Glutamine-Binding Subunit of Glutamate Synthase and Partial Reactions Catalyzed by This Glutamine Amidotransferase

(enzyme dissociation/ flavin/iron-sulfide enzyme/1-2-amino-4-oxo-5-chloropentanoate)

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ABSTRACT In the course of studies on glutamine-dependent carbamyl phosphate synthetase from Aerobacter aerogenes, we purified another protein which was found to be glutamate synthase (EC 2.6.1.53). The enzyme, obtained in apparently homogeneous form (monomer molecular weight about 227,000; s_20,w = 17.5 S), was found to be a typical glutamine amidotransferase in that it exhibits glutaminase activity and can utilize ammonia in place of glutamine as a nitrogen donor. The enzyme also catalyzes at low rates the oxidative deamination of glutamate in the presence of TPN, and it exhibits TPNH oxidase activity. The enzyme is similar to the glutamate synthase found in Escherichia coli in that it is an iron-sulfide flavoprotein. Treatment of the enzyme with sodium dodecyl sulfate or potassium thiocyanate dissociates it into nonidentical subunits exhibiting molecular weights of about 175,000 and 51,500. The glutamine-dependent activity of the enzyme is inhibited by 1-2-amino-4-oxo-5-chloropentanoic acid, but the chloroketone analog of glutamine does not affect the ammonia-dependent glutamate synthase activity. Studies with [14C]chloroketone show that the reagent binds to the heavy subunit only. Inhibition by the chloroketone and its binding to the heavy subunit are markedly reduced in the presence of L-glutamine. Sedimentation-velocity studies carried out in potassium thiocyanate indicate that iron-sulfide and flavin sites are also located on the heavy subunit. While these studies show that glutamate synthase resembles other glutamine amidotransferases in certain of its catalytic properties, the findings indicate that the light subunit of this enzyme, in contrast to that of several other glutamine amidotransferases, does not function to bind glutamine. It is of interest that the enzyme exhibits an unusually high affinity for ammonia as compared to a number of other glutamine amidotransferases. Glutamate synthase is inhibited (competitively with respect to glutamine) by low concentrations of methionine sulfone, methionine sulfoximine, and methionine sulfinate.

Earlier work in this laboratory showed that glutamine-dependent carbamyl phosphate synthetase from Escherichia coli consists of a heavy subunit which can catalyze the synthesis of carbamyl phosphate from ATP, bicarbonate, and ammonia (but not from glutamine), and a light subunit which functions to bind glutamine (1, 2). In subsequent hybridization experiments with glutamine-dependent carbamyl phosphate synthetase from Aerobacter aerogenes, it was found that the light subunit of the A. aerogenes enzyme could combine with the heavy subunit of the E. coli enzyme to form an active glutamine-dependent hybrid enzyme; similarly, an active hybrid enzyme could be prepared from the light subunit of the E. coli enzyme and the heavy subunit of the A. aerogenes enzyme.* In the course of isolating glutamine-dependent carbamyl phosphate synthetase from A. aerogenes, we noted during a gel filtration step in the procedure that another protein had also been purified (see first peak, Fig. 1). The catalytic properties of this protein were initially obscure, but we decided to pursue study of this apparently homogeneous protein after we found that it exhibited glutaminase activity and that it could be split to a heavy and a light subunit when subjected to polyacrylamide gel electrophoresis in sodium dodecyl sulfate. These observations led us to think that the new protein might be a glutamine amidotransferase, and after testing it for the activities of the several known enzymes in this category we discovered that the new protein is glutamate synthase (EC 2.6.1.53) whose major catalytic activity is to form glutamate from α-ketoglutarate and glutamine according to the following reaction (3-5):

\[
\alpha\text{-ketoglutarate} + \text{TPNH} + H^+ + L\text{-glutamine} \rightarrow 2 L\text{-glutamate} + \text{TPN}^+ 
\]

