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GLUTAMATE SYNTHASE FROM *ESCHERICHIA COLI* —AN IRON–SULFUR FLAVOPROTEIN

SEPARATION AND ANALYSIS OF NON-IDENTICAL SUBUNITS

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SUMMARY

Purified glutamate synthase from *Escherichia coli* has been disaggregated with sodium dodecylsulfate and resolved into two non-identical subunits (molecular weights 135 000 and 53 000) using agarose gel filtration in the presence of sodium dodecylsulfate. The ratio between the content of the two subunits in the native enzyme is in agreement with the ratio between subunit molecular weights suggesting that the subunits occur in equal numbers in the native enzyme. Amino acid analysis and N-terminal analysis of the separate subunits confirm their nonidentity.

INTRODUCTION

Glutamate synthase (L-glutamine:2-oxoglutarate aminotransferase (NADPH-oxidizing), EC 2.6.1.53) catalyzes the reduced pyridine nucleotide dependent conversion of α-ketoglutarate and L-glutamine to glutamate. The enzyme was discovered in *Aerobacter aerogenes* in 1970 [1] and since then its existence in a large number of microorganisms has been established [2–5]. Coupled with glutamine synthetase and transaminases, glutamate synthase provides an important pathway for the assimilation of ammonia to form the various amino acids. The enzyme appears to be of greatest physiological importance (a) when growth is limited by the availability of ammonia, (b) during nitrogen fixation and (c) in microorganisms which lack glutamate dehydrogenase.

Glutamate synthase purified to homogeneity from *Escherichia coli* contains 7.8 moles of flavin, 38.4 moles of iron and 30.4 moles of labile sulfide per mole of enzyme [6]. The molecular weight of the enzyme is 800 000 [6].

Enzyme disaggregation studies suggest that glutamate synthase is composed of at least two types of dissimilar subunits [6]. Purified glutamate synthase incubated in the presence of 1 % sodium dodecylsulfate, 8 M urea, or 6 M guanidine–HCl mi-

Abbreviation: TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

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grates as two components (molecular weights 53 000 and 135 000)* on polyacrylamide gel electrophoresis performed in the presence of 0.1% sodium dodecylsulfate. Similarly, enzyme previously incubated in 8 M urea migrates as two components on disc gel electrophoresis in the presence of 8 M urea. Spectrophotometric scans (550 nm) of Coomassie blue stained sodium dodecylsulfate polyacrylamide gels were performed [6]. Assuming a proportionality between staining intensity and protein content it was calculated that the ratio between the protein content of the high molecular weight species and that of the low molecular weight species is 2.58. This ratio is in good agreement with the ratio of the respective molecular weights (2.55) and it suggested that the two types of dissimilar subunits occur in the native protein in equimolar amounts. Gel scans were performed on a gel similar to number zero of Fig. 2. Details of these studies have been published [6].

This communication describes separation of the two types of non-identical subunits and their amino acid and N-terminal analysis. The data have been published in abbreviated form [7].

MATERIALS

Chemicals were obtained as follows: 4% agarose, BioRad Laboratories; *N*-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) and dansylamino acids, Calbiochem.; polyamide layer sheets (Chen Chin Trading Co. Ltd), Gallard Schlesinger; 1-dimethylaminonaphthalene-5-sulfonyl chloride (dansyl chloride), Schwartz/Mann. All other chemicals were of the highest grade commercially available.

METHODS

Glutamate synthase was purified to homogeneity from *E. coli* as described previously [6].

Dissociation of glutamate synthase into subunits was achieved using sodium dodecylsulfate. Purified glutamate synthase (1.5 ml containing 36.0 mg of enzyme) was dialyzed sequentially (at 30 °C for 12 h) against each of three 500 ml volumes of a solution containing 0.1 M Tris–HCl, pH 8.5, 1% sodium dodecylsulfate, 140 mM 2-mercaptoethanol. Alkylation of the disaggregated reduced enzyme was performed by dialysis for 4 h at 30 °C against 100 ml of a solution containing 0.1 M Tris–HCl, pH 8.5, 1% sodium dodecylsulfate, 0.5 M iodoacetate. Finally, the disaggregated, reduced and alkylated enzyme was exhaustively dialyzed against 10 mM sodium phosphate, pH 7.1, 0.2% sodium dodecylsulfate prior to gel filtration.

