Glutamine Synthetase from Rat Liver
PURIFICATION, PROPERTIES, AND PREPARATION OF SPECIFIC ANTISERA*

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As the initial aspect of investigations on the regulation of glutamine synthetase in rat hepatomas and in hepatoma cell culture systems, glutamine synthetase from rat liver was purified to apparent homogeneity. A single protein band is found in gels after electrophoresis in three separate systems, and a constant enzyme specific activity is found in fractions containing glutamine synthetase activity eluting from a hydroxyapatite column. The properties of crude liver enzyme and the purified protein were compared with those previously described (Tate, S. S., and Meister, A. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 781-785). No differences were found, suggesting that the protein retained essential catalytic and regulatory properties through the different purification procedures used. The apparent $K_m$ of the enzyme for L-glutamate varied over 10-fold depending upon the concentration of divalent cation used for assay. At 8 mM $Mg^{2+}$, the apparent $K_m$ for glutamate is 0.3 mM, whereas at an $Mg^{2+}$ concentration of 2 mM, the apparent $K_m$ for glutamate is 0.9 mM. Similar changes in the apparent $K_m$ for glutamate are also noted with different concentrations of $Mg^{2+}$. The inhibition of the enzyme by histidine, glutamine, and alanine differs as the concentration of divalent cation is changed. Free sulfhydryl groups appear necessary for the catalytic activity. Iodoacetamide quantitatively inactivates rat liver glutamine synthetase. The inactivation is blocked completely by the addition of substrates to the reaction mixture. No evidence for bound adenyl groups, which modify the catalytic and regulatory properties of *Escherichia coli* glutamine synthetase, or other covalent modification of rat liver glutamine synthetase, was found by ultraviolet absorption spectroscopy or after incubation of enzyme, liver extracts, and effectors which result in adenylation of *E. coli* glutamine synthetase. Antisera were obtained from rabbits immunized with purified rat liver glutamine synthetase. By immunodiffusion analysis, the antisera appear to be monospecific for rat liver glutamine synthetase; antiserum quantitatively precipitate the enzyme activity from crude rat liver and purified enzyme preparations.

Glutamine is central to intermediary metabolism because it not only serves as a required amino acid in the biosynthesis of proteins, but also is a necessary nitrogen source in other biosynthetic pathways. The amide nitrogen of glutamine is specifically incorporated into purine, pyrimidine, and pyridine nucleotides, glucosamine 6-phosphate, p-aminobenzoic acid, carboxymethyl phosphate, histidine, and tryptophan (1-7). The alpha-amino nitrogen of glutamine is used in the synthesis of amino acids from the respective alpha-keto acids by transamination (8); the alpha-ketoglutaric acid formed from glutamine is subsequently cleaved to alpha-ketoglutaric acid and ammonia. Thus, glutamine, and not ammonia, is the principal nitrogen donor for a diverse group of anabolic pathways. Although the reason for this specific role of glutamine is not fully understood, glutamine in mammals may serve to prevent the circulation of high, toxic concentrations of ammonia.

Animals do not require dietary glutamine for growth. Glutamine synthetase, which catalyzes the synthesis of L-glutamine from L-glutamate, ATP, and ammonia, is found widely distributed in animal tissues and in cell culture lines (9-16). The importance of the enzyme is suggested by the fact that glutamine, the product of the reaction catalyzed by glutamine synthetase, is the amino acid present in highest concentration in blood; high levels of glutamine are also found in mammalian heart and brain. Glutamine may be uniquely important in nervous tissue because of its relationship to y-aminobutyric acid and glutamate, which are likely mediators of synaptic transmission (17, 18).

Glutamine synthetase from microorganisms has been studied in great detail, and complex mechanisms regulating the enzyme have been described. In *Escherichia coli*, the catalytic activity of glutamine synthetase is closely correlated with the state of nitrogen balance of the cell by the post-translational enzymatic adenylylation and deadenylylation of preformed glutamine synthetase (19). *Bacillus licheniformis* (20, 21) and *Bacillus subtilis* glutamine synthetase (22-26) are also subject to complex regulation, but through mechanisms different from those encountered in *E. coli*.

