Studies on Glutamine Synthetase from Escherichia coli

FORMATION OF PYRROLIDONE CARBOXYLATE AND INHIBITION BY METHIONINE SULFOXIMINE*

(Received for publication, February 20, 1973)

ROBERTA E. WEISBROD AND ALTON MEISTER

From the Department of Biochemistry, Cornell University Medical College, New York, New York 10021

SUMMARY

Both the adenylylated and unadenylylated forms of Escherichia coli glutamine synthetase catalyze the formation of pyrrolidone carboxylate from glutamate in the presence of ATP and divalent metal ions (Mg++ or Mn++) and in the absence of ammonia. The unadenylylated enzyme catalyzes this reaction more rapidly with Mg++ than with Mn++, while the adenylylated enzyme catalyzes it more rapidly with Mn++ than with Mg++. The rates of pyrrolidone carboxylate formation catalyzed by the two forms of the E. coli enzyme are about the same or higher, relative to the corresponding rates of glutamine synthesis, than that catalyzed by ovine brain glutamine synthetase. Both forms of the E. coli enzyme are inhibited when incubated with methionine sulfoximine, ATP, and either Mg++ or Mn++; the unadenylylated enzyme, which is inhibited to about the same extent with Mg++ as with Mn++, is inhibited more rapidly than the adenylylated enzyme. L-Glutamate protects the unadenylylated enzyme (with Mg++ or Mn++) and the adenylylated form (only with Mn++) against inhibition by methionine sulfoximine. Only one of the four stereoisomers of methionine sulfoximine (L-methionine-S-sulfoximine) inhibits the enzyme. Inhibition is associated with tight binding to the enzyme of 9.2 to 11 moles of methionine sulfoximine phosphate per mole of enzyme. E. coli glutamine synthetase acts at a significant rate on several glutamate analogs that are also substrates of the brain enzyme. The data are in accord with the view that the major reaction pathway of glutamine synthesis catalyzed by the E. coli enzyme is similar to that of the reaction catalyzed by the brain enzyme, and thus involves formation and utilization of enzyme-bound γ-glutamyl phosphate and a tetrahedral intermediate or transient state whose structure is analogous to that of L-methionine-S-sulfoximine phosphate.

Recent studies in this laboratory have been concerned with the catalytic and structural properties of ovine brain glutamine synthetase (1, 2), which has a molecular weight of about 400,000 (3) and is composed of 8 apparently identical subunits which are arranged in a manner analogous to the corners of a cube (4–8). The enzyme is active toward L- and n-glutamate, α-methyl-L-glutamate, cis-cycloglutamate (cis-1-amino-1,3-dicarboxycyclohexane), and several other glutamate analogs, but it is not active toward a number of closely related compounds (1, 2, 9); it is irreversibly inhibited by L-methionine-S-sulfoximine, but not by the other three stereoisomers of methionine sulfoximine (10–13). These findings have made it possible to map the active center of the enzyme and thus to define the spatial relationships between the points on the enzyme that bind ammonia, the α-carboxyl and α-amine groups of glutamate, and the two glutamate γ-carboxyl oxygen atoms (1, 14). These studies indicate that a specific γ-carboxyl oxygen atom of enzyme-bound glutamate is phosphorylated and that L-methionine-S-sulfoximine binds to the active center in a manner such that its sulfoximine nitrogen atom lies in the position normally occupied by the N-carboxyl oxygen atom of glutamate that is phosphorylated (14). L-Methionine-S-sulfoximine thus appears to inhibit the enzyme by virtue of its ability to serve as an analog of the tetrahedral intermediate or transient state formed by reaction of ammonia with γ-glutamyl phosphate. Methionine sulfoximine is phosphorylated on the enzyme to yield methionine sulfoximine phosphate which binds tightly to the active site; such phosphorylation seems to reflect an aspect of the normal catalytic reaction, i.e. formation of enzyme-bound γ-glutamyl phosphate (10–13). When ovine brain glutamine synthetase is incubated with γ-glutamate, ATP, and Mg++ ions in the absence of ammonia there is a catalytic intermediate or transient state whose structure is analogous to that of L-methionine-S-sulfoximine phosphate.

* This work was supported by a grant from the National Institutes of Health, United States Public Health Service.
most completely dependent on Mn++.

