Rat Liver Glutamine Synthetase

PREPARATION, PROPERTIES, AND MECHANISM OF INHIBITION BY CARBAMYL PHOSPHATE*

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SUMMARY

Glutamine synthetase, purified from rat liver, is homogeneous on acrylamide gel electrophoresis and on ultracentrifugation (s20,w, 15.0 S). The enzyme, which consists of eight subunits (subunit molecular weight, 44,000), resembles ovine brain glutamine synthetase in its physical properties, amino acid composition, and substrate specificity. Complete inhibition of the liver enzyme by methionine sulfoximine is associated with the binding of 4 moles of inhibitor per mole of enzyme. The enzyme is activated by &alpha;-ketoglutarate (in the presence of Mg++ or Mn++) and is inhibited by glycine, L-alanine, L-serine, and carbamyl phosphate (with Mn++ only). Evidence is presented that inhibition by carbamyl phosphate is produced by the binding of this compound to the active site of glutamine synthetase. Thus, the enzyme can catalyze the synthesis of ATP from carbamyl phosphate and ADP. When glutamine synthetase is incubated with ADP, carbamyl phosphate, and glutamate, glutamine is formed. Enzyme inactivated by treatment with methionine sulfoximine and ATP did not utilize carbamyl phosphate. Liver glutamine synthetase also catalyzes ATP synthesis from ADP and acetyl phosphate; this reaction is competitively inhibited by glutamate. Glutamine synthetases from ovine brain, rat brain, and Escherichia coli also catalyze ATP synthesis from carbamyl phosphate and ADP.

Additional similarities (manganese ion-sensitive inhibition by glycine, alanine, and carbamyl phosphate; subunit size), they differ substantially in a number of other regulatory phenomena and in subunit structure. Such differences are probably to be expected since the glutamine synthetases of different cells are undoubtedly adapted to perform different functions. Thus, it would be expected that the function of brain glutamine synthetase would be related to the metabolism of glutamate and γ-aminobutyrate and possibly other neurotransmitter agents. On the other hand, liver glutamine synthetase probably functions in the general amino acid and nitrogen metabolism of the mammalian organism. In a previous report we briefly described the preparation of highly purified rat liver glutamine synthetase (4), and presented data indicating that the enzyme is activated by &alpha;-ketoglutarate and inhibited by glycine, alanine, and carbamyl phosphate; these studies also indicated that manganese ions play a significant role in the regulation of glutamine synthetase.

The present communication gives the details of the purification of rat liver glutamine synthetase and describes certain physical, chemical, and catalytic properties of the enzyme. It is notable that inhibition of the enzyme by methionine sulfoximine is associated with the binding of 4 moles of methionine sulfoximine per mole of enzyme (molecular weight ~360,000; eight subunits). The inhibition of the enzyme by carbamyl phosphate has been further explored and in the course of this work it was found that rat liver glutamine synthetase (as well as glutamine synthetases from brain and E. coli) can catalyze the synthesis of ATP from carbamyl phosphate and ADP. The evidence reported here indicates that carbamyl phosphate inhibits glutamine synthetase by virtue of its attachment to the acyl phosphate site in the catalytic center of the enzyme.

EXPERIMENTAL PROCEDURE

Materials

Generally labeled L-[^14C]glutamate, [P]JATP, and [S-^14C]ADP were obtained from New England Nuclear Corp. Carbamyl phosphate (lithium salt), ADP, ATP, L-methionine-SR-sulfoximine, hexokinase, and glucose 6-phosphate dehydrogenase were purchased from Sigma Chemical Co. Bovine serum albumin, ovalbumin, and chymotrypsinogen were obtained from Schwarz-Mann. L-Aspartate β-decarboxylase of Alcaligenes faecalis was prepared as described (5); the molecular weight of its monomer was determined by Bowers et al. (6). L-[14S]Methionine-SR-sulfoximine and L-[methyl-^14C]methionine-SR-sulfoximine were
prepared as described by Ronzio et al. (7, 8). 5,5'-Dithiobis-(2-nitrobenzoate) was obtained from Aldrich. Lithium acetyl phosphate was a gift from Dr. Leonard B. Spector.

**Methods**

Glutamine synthetase activity was determined either by the \(^\gamma\)-glutamyl hydroxamate synthesis assay essentially as described by Wellner and Meister (9), or by measurement of the inorganic phosphate released (10) in reaction mixtures containing ammonia. The assay mixtures (0.5 ml) usually contained imidazole-HCl buffer (0.1 M; pH 7.2), 2-mercaptoethanol (25 mM), L-glutamate (50 mM; pH 7.2), ATP (10 mM), MgCl\(_2\) (20 mM), and NH\(_4\)OH (125 mM), or NH\(_4\)Cl (40 mM). During purification of the enzyme, 20 mM ATP was used in the assay of fractions. Mn\(^{4+}\)-dependent synthetase activity was determined with 2 mM MnCl\(_2\). Inorganic phosphate and protein were determined by the methods of Fiske and Subbarow (10) and Lowry et al. (11), respectively. A unit of glutamine synthetase activity is defined as that amount of enzyme which catalyzes the synthesis of 1 \(\mu\) mole of either L-\(^\gamma\)-glutamyl hydroxamate or L-glutamine in 15 min at 37\(^\circ\)C.

The \(^\gamma\)-glutamyl transferase activity of glutamine synthetase was determined essentially as described by Levintow (12). The assay mixtures (0.5 ml) contained imidazole-HCl buffer (0.1 M; pH 7.2), L-glutamate (50 mM), MnCl\(_2\) (0.2 mM), NH\(_4\)OH (62.5 mM), sodium arsenate (10 mM), and ADP (0.4 mM). Incubation was carried out for 15 min at 37\(^\circ\)C, at the end of which time the \(^\gamma\)-glutamyl hydroxamate formed was determined.