We have purified glutamine synthetase from rat liver to apparent homogeneity by a procedure different from that reported by Tate et al. (27, 28) and present properties of the enzyme which have not been described previously. Comparison of the properties of crude enzyme, our purified enzyme, and the properties of glutamine synthetase as reported by Tate et al. showed no differences in the three preparations;
the purification procedures therefore do not appear to modify essential catalytic and regulatory properties of glutamine synthetase. These studies provide the basis for subsequent investigations on the in vivo regulation of the enzyme in rat liver and in hepatoma, and they complement other investigations of glutamine synthetase from rat liver (27-31) and sheep brain (29). Preliminary results of this work have been published (30, 31).

**MATERIALS AND METHODS**

**Animals**—Glutamine synthetase was isolated from the livers of adult male Sprague-Dawley rats which were maintained on Mouse/Rat Diet, 4% fat (Teklad Mills) and tap water until they were killed.

**Cell Fractionation**—Cell fractions were prepared by a modification of the method of Curtoyes and Weiss (29). Rat liver was homogenized in an Omni-Mixer (Ivan Sorval, Norwalk, Conn.) in 8 volumes (v/w) of 0.25 M sucrose, 2 mM 2-mercaptoethanol, and 0.2 M EDTA. After separating the nuclei and mitochondria, the microsomal fraction was collected by centrifugation at 144,000 × g for 1 h. The nuclei/mitochondria and the microsomes were each resuspended in 0.25 M sucrose solution, washed, ground in a Teflon tissue grinder, and assayed for glutamine synthetase activity by the γ-glutamyltransferase assay.

**Reagents**—Amino acids and urea were obtained from Schwarz/Mann, Orangeburg, N. Y.; nucleotides were purchased from P-L Biochemicals, Inc., Milwaukee, Wis.; Sodium dodecyl sulfate was obtained from Fisher Scientific Co., Fair Lawn, N. J.; Acrylamide, N,N,N',N'-tetramethylethylenediamine, and 2-mercaptoethanol were obtained from Eastman Kodak Co., Rochester, N. Y.; γ-Glutamylhydroxamate was obtained from Sigma Chemical Co., St. Louis, Mo. All other reagents were of the highest grade commercially available.

**Assays of Glutamine Synthetase Activity**—Glutamine synthetase activity was measured by the γ-glutamyltransferase assay of Leibowitz (33). The reaction consisted of 50 mM imidazole HCl (pH 7.2), 50 mM L-glutamate, 40 mM NaCl, 2 mM MnCl₂, and 10 mM ATP. After incubation at 37°C, the reaction was stopped by the addition of 1 ml of a solution of 2.42% FeCl₃ and 1.45% trichloroacetic acid in 1.82 % HzSO₄. Insoluble material was removed by centrifugation, and the absorbance at 540 nm was read with a Gilford 300-N spectrophotometer. One unit of enzyme activity is that amount of enzyme which catalyzes the production of 1 μmol of γ-glutamylhydroxamate in 1 h. The γ-glutamyltransferase assay was used to measure glutamine synthetase activity in crude and partially purified enzyme preparations.

Enzyme activity was also measured by the biosynthetic assay of Boyer et al. (34), in a 0.4 ml reaction mixture containing 100 mM imidazole HCl (pH 7.2), 50 mM L-glutamate, 40 mM NH₄Cl, 2 mM MnCl₂, and 10 mM ATP. After incubation at 37°C, the reaction was stopped by the addition of 1.8 ml of a freshly prepared solution of 0.8% FeCl₃ in 0.3 N H₂SO₄. Color development was initiated by the addition of 0.15 ml of a solution of 6.8% ammonium molybdate in 7.5 N H₂SO₄. Insoluble material was removed by centrifugation, and the absorbance at 660 nm was read in a Gilford 300-N spectrophotometer. The biosynthetic assay was used for measurement of glutamine synthetase activity in experiments in which purified enzyme was studied.

**Ultraviolet Absorption Spectroscopy**—The ultraviolet absorption spectrum of purified rat liver glutamine synthetase (protein concentration = 5.7 mg/ml; specific activity = 12,500 units/mg) was obtained with a Cary 14 recording spectrophotometer. The standard enzyme buffer was used as the reference blank.