The two forms differ also with respect to susceptibility to inhibition by various metabolites. Thus, alanine and glycine are more inhibitory to the unadenylated form, while tryptophan, histidine, AMP, CTP, glucosamine 6-phosphate, and carbamyl phosphate are more inhibitory to the adenylylated form. In contrast to the E. coli enzyme, ovine brain glutamine synthetase has not been found in an adenylylated form, nor is it inhibited by the several inhibitors of the E. coli enzyme (3). 2

Although there are significant structural and regulatory differences between the glutamine synthetases of brain and E. coli, it seemed of importance to examine certain properties of the E. coli glutamine synthetase which might be considered to reflect aspects of the mechanism of the reaction catalyzed and the nature of the active center of the enzyme. Since both enzymes catalyze the same chemical reaction, one might expect that the mechanisms of the reactions catalyzed by the two enzymes would be similar. However, it was recently suggested that the mechanisms of the reactions catalyzed by the brain enzyme and the adenylylated form of the E. coli enzyme are significantly different, and it was stated (30) that the latter enzyme does not catalyze the formation of pyrrolidone carboxylate under conditions in which the brain enzyme was reported to do so (15, 16). In the present study, some of the properties of the active site which have been studied previously in the brain enzyme have been examined in the adenylylated and unadenylated forms of E. coli glutamine synthetase. Data reported here show that both forms of the E. coli enzyme can catalyze the formation of pyrrolidone carboxylate and that both are inhibited by methionine sulfoximine. Several studies have also been carried out on the substrate specificity of the E. coli enzyme.

EXPERIMENTAL PROCEDURE

Materials—Preparations of E. coli glutamine synthetase (E66,4 and E6,) were isolated as described by Woolfolk et al. (19); preparations essentially equivalent in specific activity and state of adenylylation to those reported by these workers were obtained. E6,4 preparations were treated with E. coli deadenylating enzyme (32, 33) to yield almost completely unadenylated enzyme (E6). E6,4-12 preparations were treated with E. coli adenylylating enzyme (34, 35) to achieve preparations of the enzyme that were virtually fully adenylylated (E6,12). Determinations of the states of adenylylation of the several enzyme preparations were carried out by the spectrophotometric procedure and the enzymatic method of Shapiro and Stadtman (31); good agreement was obtained between the two methods.

L-Glutamic acid, L-glutamine, L-methionine-SR-sulfoximine, sodium ATP, sodium phosphoenolpyruvate, α-methyl- DL-glutamic acid, DPNH, pyruvate kinase, and lactate dehydrogenase were obtained from Sigma Chemical Corp. d-Glutamic acid (15), threo-γ-methyl-L-glutamic acid (30), cis-β-methylcyclohexylamine acid (cis-1-amino-1,3-dicarbocyclohexane) (9), d-methionine- SR-sulfoximine (11), L-methionine-S-sulfoximine (13), L-methionine- R-sulfoximine (13), and L-methyl-dL-methionine-SR-sulfoximine (11) and L-methionine-SR-sulfoximine phosphate (12) were products previously prepared in this laboratory. L-[1-14C]-Glutamic acid (generally labeled) and L-[1-14C]glutamine acid were obtained from New England Nuclear Corp. D-[1-14C]Glutamic acid was prepared by the action of L-glutamate decarboxylase on L-[1-14C]glutamate (15).

Methods—Glutamine synthetase activity was determined in reaction mixtures (final volume, 1.0 ml) containing enzyme, imidazole-HCl buffer (pH 7.0; 50 μmoles), either MgCl2 (50 μmoles) or MnCl2 (50 μmoles), sodium ATP (7.6 μmoles), NH4Cl (50 μmoles), and sodium L-glutamate (100 μmoles). (In studies with racemic glutamate analogues the amino acid concentration was doubled.) After incubation at 37° for 15 min, the mixtures were deproteinized by adding 0.1 ml of 20% perchloric acid followed by centrifugation; the orgamic phase formed was determined by the method of Fiske and SubbaRow (37). In some experiments the formation of ADP was determined as described (16, 38) using the coupled pyruvate kinase and lactate dehydrogenase reactions. γ-Glutamyltransferase activity was determined using the conditions described by Woolfolk et al. (19). Protein was determined as described by Lowry et al. (39).