Several glutamine amidotransferases [e.g., carbamyl phosphate synthetase (1, 2), anthranilate synthetase (6), p-amino-benzoate synthetase (7)] are composed of a heavy and a light subunit, and there is strong evidence in each case that the light subunit has the function of binding glutamine; the amide nitrogen is then transferred to the heavy subunit for use in the corresponding synthesis reactions. We therefore expected that the light subunit of A. aerogenes glutamate synthase would contain the glutamine binding site of this enzyme. However, as described here, we found that the glutamine binding site of glutamate synthase is located on the heavy subunit. Miller and Stadtmann (8, 9) had previously shown that glutamate synthase from E. coli is an iron-sulfide flavoprotein. We have found that the A. aerogenes glutamate synthase is also an iron-sulfide flavoprotein and that a substantial fraction of the iron and flavin (FMN and FAD) are located on the heavy subunit of the enzyme. In the course of this work we also found that highly purified glutamate synthase preparations (apparently free of glutamate dehydrogenase) can catalyze the reductive amination of α-ketoglutarate with ammonia; although the enzyme is much more active with glutamine than with ammonia, it is of interest that its affinity for ammonia is relatively high.

EXPERIMENTAL

Materials. Aerobacter aerogenes (1/4 through log phase) was grown at the New England Enzyme Center on minimal salt medium (10). L-2-Amino-4-oxo-5-chloro-[5-14C]pentanoic acid was prepared as described (11). L-Glutamine, N,N-dimethyl-p-phenylenediamine·HCl, DPNH, TPNH, TPN, deamido-

DPNH, and glutamate dehydrogenase were obtained from Sigma. The amino acids were obtained from Schwarz-Mann. Acrylamide and bis-acrylamide (electrophoresis grade) were obtained from Eastman. Sodium dodecyl sulfate (Sequanol grade) was obtained from Pierce. 1,10-Phenanthroline was obtained from Matheson, Coleman and Bell. \(\alpha\)-Ketoglutaric acid and Triton X-100 were purchased from Calbiochem. KSCN was obtained from Baker. Liquifluor tolueno concentrate and \(\alpha\)-keto[U-\(^{14}\)C]glutaric acid (2.5 Ci/mol) were obtained from New England Nuclear Corp.

**Methods.** Glutamate synthase activity was assayed at 23-24\(^\circ\) C in three ways: (a) 10-40 \(\mu\)l of enzyme solution in 0.2 M potassium phosphate (pH 7.6) containing 0.5 mM EDTA was incubated in a final volume of 0.5 ml containing 0.35 mM TPNH, 5 mM \(\alpha\)-ketoglutarate, 100 mM Tris-Cl (pH 7.8), and 5 mM L-glutamine; initial velocity was obtained from the decrease in absorbance at 340 nm. Ammonia-dependent glutamate synthase and glutamate dehydrogenase activities were assayed in a similar manner using 100 mM NH\(_4\)Cl in place of glutamine. (b) The reaction mixture given in (a) was used except that \(\alpha\)-keto[U-\(^{14}\)C]glutamate (80,000-100,000 cpm/\(\mu\)mole) was added (final volume, 0.3 ml). After 1.5-2 min, the reaction was stopped by adding 0.1 ml of 1 N HCl and the solution was placed at 0\(^\circ\) C for 10 min. After neutralization with 0.1 ml of 1 M Tris, the mixture was placed on a Dowex-1 acetate column; \(^{14}\)C-glutamate was eluted with 0.3 N acetic acid, which does not elute \(\alpha\)-ketoglutarate. (c) The reaction mixture given in (a) was used and the glutamate formed was determined with a Durrum model 500 amino acid analyzer. TPNH oxidase activity was assayed as in (a) without added nitrogen donor. The reaction of TPN and L-glutamate to form TPNH was carried out with reaction mixtures containing enzyme, 1.1 mM TPN, 10 mM L-glutamate, and 100 mM Tris-Cl (pH 7.8) in a final volume of 0.5 ml; the increase in absorbance at 340 nm was followed. Glutaminase was assayed at 37\(^\circ\) C in reaction mixtures (final volume, 0.3 ml) containing enzyme, 10 mM L-glutamate, 100 mM Tris-Cl (pH 7.8). After 5 min, 0.1 ml of 1 N HCl was added, and the solution was placed at 0\(^\circ\) C for 10 min. The reaction mixture was neutralized with 0.1 ml of 1 M Tris and the glutamate formed was determined by the DPNH-glutamate dehydrogenase assay (12). Carbamyl phosphate synthetase activity was determined by quantitating the amount of ADP formed, as previously described (10). Iron was measured by the 1,10-phenanthroline method (13). Labile sulfide was determined by the formation of methylene blue from \(N,N\)-dimethyl-p-phenylenediamine (14). Flavin was determined fluorometrically. The enzyme (in 0.15 M potassium phosphate, pH 6.8) was boiled in the dark for 5 min to release bound flavin. After centrifugation of precipitated protein, a portion of the supernatant was brought to pH 1 by adding 6 N HCl and was boiled for 15 min in the dark to convert FAD to FMN. The solution was then cooled and neutralized with NaOH. The FAD and FMN content of the enzyme were calculated from the fluorescence observed before and after boiling in acid; appropriate standardization was carried out with solutions containing various concentrations of FMN and FAD. Sedimentation velocity studies were done as described (15) in a model E analytical ultracentrifuge equipped with a RTIC unit, electronic speed control, photoelectric scanner, and a parabolic mirror. Runs were monitored at 280, 450, and 550 nm. Sedimentation coefficients were determined by following the movement with time of the 50% position of apparent boundaries. Polyacrylamide gel scans were done with a Caneco G model microdensitometer equipped with a filter with peak transmittance at 660 nm; half band width, 60 nm. Enzyme solutions were concentrated by ultrafiltration through XM-50 membranes. Protein was determined by the method of Lowry et al. (16).