Agarose gel filtration in the presence of sodium dodecylsulfate was performed as suggested by Fish et al. [8]. The biuret reaction was performed as described by Layne [9] using bovine serum albumin as standard.

Sodium dodecylsulfate–polyacrylamide gel electrophoresis was performed as described by Weber and Osborn [10] with the exception that 25 mM TES–Tris, pH 7.1, was substituted for sodium phosphate buffer for both gel polymerization and for the electrode buffer. This modification, suggested by Dr John Baker (personal com-

* Molecular weights are averages from five determinations. Molecular weight range was 125 000–140 000 for the high molecular weight subunit and 50 000–56 000 for the low molecular weight subunit.

munication), reduced by 75% the time required and by 80% the current required for electrophoresis. The modification resulted in no change in relative migration of glutamate synthase subunits or in the relative migration of bovine serum albumin or ovalbumin standards as compared with electrophoresis using sodium phosphate buffer. Gels were stained for protein with Coomassie blue.

Amino acid analysis was performed as described previously [6]. Protein samples in 6 M HCl were sealed in evacuated tubes and hydrolyzed at 106–110 °C for 24 h after which HCl was removed by rotary evaporation and samples were analyzed using a Beckman Model 121 automatic amino acid analyzer equipped with an Infotronics Model CRS-210 integrator. The protein content of samples was estimated by the method of Lowry et al. [11] using bovine serum albumin as standard.

N-terminal amino acid analysis was performed as described by Gros and Labouesse [12] with the following modifications. The dansylation reaction was performed in the presence of 1.3% sodium dodecylsulfate, 0.12 M sodium phosphate instead of 8 M urea, 0.1 M phosphate buffer. The dansyl derivatives of amino acids were liberated from the protein by hydrolysis at 106 °C for 18 h in 6 M HCl in sealed evacuated tubes. Dansylated amino acids were separated by two dimensional thin-layer chromatography on polyamide layer sheets using 1% NH_4OH for the first dimension and a mixture of heptane–formic acid–butanol (10:1:10; v/v/v) for the second dimension [13]. Due to the presence of large amounts of ε-dansyl lysine, ether extraction of the hydrolyzed sample was necessary prior to chromatography. N-terminal analysis of bovine serum albumin and lysozyme gave the expected results using the procedures described. Quantitation of dansyl amino acids was not attempted.

RESULTS

Agarose gel filtration

The non-identical subunits of purified glutamate synthase were separated by gel filtration of disaggregated enzyme. Fig. 1 shows the elution profile of sodium dodecylsulfate disaggregated, reduced and alkylated glutamate synthase from

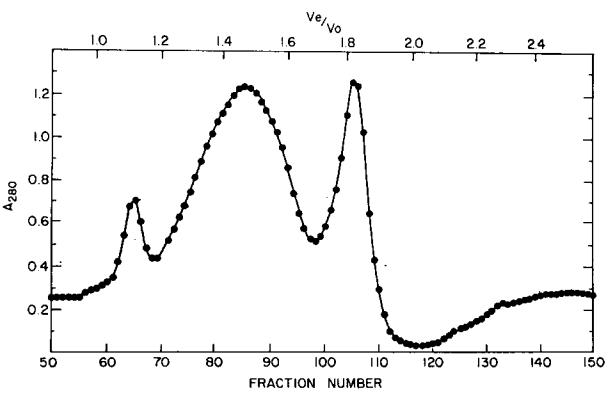

Fig. 1. Elution profile of sodium dodecylsulfate disaggregated glutamate synthase from a 4% agarose column (91.5 cm × 6 cm). The sample (1.4 ml) contained 33 mg of sodium dodecylsulfate disaggregated glutamate synthase after prior reduction with 2-mercaptoethanol and alkylation with iodoacetate. Buffer contained 10 mM sodium phosphate, pH 7.1, 0.2% sodium dodecylsulfate. Flow rate, 4 ml/cm² per h. Fractions are 1.2 ml; V_e/V_0, ratio of elution volume to void volume.