The formation of [14C]pyrrolidone carboxylate was determined as previously described (16) in reaction mixtures containing [14C]-glutamate, [14C]Glutamate and [14C]pyrrolidone carboxylate were separated by paper chromatography in a solvent consisting of butanol-acetic acid-water (4:1:1, v/v). The enzyme preparations and reagents were freed of ammonia (15), and controls were carried out to verify the fact that no more than trace amounts of glutamine had been formed. In some experiments the reaction was followed by determining the ADP formed in the absence of ammonia using the coupled pyruvate kinase-lactate dehydrogenase system (16).

The binding of methionine sulfoximine to the enzyme was determined as previously described (10-12) by incubating the enzyme (0.3 to 2 mg) in reaction mixtures (final volume, 0.5 to 0.75 ml) containing imidazole-HCl buffer (pH 7.0; 50 mM), NaCl (5 mM), or MgCl2 (50 mM), sodium ATP (10 mM), 2-mercaptoethanol (1 mM), and [14C]methionine-SR-sulfoximine (5 mM, 1.6 × 10⁶ cpm per μmole). After incubation for 1 to 2 hours at 37°, which time less than 1% of the initial activity remained, the reaction mixture was applied to a column (0.8 × 18 cm) of Sephadex G-50 at 23-25° which had been equilibrated with 0.05 M imidazole-HCl buffer (pH 7.0). Elution was carried out with the same buffer (flow rate, 0.3 to 0.5 ml per min) and fractions of 0.35 ml were collected at 4°; the fractions were analyzed for protein (39) and radioactivity by scintillation counting.

The nature of the bound methionine sulfoximine was investigated by heating samples of the inactivated enzyme at 100° for 2 min; the denatured protein was removed by centrifugation at 4°, and the supernatant solution, which contained virtually all of the radioactivity, was concentrated in vacuo at 24° and then subjected to paper electrophoresis for 2½ hours in 0.5 M acetic acid (pH 2.5) at 40 volts per cm (11). Methionine sulfoximine migrated 7.5 to 8.1 cm in the direction of the cathode and methionine sulfoximine phosphate migrated 0.9 to 3.6 cm toward the anode under these conditions.

RESULTS

Catalytic Formation of Pyrrolidone Carboxylate—When the enzyme (E6,4 or E6,) was incubated with ATP, L-glutamate, and either Mg++ or Mn++ in the absence of ammonia, there was catalytic formation of pyrrolidone carboxylate. As previously observed in studies on brain glutamine synthetase (15), the
The formation of pyrrolidone carboxylate in the presence of Mn++ was much more rapid than with Mg++ (Fig. 2). Conversely, in the studies with adenylylated enzyme (Fig. 1B), pyrrolidone carboxylate synthesis was much more rapid with Mn++ than with Mg++. Earlier work (18-23) has shown that the adenylylated enzyme is much less active in the synthesis reaction with Mg++ than it is with Mn++. In the studies presented in Table I, in which a higher concentration of glutamate was employed, the formation of pyrrolidone carboxylate followed the same general pattern as that described in Fig. 1. However, under these conditions, a low but measurable rate of pyrrolidone carboxylate formation was found with the adenylylated enzyme in the presence of Mg++. The unadenylated enzyme catalyzed pyrrolidone carboxylate formation in the presence of Mn++ at a rate that was about 1% of that of the corresponding synthesis reaction. The rate of pyrrolidone carboxylate formation catalyzed by the unadenylated enzyme with Mg++ and the rates of pyrrolidone carboxylate formation catalyzed by the adenylylated form of the enzyme with either Mg++ or Mn++ were about 0.1% of the corresponding synthesis reactions. It is thus evident that both forms of E. coli glutamine synthetase catalyze pyrrolidone carboxylate formation at rates which are, relative to the corresponding synthesis reactions, about the same or higher than those observed under similar conditions with ovine brain glutamine synthetase (15, 16).

The rates of pyrrolidone carboxylate formation from n-glutamate were determined under the experimental conditions described in Fig. 1 for the unadenylated enzyme (E0.8) and the adenylylated (E11.5) forms of the enzyme by methionine sulfoneimine. The reaction mixtures contained enzyme (E0.8, 20 to 40 μg; E11.8, 0.5 μg), imidazole-HCl buffer (pH 7.0; 50 mM), MgCl2 (50 mM) or MnCl2 (5 mM), NH4Cl (50 mM in B only), sodium ATP (7.6 mM), KCl (90 mM), sodium phosphate-pyruvate (2 mM), DPNH (0.08 mM), lactate dehydrogenase (22 units), and pyruvate kinase (10.4 units) in a final volume of 1.0 ml. The rates of DPNH oxidation were determined at 37°.