**RESULTS**

**Purification of the Enzyme.** Glutamate synthase was obtained by the procedure previously used for the isolation of carbamyl phosphate from E. coli (10). This method involves sonication, heating in the presence of glutamine, treatment with protamine sulfate, ammonium sulfate fractionation, and chromatographies on DEAE-Sephadex and Sephadex G-200. Glutamate synthase is eluted in the void volume of the latter column and thus is separated from carbamyl phosphate synthetase (Fig. 1). Glutamate dehydrogenase activity eluted in a position identical to that of carbamyl phosphate synthetase. To remove traces of glutamate dehydrogenase and carbamyl phosphate synthetase, we further purified glutamate synthase by repeated chromatography on Sephadex G-200; an apparently homogeneous preparation was obtained that is more than 600-fold purified. After Sephadex G-200 chromatography, the enzyme was concentrated by ultrafiltration. Storage of the enzyme in 0.2 M potassium phosphate buffer (pH 7.6) containing 0.5 mM EDTA at 0\(^\circ\) C led to gradual loss of glutamine-dependent activity (about 20-25%/week); partial protection against such loss was achieved by adding 5 mM dithiothreitol.

**Catalytic Properties of the Enzyme** (Table 1). The enzyme catalyzed formation of 2 moles of glutamate for each mole of TPNH and \(\alpha\)-ketoglutarate utilized; studies with \(\alpha\)-keto-\[^{14}\]C\]glutarate showed that 1 mole of glutamate is formed from \(\alpha\)-ketoglutarate. The apparent \(K_m\) values for TPNH, glutamine, and \(\alpha\)-ketoglutarate are 0.012, 0.3, and 0.3 mM, respectively. Neither DPNH nor deamido-DPNH substituted for TPNH.

It was found that ammonium chloride could substitute for glutamine as a nitrogen donor; in this reaction (Table 1,b)
TABLE 1. Reactions catalyzed by glutamate synthase