**Gel Electrophoresis**—See supplemental material.¹

**Rabbit Anti-glutamine Synthetase Antibody**—Rabbit anti-glutamine synthetase antisera were produced by two subcutaneous injections, 3 weeks apart, of purified rat liver glutamine synthetase in complete Freund's adjuvant, using the methods outlined by Brown (35). Prior to injection, the enzyme was subjected to gel filtration with agarose A-0.5m. The enzyme specific activity was not significantly increased by this procedure, but several minor contaminating proteins were removed, as determined by standard disc gel electrophoresis or gel electrophoresis in SDS. Booster injections of purified enzyme were given intravenously. Antiserum was obtained by bleeding animals from an ear vein and was partially purified according to the method of Brown (35). After removal of the clot and centrifugation to remove cells, the antiserum was heated at 56°C for 30 min, the γ-globulin fraction was precipitated by 50% ammonium sulfate, and the precipitate was resuspended in a 0.5 volume of 0.9% NaCl, dialyzed against potassium phosphate, 10 mM, pH 7.0, and the IgG fraction was obtained by passage of the antiserum over DEAE-cellulose, equilibrated also with potassium phosphate (36). The partially purified antiserum was dialyzed against 0.9% NaCl and stored frozen without loss of activity. Double immunodiffusion analysis of the partially purified antiserum and enzyme preparations was performed by the Ouchterlony method.

**RESULTS**

**Subcellular Distribution of Rat Liver Glutamine Synthetase**—The subcellular distribution of rat liver glutamine synthetase was studied by centrifugation analysis in isotonic sucrose. Table I demonstrates that approximately 80% of the enzyme activity is recovered in the microsomal fraction; the remainder of the activity is distributed equally between the nuclei/mitochondria and the cytosol. Washed microsomes were incubated with potassium chloride of increasing ionic strength to evaluate the release of the enzyme into the supernatant. At 100 mM KCl, 70% of the enzyme is dissociated from the microsomal particles; at 150 mM KCl, the dissociation is complete (data not shown). The enzyme thus appears to be bound to the microsomal fraction largely by electrostatic binding and at physiological salt concentrations it is released into solution from the microsomal fraction.

**Purification of Rat Liver Glutamine Synthetase**—See supplemental material.¹

**Properties of Purified Rat Liver Glutamine Synthetase**—An approximate molecular weight of 43,000 for the subunit was obtained in SDS-gel electrophoresis by comparison with the observed mobilities of proteins of known molecular weights. Analysis of the intact enzyme by sucrose gradient sedimentation techniques suggested an approximate molecular weight of 360,000, consistent with that reported by Tate et al. (27-29). These authors also reported a molecular weight of 47,000 for the enzyme dissociated in SDS. Other properties of the purified enzyme were compared with those reported by Tate et al. (27-29) and with those of crude enzyme.

No differences were found between the preparations in the ratio of activity with Mn²⁺ (2 mm) and with Mg²⁺ (50 mM), the inhibition of the enzyme by alanine and glycine, and the apparent Km for L-glutamate. These results strongly suggest that the enzyme purified independently in two laboratories retains the important catalytic and in vitro regulatory properties described previously and is not modified in preparation.

In E. coli, glutamine synthetase is enzymatically adenylylated or deadenylated in response to excess or limited availability of nitrogen during growth; adenylylated glutamine synthetase differs from nonadenylylated enzyme in intrinsic specific activity, in divergent cation specificity, and in responsiveness to feedback inhibitors (19). To determine whether or not enzymatic adenylylation of glutamine synthetase occurred in rat liver, we examined the ultraviolet absorption spectra of multiple preparations of glutamine synthetase. The spectra were identical and typical of many proteins. The A₂₈₀/A₃₆₀ nm ratio of the purified enzyme is 1.71, and, in the absence of

¹ Some of the Methods (include 1 figure and 1 table) are presented in miniprint at the end of this paper. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document 77M-587, cite author(s), and include a check or money order for $1.00 per set of photocopies.

² The abbreviation used is: SDS, sodium dodecyl sulfate.
increased absorbance at 260 nm, indicates that significant amounts of enzyme-bound adenylyl groups are not present in the purified enzyme. Furthermore, incubation of crude enzyme preparations and purified enzyme with L-glutamate, α-ketoglutarate, L-glutamine, ammonium chloride, or 3′,5′-cyclic AMP failed to modify catalytic activity assayed with either Mg$^{2+}$ or Mn$^{2+}$, or the responsiveness of the enzyme to the inhibitors L-alanine, L-histidine, and L-glutamine (data not shown).

The amino acid composition of the purified enzyme was analyzed and found to be in agreement with published results (28). Using the standard Edman degradation technique with a Beckman Sequenator, we made attempts to determine the NH$_2$-terminal residues of the purified enzyme. No amino acids were released from the reduced, carboxymethylated protein. No further experiments have been done to establish the nature of the blocked NH$_2$ terminus.