Polyacrylamide gel electrophoresis in the absence and in presence of sodium dodecyl sulfate were performed by the methods of Davis (13) and Weber and Osborn (14). The buffer systems used have been described (4).

Amino acid analyses were carried out on samples of the enzyme that were freed of 2-mercaptoethanol by dialysis and then hydrolyzed in 6 N HCl at 110\(^\circ\)C for 24 to 72 hours, with a Beckman amino acid analyzer model 120 C by the method of Spackman et al. (16). The half-cystine residues were determined after performic acid oxidation of the enzyme (16). Tryptophan was determined by the spectrophotometric method of Edelhoch (17). Sulfhydryl group determinations were performed by titrating enzyme samples with 5,5'-dithiobis(2-nitrobenzoate) by the procedure of Eillman (18).

The binding of [\(^3\)H]ATP to rat liver glutamine synthetase was measured by equilibrium dialysis as described by Myer and Schellman (19), using Lucite cells (The Chemical Rubber Co., Cleveland, Ohio) having a total capacity of 2 ml. The cell was divided into two equal compartments by a semipermeable membrane (3 cm in diameter) cut from cellulose dialysis tubing.

The formation of ATP from carbamyl phosphate and ADP was determined in reaction mixtures (final volume, 20 \(\mu\)l) containing imidazole-HCl buffer (50 mM; pH 7.2), carbamyl phosphate (10 mM), either magnesium chloride (10 mM) or manganese chloride (1 mM), [\(^{3}\)H]ADP (5 \(\mu\)M; 200,000 cpm per \(\mu\)mole), and glutamine synthetase. After incubation at 37\(^\circ\)C for 30 min; a Spinco model R Electrophoresis cell was used. The dried paper strips were treated with ninhydrin solution (0.25% in acetone) to locate the glutamate and glutamine areas, which were cut out; the radioactivity present was determined as described above.

### Purification of Rat Liver Glutamine Synthetase

The procedure used for the isolation of rat liver glutamine synthetase is similar to that described for the isolation of ovine brain glutamine synthetase (21). Sprague-Dawley rats (250 to 300 g) were used; unless otherwise stated all steps were carried out at 4\(^\circ\)C.

The rats were killed by decapitation and exsanguinated; the livers were excised and homogenized in a Waring Blender with 4 volumes of 0.15 M KCl containing 5 mM 2-mercaptoethanol and 1 mM EDTA (pH 7.2). The homogenate was centrifuged at 0\(^\circ\)C for 30 min at 16,000 \(\times\) g. The supernatant solution was cooled and processed through the acid precipitation (Step 2, Table I) and hydroxylapatite chromatography (Step 3) steps as described by Ronzio et al. (21). The fractions containing the enzyme were pooled and solid ammonium sulfate added to yield 0.5 of saturation. Stirring was continued for 30 min at 4\(^\circ\)C; the solution was then centrifuged at 16,000 \(\times\) g for 40 min. The precipitated protein was dissolved in 15 ml of buffer containing 0.01 M imidazole-HCl, 0.002 M Na\(_2\)EDTA, and 0.005 M 2-mercaptoethanol. The pH of this buffer solution at 4\(^\circ\)C was 7.9. The protein solution was dialyzed for 18 hours against 2 liters of this buffer. The solution was then run into a DEAE-cellulose column (Whatman DE-52, microgranular, previously swollen, were cut out and placed in 10 ml of liquid scintillation medium and then counted in a Nuclear Chicago liquid scintillation counter. In some experiments ATP was determined enzymatically as indicated in the text.

The formation of glutamine from L-glutamate, ADP, and carbamyl phosphate was studied in reaction mixtures whose composition is given in the paragraph above except that unlabeled ADP and generally labeled L-[\(^{14}\)C]glutamate (final concentration 5 mM; 40,000 cpm per \(\mu\)mole) were used. After incubation at 37\(^\circ\)C the reactions were terminated by addition of 5 \(\mu\)l of 10% trichloroacetic acid. Unlabeled glutamate and glutamine were added as carriers and aliquots (20 \(\mu\)l) of the mixtures were applied to strips of Whatman No. 3MM paper (1 \(\times\) 12 cm). Electrophoresis was carried out at 25\(^\circ\)C in 0.05 M sodium acetate buffer (pH 5.5) with a potential gradient of 30 volts per cm for 30 min; a Spincos model R Electrophoresis cell was used. The dried paper strips were treated with ninhydrin solution (0.25% in acetone) to locate the glutamate and glutamine areas, which were cut out; the radioactivity present was determined as described above.

### Purification of Glutamine Synthetase from Rat Liver

#### Table I

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Protein</th>
<th>Activity</th>
<th>Specific Activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration</td>
<td>Total</td>
<td>Total units</td>
<td>concentration</td>
<td>%</td>
</tr>
<tr>
<td>1. Liver extract</td>
<td>(960)</td>
<td>25.1</td>
<td>24,100</td>
<td>11,100</td>
<td>0.47</td>
</tr>
<tr>
<td>2. Acid precipitation</td>
<td>277</td>
<td>4.91</td>
<td>1,368</td>
<td>8,100</td>
<td>5.90</td>
</tr>
<tr>
<td>3. Hydroxylapatite chromatography</td>
<td>370</td>
<td>0.46</td>
<td>170</td>
<td>5,900</td>
<td>34.7</td>
</tr>
<tr>
<td>4. DEAE-cellulose chromatography</td>
<td>55</td>
<td>0.51</td>
<td>28.0</td>
<td>2,720</td>
<td>97</td>
</tr>
<tr>
<td>5. Chromatography on Sephadex G-100</td>
<td>9.3</td>
<td>1.77</td>
<td>16.8</td>
<td>2,260</td>
<td>134</td>
</tr>
</tbody>
</table>