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Specific activity (μmol/hr per mg of protein)</th>
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<tbody>
<tr>
<td>(a) α-Ketoglutarate + TPNH + H⁺ +</td>
<td>1100</td>
</tr>
<tr>
<td>l-glutamine → 2 l-glutamate + TPN⁺</td>
<td></td>
</tr>
<tr>
<td>(b) α-Ketoglutarate + TPNH + H⁺ +</td>
<td>48</td>
</tr>
<tr>
<td>NH₃ → l-glutamate + TPN⁺</td>
<td></td>
</tr>
<tr>
<td>(c) l-Glutamine + H₂O → l-glutamate + NH₃</td>
<td>99</td>
</tr>
<tr>
<td>(d) TPNH + H⁺ + 1/2 O₂ → TPN⁺ + H₂O</td>
<td>1.1</td>
</tr>
<tr>
<td>(e) TPN⁺ + l-glutamate → NH₃ + TPNH + H⁺ + α-ketoglutarate</td>
<td>3.3</td>
</tr>
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Activities were determined as described under Methods. In reactions (a) and (b), the stoichiometry was established by following three different conversions: the oxidation of TPNH (spectrophotometrically at 340 nm), the formation of l-glutamate (amino acid analyzer), and the conversion of α-keto[¹⁴C]glutamate to l-[¹⁴C]glutamate.

1 mole of glutamate was formed for each mole of TPNH and α-ketoglutarate used. The pH optimum for the ammonia-dependent reaction is about 9 while that for the glutamine-dependent reaction is about 7.8. The apparent Kₘ value for NH₄Cl varied from 0.5 to 2 mM with different preparations which were judged to be free of glutamate dehydrogenase by virtue of apparent chromatographic separation. The purified enzyme also exhibited appreciable glutaminase activity and a low level of TPNH oxidase activity. It also slowly catalyzed the oxidative deamination of glutamate (Table 1, e), a reaction that may be considered as a partial reversal of the overall reaction catalyzed by glutamate synthase. The partial reactions were demonstrable after repeated chromatography on Sephadex G-200 and Sepharose 6B. It thus appears likely that these activities are catalyzed by the synthase itself and, therefore, reflect steps in the mechanism of the overall reaction.

Fig. 2. Scans of polyacrylamide gel electrophoresis. (A) The enzyme was run in a 4% gel in 0.1 M Tris-acetate (pH 8). (B) The enzyme was boiled for 5 min in a solution containing 1% sodium dodecyl sulfate, 1% mercaptoethanol, and 0.1 M Tris-HCl (pH 8.5) and was then run in a 4.5% gel in 0.1% sodium dodecyl sulfate and 0.1 M Tris-acetate (pH 8). Each gel was about 100 mm in length.

Characterization of Glutamate Synthase Subunit

<table>
<thead>
<tr>
<th></th>
<th>Physical constants of glutamate synthase</th>
</tr>
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<tbody>
<tr>
<td>1. Sedimentation coefficient, s₂₀/u</td>
<td>17.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>(a) at 280 nm; 0.36 mg/ml</td>
</tr>
<tr>
<td></td>
<td>(b) at 280 nm in 1.2 M KSCN; 0.70 mg/ml (total)</td>
</tr>
<tr>
<td></td>
<td>Heavy subunit</td>
</tr>
<tr>
<td></td>
<td>Light subunit</td>
</tr>
<tr>
<td></td>
<td>(c) at 450 nm, in 1.2 M KSCN; 0.80 mg/ml</td>
</tr>
<tr>
<td></td>
<td>(d) at 550 nm, in 1.2 M KSCN; 0.80 mg/ml</td>
</tr>
<tr>
<td>2. Molecular weight of heavy subunit</td>
<td>175,000</td>
</tr>
<tr>
<td>3. Molecular weight of light subunit</td>
<td>51,500</td>
</tr>
<tr>
<td>4. Moles flavin (FMN + FAD)/227,000 g</td>
<td></td>
</tr>
<tr>
<td>of enzyme</td>
<td>1.7*</td>
</tr>
<tr>
<td>5. Moles FMN/moles FAD</td>
<td>1.3*</td>
</tr>
<tr>
<td>6. Moles iron/227,000 g of enzyme</td>
<td>6.7†</td>
</tr>
<tr>
<td>7. Moles labile sulfide/227,000 g of</td>
<td>13.0†</td>
</tr>
<tr>
<td>enzyme</td>
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Physical Properties (Table 2). The purified enzyme gave a single band on polyacrylamide gel electrophoresis (17) in 0.1 M Tris-acetate (pH 8.0) (Fig. 2A). It also gave a symmetrical boundary in sedimentation velocity runs in the analytical ultracentrifuge. The sedimentation coefficient, determined at relatively low protein concentration, is 17.6 S. However, in the presence of sodium dodecyl sulfate (18), two bands were seen on polyacrylamide gel electrophoresis; these exhibited molecular weights of about 175,000 and 51,500 (Table 2). The relative intensity of the higher molecular weight band to that of the lower molecular weight band after staining with Coomassie blue was about 3.5 to 1 (Fig. 2B). After the enzyme had been in a buffer containing 1.2 M KSCN for 1 hr at 4°, two boundaries, which exhibited sedimentation coefficients of about 8.3 S and 3.4 S, were observed in the ultracentrifuge (Table 2). Such treatment of the enzyme led to loss of catalytic activity, which was not restored by removal of thiocyanate. The correspondence of the observed boundaries with the heavy and light subunits of the enzyme was also shown by Sephadex G-200 chromatography in 1.2 M KSCN. Polyacrylamide gel electrophoresis of different fractions obtained from the column effluent confirmed dissociation into subunits.