**Modification of Catalytic Properties of Rat Liver Glutamine Synthetase by Divalent Cations—**As described previously (27-31), rat liver glutamine synthetase has an absolute requirement for divalent cation for catalytic activity. When ATP is present at 10 mM during assay, optimum catalytic activity is found at 2 mM Mn$^{2+}$, at 12 mM Mg$^{2+}$, and at 20 mM Co$^{2+}$. The intrinsic specific activity of the enzyme is 4- to 5-fold higher in assays with Mg$^{2+}$ than when the assay is done with Mn$^{2+}$. For enzyme assays at 2 mM Mn$^{2+}$, the apparent $K_m$ for L-glutamate is 5 mM. Further experiments have demonstrated, however, that the apparent $K_m$ for glutamate varies with the concentration of divalent cation used. Fig. 1A demonstrates the glutamate substrate saturation curves for rat liver glutamine synthetase assayed at two different concentra-

**Table 1**

<table>
<thead>
<tr>
<th>Subcellular distribution of rat liver glutamine synthetase: Fractionation of rat liver homogenate</th>
<th>Activity recovered %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei/mitochondria</td>
<td>11</td>
</tr>
<tr>
<td>Microsomes</td>
<td>78</td>
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<td>Cytosol</td>
<td>12</td>
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**Fig. 1.** A, substrate saturation curves for L-glutamate at 2 and 8 mM Mn$^{2+}$. Double reciprocal plots are shown in the inset. Enzyme activity was determined in the biosynthetic assay; the concentrations of L-glutamate are shown in the figure. Initial velocity is given in terms of units (see "Materials and Methods"). ○—○, 2 mM MnCl$_2$; ●—●, 8 mM MnCl$_2$. B, as in A except that 10 and 50 mM Mg$^{2+}$ were used. ○—○, 10 mM Mg$^{2+}$; ▲—▲, 50 mM Mg$^{2+}$.
tions of Mn\textsuperscript{2+}. The respective double reciprocal plots are shown in the inset of Fig. 1A. The apparent $K_m$ for glutamate is 0.3 mM when Mn\textsuperscript{2+} is 8 mM; at 2 mM Mn\textsuperscript{2+} the value is 5.3 mM, more than a 10-fold increase above the apparent $K_m$ for glutamate when Mn\textsuperscript{2+} is 8 mM. Although not shown here, an intermediate value for the apparent $K_m$ is noted when the glutamate substrate saturation curve is obtained with 5 mM Mn\textsuperscript{2+}. Furthermore, at 8 mM Mn\textsuperscript{2+}, the glutamate substrate saturation curve is no longer hyperbolic at high concentrations of L-glutamate; apparent inhibition by substrate is present. Similar phenomena were observed when the glutamate substrate saturation curves were generated at different concentrations of Mg\textsuperscript{2+}. At 50 mM Mg\textsuperscript{2+}, the apparent $K_m$ for glutamate is 1.6 mM, whereas at 10 mM the apparent $K_m$ is 5.0 mM (Fig. 1B). Experiments to determine the apparent $K_m$ for ammonia were done at different concentrations of divalent cation. The apparent $K_m$ for ammonia was found to be 0.3 mM at 2, 5, and 8 mM Mn\textsuperscript{2+} (data not presented).

Competitive inhibitors of enzymes are increasingly effective as the concentration of substrate is reduced. With rat liver glutamine synthetase, raising the apparent $K_m$ of the enzyme for L-glutamate by lowering the divalent cation concentration should also increase the effectiveness of competitive inhibitors at any given inhibitor concentration. Histidine and glutamine, which act as apparent competitive inhibitors with respect to glutamate in the Mn\textsuperscript{2+} assay, are shown in Fig. 2 A and B to be more effective in inhibiting glutamine synthetase at 2 mM Mn\textsuperscript{2+} than at 8 mM Mn\textsuperscript{2+}. Furthermore, although the data are not shown, alanine, which is more effective as an inhibitor when glutamate is present in saturating concentrations, is less effective as an inhibitor when the Mn\textsuperscript{2+} concentration is 2 mM rather than 8 mM. The difference in inhibition of glutamine synthetase at 2 mM Mn\textsuperscript{2+} relative to the inhibition at 8 mM Mn\textsuperscript{2+} by histidine and glutamine is greatly magnified above that shown in Fig. 2 if the comparison is made at glutamate concentrations at or near the apparent $K_m$ of the enzyme for glutamate, rather than at saturating concentrations of glutamate, as shown in Fig. 2.