*From 220 g of rat liver; see the text for details.*
2.5 x 8 cm) which was previously equilibrated with the same buffer. The column was washed with 100 ml of buffer followed by elution with 0.02 M imidazole-HCl buffer containing 0.002 M EDTA and 0.005 M 2-mercaptoethanol (pH adjusted to 6.2 at 4°C by addition of citric acid). Fractions of 4 to 5 ml were collected (Step 4, Table I); the active fractions were pooled and glycerol was added to a final concentration of 10% (v/v). The solution was concentrated by ultrafiltration through dialysis tubing in vacuo to about 6 to 7 ml.

The concentrated solution was applied to the top of a column of Sephadex G-100 (2.5 x 90 cm) which was previously equilibrated with 0.15 M KCl buffer (pH 7.2) containing 0.001 M EDTA and 0.005 M 2-mercaptoethanol. The column was eluted with the same buffer at a flow rate of about 20 ml per hour. Fractions of about 5 ml were collected and those exhibiting a specific activity greater than 120 were pooled and concentrated by ultrafiltration through dialysis tubing in vacuo (Step 5).

A summary of the purification is given in Table I. The procedure has been repeated six times with similar results. Preparations with specific activities of 130 to 140 units per mg have been obtained (about 300-fold purification) in about 20% yield. The specific activities of the liver enzyme preparations have been consistently about 80% of those of ovine and other brain enzyme preparations (160 to 180 units per mg).

Glutamine Synthetases from Other Sources—Glutamine synthetase was isolated from rat brain as follows. Frozen rat (Sprague-Dawley) brains (294 g from 150 rats) were thawed at 4°C, homogenized, and extracted as described above for the liver enzyme. A purification procedure very similar to that used for liver glutamine synthetase was followed. A preparation that exhibited a specific activity of 160 was obtained in low (8%) yield. The ovine and human brain glutamine synthetases were isolated from brain acetone powders essentially as described for the ovine brain enzyme by Ronzio et al. (21); the modifications of the DEAE-cellulose step and the Sephadex G-100 gel filtration step described above for the rat liver enzyme preparation were used. Glutamine synthetase of peas was isolated by Dr. Y. Tada of this laboratory. E. coli glutamine synthetase (22-24) was kindly made available to us by Dr. Roberta Weisbrod and Mrs. Susan Lord Lundt of this laboratory.

RESULTS

Physical and Chemical Properties of Rat Liver Glutamine Synthetase—The purified rat liver enzyme preparation migrated as a single band when subjected to electrophoresis on 4% polyacrylamide gels at pH values of 7.2, 8.0, and 8.5. Similar results were obtained with the purified glutamine synthetases isolated from rat brain, ovine brain, and human brain; there was no significant difference between the migration of these enzymes and that of the rat liver enzyme. Analytical ultracentrifugation studies (carried out in 0.15 M KCl, 5 mM 2-mercaptoethanol, and 1 mM EDTA (pH 7.2); protein concentration 0.5 mg per ml) led to a sedimentation coefficient (s20, w) of 15.0 S for the rat liver enzyme. This value is substantially the same as that determined for the ovine brain enzyme (25, 26). As cited previously (4), the rat liver enzyme and the ovine brain enzyme both exhibited morphology in the electron microscope consistent with an octameric structure (27, 28).

The molecular weights of the monomeric units of the rat liver and sheep brain glutamine synthetases were determined by electrophoresis in 6% polyacrylamide gels carried out in the presence of 0.1% sodium dodecyl sulfate. A representative molecular weight calibration curve is given in Fig. 1. The dissociated monomeric units of the rat liver and ovine brain enzymes moved as single discrete bands; molecular weights of 44,000 ± 2,000 and 49,000 ± 3,000, respectively, were calculated from these data. Virtually identical results were obtained in gel electrophoresis studies carried out on 5% polyacrylamide gels in the presence of 0.1% sodium dodecyl sulfate.

The amino acid compositions of the rat liver and rat brain glutamine synthetases are given in Table II; the values previously determined (21) for the ovine brain enzyme are included for comparative purposes. Perusal of these data indicates that the three glutamine synthetases exhibit an impressively similar amino acid composition.

Titration of the liver enzyme with 5,5'-dithiobis(2-nitrobenzoate) at pH 7.2 showed that about three sulfhydryl groups per subunit reacted rapidly (Fig. 2; Curve 1); a slower reaction also took place in which the equivalent of two additional sulfhydryl groups reacted after 100 min. When the titration was carried out in the presence of Mn2+ (Curve 2) the slower phase of reaction decreased substantially, and when the titration was carried out in the presence of both Mn2+ and ATP (Curve 3) there was no additional reaction after the initial burst. Curves 1A and 3A (Fig. 2) describe the γ-glutamyltransferase activity of the enzyme titrated with the reagent as described in Curves 1 and 3, respectively; under these conditions there was virtually no change in transferase activity over a period of 40 min. When the titration was carried out in the presence of 5 mM guanidine HCl, all enzymatic activity was lost, and the equivalent of about 11 moles of sulfhydryl reacted (Curve 4); this result, and the finding of 12 half-cystine residues on amino acid analysis (Table II) suggest that the enzyme does not have disulfide linkages.