![Graph](http://www.jbc.org/)

**FIG. 1 (top).** Formation of pyrrolidone carboxylate from L-glutamate catalyzed by the unadenylated and adenylylated forms of the enzyme. The reaction mixtures (final volume, 0.4 ml) contained enzyme (E0.8, 0.15 mg; E11.8, 0.5 mg), imidazole-HCl buffer (pH 7.0; 25 mM), sodium L-[^14]C glutamate (5.3 mM; 155 μCi per amole), sodium ATP (7.5 mM), and either MgCl2 (50 mM; open circles), or MnCl2 (5 mM; closed circles). The reaction mixtures were incubated at 37° and the formation of [^14]C pyrrolidone carboxylate was then determined as described under “Methods.”

**FIG. 2 (bottom).** Inhibition of the unadenylated (E0.8) and the adenylylated (E11.5) forms of the enzyme by methionine sulfoneimine. Reaction mixtures contained the enzyme (E0.8, 20 μg; E11.8, 14.8 μg), imidazole-HCl buffer (pH 7.0; 50 mM), sodium ATP (10 mM), either MgCl2 (20 mM), or MnCl2 (5 mM), 2-mercapto-ethanol (20 mM), and L-methionine-35S-sulfoximine, 7.5 μM (E0.8), 25 μM (E11.8, Mn++), 1 mM (E11.8, Mg++). The reaction mixtures were incubated at 37°; aliquots (0.05 ml) were then removed and assayed for γ-glutamyltransferase activity in a final volume of 1.0 ml as described under “Methods.”

**Table I**

<table>
<thead>
<tr>
<th>Enzyme form</th>
<th>Metal ion</th>
<th>(A) Pyrrolidone carboxylate formation</th>
<th>(B) Glutamine synthesis</th>
<th>A/B X 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>E0.7</td>
<td>Mg++</td>
<td>1.07</td>
<td>1400</td>
<td>0.08</td>
</tr>
<tr>
<td>E0.7</td>
<td>Mn++</td>
<td>0.38</td>
<td>35</td>
<td>1.1</td>
</tr>
<tr>
<td>E11.8</td>
<td>Mg+++</td>
<td>0.06</td>
<td>80</td>
<td>0.1</td>
</tr>
<tr>
<td>E11.8</td>
<td>Mn+++</td>
<td>0.40</td>
<td>380</td>
<td>0.1</td>
</tr>
</tbody>
</table>

23) on the corresponding synthesis reactions, which proceed much more rapidly with Mg++ than with Mn++. Conversely, in the studies with adenylylated enzyme (Fig. 1B), pyrrolidone carboxylate synthesis was much more rapid with Mn++ than with Mg++; earlier work (18-23) has shown that the adenylylated enzyme is much less active in the synthesis reaction with Mg++ than it is with Mn++.

The formation of pyrrolidone carboxylate was accompanied by stoichiometric formation of ADP. The rates of formation of pyrrolidone carboxylate varied and were a function of whether or not the enzyme was adenylylated, and whether Mg++ or Mn++ were present; they were also influenced by the concentration of glutamate. The formation of pyrrolidone carboxylate was, under the conditions employed here, linear with time and proportional to the concentration of enzyme. In the studies described in Fig. 1, the formation of L[^14]Cpyrrolidone carboxylate was determined in reaction mixtures containing L[^14]C glutamate and either unadenylated enzyme (E1.5) or adenylylated enzyme (E11.5). The formation of pyrrolidone carboxylate with unadenylated enzyme was more rapid with Mg++ than with Mn++ (Fig. 1A), and thus parallels the findings of Stadtman and colleagues (18, 22).
established rapidly with 7.5 μM L-methionine sulfoximine; the rate of inhibition was about the same in the presence of Mg\(^{2+}\) as it was with Mn\(^{2+}\). Methionine sulfoximine also inhibited the adenylylated form of the enzyme (E11.4) in the presence of Mn\(^{2+}\), but the rate of inhibition was considerably less than observed with the unadenylated form of the enzyme. The adenylylated form of the enzyme was also inhibited by methionine sulfoximine in the presence of Mg\(^{2+}\), but much less so than observed with Mn\(^{2+}\); thus, the inhibition curve shown in Fig. 2 (E11.4; squares) was obtained with 1 mM methionine sulfoximine. It is notable that the adenylylated form of the enzyme is much more susceptible to inhibition by methionine sulfoximine in the presence of Mn\(^{2+}\) than with Mg\(^{2+}\); this finding parallels the glutamine synthetase activities found under these conditions with these metal ions (18-25). On the other hand, the observation that the unadenylated enzyme is inhibited by methionine sulfoximine at about equal rates in the presence of either Mn\(^{2+}\) or Mg\(^{2+}\) does not seem to follow the metal ion specificity pattern observed in the glutamine synthetase reaction.