a 4% agarose column. Fig. 2 shows the results of sodium dodecylsulfate–polyacrylamide gel electrophoresis performed on the starting material (O) and on selected fractions from the agarose column. Prior to the application of any protein, the column effluent contained an unknown material having an absorbance of approximately 0.26 at 280 nm when compared to the $A_{280\ nm}$ of the column reservoir buffer. The material did not contain protein as determined by the method of Lowry et al. [11]. It was not biuret reactive and it did not contain detectable levels of amino acids after hydrolysis in 6 M HCl. This material continued to elute from the column even after the column had been washed with more than four column bed volumes of buffer and during elution of the glutamate synthase subunits. The decrease in $A_{280\ nm}$ following the third protein elution peak of Fig. 1 (Fractions 111–140) suggests that some of the non-protein material was bound by the glutamate synthase subunit(s) [14]. The ultraviolet absorption spectrum (between 250 and 350 nm) of the sample applied to the agarose column was compared with the spectrum of the column effluent prior to sample application and to the spectra of separately pooled elution peak fractions. The spectra ($A_{280\ nm}/A_{260\ nm}$ ratio) suggest that the non-protein, 280-nm absorbing material was associated with all protein-containing column fractions. Attempts to dissociate this material from the protein samples by acetone precipitation and dialysis were not successful.

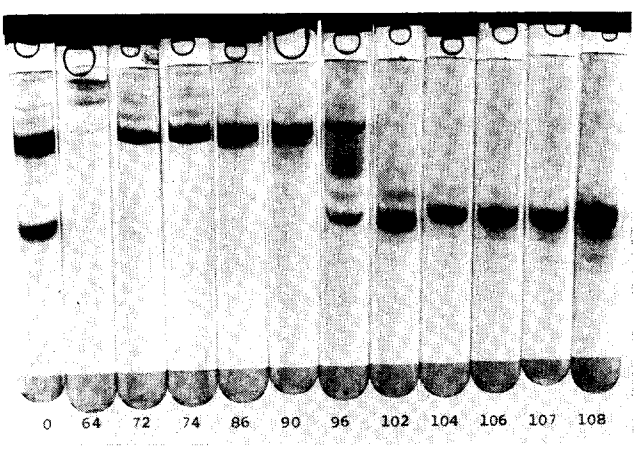

Fig. 2. Sodium dodecylsulfate–polacrylamide gel electrophoresis performed on the original sample applied to the agarose column (Fig. 1) and on selected fractions from the column. Gel number corresponds to column fraction number in Fig. 1.

Protein content of column elution peaks was estimated from the areas between the elution profile (Fig. 1) and a horizontal line through 0.26 on the ordinate of Fig. 1. This estimate is based on two assumptions: (a) there is a constant amount of non-protein, 280-nm absorbing material in each fraction and (b) the tyrosine and tryptophan content relative to protein in each subunit of glutamate synthase is constant. The first protein eluted from the column after application of the disaggregated glutamate synthase accounts for about 8% of the total protein eluted and may represent an impurity in the enzyme or non-disaggregated enzyme (Gel 64, Fig. 2). The second and third proteins eluted are the two types of dissimilar subunits of glutamate synthase

as determined from their mobilities on sodium dodecylsulfate–polyacrylamide gel electrophoresis (Fig. 2). The ratio between the areas under the second and third elution peaks is 2.9 to 1. The absorbance at 280 nm per mg of protein [11] for pooled Fractions 76–90 and 104–108 (Fig. 1) was 0.93 and 1.27, respectively. When the areas under the second and third elution peaks are corrected for protein content the ratio between them is 2.15 to 1. In either case, there is reasonable agreement with the ratio between the subunit molecular weights (2.23–2.80) determined from sodium dodecylsulfate–polyacrylamide gel electrophoresis [6]. This again suggests a 1 to 1 stoichiometry for the dissimilar subunits in the holoenzyme.