Substrate Specificity of Rat Liver Glutamine Synthetase—The apparent Km value for L-glutamate in the presence of Mg2+ or Mn2+ and ammonium chloride was 5 mM. The apparent Vm values for ATP with Mg2+ was 2.3 mM. The Vmax values with Mg2+ and Mn2+ were, respectively, 160 and 30 units per mg of
cant differences between these enzymes in their affinity for ATP.  

The available data thus indicate that there are significantly, and that about 4 additional moles of ATP bind less tightly, and that about 8 moles of ATP per mole of enzyme (molecular weight, 400,000) bind.

Figs. 4. 5, and 6 represent the same experiment except that MnCl₂ (1 mM) and MnCl₂ (1 mM) plus ATP (0.2 mM), respectively, were added. The ordinate gives the calculated moles of sulphhydryl groups per 45,000 g of enzyme. Curve 4 was obtained in the presence of 5 M guanidine-HCl. Curve 4A and 4A give the γ-glutamyltransferase activities of the enzyme treated with reagent as described in Experiments 1 and 3, respectively.

TABLE II  
Amino acid composition of rat liver, rat brain, and ovine brain glutamine synthetases

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Amount of amino acid per 45,000 g of enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rat liver*</td>
</tr>
<tr>
<td>-----------------</td>
<td>------------</td>
</tr>
<tr>
<td>Lysine</td>
<td>20</td>
</tr>
<tr>
<td>Histidine</td>
<td>13</td>
</tr>
<tr>
<td>Arginine</td>
<td>22</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>48</td>
</tr>
<tr>
<td>Threonine</td>
<td>20*</td>
</tr>
<tr>
<td>Serine</td>
<td>25*</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>35</td>
</tr>
<tr>
<td>Proline</td>
<td>23</td>
</tr>
<tr>
<td>Glycine</td>
<td>42</td>
</tr>
<tr>
<td>Alanine</td>
<td>28</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>12*</td>
</tr>
<tr>
<td>Valine</td>
<td>20*</td>
</tr>
<tr>
<td>Methionine</td>
<td>12</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>23*</td>
</tr>
<tr>
<td>Leucine</td>
<td>25</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>17</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>21</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>6*</td>
</tr>
</tbody>
</table>

* Averaged values to the nearest integer for 24-, 48-, and 72-hour hydrolyses, except as noted.

† Values taken from (21) and recalculated for 45,000 g of enzyme.

* Extrapolated.

$^\dagger$ As cysteic acid; 24-hour hydrolysis (16).

$^\ddagger$ 72-hour hydrolyzed.

$^\circ$ Determined spectrophotometrically (17).

Relative activities of various substrates of rat liver glutamine synthetase

Activity was determined by the γ-glutamyl hydroxamate assay (NH₄OH) or by measurement of the phosphate formed (NH₄). The reaction mixtures contained 0.1 M imidazole buffer (pH 7.2), 25 mM 2-mercaptoethanol, 40 mM amino acid, 10 mM ATP, 125 mM NH₄OH or 100 mM NH₄Cl, and 20 mM MgCl₂ or 2 mM MnCl₂. The values given for synthetase activity are relative to those found with L-glutamate (arbitrarily designated as 100). The figures in parentheses indicate the relative activities obtained with brain glutamine synthetase (from Reference 2).

$\dagger$ Dr. S. S. Tate, unpublished studies in this laboratory.

Binding of Methionine Sulfoximine to Enzyme—Earlier studies in this laboratory showed that the inhibition of glutamine synthetase by methionine sulfoximine is associated with the phosphorylation of this compound and its tight attachment to the active site of the enzyme (7, 8, 30). As summarized in Table IV, a number of experiments were carried out in which the rat liver enzyme was incubated with either S- or methyl-³⁵C-labeled...
mole of enzyme. The sigmoidal relationship between inhibition and the amount of methionine sulfoximine bound to the enzyme was studied by incubating the enzyme with methionine sulfoximine and ATP. The relationship between the extent of inhibition and the amount of methionine sulfoximine per mole of enzyme suggests the possibility of cooperativity between the substrate binding sites of different subunits.

**Effect of Various Compounds on Activity of Glutamine Synthetase**—Table V summarizes studies in which the effects of α-ketoglutarate, several amino acids, carbamyl phosphate, and adenosine 5′-monophosphate were studied on the activity of glutamine synthetase from rat liver and several other sources. α-Ketoglutarate activated rat liver glutamine synthetase in the presence of 

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

**Table IV**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>[35S] or [14C]Methionine sulfoximine</th>
<th>Binding of methionine sulfoximine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/mole enzyme</td>
<td>Mg++</td>
</tr>
<tr>
<td>1</td>
<td>0.1 [35S]</td>
<td>4.1</td>
</tr>
<tr>
<td>2</td>
<td>0.5 [35S]</td>
<td>4.4</td>
</tr>
<tr>
<td>3</td>
<td>1.6 [methyl-[14C]]</td>
<td>4.0</td>
</tr>
<tr>
<td>4</td>
<td>2.5 [35S]</td>
<td>4.1</td>
</tr>
</tbody>
</table>

methionine sulfoximine, ATP, and either Mn++ or Mg++; incubation was followed by extensive dialysis or gel filtration in order to remove low molecular weight substances. As indicated in Table IV, close to 4 moles of methionine sulfoximine were bound per mole of inactivated enzyme. Earlier studies on the ovine brain enzyme, when recalculated for a molecular weight of 400,000, indicated the binding of 5.7 to 6.1 moles of methionine sulfoximine per mole of enzyme. The relationship between the extent of inhibition and the amount of methionine sulfoximine bound to the enzyme was studied by incubating the enzyme with methionine sulfoximine and ATP. The concentrations of ATP and the incubation period were chosen such that the reaction of the enzyme with methionine sulfoximine and ATP had stopped in each case prior to analysis. After removal of excess reagents the binding of methionine sulfoximine and the residual γ-glutamyltransferase activity were determined. The results of this study, given in Fig. 5, indicate that essentially complete inactivation is associated with the binding of 4.0 to 4.2 moles of methionine sulfoximine per mole of enzyme. The sigmoidal relationship between inhibition and the binding of methionine sulfoximine suggests the possibility of cooperativity between the substrate binding sites of different subunits.