The absorption spectrum of the enzyme, which is similar to that reported for the E. coli glutamate synthase (8, 9), exhibited maxima at 278, 375, and 447 nm, as well as a broad shoulder of low absorbance extending above 500 nm. Consistent with this spectrum and in general agreement with the findings on the E. coli enzyme (8, 9), glutamate synthase was shown to contain iron (mainly ferrous), sulfur, and both FMN and FAD (Table 2). The presence of both FMN and FAD was confirmed by silica gel chromatography.

In order to establish which subunit contains the flavin and iron-sulfide binding sites, we performed sedimentation velocity
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FIG. 3. Inhibition of glutamine-dependent glutamate synthase by L-2-amino-4-oxo-5-chloropentanoic acid. Enzyme (0.55 mg/ml; 0.5 mM KSCN) was preincubated at 23° with 55 μM chloroketone in the presence and absence of 10 mM glutamine. At the indicated intervals 25 μl aliquots were assayed in a final volume of 0.5 ml by following the decrease in absorbance of TPNH at 340 nm. The effect of the chloroketone during the assay was negligible.

runs in 1.2 M KSCN (0.2 M potassium phosphate (pH 7.6) containing 0.5 mM EDTA) and observed ultraviolet absorbance at 450 and 550 nm. At 450 nm both iron-sulfide and flavin absorb, whereas at 550 nm the absorbance is due only to the iron–sulfide linkages (19). Titration with dithionite indicated that about 60% of the sedimenting absorbance was present in a single boundary, which exhibited a sedimentation coefficient virtually identical to that of the heavy subunit observed at 280 nm. The most straightforward conclusion is that most, but not necessarily all, of the iron–sulfide and flavin binding sites are located on the heavy subunit.

Inhibition of Glutamine-Dependent Activity by L-2-Amino-4-oxo-5-chloropentanoate. When the native enzyme was preincubated with a low concentration (55 μM) of L-2-amino-4-oxo-5-chloropentanoate (20), there was rapid loss of glutamine-dependent activity (Fig. 3); addition of 10 mM L-glutamine provided substantial protection against inactivation. The glutaminase activity of the enzyme was similarly inhibited by the chloroketone and protected by glutamine. However, the chloroketone had no effect on the ammonia-dependent activity. When the enzyme was incubated with [14C]chloroketone under similar conditions, and then subjected to polyacrylamide gel electrophoresis in sodium dodecyl sulfate, 90% of the radioactivity was found in the heavy subunit (Fig. 4). The radioactivity incorporated into the protein under these conditions corresponds to the binding of 1.1 mole of analog per 227,000 g of enzyme. The addition of glutamine reduced the incorporation of radioactivity by 52%. Thus, a site interacting with both glutamine and the chloroketone glutamine analog is localized on the 175,000 molecular weight subunit.