Amino acid analysis

Agarose column Fractions 62–66, 76–90 and 104–108 were pooled separately. Protein from the original sample and from the pooled column fractions was precipitated with acetone (90% by vol.). The precipitated protein was hydrolyzed and amino acid analysis was performed as described in Methods.

As shown in Table I, the high molecular weight subunit (pooled Fractions

TABLE I

AMINO ACID COMPOSITION OF GLUTAMATE SYNTHASE SUBUNITS

Amino acid	Holoenzyme*		High molecular weight (135 000) subunit**		Low molecular weight (53 000) subunit***	
	% by weight	Moles per 188 000 g of protein	% by weight§§	Moles per 135 000 g of protein	% by weight	Moles per 53 000 g of protein
Lysine	5.40	69.2	5.40	49.7	6.32	22.8
Histidine	1.82	22.1	1.98	17.2	1.44	4.9
Arginine	8.83	95.3	7.88	61.1	8.95	27.2
Aspartic acid	10.41	147.1	10.24	103.8	11.74	46.7
Threonine	4.30	67.8	4.45	50.5	4.08	18.2
Serine	3.66	65.4	3.93	50.5	3.08	15.5
Glutamic acid	13.71	172.8	12.89	116.7	17.76	63.1
Proline	4.19	68.4	3.62	42.5	4.81	22.1
Glycine	5.50	137.6	5.22	93.9	6.81	48.1
Alanine	6.69	141.1	6.83	103.5	6.41	38.2
Cysteine§	1.28	15.7	1.04	9.17	2.06	7.11
Valine	6.47	103.8	5.32	61.2	8.32	37.6
Isoleucine	5.11	73.2	4.99	51.3	5.67	22.9
Leucine	9.75	139.8	10.59	109.0	7.80	31.5
Tyrosine	6.17	46.1	5.03	37.5	4.15	12.1
Phenylalanine	5.59	63.6	5.71	46.6	6.61	21.2
Total	98.88		95.12		106.01	

* Aliquot analyzed was taken from enzyme prepared for agarose column chromatography as described in the legend to Fig. 1.

** Aliquot analyzed was taken from pooled Fractions 76–90 of Fig. 1.

*** Aliquot analyzed was taken from pooled Fractions 104–108 of Fig. 1.

§ Determined as *S*-carboxymethylcysteine.

§§ The data presented are the results of the analysis of a single set of hydrolysates (see Methods). The percent by weight for each amino acid is determined by dividing the mass of the amino acid found in the sample by the total mass of protein in the sample.

76–90 of Fig. 1) differs from the low molecular weight subunit (pooled Fractions 104–108 of Fig. 1) in its relative content of several amino acids. Most notably the relative content of cysteine (as *S*-carboxymethylcysteine), valine, glutamic acid, proline and glycine is greater in the low molecular weight subunit than in the high molecular weight subunit. The opposite is true for the amino acids histidine, serine and leucine. It is noteworthy that the weighted average amino acid content (based on subunit molecular weight) for any given amino acid in the two subunits is in good agreement with the content of that amino acid in the disaggregated enzyme prior to resolution into subunits showing that the data are internally consistent*. The data support the conclusion that the two types of subunits differ in primary structure.