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Present the presence of Mg++. Similar enhancement of glycine inhibition was present. Thus, as shown in Fig. 6, the presence of phosphate changes of a solution containing 0.15 M KCl and 2 mM EDTA (pH 7.2). Determinations were then diazylzed exhaustively at 4°C against several changes of a solution containing 0.15 M KCl and 2 mM EDTA (pH 7.2). Determinations were then made of the r-glutamyltransferase activity, protein, and amount of bound 14C.

Mechanism of Inhibition of Glutamine Synthetase by Carbamyl Phosphate—As indicated above, carbamyl phosphate inhibits glutamine synthetase from rat liver and other sources markedly in the presence of Mn++. While glycine and L-alanine did not inhibit the liver enzyme appreciably in the presence of Mg++, it was found that these amino acids inhibited substantially when phosphate was also present. Thus, as shown in Fig. 6, the presence of phosphate led to a considerable enhancement of the inhibition by glycine in the presence of Mg++. Similar enhancement of glycine inhibition by phosphate was observed in the presence of Mn++. It is notable that phosphate itself inhibits glutamine synthetase in the presence of either Mg++ or Mn++. Inhibition of glutamine synthetase by inorganic phosphate would be expected since the reaction is freely reversible (32, 33). As discussed below, phosphate probably binds to the enzyme at or close to the site that binds the terminal phosphoryl group of ATP. In studies under conditions similar to those used in Fig. 6 in which the ATP concentration was varied between 1 and 6 mM, inhibition by inorganic phosphate (10 mM) was found to be competitive with respect to ATP.

Mechanism of Inhibition of Glutamine Synthetase by Carbamyl Phosphate—As indicated above, carbamyl phosphate inhibits glutamine synthetase from rat liver and other sources markedly in the presence of Mn++. While carbamyl phosphate is known to decompose to yield inorganic phosphate and cyanate at values of pH from about 6 to 9 (34), our studies indicate that carbamyl phosphate is considerably more inhibitory than equivalent concentrations of inorganic phosphate. Thus, with 5 mM phosphate we observed 22% inhibition, while 32% inhibition was observed with 5 mM carbamyl phosphate. No inhibition of the enzyme was found with 5 mM cyanate. It therefore seems unlikely that all of the inhibition observed in the presence of added carbamyl phosphate can be ascribed to the presence or formation of inorganic phosphate. The structural similarity between carbamyl phosphate, 3-glutamyl phosphate, and the tetrahedral intermediate 3-glutaminyl phosphate (postulated to be formed by reaction of ammonia with 3-glutamyl phosphate (1-3)), led us to

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Mechanism of Inhibition of Glutamine Synthetase by Carbamyl Phosphate—As indicated above, carbamyl phosphate inhibits glutamine synthetase from rat liver and other sources markedly in the presence of Mn++. While carbamyl phosphate is known to decompose to yield inorganic phosphate and cyanate at values of pH from about 6 to 9 (34), our studies indicate that carbamyl phosphate is considerably more inhibitory than equivalent concentrations of inorganic phosphate. Thus, with 5 mM phosphate we observed 22% inhibition, while 32% inhibition was observed with 5 mM carbamyl phosphate. No inhibition of the enzyme was found with 5 mM cyanate. It therefore seems unlikely that all of the inhibition observed in the presence of added carbamyl phosphate can be ascribed to the presence or formation of inorganic phosphate. The structural similarity between carbamyl phosphate, 3-glutamyl phosphate, and the tetrahedral intermediate 3-glutaminyl phosphate (postulated to be formed by reaction of ammonia with 3-glutamyl phosphate (1-3)), led us to
it could be estimated that it is less than 0.5 \text{ mM} or about 10\% of the system was too low to determine accurately by this method, but for glutamate (5 \text{ mM}). The corresponding value for the Mn\+2 + phosphate of 6.3 \text{ mM}, a value which is slightly higher than that obtained led to an apparent $K_A$ value for carbamyl phosphate. The $V_{\text{max}}$ values in the presence of Mn\+2 + but not in the presence of Mg\+2 affected ATP synthesis.

The possibility of ATP formation under these conditions is equivalent to about 2\% of the rate of glutamine synthesis catalyzed by this amount of enzyme. Addition of glutamate (Experiment 2) or of ATP (Experiment 3) decreased the formation of ATP from ADP and carbamyl phosphate. On the other hand, addition of NH\+4 (Experiment 4) did not affect the formation of ATP appreciably. Addition of L-alanine, which inhibits glutamine synthesis in the presence of Mn\+2 + but not in the presence of Mg\+2 affected ATP synthesis from ADP and carbamyl phosphate in the presence of Mn\+2 + but not in the presence of Mg\+2. When enzyme inactivated by prior treatment with methionine sulfoximine and ATP was used (Experiment 6), no synthesis of ATP occurred.

These findings suggest that the formation of ATP from carbamyl phosphate and ADP involves some of the same enzyme sites that are used for glutamine synthesis. Since carbamyl phosphate inhibits glutamine synthesis more effectively in the presence of Mn\+2 + than in the presence of Mg\+2, it seems probable that the affinity of the enzyme for carbamyl phosphate is greater with Mn\+2. The effect of varying carbamyl phosphate concentration on ATP synthesis from ADP was studied in the presence of Mg\+2 (Fig. 7); the data obtained led to an apparent $K_m$ value for carbamyl phosphate of 6.3 \text{ mM}, a value which is slightly higher than that for glutamate (5 \text{ mM}).