Modification of Rat Liver Glutamine Synthetase by Iodoacetamide—The interactions between substrates and enzyme were defined further by experiments in which the enzyme was modified in the presence and absence of substrates by incubation with the sulfhydryl-reactive compound iodoacetamide. Enzyme activity was rapidly and irreversibly lost when the purified enzyme was incubated with 25 mM iodoacetamide in the absence of substrates (Fig. 3). The effects of the addition of substrates or divalent cations to the incubation mixture on the rate of enzyme inactivation by iodoacetamide are also shown in Fig. 3. Glutamate (50 or 100 mM) in the incubation mixture consistently results in a slight decrease in the rate of inactivation (data not shown). However, Mn\textsuperscript{2+} (2 or 8 mM) significantly retards inactivation of the enzyme by iodoacetamide; the higher concentration of Mn\textsuperscript{2+} is slightly more effective than the lower in preserving enzyme activity. Addition of both Mn\textsuperscript{2+} (2 or 8 mM) and L-glutamate (50 mM) to the incubation mixture results in even greater protection from inactivation by iodoacetamide. Addition of ATP (10 mM) with 2 mM Mn\textsuperscript{2+} and 50 mM L-glutamate completely protects the enzyme activity against inactivation by iodoacetamide for the 60-min period of incubation. Ammonia alone, or in combination with other substrates, has no effect on the rate of inactivation (data not shown). ATP when present alone results in a loss of activity greater than that observed with enzyme and iodoacetamide alone. However, either 2 or 8 mM Mn\textsuperscript{2+}, when added with ATP (10 mM), affords substantial protection when compared to enzyme incubated without added substrates.

**Fig. 3.** Effect of substrates on the inactivation of purified glutamine synthetase by 25 mM iodoacetamide. 0.12 mg of purified enzyme was incubated at 37°C with 100 mM imidazole (pH 8), 1 mM 2-mercaptoethanol, 1 mM EDTA, 25 mM iodoacetamide, and one or more substrates at the concentrations specified. Aliquots were removed at the times shown and assayed by the γ-glutamyltransferase assay. The results are expressed as a percentage of the activity of a control sample incubated in the absence of iodoacetamide.

**Fig. 2 A, effect of histidine on the catalytic activity of purified rat liver glutamine synthetase at 8 mM Mn\textsuperscript{2+} and at 2 mM Mn\textsuperscript{2+}.** Enzyme activity was determined in the biosynthetic assay. O---O, percentage activity at 2 mM Mn\textsuperscript{2+} at the histidine concentrations shown; •---•, percentage activity at 8 mM Mn\textsuperscript{2+}. **B, effect of glutamine on the catalytic activity.** Details as in A.
To determine whether enzyme that has been modified by iodoacetamide has altered kinetic properties in comparison to unmodified enzyme, we incubated enzyme with iodoacetamide and terminated the reaction with 2-mercaptoethanol when enzyme activity had been reduced to approximately 50% of the control. Both the native and the iodoacetamide-treated enzyme were compared according to several criteria: the ratio of activity in the Mn²⁺-catalyzed biosynthetic reaction compared to that of Mg²⁺-catalyzed reaction, the ratio of specific activity in the transferase reaction compared to that in the biosynthetic reaction, the apparent $K_m$ for L-glutamate in the Mn²⁺-catalyzed reaction, and the inhibition of catalytic activity by L-alanine (5 and 20 mM), L-histidine (5 and 20 mM), and L-glutamine (5 and 20 mM). No differences were seen between the native enzyme and the iodoacetamide-treated enzyme in these studies, except for the expected proportionate decrease in the $V_{\text{max}}$ of the reaction in which iodoacetamide-treated enzyme was used.

Anti-rat Liver Glutamine Synthetase Antisera—As detailed under “Materials and Methods,” purified rat liver glutamine synthetase was used for the preparation of antisera in rabbits, and the antisera were partially purified before use. To assess the specificity of these antisera for the enzyme, we performed double diffusion and quantitative precipitin studies. Fig. 4 shows an immunodiffusion pattern in which antiserum in the center well was reacted with partially purified and fully purified glutamine synthetase preparations. In both cases, a single line of precipitation was observed. The antiserum thus reacts with only one major antigenic protein in the enzyme preparations under the experimental conditions used. The smooth fusion of the precipitin lines without spur formation indicates apparent antigenic identity of the proteins in the different preparations. This result supports the gel electrophoresis studies, which suggest that the enzyme preparation is highly pure and demonstrates that, within the limits of immunodiffusion analysis, the enzyme elicits the production of an apparently monospecific antibody in rabbits.