Studies on ovine brain glutamine synthetase have indicated that D-methionine SR-sulfoximine is inactive as an inhibitor of this enzyme and that of the two L-methionine sulfoximine stereoisomers, only L-methionine-S-sulfoximine inhibits the enzyme (13). In the present studies, when D-methionine SR-sulfoximine (0.5 mM) was tested as an inhibitor using the conditions described in Fig. 2, no inhibition was observed. L-Methionine-S-sulfoximine was about twice as active as an inhibitor as L-methionine-SR-sulfoximine (7.5 μM). L-Methionine-R-sulfoximine was also tested at a concentration of 0.5 mM under these conditions and about 12% inhibition of activity was observed after 2 min; however, this preparation of L-methionine-R-sulfoximine is known to contain 0.5 to 1% of the corresponding S-stereoisomer (13,40), and the amount of inhibition observed is consistent with this level of contamination. Thus, it was found that L-methionine SR-sulfoximine at a concentration of 5 μM inhibited the enzyme to about the same extent as did the L-methionine-R-sulfoximine preparation (at a concentration of 0.5 mM). It may therefore be concluded that E. coli glutamine synthetase is inhibited by the same stereoisomer of methionine sulfoximine, i.e. L-methionine-S-sulfoximine, that inhibits glutamine synthetase from ovine brain.

As indicated in Table II, L-glutamate protected the unadenylated enzyme against inhibition by methionine sulfoximine in the presence of either Mg\(^{2+}\) or Mn\(^{2+}\). Such protection indicates that glutamate binds to the unadenylated enzyme in the presence of Mg\(^{2+}\); this finding is expected since L-glutamate is a substrate of the unadenylated enzyme with Mg\(^{2+}\). The protection by glutamate of the unadenylated enzyme in the presence of Mn\(^{2+}\) indicates that glutamate can bind to the unadenylated enzyme in the presence of Mn\(^{2+}\); however, such bound glutamate evidently cannot react to form glutamine at a substantial rate. Glutamate also protected the adenylylated enzyme against inhibition by methionine sulfoximine in the presence of Mn\(^{2+}\), but glutamate did not have a significant effect on the inhibition observed in the presence of Mg\(^{2+}\).

The amount of methionine sulfoximine bound to the enzyme was determined in experiments in which L-[\(^{14}C\)]methionine SR-sulfoximine was incubated with the enzyme in the presence of ATP and metal ion and then applied to the top of a column of Sephadex as described under “Methods.” Under the conditions employed, the protein eluted in a band that was well separated from compounds of low molecular weight (Fig. 3). The fractions containing the inactivated enzyme were pooled and determinations of protein and radioactivity were carried out. As indicated in Table III, between 9.2 and 11 moles of methionine sulfoximine were bound per mole of enzyme in studies carried out on enzyme preparations of varying degrees of adenylylation in the presence of either Mn\(^{2+}\) or Mg\(^{2+}\). In order to determine the nature of the enzyme-bound methionine sulfoximine, preparations of the inactivated labeled enzyme were heated for 2 min at 100° (10-12); after removal of the denatured protein, the supernatant solution was subjected to paper electrophoresis as described under “Methods.” In these studies, 98% of the radioactivity moved with authentic methionine sulfoximine phosphate while the remainder moved with methionine sulfoximine. These studies, which indicate that methionine sulfoximine binds to E. coli glutamine synthetase in the form of methionine sulfoximine phosphate, are analogous to observations made previously on ovine brain glutamine synthetase (10-12).

