obtained with a Perkin Elmer model 137 instrument, showed absorption peaks (microns) at 2.7 to 3.5 (α-NH3, and —OH), 6.0 (CO2, and PO—OH), 6.8 (α-NH3, and CO—NH—), 7.35 (CO—NH—, and NH—CH2—CO), 8.05 (CO—NH—), 8.65 and 9.15 (C—C), 9.40 and 10.55 (PO—OH), and 11.85 and 13.0 (—NH2 and C—C).

The proton NMR spectrum for the product in D2O using a Varian T-60 instrument was characterized by a triplet at 8.13 for the α proton, a doublet at 8.36 for the β protons, and a doublet (J = 8.6 Hz) at 8.50 for the methylene bridge protons. Splitting by protons on adjacent nitrogens was not observed, apparently due to rapid exchange with D2O, possibly catalyzed by the acidic phosphonate 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 3000 instrument interfaced to a model 6000 data system. Direct chemical ionization analysis with methane carrier gas showed mass peaks for the parent ion plus H2, plus CH2, and plus C2H4, as well as the key fragments for (HO2CCH2NH2Cl)− and (CH2N2CO2H)0.02, 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 spectrophotometric (12) or fluorometric (13) approach. The label used for the parent ion plus H2, plus CH2, and plus C2H4, as well as the key fragments for (HO2CCH2NH2Cl)− and (CH2N2CO2H)0.02, and derivatives thereof. Taken together, these results are consistent with the assigned structure for the product.

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Inhibition Studies—In steady state kinetic experiments involving variation of a substrate concentration at fixed inhibitor levels, the values of observed velocity, v, and [S] were plotted as \( \frac{1}{v} \) versus \( \frac{1}{[S]} \) and as \( \frac{v}{v_0} \) versus \( [S] \), from which values of \( K_m \) and \( K_i \) (app) were calculated. Since both inhibitors gave either competitive or noncompetitive (mixed) inhibition patterns with different enzymes or conditions, calculation of the competitive component (\( K_i \)) was necessary to allow comparisons among these \( K_i \) values. Replots of the slopes of Lineweaver-Burk plots versus inhibitor concentration gave linear plots with x intercepts equal to \( -K_i \) (15-17). This technique has been proven valid in similar comparisons of \( K_i \) values for intermediate analogs with acocitase (17).

The rates of inhibitor-induced enzyme inactivation with PA,LA or Met(O)NH were observed by removal of small samples of enzyme from the incubation mixture at appropriate time intervals. These samples were assayed as a group at the appropriate temperature after addition of 2-fold concentrated assay mixture.

Arrhenius Plots—Variation of temperature was accomplished

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with either a Haake water bath or Tecum Dri-Block devices, each thermostated to ±0.2 °C 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 (s⁻¹) by 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 ¹³N-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 NH₄Cl, 10 mM L-glutamate, and 50 mM MgCl₂. Analysis for ammonia before and after such treatment was by the Nessler microdetermination of San Pietro (18) that detected NH₃ at >10⁻⁶ M. Nucleotide was detected as ribose in the orcinol/H₂SO₄ colorimetric procedure of Brueckner (19), used at micro scale and sensitive to ribose at >10⁻⁹ M. Dowex treatment after enzyme reaction removed NH₃ or nucleotide to below the levels of detection in all cases, and that corresponds to <0.1 Kₐ 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 y-glutamyl-phosphate is readily formed by glutamine synthetase in a reaction such as

$$E + \text{Glu} \rightleftharpoons (E,\text{Glu}-\text{ATP}) \rightleftharpoons (E,\text{Glu} \cdot \text{P}) \rightleftharpoons (E,\text{Gln} \cdot \text{P}) \rightleftharpoons E + \text{Gln} + \text{ATP}$$

one should be able to observe both an ammonia- and glutamate-independent, glutamate-dependent ADP-ATP exchange, and a nucleotide-independent, P₇-dependent NH₃-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, NH₃-Gln, ADP-ATP, and P₇-ATP. Very striking differences between the bacterial and plant enzymes are immediately apparent. The unadenylated Escherichia coli enzyme used here, as with the adenylylated enzyme studied earlier (3), catalyzes no appreciable exchanges without all substrates bound. In contrast, the pea seed enzyme catalyzes 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–substrate 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. coli enzyme, since random order substrate binding mechanisms were found for both the adenylylated and unadenylated forms (2, 3). To test hypothesis b, the binding of a nonreactive structural analog of γ-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. 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 NH₃⁺. The active site may be specific for neutral NH₃ rather than NH₃⁺ ion.