The precipitation of rat liver glutamine synthetase with antisera was evaluated quantitatively (Fig. 5). Increasing...
amounts of the antiserum were added to fixed levels of enzyme. After incubation and centrifugation of the insoluble enzyme, the supernatant was assayed for glutamine synthetase activity. A progressive loss of activity was demonstrated as increasing amounts of antiserum were added; essentially, the entire enzyme activity was removed from the supernatant by appropriate levels of antiserum. When the immune precipitate was resuspended in buffer and the resulting suspension assayed, the enzyme-antibody complex contained all of the original enzyme activity; thus, the formation of the complex appears not to result in loss of enzyme activity. For a further assessment of the use of the antibody in precipitin reactions with crude enzyme extracts, we obtained quantitative precipitation curves by using 25 μl of antiserum and either purified or partially purified enzyme (Fig. 6). Twenty-five microliters of antiserum completely precipitated 1400 units of enzyme activity from each enzyme preparation, and precipitates with essentially equal protein content resulted in each instance. These data also support the high specificity of the antibody for the enzyme and establish the usefulness of the antibody preparation for precipitating enzyme from partially purified preparations of rat liver glutamate synthetase.

**DISCUSSION**

Purified rat liver glutamine synthetase was judged to be nearly homogeneous on the basis of the demonstration of a single band in standard polyacrylamide gel electrophoresis and in gel electrophoresis in SDS or urea. A constant specific activity is found across the activity peak eluted during hydroxyapatite chromatography. Furthermore, rabbit antibody prepared against purified rat liver glutamine synthetase appears monospecific, as shown by the presence of a single precipitin line in immunodiffusion studies with both partially purified and purified enzyme. The purified enzyme thus behaves as a single antigenic species.

Glutamine synthetase is associated with the microsomal particles when rat liver is fractionated in isotonic sucrose, as was noted by Wu (37). The release of glutamine synthetase from the microsomal fraction in physiological salt concentrations suggests, however, that the synthesis of glutamine in vivo may occur when the enzyme is free in the cytosol, unassociated with the membrane fraction. The partition of glutamine synthetase from glutaminase, a mitochondrial enzyme (32), may serve as one physiological barrier to the futile cycling of glutamine and glutamate which occurs at the expense of 1 mol of cellular ATP per mol of glutamine synthesized. The separate intracellular loci of glutamine synthesis and degradation may be of importance in the cellular regulation of glutamine metabolism.

As is true of glutamate synthetase isolated from microorganisms and sheep brain (19–26, 29), divalent cations have a profound effect on glutamine synthetase activity in rat liver. As reported elsewhere (27–31), the enzyme, when studied at optimal concentrations of divalent cations, is more active with Mg2+ than with Mn2+. The inhibition of catalytic activity by various effectors also is influenced by the divalent cation used for assay. Inhibition of glutamine synthetase by alanine, histidine, and glutamine is observed only in the Mn2+-dependent assay system and not in the Mg2+-dependent assay (27, 29–31). Our observation that the apparent Km for glutamate increases sharply as the concentration of divalent cation is reduced seems equally important with respect to cellular regulation. If the Mn2+ concentration is lowered from 8 mM to 2 mM, the apparent Km for glutamate increases from 0.3 mM to 5 mM. Further, the inhibition of activity by high concentrations of glutamate, when the enzyme is assayed at 8 mM Mn2+, is no longer observed at 2 mM Mn2+. Similar phenomena were noted when glutamate substrate saturation curves were obtained at different concentrations of Mg2+. No such changes occur when the substrate saturation curve for ammonia is generated at different concentrations of Mn2+. The apparent increase in affinity of the enzyme for its substrate at the higher concentrations of divalent cation seems likely to reflect a conformational change in the tertiary and quaternary structure of the enzyme mediated by divalent cation. This interpretation is supported by the data which show a reduction in sulfhydryl reactivity when Mn2+ was present during the incubation of enzyme and iodoacetamide. Such conformational changes have been documented with glutamine synthetase from E. coli. In the absence of divalent cations, the enzyme assumes a “relaxed,” catalytically inactive form that is converted to the active, “taut” form by either Mn2+ or Mg2+ (38). Alternatively, a second, high affinity glutamate binding site in the rat liver enzyme might become available at higher concentrations of Mn2+. Studies to distinguish these possibilities by measuring the direct binding of enzyme and substrate will be done. These findings may be relevant to the in vivo regulation of rat liver glutamine synthetase. At the low levels of divalent cation found in mammalian cells, the activity of the enzyme would be influenced substantially by the intracellular levels of glutamate. Also, as the concentration of divalent cation is lowered and thus the apparent Km for glutamate increased, those inhibitors of enzyme activity that are competitive or partially competitive with glutamate should be increasingly effective. As shown in Fig. 2, glutamine and histidine, previously shown by kinetic criteria to be competitive with glutamate when studied with Mn2+ (30, 31), are far more potent inhibitors of purified rat liver glutamine synthetase at 2 mM Mn2+ than at 8 mM Mn2+. The effectiveness of these inhibitors is further enhanced when glutamate is reduced from saturating levels to levels at or near the apparent Km.