Amino-terminal analysis

Amino-terminal analysis was performed on the holoenzyme and on the separated subunits (see Methods). A single amino terminal (serine) was identified in the holoenzyme. Serine was identified as the N-terminal amino acid in the low molecular weight subunit (molecular weight 53 000) isolated as described in the legend to Fig. 1. No amino terminal amino acid could be identified in the high molecular weight subunit (molecular weight 135 000) suggesting that the amino terminal might be blocked in this subunit. These data further support the conclusion that the two types of subunits of glutamate synthase differ in primary structure.

DISCUSSION

Non-identity of the two types of subunits of glutamate synthase seems unequivocal in view of amino acid and N-terminal analyses. In addition, available data suggest that the non-identical subunits occur in equal numbers in the native enzyme.

Savageau et al. [5] have suggested the possibility that glutamate synthase and glutamate dehydrogenase might exist as a "bifunctional" complex in *E. coli* and that "the activity of the complex could arise from bifunctional subunits, or from a collection of subunits with separate activities or simply from the association of subunits". In this context, it seems worthwhile to point out that the glutamate synthase used for the studies outlined in this communication was separated from glutamate dehydrogenase activity during the purification procedure (see Methods and ref. 7). There is virtually no glutamate dehydrogenase activity in purified glutamate synthase [6]. Thus, it seems highly unlikely that the glutamate dehydrogenase activity to which Savageau et al. [5] refer resides in either of the glutamate synthase subunits.

The site of flavin binding to glutamate synthase remains to be determined since disaggregation of the enzyme in sodium dodecylsulfate resulted in dissociation of all bound flavin. Similarly, iron was not detectable by atomic absorption spectroscopy in either of the subunits after sodium dodecylsulfate disaggregation. No clue as to the binding site of flavin has been obtained; however, the relatively higher content of cysteine residues in the subunit of molecular weight 53 000 suggests the possibility that this subunit might be the site of iron and labile sulfide binding.

* The holoenzyme content of tyrosine and phenylalanine (6.17 and 5.59% by wt, respectively) differs from that reported previously [6] (3.69 and 4.83% by weight, respectively). This is probably explained by the fact that phenol (which prevents destruction of aromatic amino acids during hydrolysis) was not present during hydrolysis in the earlier study [6], but it was included for this study.

The data presented provide additional support for the conclusion, proposed by Miller and Stadtman [6], that glutamate synthase is composed of eight subunits, four of each of two types of non-idential subunits of molecular weights 135 000 and 53 000. Data presented previously [6] suggest that the purified enzyme might be an aggregate of four catalytically active dimers, each composed of one of each of the two types of dissimilar subunits, eight iron atoms, eight labile sulfide atoms, and two flavin molecules.

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REFERENCES

1 Tempest, D. W., Meers, J. L. and Brown, C. M. (1970) Biochem. J. 117, 405–407
2 Meers, J. L., Tempest, D. W. and Brown, C. M. (1970) J. Gen. Microbiol. 64, 187–194
3 Elmerich, C. and Aubert, J. (1971) Biochem. Biophys. Res. Commun. 42, 371–376
4 Nagatani, A., Shimizu, M. and Valentine, R. C. (1971) Arch. Microbiol. 79, 164–175
5 Savageau, M. A., Kotre, A. M. and Sakamoto, N. (1972) Biochem. Biophys. Res. Commun. 48, 41–47
6 Miller, R. E. and Stadtman, E. R. (1972) J. Biol. Chem. 247, 7407–7419
7 Miller, R. E. (1973) The Enzymes of Glutamine Metabolism (Prusiner, S. and Stadtmen, E. R., eds), p. 183, Academic Press, New York
8 Fish, W. W., Mann, K. G. and Tanford, C. (1969) J. Biol. Chem. 244, 4989–4994
9 Layne, E. (1957) Methods Enzymol. 3, 447–448
10 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
11 Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
12 Gros, C. and Labouesse, B. (1969) Eur. J. Biochem. 7, 463–470
13 Mihalyi, E. and Albert, A. (1971) Biochemistry 10, 237–242
14 Hummel, J. P. and Dreyer, W. J. (1962) Biochim. Biophys. Acta 63, 532–534