Binding of P₇-A—The synthesis of a nonreactive analog of γ-Glu-P (I), namely 3-(phosphonoacetylamo)-L-alanine, abbreviated PA₇-A (II), is described under “Experimental Procedures.”

Enzyme binding of PA₇-A was first studied by steady-state

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Table 1

<table>
<thead>
<tr>
<th>Additions (nm)</th>
<th>Relative exchange rates</th>
<th>Pea seed enzyme</th>
<th>E. coli enzyme</th>
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<tr>
<td>NH₃</td>
<td>Glu</td>
<td>ATP</td>
<td>Gln</td>
</tr>
<tr>
<td>0.5</td>
<td>5</td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td>0.5</td>
<td>5</td>
<td>0</td>
<td>25</td>
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<tr>
<td>0.5</td>
<td>0</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td>25</td>
</tr>
</tbody>
</table>

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1 All previous reports involved the use of impure enzymes. Data of Graves and Boyer (34) were for the V₄-ATP and Glu-Gln exchanges, not pertinent to γ-Glu-P formation, but do not eliminate or support its existence. Both Elliott (8) and LeVintow and Meister (45) reported a dependence on ADP and P₇ or As₅ of γ-Glu-NH₂OH formation from NH₂OH and Glu. Stumpf and Loomis (46) reported that this transferase activity depended on added Mn²⁺ and P₇ or As₅, but not on ATP, ADP, or CoA.
kinetics with PA\textsubscript{LA} versus L-glutamate as the varied substrate. PA\textsubscript{LA} was allowed to interact with each enzyme in several different ways: first, by addition of enzyme to assay mixtures containing PA\textsubscript{LA} and substrates; second, by preincubation of enzyme with PA\textsubscript{LA} at the assay temperature for 15 min prior to addition of this enzyme to assay mixture containing substrates and an identical concentration of PA\textsubscript{LA}. Each of these procedures was also carried out in the presence and absence of ADP and MgCl\textsubscript{2}. The data of Table II, part A, indicate markedly different behavior of the \textit{E. coli} and pea seed enzymes.

With the \textit{E. coli} enzyme, the $K_i$ of PA\textsubscript{LA} (3 mM) is comparable to the $K_m$ of L-glutamate (2.5 mM). Inhibition patterns and slope replots showed that PA\textsubscript{LA} is competitive versus L-glutamate, and preincubation of PA\textsubscript{LA} with enzyme does not alter the $K_i$ value. Added MgADP enhances the affinity of enzyme for PA\textsubscript{LA}, 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 PA\textsubscript{LA} with \textit{E. coli} glutamine synthetase. The perturbation of the ESR spectrum of bound Mn(II) in the tight (n\textsubscript{5}) sites of the enzyme by PA\textsubscript{LA} is almost indistinguishable from that observed with L-glutamate.\textsuperscript{2} Second, fluorescence changes induced in the enzyme by bound PA\textsubscript{LA} alone are not distinguishable from those induced by L-glutamate alone.

PA\textsubscript{LA} interacts with the pea seed enzyme very differently. With no preincubation in the absence of MgADP, PA\textsubscript{LA} 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 $\mu$M upon preincubation with PA\textsubscript{LA} and MgADP. The competitive constants, $K_a$, 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 PA\textsubscript{LA} 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 PA\textsubscript{LA} and enhanced by MgADP as shown in Fig. 1.

An alternative method of determining binding constants for PA\textsubscript{LA} with the pea seed enzyme was to observe the rate of the PA\textsubscript{LA}-induced inhibition as a function of PA\textsubscript{LA} concentration, in the presence and absence of ADP and MgCl\textsubscript{2}. These data, shown in Table II, part B, indicate that the concentrations of PA\textsubscript{LA} required for the half-maximal rate of activity inhibition, called $I_{0.5}$, agree reasonably well with the $K_a$ values calculated from steady state kinetics (part A). These inactivation rate studies indicate that MgADP lowers the $I_{0.5}$ value rather than increasing the maximal rate of inactivation by PA\textsubscript{LA}, $k_i$ (max).

**Nature of PA\textsubscript{LA} Inhibition of Pea Seed Enzyme — PA\textsubscript{LA} 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 $\delta$-carboxyl group of PA\textsubscript{LA}. The data of Table III indicate that the PA\textsubscript{LA}-induced inhibition is completely reversible. Enzyme that was first inhibited by PA\textsubscript{LA} 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 PA\textsubscript{LA} is tight but reversible.