The corresponding value for the Mn\+2 system was too low to determine accurately by this method, but it could be estimated that it is less than 0.5 \text{ mM} or about 10\% of the $K_m$ value for glutamate. The $V_{\text{max}}$ values in the presence of Mg\+2 and Mn\+2, respectively, were 2.6 and 0.41 amoles of ATP formed per mg of enzyme.

### Table VI

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Components of reaction mixture</th>
<th>ATP formed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ADP + CP</td>
<td>28.2 \text{ amoles}</td>
</tr>
<tr>
<td>1</td>
<td>ADP + CP + L-glutamate (5 \text{ mM})</td>
<td>7.8 \text{ amoles}</td>
</tr>
<tr>
<td>2</td>
<td>ADP + CP + ATP (5 \text{ mM})</td>
<td>10.4 \text{ amoles}</td>
</tr>
<tr>
<td>3</td>
<td>ADP + CP + NH\textsubscript{4}Cl (10 \text{ mM})</td>
<td>27.4 \text{ amoles}</td>
</tr>
<tr>
<td>4</td>
<td>ADP + CP + L-alanine (20 \text{ mM})</td>
<td>28.8 \text{ amoles}</td>
</tr>
<tr>
<td>5</td>
<td>ADP + CP</td>
<td>0 \text{ amoles}</td>
</tr>
</tbody>
</table>

The inset gives the double reciprocal plot of the data.

### Table VII

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Components of assay mixture</th>
<th>Formation of Glutamine and ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ADP + CP (10 \text{ mM})</td>
<td>0 \text{ amoles}</td>
</tr>
<tr>
<td>1</td>
<td>ADP + CP (10 \text{ mM}) + L-glutamate</td>
<td>7.50 \text{ amoles}</td>
</tr>
<tr>
<td>2</td>
<td>ADP + CP (20 \text{ mM})</td>
<td>0 \text{ amoles}</td>
</tr>
<tr>
<td>3</td>
<td>ADP + CP (20 \text{ mM}) + L-glutamate</td>
<td>8.61 \text{ amoles}</td>
</tr>
<tr>
<td>4</td>
<td>ADP + L-glutamate</td>
<td>1.10 \text{ amoles}</td>
</tr>
</tbody>
</table>

The reaction mixtures (final volume, 20 \text{ µl}) contained, as indicated, [14C]ADP (5 \text{ µM}), carbamyl phosphate (10 \text{ mM}), MgCl\textsubscript{2} (10 \text{ mM}) or MnCl\textsubscript{2} (1 \text{ mM}), imidazole-HCl buffer (50 \text{ mM}; pH 7.2), and rat liver enzyme (9 \text{ µg}). After incubation at 37\textdegree for 30 min, 5 \text{ µl} of 10\% trichloroacetic acid were added, and ATP was separated from ADP and determined as described under “Methods.”

The enzyme was inactivated by preliminary incubation with methionine sulfoximine, ATP, and Mg\+2, and then extensively dialyzed.

### Consideration

Consider the possibility that carbamyl phosphate might bind to the active center of the enzyme at the acyl phosphate binding site. If such were the case, it might be expected that glutamine synthetase could catalyze the synthesis of ATP from carbamyl phosphate and ADP. This possibility was examined by carrying out the experiments described in Table VI. In these studies, the liver enzyme was incubated with ADP, carbamyl phosphate, and either Mg\+2 or Mn\+2. As indicated in Experiment 1, substantial formation of ATP was observed; the rate of ATP formation under these conditions is equivalent to about 2\% of the rate of glutamine synthesis catalyzed by this amount of enzyme. Addition of glutamate (Experiment 2) or of ATP (Experiment 3) decreased the formation of ATP from ADP and carbamyl phosphate.

Although the enzyme does not catalyze the synthesis of carbamyl phosphate from bicarbonate, ammonia (or glutamine), and ATP, it can, as expected, catalyze the synthesis of glutamine from L-glutamate, carbamyl phosphate, and ADP. Thus, as indicated in Table VII, addition of L-glutamate (Experiments 2 and 4) to reaction mixtures containing carbamyl phosphate and ADP (Experiments 1 and 3) decreased the synthesis of ATP substantially and led to appreciable synthesis of glutamine. The formation of glutamine under these conditions may be ascribed to utilization of ammonia formed from spontaneous hydrolysis of carbamate.

### References

- The reaction mixtures (final volume, 20 \text{ µl}) contained [14C]ADP (5 \text{ µM}), MgCl\textsubscript{2} (10 \text{ mM}), L-[14C]glutamate (5 \text{ mM}), carbamyl phosphate (10 or 20 \text{ mM}), imidazole-HCl buffer (50 \text{ mM}; pH 7.2), and rat liver enzyme (9 \text{ µg}).

- The reaction mixtures (final volume, 20 \text{ µl}) contained, as indicated, [14C]ADP (5 \text{ µM}), MgCl\textsubscript{2} (10 \text{ mM}), L-[14C]glutamate (5 \text{ mM}), carbamyl phosphate (10 or 20 \text{ mM}), imidazole-HCl buffer (50 \text{ mM}; pH 7.2), and rat liver enzyme (9 \text{ µg}).