**Table II**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Reaction components</th>
<th>Percentage of initial activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Without glutamate</td>
</tr>
<tr>
<td>1</td>
<td>E5.1, methionine sulfoximine, Mg(^{2+})</td>
<td>38</td>
</tr>
<tr>
<td>2</td>
<td>E5.1, methionine sulfoximine, Mn(^{2+})</td>
<td>36</td>
</tr>
<tr>
<td>3</td>
<td>E11.4, methionine sulfoximine, Mn(^{2+})</td>
<td>65</td>
</tr>
<tr>
<td>4</td>
<td>E11.5, methionine sulfoximine, Mg(^{2+})</td>
<td>39</td>
</tr>
</tbody>
</table>

**Fig. 3.** Binding of methionine sulfoximine to Escherichia coli glutamine synthetase. The enzyme (E5.1; 1.1 mg) was incubated with ATP, [\(^{14}C\)]methionine sulfoximine and MnCl\(_2\), and then chromatographed on a column of Sephadex G-50 (see "Methods"); fractions of 0.3 ml were collected.
The enzyme was incubated at 37° with [35S] methionine sulfoximine, MgCl₂ (or MnCl₂), and ATP for 1 to 2 hours (inhibition >90%), and the reaction mixtures were then subjected to gel filtration as described in Fig. 3. The experimental details are given under “Methods.”

### Table III

**Binding of methionine sulfoximine to enzyme**

<table>
<thead>
<tr>
<th>Enzyme form</th>
<th>Metal ion</th>
<th>Enzyme</th>
<th>Methionine sulfoximine bound</th>
<th>Methionine sulfoximine bound per mole of enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>E₁₀⁺</td>
<td>Mg⁺⁺</td>
<td>I₁</td>
<td>mole</td>
<td>mole</td>
</tr>
<tr>
<td>E₁₁⁺</td>
<td>Mn⁺⁺</td>
<td>0.93</td>
<td>8.7</td>
<td>9.4</td>
</tr>
<tr>
<td>E₂⁺⁺</td>
<td>Mg⁺⁺</td>
<td>0.92</td>
<td>10.1</td>
<td>11</td>
</tr>
<tr>
<td>E₃⁺⁺</td>
<td>Mg⁺⁺</td>
<td>0.93</td>
<td>10.1</td>
<td>11</td>
</tr>
<tr>
<td>E₄⁺⁺</td>
<td>Mg⁺⁺</td>
<td>0.73</td>
<td>7.45</td>
<td>10</td>
</tr>
<tr>
<td>E₅⁺⁺</td>
<td>Mn⁺⁺</td>
<td>0.52</td>
<td>4.8</td>
<td>9.2</td>
</tr>
<tr>
<td>E₆⁺⁺</td>
<td>Mn⁺⁺</td>
<td>1.15</td>
<td>12.5</td>
<td>11</td>
</tr>
<tr>
<td>E₇⁺⁺</td>
<td>Mg⁺⁺</td>
<td>0.17</td>
<td>1.7</td>
<td>10</td>
</tr>
<tr>
<td>E₈⁺⁺</td>
<td>Mg⁺⁺</td>
<td>2.1</td>
<td>22.5</td>
<td>11</td>
</tr>
</tbody>
</table>

### Table IV

**Activity of unadenylated and adenylated forms of E. coli glutamine synthetase toward several glutamate analogs**

Amide synthesis was determined as described under “Methods”; the specific activities of the enzyme preparations (with L-glutamate) were 910, 395, 425, and 224 units per mg, respectively, for E₁₀, E₁₁, E₁, and E₂.

<table>
<thead>
<tr>
<th>Amino acid substrate</th>
<th>Relative amide synthesis activity</th>
<th>E₁₀⁺ (Mg⁺⁺)</th>
<th>E₁₁⁺ (Mg⁺⁺)</th>
<th>E₂⁺⁺ (Mg⁺⁺)</th>
<th>E₃⁺⁺ (Mn⁺⁺)</th>
<th>E₄⁺⁺ (Mn⁺⁺)</th>
<th>Cow brain glutamine synthetase</th>
</tr>
</thead>
<tbody>
<tr>
<td>t-Glutamate</td>
<td>(100)</td>
<td>(100)</td>
<td>(100)</td>
<td>(100)</td>
<td>(100)</td>
<td>(100)</td>
<td></td>
</tr>
<tr>
<td>d-Glutamate</td>
<td>1.2</td>
<td>0.9</td>
<td>1.3</td>
<td>13</td>
<td>27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Methyl-DL-glutamate</td>
<td>4.7</td>
<td>3.3</td>
<td>2.3</td>
<td>32</td>
<td>75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>threeo-γ-methyl-L-glutamate</td>
<td>7.1</td>
<td>11</td>
<td>8.0</td>
<td>30</td>
<td>27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cis-DL-cycloglutamate</td>
<td>1.8</td>
<td>15</td>
<td>11</td>
<td>40</td>
<td>20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* From Reference 2.