The possibility of a covalent reaction between the pea seed enzyme and PA\textsubscript{LA} 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 PA\textsubscript{LA} 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 x 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 in 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 PA\textsubscript{LA}-inhibited enzyme, (b) nonbound small molecules from the PA\textsubscript{LA}-inhibited enzyme from the Bio-Gel P-6 column, and (c) small molecules released from PA\textsubscript{LA}-inhibited enzyme by incubation with no excess PA\textsubscript{LA} and 0.2 M KCl, as well on authentic PA\textsubscript{LA} and L-$\alpha$, $\beta$-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. PA\textsubscript{LA} and L-$\alpha$, $\beta$-diaminopropionic acid clearly migrate with distinctly different $R_f$ values. In the initial incubation mixture among the nonbound small molecules (A), there is not detectable $\epsilon$-Met(O,$\epsilon$) was reported previously (4, 20-22). Inhibition by L-Met(O$\epsilon$) was completely and rapidly reversible with both enzymes, but inhibition by Met(O$\epsilon$)(NH$\epsilon$) was apparently irreversible and.

* F. C. Wedler and D. J. Villafranca, unpublished results.

**Glutamine Synthetase Reaction Mechanisms**

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The binding of L-methionine-SR-sulfoxamine with the \textit{E. coli} and pea seed glutamine synthetases was studied by several kinetic approaches. First, fixed levels of Met(O$\epsilon$)(NH$\epsilon$) 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 $v/S$ plots indicate that the pea seed enzyme exhibits noncompetitive behavior, whereas the \textit{E. coli} enzyme undergoes a large change in $K_m$ (Glu) at 5 to 10 $\mu$M Met(O$\epsilon$)(NH$\epsilon$), at which level of inhibitor the pattern is competitive, but changes to noncompetitive at 25 $\mu$M and higher Met(O$\epsilon$)(NH$\epsilon$). Values of $K_i$ and $K_a$ 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$\epsilon$)(NH$\epsilon$) and also L-Met(O$\epsilon$), with both enzymes in the presence of ATP and MgCl\textsubscript{2}, with removal of samples at various times for assay. Related experiments with ovine brain and \textit{E. coli} enzyme were reported previously (4, 20-22). Inhibition by l-Met(O$\epsilon$) was completely and rapidly reversible with both enzymes, but inhibition by Met(O$\epsilon$)(NH$\epsilon$) was apparently irreversible and,
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° (cf. Fig. 1).

<table>
<thead>
<tr>
<th>Source</th>
<th>Additions (mM)</th>
<th>Type inhibition pattern</th>
<th>Constant</th>
<th>Additions (mM)</th>
<th>Type inhibition pattern</th>
<th>Constant</th>
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<tbody>
<tr>
<td>E. coli</td>
<td>PA &amp; LA (1.31, 2.62)</td>
<td>C</td>
<td>Kᵢ = 3000</td>
<td>PA &amp; LA (2.62)</td>
<td>C</td>
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<tr>
<td>(37°)</td>
<td>PA &amp; LA (0.55) + MgADP (0.02)</td>
<td>C</td>
<td>Kᵢ = 910</td>
<td>PA &amp; LA (0.55) + MgADP (0.02)</td>
<td>C</td>
<td>Kᵢ = 910</td>
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<tr>
<td>Pea seed</td>
<td>PA &amp; LA (1.31, 2.62)</td>
<td>C</td>
<td>Kᵢ = 700</td>
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<td>Kᵢ = 330</td>
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<tr>
<td>(30°)</td>
<td>PA &amp; LA (0.55) + MgADP (0.02)</td>
<td>NC</td>
<td>Kᵢ = 500</td>
<td>PA &amp; LA (0.55) + MgADP (0.02)</td>
<td>NC</td>
<td>Kᵢ = 310</td>
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B. Additions | Constants | 10⁴ kₐ (max) |
<table>
<thead>
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<th></th>
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<tr>
<td>PA &amp; LA</td>
<td>500</td>
<td>8.3</td>
</tr>
<tr>
<td>PA &amp; LA + ADP (0.1) + MgCl₂ (50)</td>
<td>150</td>
<td>7.7</td>
</tr>
</tbody>
</table>

* No preincubation had taken place.
* C = competitive; NC = noncompetitive.
* Kᵢ = competitive component of noncompetitive inhibition (see text).
* Preincubation had taken place.
* Iₐₕ = 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°, as assayed by biosynthetic activity.

TABLE III

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°, 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° for 24 h. Controls included enzyme with PA & LA omitted. Per cent activity was calculated from measured specific activity (units/mg).