### Fig. 7

Effect of carbamyl phosphate concentration on ATP synthesis. The reaction mixtures (20 \text{ µl}) contained 5 \text{ mM} [14C]ADP, 10 \text{ mM} MgCl\textsubscript{2}, 8.5 \text{ µg} of liver enzyme, and carbamyl phosphate as indicated. The inset gives the double reciprocal plot of the data.
Fig. 8. Synthesis of ATP catalyzed by rat liver glutamine synthetase; reversal of glutamine synthetase and phosphorylation of ADP by carbamyl phosphate and acetyl phosphate. A, the reaction mixture (final volume, 0.1 ml) contained imidazole-HCl buffer (0.1 m; pH 7.2), ADP (10 mM), MgCl₂ (20 mM), and rat liver enzyme (10 µg). Experiment 1 also contained l-glutamine (40 mM) and potassium phosphate (10 mM). Experiments 2 and 3 contained carbamyl phosphate (10 mM), and acetyl phosphate (10 mM), respectively. B, The reaction mixtures (final volume, 0.01 m) contained imidazole-HCl buffer (0.1 m; pH 7.2), ADP (10 mM), MnCl₂ (1 mM), and 32 µg of enzyme. In Experiment 1, l-glutamine (40 mM) and potassium phosphate (10 mM) were added. In Experiment 2, carbamyl phosphate (20 mM) was added; in Experiment 3, L-glutamine (40 mM), potassium phosphate (10 mM), and carbamyl phosphate (20 mM) were added. Incubation was carried out at 37°C and was terminated by adding 50 µl of 1 M HCl and allowing the solutions to stand at 0°C for 10 min; then 50 µl of 1 M Tris were added followed by 0.8 ml of a solution containing Tris-HCl buffer (0.1 m; pH 7.6), d-glucose (30 mM), bovine serum albumin (2.5 µg), MgCl₂ (0.25 mM), TPN⁺ (1.25 mM), and 6 units each of hexokinase and glucose-6-phosphate dehydrogenase. This solution was incubated at 37°C, and after no further increase in absorbance at 340 nm was observed, the formation of ATP was calculated from the amount of TPNH formed.

Under the conditions employed, carbamyl phosphate was not hydrolyzed by glutamine synthetase. Incubation of the enzyme with carbamyl phosphate, glutamate, and metal ions did not lead to glutamine formation, nor did incubation of the enzyme with carbamyl phosphate and methionine sulfoximine cause inhibition. The enzyme did not catalyze urea formation from carbamyl phosphate and ammonia (or glutamine).

In the course of these studies, it was found that acetyl phosphate could be substituted for carbamyl phosphate in the ATP synthesis reaction. As indicated in Fig. 8 (A, Curve S) the formation of ATP from ADP and acetyl phosphate takes place at about 50% of the rate observed with ADP and carbamyl phosphate (Curve 2). Both of these reactions take place at rates which are much slower than the reversal of glutamine synthetase (Curve 1). The reactions shown in Fig. 8A were carried out in the presence of Mg⁺⁺. As indicated in Fig. 8B, in the presence of Mn⁺⁺, the synthesis of ATP from carbamyl phosphate and ADP (Curve 2) takes place at about 50% of the rate of the reverse synthesis reaction (Curve 1). When carbamyl phosphate was added to the reverse synthesis system (Curve 8) the rate of ATP formation was somewhat less than that of the reverse synthesis reaction (Curve 1). The findings, which show that the rates of ATP synthesis from glutamine, P₁, and ADP and from carbamyl phosphate and acetyl phosphate are not additive, are in accord with the view that both reactions are catalyzed by the same enzyme sites. Additional evidence in support of this conclusion is given in Fig. 9, which describes the effect of L-glutamate on the synthesis of ATP from ADP and acetyl phosphate. Glutamate inhibited competitively; the Kᵢ value for L-glutamate is 4.5 mM, a value similar to the Kᵢ determined for glutamine synthetase (5.5 mM). A satisfactory experiment of this type with carbamyl phosphate could not be done because formation of ammonia led to ATP utilization for glutamine synthetase (see Table VII). Incubation of the enzyme with ADP and either amidophosphate, creatine phosphate, phosphoenolpyruvate or ethanolamine phosphate did not lead to ATP synthesis.

The ability to catalyze ATP synthesis from carbamyl phosphate and ADP is not only a property of rat liver synthetase. Thus, as indicated in Table VII, purified synthetases obtained from rat brain, ovine brain, and E. coli can also catalyze this reaction.

**DISCUSSION**

Although glutamine synthetase activity has been found in a variety of mammalian tissues (see, for example, References...
35–37), brain glutamine synthetase seems to be the only mammalian glutamine synthetase which has previously been extensively purified and studied (21, 25, 29, 33). Partially purified preparations of rat liver glutamine synthetase have been obtained (38, 30). The present investigation of highly purified rat liver glutamine synthetase and previous work in this laboratory on this enzyme preparation (4, 40) show that there are many similarities between the brain enzyme and the rat liver enzyme, but also some significant differences. The liver enzyme resembles the brain enzyme in subunit size, number, and arrangement, electrophoretic mobility on polyacrylamide gels, and amino acid composition. Additional similarities include substrate specificity and susceptibility to irreversible inhibition by methionine sulfoximine.