### Activity toward Glutamate Analogs

The data given in Table IV show that the activity of E. coli glutamine synthetase toward several glutamate analogs varies with different states of adenyl- ylation. The unadenylated form is the most specific for t-glutamate. The activity of the adenylated forms (E₁₀, E₁₁) toward these analogs of glutamate is not very greatly different from that observed with the ovine brain enzyme (2); partially adenylated enzyme (E₃) acted on all of the substrates at an appreciable rate. Ginsburg et al. (41) studied the activity of E. coli glutamine synthetase toward d-glutamate and found that activity in the presence of Mn⁺⁺ increased as the extent of aden- ylation decreased; in the presence of Mg⁺⁺ there was relatively low activity toward d-glutamate with enzymes exhibiting various degrees of adenylolation. The present findings on d-glutamate are consistent with those of Ginsburg et al. (41).

### DISCUSSION

The finding that E. coli glutamine synthetase catalyzes the formation of pyrrolidone carboxylate from glutamate (in the absence of ammonia) is in accord with the view that the reaction catalyzed by this enzyme, like that catalyzed by the brain enzyme, involves intermediate formation of enzyme-bound γ-glutamyl phosphate. It is notable that the observed rates of pyrrolidone carboxylate formation by the E. coli enzyme are about the same or higher, relative to the rates of glutamine synthesis, as that found for the brain enzyme. Although Wedler and Boyer (30) were unable to detect pyrrolidone carboxylate formation by an adenylated form (E₁₁) of the E. coli enzyme, it is not certain that these workers succeeded in excluding ammonia from their reaction mixtures; thus, they found (in experiments with [³⁵S]glutamate) peaks for [³⁵S]glutamate and [³⁵S]glutamine on chromatography of reaction mixtures. Wedler and Boyer (30) state that they did not find pyrrolidone carboxylate formation under the conditions used in this laboratory in studies on the ovine brain enzyme (15, 16), however, they did not report studies of their own on the ovine brain enzyme. It should be noted that the conditions used by Wedler and Boyer (30) differ from those employed in the brain enzyme studies in that they added both Mn⁺⁺ and Mg⁺⁺ to the reaction mixtures, while only Mg⁺⁺ ions were added in the brain enzyme work. It is difficult to predict from currently available data the effects of the simultaneous presence of Mg⁺⁺ and Mn⁺⁺ on the formation of pyrrolidone carboxylate by an enzyme preparation such as that used by Wedler and Boyer (30) which contained about 17% unadenylated subunits. However, the present studies show that the rate of pyrrolidone carboxylate formation catalyzed by the fully adenylated form of the enzyme in the presence of Mg⁺⁺ is quite low and might therefore, under some conditions, be difficult to detect.