<table>
<thead>
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<th>% activity</th>
<th>Additions</th>
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<tr>
<td></td>
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<tr>
<td>Initially</td>
<td>100</td>
</tr>
<tr>
<td>After incubation</td>
<td>100</td>
</tr>
<tr>
<td>After dialysis</td>
<td>100</td>
</tr>
</tbody>
</table>

Time-dependent (Fig. 4). Added L-glutamate slowed down the inhibition reactions with Met(O)(NH)₂, as expected, but only in the case of the E. coli enzyme did added NH₃Cl have any effect, namely, inhibition of the rate of activity loss. Met(O)(NH)₂ 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)₂ 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)₂ with the pea enzyme is not in agreement with the earlier suggestion by Gass and Meister (23) that the methyl group of these 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)₂ to the two enzymes were also obtained by observing the rate of inhibition by Met(O)(NH)₂ plus MgATP as a function of Met(O)(NH)₂ concentration. One may then plot the first order rates, kₐ (s⁻¹), versus [Met(O)(NH)₂] or as double-reciprocal plots. From these data for each enzyme, one may calculate Iₐₕ the [Met(O)(NH)₂] required to achieve the half-maximal inhibition rate, kₐ (max). One may then compare kₐ (max) with the rate constants for net turnover and Iₐₕ with the Kᵢ and Kᵢₐ values from steady state experiments. Such comparisons (Table IV), show that the Iₐₕ values are significantly higher than the Kᵢ or Kᵢₐ 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)₂ 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. kₐ (max) values are approxi-
approximately 10^3 slower than turnover rates (see Fig. 6).

Of particular interest is the strikingly low value of \( K_i \) for Met(O)(NH) with the E. coli enzyme, fully 3 orders of magnitude below the \( K_a \) of L-glutamate (10), and more than 2 orders of magnitude below the \( K_a \) values for Met(O)(NH) with the pea seed enzyme. This value has been confirmed independently by titration of unadenylated 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 \( n_1 \) 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 \( K_i \) values (\( K_i \)) 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 \( \mu \)M at zero incubation time to a value of 25 \( \mu \)M at 20 min. Since \( K_i \) 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(O)2** — When steady state kinetic studies with L-glutamate as a competitor of L-methionine sulfone were

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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 \( VS \) plots. The concentrations of inhibitor (micromolar) in each experiment are shown adjacent to each line; \( b \) represents zero. Assay incubation conditions were (A) 10 min, 30°C, and (B) 15 min, 37°C. \( V \) is \( \Delta \Delta s \)/unit time (see "Experimental Procedures").**

**Fig. 4. Time dependence for the inhibition of biosynthetic activity of the (A) pea seed and (B) Escherichia coli glutamine synthetase (GS) enzymes upon incubation with ATP, MgCl\(_2\), and either L-Met(O)\(_2\) (circles) or Met(O)(NH) (triangles, diamonds, and squares).** The pea enzyme (0.4 mg) was incubated at 30°C with 0.625 mM Met(O)\(_2\) or 0.1 mM Met(O)(NH). The E. coli enzyme (2 mg) was incubated at 37°C with 4 mM L-Met(O)\(_2\) or 0.01 mM Met(O)(NH). Each inhibition reaction of 1.0 ml also contained, in micromoles: 50 imidazole\(\cdot\)HCl, pH 7.3, 50 MgCl\(_2\), and 7.5 ATP. Ammonia and L-glutamate, when added, were at 10 mM and 5 mM, respectively. At the indicated times, 10-\(\mu\)l samples were withdrawn, diluted 100\(\times\) in 0° buffer, and assayed by the standard biosynthetic reaction procedures (see "Experimental Procedures").

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**TABLE IV**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Inhibitor</th>
<th>( K_i ) (( \mu \text{M} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli (Es)</td>
<td>L-glutamate</td>
<td>1.5 ( \mu \text{M} )</td>
</tr>
<tr>
<td>E. coli (Es)</td>
<td>NH(_2)</td>
<td>0.2 ( \mu \text{M} )</td>
</tr>
<tr>
<td>E. coli (Es)</td>
<td>ATP</td>
<td>NC</td>
</tr>
<tr>
<td>Pea seed</td>
<td>L-glutamate</td>
<td>200 ( \mu \text{M} )</td>
</tr>
<tr>
<td>Pea seed</td>
<td>NH(_2)</td>
<td>NC</td>
</tr>
<tr>
<td>Pea seed</td>
<td>ATP</td>
<td>NC</td>
</tr>
</tbody>
</table>

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Met(O)(NH) than with the R isomer. Binding constants (either \( K_i \), \( K_a \), or \( I_1 \), values) were not determined. Such parameters, determined for the SR mixture may be equal to or higher than the values obtained with pure R or pure S compound. As discussed above, the isomer that reacts with ATP most rapidly in the enzyme active site may not be that which is bound most tightly.