Glutamine synthetase from bacteria exhibits other regulatory properties that also differ with the divalent cations and the concentrations of the specific cation used. In E. coli, enzyme-mediated adenylation converts glutamine synthetase from a more active to a less active form, changes the enzyme from an Mg2+-dependent to an Mn2+-dependent form, and modifies the responsiveness of the enzyme to feedback inhibitors (19, 38–42). In B. subtilis, inhibition of glutamine synthetase by L-glutamine was marked when Mg2+ served as the divalent cation, but was minimal when Mn2+ instead of Mg2+ was used. However, noninhibitory concentrations of AMP convert glutamine to a very powerful inhibitor of the B. subtilis enzyme in an Mn2+ assay. The effects of AMP and glutamine are reciprocal and strongly synergistic (28).

As noted above, striking changes in the kinetic properties of E. coli glutamine synthetase occur when the enzyme is adenyllylated by the glutamine-stimulated adenylyltransferase (19, 38–42). To seek in rat liver an analogous reaction to the enzyme-mediated adenylation of glutamine synthetase characteristic of the enzyme in E. coli, we examined the ultraviolet absorption spectrum of the purified enzyme for the presence of enzyme-bound adenylyl groups. No nucleotide moiety was present, as judged by the absence of unusual absorbance at 260 nm. To seek possible other post-translational modifications of rat liver glutamine synthetase, we exposed crude extracts and purified enzyme to conditions in vitro which result in the adenyllylation enzyme-glutamine synthetase. No changes in the catalytic activity, divalent cation specificity, or responsiveness of the enzyme to inhibitors in vitro were found. In addition, cyclic AMP in the incubation mixture did not modify the activity of the enzyme. Thus, we could detect no mechanism in crude extracts for the adenyllylation or similar modification of rat liver glutamine synthetase, using condi-
tions analogous to those employed for enzymatic adenylylation of E. coli glutamine synthetase. In this respect, the enzyme from rat liver is similar to that from B. subtilis and unlike that from E. coli.

The inactivation of glutamine synthetase by iodoacetamide suggests that one or more free sulfhydryl groups are near or at the active site of the enzyme. This view is strengthened by the observation that substrates protect the enzyme against inactivation by iodoacetamide. At the concentrations of substrates used in the assay, complete protection of enzyme activity is found at iodoacetamide concentrations that result in complete loss of activity when substrates are omitted from the incubation procedure. The studies also indicate that glutamate, Mn\textsuperscript{2+}, and ATP each may bind to the enzyme independently of the binding of other substrates, since each alone significantly and reproducibly modifies the inactivation of glutamine synthetase by iodoacetamide. Proposed catalytic mechanisms for glutamine synthetase which require binding of ATP to the enzyme before glutamate can bind (29) seem contradicted by the evidence presented here, since glutamate alone protects the enzyme against inactivation by iodoacetamide. These results do not establish, however, that ATP bound to the enzyme is without influence on the subsequent binding of glutamate. In fact, the striking protective effect of glutamate and ATP together may be interpreted as evidence that the binding of one substrate to the enzyme significantly influences the binding of the other. Determination of the number of essential sulfhydryl groups per enzyme molecule is currently underway. Preliminary results indicate that 4 to 5 cysteine residues per subunit are modified when enzyme is incubated with iodoacetamide in the absence of added substrate (courtesy of Dr. Robert Heinrikson, Department of Biochemistry, The University of Chicago).