We have observed that L-methionine, which inhibits the E. coli enzyme (8, 9), also inhibits the glutamine-dependent activity of the A. aerogenes enzyme. However, L-methionine sulfone, L-methionine-SR-sulfoxime, and L-methionine-SR-sulfone were found to be much more potent inhibitors than methionine. Thus, the respective concentrations necessary for 50% inhibition (with 0.5 mM glutamine and saturating concentrations of the other substrates) are 0.005, 0.35, and 0.7 mM, compared to 6 mM for L-methionine. All of these compounds were shown to inhibit competitively with respect to L-glutamine. In this respect glutamate synthase differs from carbamyl phosphate synthetase, which is not appreciably inhibited by methionine sulfoxime and methionine sulfone. It seems of interest that both glutamate synthase and glutamine synthetase are inhibited by these methionine derivatives; however, inhibition of the latter enzyme involves phosphorylation of the inhibitor as well as substrate competition.

**DISCUSSION**

That the synthesis of glutamate can be catalyzed by a glutamine-dependent enzyme was first shown in A. aerogenes (3) and later in other prokaryotes (4, 5, 8, 9, 21) and in yeast (22, 23). When the reaction catalyzed by glutamate synthase is coupled with that catalyzed by glutamine synthetase, the net result is the synthesis of L-glutamate from ammonia and α-ketoglutarate. These two enzymes functioning together provide an efficient pathway for L-glutamate biosynthesis, since the pathway is essentially irreversible and can operate effectively at low concentrations of ammonia. Only in the presence of relatively high concentrations of ammonium salts does the freely reversible reaction of ammonia and α-keto-
glutamate catalyzed by glutamate dehydrogenase become a major pathway of L-glutamate biosynthesis. In certain bacteria the only means of L-glutamate synthesis is through the glutamate synthase reaction (21, 24).

The present studies show that glutamate synthase exhibits a number of properties that are characteristic of other glutamine amidotransferases. Thus, the enzyme can bind and hydrolyze glutamine in the absence of other substrates. L-2-Amino-4-oxo-5-chloropentanoic acid, a chloroketone analog of glutamine, inhibits the glutamine-dependent synthesis reaction as well as the glutaminase activity, but does not affect the ammonia-dependent activity. In this respect the present findings are analogous to those on glutamine-dependent carbamyl phosphate synthetase (11, 20) and asparagine synthetase (25). It is notable that, like other glutamine amidotransferases, glutamate synthase can utilize ammonia in place of glutamine. The maximal activity of *A. aerogenes* enzyme with ammonia is only 5% of that observed with glutamine, but it is of interest that the enzyme evidently has a relatively high affinity for ammonia. The yeast enzyme (23) is about as active with ammonia (relative to its activity with glutamine) as the *A. aerogenes* enzyme, while the *E. coli* enzyme (8, 9) is essentially inactive with ammonia. It cannot be unequivocally excluded that this activity is catalyzed by a trace of contaminating glutamate dehydrogenase, but this possibility appears to be minimized by the fact that the ammonia-dependent activity persists even after repeated chromatography on Sephadex G-200 and Sepharose 6B.

A number of the characteristics of the glutamate synthase from *A. aerogenes* described here are similar to those reported for the *E. coli* enzyme (8, 9). It seems significant that two different purification schemes yield apparently homogeneous preparations which exhibit two bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In each case, the relative staining intensities of the two bands suggests a one-to-one molar ratio of the chains. These results make less likely the possibility that the light subunit is produced from a contaminating protein. It is of interest that the light subunits from the two bacteria have virtually identical molecular weights, whereas the heavy subunit from the *A. aerogenes* enzyme is significantly larger (molecular weight: 175,000) than that from the *E. coli* enzyme (molecular weight: 135,000).

The present work indicates that the heavy subunit of the *A. aerogenes* enzyme contains the binding sites for glutamine, flavin, and iron–sulfide. That a small fraction of the iron–sulfide or flavin may be located on the light subunit cannot be definitely excluded by the sedimentation velocity data presented here. The catalytic significance of the light subunit requires further study. Although dissociation of the enzyme by treatment with 1.2 M potassium thiocyanate led to loss of synthase activity, it was not restored by removal of thiocyanate. We have recently found that addition of dithiothreitol, 2-ketoglutarate, and L-glutamine protects against inactivation by thiocyanate to some extent. This suggests that it may be possible to achieve dissociating conditions under which enzymatic activity is retained, thus making it feasible to approach directly the problem of the function of the light subunit.

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