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carried out, as was done for Met(O)(NH) in Fig. 3. \( K_i \) values were obtained. The \( K_i \) for L-Met(O)\(_2\) for the pea seed enzyme\(^6\) was 620 \( \mu \text{M} \). \( K_i \) for the E. coli enzyme was 240 \( \mu \text{M} \). These values are 100-fold higher than \( K_i \) for the E. coli enzyme with \( \text{Met(O)}_2 \) versus L-glutamate was also observed by Ronzio *et al.* (22).
Met(O)(NH), but only 1.6-fold higher than the $K_w$ 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(O$_2$) 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 titratable 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, 95% of this was recovered upon dialysis overnight against buffer (pH 7.4, 50 mM imidazole 100 mM KCl, 4$^o$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$^o$ 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$^o$, however.

Calculated values of other activation parameters are presented in Table VI. Comparing these values, it is clear that $\Delta S^*$ and the rate-limiting step are dominated by different activation parameters: $\Delta H^*$ for the $E. \ coli$ system but $T\Delta S^*$ 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$^o$ 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 via in the main reaction pathway. Based largely on demonstrations that glutamine synthetase can form phosphorylated derivatives of Met(O)(NH)$_2$, 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 5-oxoproline" (28). Indeed, unusual stability of...
Experimental Procedures" for details. Substrate levels used were NH$_3$Cl; above 37°, the levels were twice these, except that MgCl$_2$ was held at 50 mM.

That Met(O)(NH)$_3$ can displace L-glutamate from the E. coli enzyme (5) also tends to support an enzyme isomerization induced either by Met(O)(NH)$_3$, or nucleotide, or both. This plus the data of Table V indicate that the affinity of Met(O)(NH)$_3$ 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)$_3$ 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 $V_{\max}$ values (33).

Hypotheses — From the present data, we propose that the minimum differences between geometries of the most stabilized intermediates for each of the two enzymes studied are those for a trigonal versus a tetrahedral geometry at the 5-atom. The respective band angles between the ammonia nitrogen and the carbon-oxygen at C-5 (90° or $\sim$109°) are critical, since optimal attack on $\geq$O occurs with the nitrogen perpendicular to the plane of the trigonal carboxylate. Recent experiments by Stokes and Boyer* with $^{14}$C exchange between Pi-Glu and Pi-Glu indicate that the trigonal $\gamma$-carboxylate of glutamate has little rotational freedom when bound to the pea seed enzyme (34), but that its rotational freedom is much greater in the E. coli enzyme active site.

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 present 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 metaphosphate indicate poor stabilization of the trigonal bipyramidal form of the $\gamma$-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 $\gamma$-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.

* B. Stokes and P. D. Boyer, personal communication.
Recent magnetic resonance studies with the E. coli enzyme suggest that metal ion in the n, site is very close to and interacts with the ω-carboxylate of L-glutamate (or Met(O)(NH))(24, 35, 36). NMR relaxation studies with 5-[^15]N-labeled glutamate suggest that the Mn(II)-C5 distance is small enough to allow interaction via a water bridge.3 Thus, in the E. coli enzyme the electrophilic Mn(II) may be exactly positioned (37) so as to stabilize the tetrahedral intermediate (Fig. 7B). 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 intermediates (37) so as to stabilize the tetrahedral intermediate (Fig. 7B). The formation of stable tetrahedral intermediates in the active sites of proteolytic enzymes has been observed by high resolution spectroscopic techniques (38, 39).

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).

Acknowledgments—We wish to thank Dr. Larry Hendry for the gas chromatography-mass spectrometry determinations on PA\textsubscript{LA}. Dr. J. J. Villafranca for the magnetic resonance experiments with Met(O)(NH)\textsubscript{2} and PA\textsubscript{LA}, and Dr. Robert Reeves for the mass spectral analyses on ^15N-labeled N\textsubscript{2} derived from glutamate. Helpful comments from Drs. P. D. Boyer, W. W. Cleland, and J. Westley are gratefully acknowledged.

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Catalytic mechanisms of glutamine synthetase enzymes. Studies with analogs of possible intermediates and transition states.

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