When injected into rabbits, purified rat liver glutamine synthetase elicited the production of antibodies which are specific for the enzyme protein. The antibodies are of the precipitating variety, and appropriate amounts of antiserum precipitate all of the enzyme activity from purified or partially purified enzyme preparations. Since the resuspended immune precipitate was fully active catalytically, the binding of antibody to the enzyme molecule does not appear to block the active site of the enzyme. The single precipitin lines observed in the immunodiffusion studies indicate that the glutamine synthetase used as antigen was of high purity and was essentially a single antigenic protein. The specificity of the quantitative precipitin and immunodiffusion experiments suggests that glutamine synthetase is the only protein precipitated from partially purified enzyme preparations. The results of experiments in which antibody is used in the study of the regulation in vivo of rat liver, rat hepatoma, and hepatoma cell culture line glutamine synthetase are presented in the accompanying papers (47, 48).

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Glutamine Synthetase from Rat Liver

METHODS

Purification of glutamine synthetase—Rats were killed by decapitation and the livers removed for homogenization in 10 vol of 0.25 M sucrose (15 min) of homogenate was centrifuged at 15,000 g for 15 min. The supernatant was centrifuged at 105,000 g for 60 min, and the supernatant was reconstituted to a final volume of 8.9 l. The supernatant was dialyzed against 200 ml of 0.2 M sucrose (15 ml) for 15 min, and the supernatant was reconstituted to a final volume of 200 ml of 0.2 M sucrose (15 ml).

STEP 1. Acid precipitation—The supernatant was adjusted to pH 7.0 by the addition of 0.2 M hydrochloric acid. The precipitate was collected by centrifugation at 3,000 g for 15 min. The precipitate was then dissolved in 10 ml of 0.2 M sucrose (15 min), and the supernatant was reconstituted to a final volume of 10 ml of 0.2 M sucrose (15 min). The precipitate was then dissolved in 10 ml of 0.2 M sucrose (15 min), and the supernatant was reconstituted to a final volume of 10 ml of 0.2 M sucrose (15 min).

STEP 2. Acetic acid precipitation—The supernatant was adjusted to pH 5.0 by the addition of 0.2 M acetic acid. The precipitate was collected by centrifugation at 3,000 g for 15 min. The supernatant was reconstituted to a final volume of 10 ml of 0.2 M sucrose (15 min). The precipitate was then dissolved in 10 ml of 0.2 M sucrose (15 min), and the supernatant was reconstituted to a final volume of 10 ml of 0.2 M sucrose (15 min).

STEP 3. Ethanol precipitation—The supernatant was adjusted to pH 5.0 by the addition of 0.2 M hydrochloric acid. The precipitate was collected by centrifugation at 3,000 g for 15 min. The supernatant was reconstituted to a final volume of 10 ml of 0.2 M sucrose (15 min). The precipitate was then dissolved in 10 ml of 0.2 M sucrose (15 min), and the supernatant was reconstituted to a final volume of 10 ml of 0.2 M sucrose (15 min).

STEP 4. Differential centrifugation—The supernatant was adjusted to pH 5.0 by the addition of 0.2 M hydrochloric acid. The precipitate was collected by centrifugation at 3,000 g for 15 min. The supernatant was reconstituted to a final volume of 10 ml of 0.2 M sucrose (15 min). The precipitate was then dissolved in 10 ml of 0.2 M sucrose (15 min), and the supernatant was reconstituted to a final volume of 10 ml of 0.2 M sucrose (15 min).

FIGURE 1. Glutamine synthetase from rat liver. Purification of activity—The supernatant was adjusted to pH 5.0 by the addition of 0.2 M hydrochloric acid. The precipitate was collected by centrifugation at 3,000 g for 15 min. The supernatant was reconstituted to a final volume of 10 ml of 0.2 M sucrose (15 min). The precipitate was then dissolved in 10 ml of 0.2 M sucrose (15 min), and the supernatant was reconstituted to a final volume of 10 ml of 0.2 M sucrose (15 min).

Table 1

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<th>Step</th>
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<th>Purification</th>
<th>Recovery</th>
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</table>

One unit of activity is defined as the amount of enzyme which catalyzes the formation of 1 μmole of glutamine per hour.
Glutamine synthetase from rat liver. Purification, properties, and preparation of specific antisera.
T F Deuel, M Louie and A Lerner


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