It is notable that the rat liver enzyme is much more susceptible to activation by α-ketoglutarate and to inhibition by certain amino acids than the brain enzyme. Inhibition of the liver enzyme by amino acids is more marked in the presence of Mn++, and with Mg++ inhibition by both inorganic phosphate and glycine is greater than the sum of the inhibitions observed with phosphate and glycine separately. The inhibition of the liver enzyme by inorganic phosphate alone may be ascribed, at least in part, to the reversibility of glutamine synthesis (32). Thus, evidence was obtained for competitive inhibition between phosphate and ATP. The computer studies of the active site of glutamine synthetase (3) are in accord with the view that inorganic phosphate binds to the enzyme site that is normally in contact with the terminal phosphoryl group of ATP. However, the data do not exclude the possibility that inorganic phosphate may also inhibit the enzyme by other mechanisms, such as complex formation with metal ions. It is possible that the inhibition of glutamine synthetase by inorganic phosphate has physiological significance. The well known activation of certain glutaminases by inorganic phosphate suggests that phosphate may offer reciprocal control of glutaminase and glutamine synthetase by activating the former and inhibiting the latter enzyme. It is apparent that uncontrolled coupling of glutaminase and glutamine synthetase represents a potentially wasteful metabolic situation, which must be normally prevented in vivo either by compartmentalization or by a control mechanism of some sort.

The potential metabolic significance of the inhibition of liver glutamine synthetase by glycine, L-alanine, and L-serine was emphasized previously in relation to the activities of other enzymes that are involved in the metabolism of glutamine (4). Thus, the utilization of the α-amino group of glutamine by rat liver glutamine transaminase, for which glyoxylate, pyruvate, and hydroxy-pyruvate are amongst the best α-keto acid substrate (41) may be controlled by inhibition of liver glutamine synthetase. Similarly, inhibition of glutamine synthetase by carbamyl phosphate may serve to control the amount of glutamine available for pyrimidine biosynthesis, at least by the glutamine-dependent carbamyl phosphate synthetase pathway.

The data presented here indicate that carbamyl phosphate inhibits glutamine synthetase by interacting with the acyl phosphate binding site at the active center of this enzyme. There are now several lines of evidence for the intermediate formation of an acyl phosphate in glutamine synthesis. Thus, glutamine synthetase (from ovine brain (42) and from E. coli (43)) catalyzes the formation of pyrrolidone carboxylate from glutamate in the absence of ammonia. In the studies on the brain enzyme it was shown that the binding of glutamate is associated with cleavage of ATP to ADP (42). In addition, the enzyme can use β-amino-glutaryl phosphate for the synthesis of ATP (44), and it catalyzes the phosphorylation of methionine sulfoximine (7, 8, 30). More recently it was shown that the enzyme can catalyze the net formation of cycloglutamyl phosphate from cycloglutamate and ATP (45). The structural analogy between γ-glutamyl phosphate, the postulated tetrahedral γ-glutaminyl phosphate intermediate (1, 3) and carbamyl phosphate (Fig. 10) suggested that carbamyl phosphate might bind to the same sites that bind these intermediates in the normal synthesis of glutamine. The data seem to support this possibility. Thus, the synthesis of ATP from carbamyl phosphate and ATP is catalyzed by the rat liver enzyme and also by other glutamine synthetases (E. coli, rat brain, ovine brain; Table VIII). This reaction may be considered as the reversal of the first step of glutamine synthesis, i.e. formation of the acyl phosphate from glutamate and ATP. The ability of glutamine synthetase to catalyze the formation of ATP from carbamyl phosphate and ADP makes possible a net synthesis of glutamine in reaction mixtures containing the enzyme, glutamate, carbamyl phosphate, and ADP.6 Whereas the rat liver and ovine brain glutamine synthetases were found to be inactive in catalyzing the synthesis of carbamyl phosphate from bicarbonate, ammonia (or glutamine), and ATP, it should be noted that carbamyl phosphate synthetases from various sources can also catalyze formation of ATP from carbamyl phosphate and ADP (46–48).

It is curious that although the rat liver enzyme has eight subunits, it can be completely inhibited by the binding of only about 4 moles of methionine sulfoximine. In addition, the studies on ATP binding indicate that a maximum of 5 moles of ATP bind to the enzyme. In this respect the liver enzyme differs from the ovine brain enzyme which can bind about 8 moles of ATP tightly and whose inhibition by methionine sulfoximine is accompanied by binding of substantially more than 4 moles of inhibitor per mole of enzyme. It may be relevant to note that the isolated enzyme preparations from brain have been found to be about 20% more active than those from liver. While the explanation of the binding phenomena observed with the rat liver enzyme requires further study, several possible explanations for the findings may be considered. Thus, binding of inhibitor to half of the active sites may affect the remaining sites in some manner as to make them unavailable for combination with substrate or inhibitor. A number of interesting speculations along these lines are possible (see, for example, Reference 49). However, the

The finding that acetyl phosphate can also be used by the enzyme for ATP synthesis would seem to be consistent with the structural analogies cited above (Fig. 10). It is possible that the methyl group of acetyl phosphate and the amino group of carbamyl phosphate occupy the same enzyme site. This would be analogous to the postulate discussed earlier (3) that the methyl group of methionine sulfoximine attaches to the ammonia binding site of the enzyme.
finding that the binding of ATP is substantially less than 8 moles per mole of enzyme would be consistent with the possibility that some of the subunits of the isolated enzyme are not active, perhaps because of inactivation during preparation. Another possibility is that about half of the active sites do not function in the synthesis of glutamine, but serve instead as binding sites for amino acid inhibitors such as glycine, L-alanine, and L-serine. Presumably the binding of such amino acids to these allosteric sites can inhibit the activity of the substrate sites of the enzyme. According to this interpretation, the enzyme normally uses only four subunits for catalysis; the remaining four may have been modified to function in a regulatory capacity. It is notable that β-alanine and d-alanine are among the most inhibitory of the amino acid inhibitors such as glycine, L-alanine, and L-serine. Although the available data indicate that the subunits of rat liver glutamine synthetase have the same molecular weight, there is insufficient evidence at hand to conclude that the subunits are identical.

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Suresh S. Tate, Fang-Yun Leu and Alton Meister


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