Explanations for the much higher relative rate of pyrrolidone carboxylate formation with the unadenylated enzyme in the presence of Mn⁺⁺ and for the much higher relative rate at which this form of the enzyme catalyzes the cyclization of d-glutamate are not yet evident. Although the unadenylated form of the enzyme is essentially inactive in the presence of Mn⁺⁺ in catalyzing glutamine synthesis from L-glutamate, this form of the enzyme is about as active as the fully adenylated form in catalyzing the cyclization of L-glutamate (Table I). Since both the formation of pyrrolidone carboxylate and of glutamine require binding to the enzyme of glutamate and ATP and cleavage of the latter to ATP, the relative inactivity of the unadenylated form of the enzyme in glutamine synthesis would seem to indicate that ammonia does not effectively bind or react in the presence of Mn⁺⁺, or that glutamine is not readily released from the enzyme. Presumably, one or more of the latter events occur less readily with Mn⁺⁺ and more readily with Mg⁺⁺. Additional evidence that the unadenylated enzyme can bind glutamate in the presence of Mn⁺⁺ is the finding that glutamate protects this form of the enzyme (in the presence of Mn⁺⁺) from inactivation by methionine sulfoximine. The very low rate of pyrrolidone carboxylate formation catalyzed by the adenylated form of the enzyme in the presence of Mg⁺⁺ seems to be consistent with the mability of this form of the enzyme to catalyze appreciable glutamine synthesis with Mg⁺⁺. These observations and the finding that glutamate does not protect the adenylated form of the enzyme against methionine sulfoximine in the presence of Mg⁺⁺ indicate that this form of the enzyme does not bind glut-
nine sulfoximine inhibits by acting as an analog of the tetrahedral inhibition of the E. coli enzyme by methionine sulfoximine in-
the tetrahedral intermediate.
The observation that methionine sulfoximine inhibits E. coli glutamine synthetase also indicates that the reaction catalyzed by this enzyme, like that catalyzed by the brain enzyme, involves an acyl phosphate intermediate. The present data show that inhibition of the E. coli enzyme by methionine sulfoximine in-
volves phosphorylation of the inhibitor and tight binding of the methionine sulfoximine phosphate formed to the enzyme. About 9 to 11 moles of inhibitor are bound per mole of enzyme; this is in accord with the findings of Stadtman and his colleagues (18–
25) that the enzyme has 12 apparently identical subunits. It is of interest that the unadenylylated form of the enzyme, which is more active in glutamine synthesis than the adenylylated form, is more readily inactivated by methionine sulfoximine than is the adenylylated form. Data cited above (14) indicate that methio-
nine sulfoximine inhibits by acting as an analog of the tetrahedral intermediate or transient state formed in the normal glutamine
synthesize reaction. Assuming this to be true, it would seem that the analog as well as the tetrahedral intermediate fit better into the active site of the unadenylylated enzyme than into that of the
adenylylated enzyme. Thus, those structural features of the enzyme which limit the activity of the adenylylated form may also serve to decrease the rate of its inhibition by an analog of the tetrahedral intermediate.
The present studies offer evidence that the mechanism of the reaction catalyzed by E. coli glutamine synthetase closely resembles that catalyzed by brain glutamine synthetase. The findings indicate that both enzymes catalyze reactions in which glutamate
is phosphorylated on the enzyme and suggest that an intermediate is formed in both reactions whose structure closely resembles methionine sulfoximine. This implies that the enzymes probably have a number of common structural features, especially in the region of the active site. Indeed, the finding that the same dia-
stereoisomer of L-methionine sulfoximine inhibits both enzymes and the observation that they exhibit relatively similar substrate specificity support this view.

It seems probable that the changes in catalytic activity exhibited by E. coli glutamine synthetase as a result of adenylyla-
tion or of the binding of inhibitors reflect the occurrence of changes in the structure of the active center which, although they may alter the affinity for substrate or the rates of formation or utilization of enzyme-bound intermediates, probably do not af-
flect the major reaction pathway. The present studies suggest that useful information about the mechanisms involved in feedback inhibition of the enzyme by various glutamine metabolites might be obtained from detailed studies of the effects of these inhib-
itors on other reactions catalyzed by the enzyme, e.g. pyrroli-
dione carboxylate formation, phosphorylation of methionine sulfoximine, cycloglutamyl phosphate synthesis (17), synthesis of ATP from ADP and carbamyl phosphate (29) or β-glutamyl phosphate (42).

REFERENCES
2. MEISTER, A. (1969) Harvey Lect. 63, 139–175
chemistry 9, 2089–2103
891
chemistry 8, 1066–1075
15. KRISHNASWAMY, P. R., PAMIZIANO, V., and MEISTER, A. (1962) J. Biol. Chem. 237, 2932–2940
16. WELLSNER, V. P., ZOURIS, M., and MEISTER, A. (1966) Bio-
chemistry 5, 3069–3074
chemistry 10, 3186–3189
Symp. Biol. 21, 378–396
27. HOLZER, H., MECKE, D., WULFF, K., LIESS, K., and HEIL-
Enzymol. 17A, 922–927
2432
42. KLEIDHOU, E., WELLSNER, V. P., and MEISTER, A. (1964) Bio-
chemistry 3, 821–828
43. MEISTER, A. (1973) in The Enzymes (BOYER, P. D., ed.) 3rd
Studies on Glutamine Synthetase from *Escherichia coli* : FORMATION OF PYRROLIDONE CARBOXYLATE AND INHIBITION BY METHIONINE SULFOXIMINE

Roberta E. Weisbrod and Alton Meister


Access the most updated version of this article at [http://www.jbc.org/content/248/11/3997](http://www.jbc.org/content/248/11/3997)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/248/11/3997.full.html#ref-list-1](http://www.jbc.org/content/248/11/3997.full.html#ref-list-1)