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Glutamate Dehydrogenase from *Escherichia coli*: Purification and Properties

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Glutamate dehydrogenase (L-glutamate:$NADP^+$ oxidoreductase [deaminating], EC 1.4.1.4) has been purified from *Escherichia coli* B/r. The purity of the enzyme preparation has been established by polyacrylamide gel electrophoresis, ultracentrifugation, and gel filtration. A molecular weight of 300,000 ± 20,000 has been calculated for the enzyme from sedimentation equilibrium measurements. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate and sedimentation equilibrium measurements in guanidine hydrochloride have revealed that glutamate dehydrogenase consists of polypeptide chains with the identical molecular weight of 50,000 ± 5,000. The results of molecular weight determination lead us to propose that glutamate dehydrogenase is a hexamer of subunits with identical molecular weight. We also have studied the stability and kinetics of purified glutamate dehydrogenase. The enzyme remains active when heat treated or when left at room temperature for several months but is inactivated by freezing. The Michaelis constants of glutamate dehydrogenase are 1,100, 640, and 40 μM for ammonia, 2-oxoglutarate, and reduced nicotinamide adenine dinucleotide phosphate, respectively.

Glutamic acid, as the donor of an α-amino group in transaminase reactions, is a key metabolite for the biosynthesis of amino acids. There are two enzymes in *Escherichia coli* responsible for the biosynthesis of glutamic acid (13, 14, 16). One is glutamate dehydrogenase (L-glutamate:$NADP^+$ oxidoreductase [deaminating], EC 1.4.1.4) that catalyzes the reversible reaction:

$$\text{2-oxoglutarate} + NH_4^+ + \text{NADPH} \rightleftharpoons \text{L-glutamate} + \text{water} + NADP^+ \quad \text{(i)}$$

The other is glutamate synthase (L-glutamate:$NADP^+$ oxidoreductase [deaminating, glutamine-forming], EC 1.4.1.X) that carries out the virtually irreversible reaction of amide transfer:

$$\text{2-oxoglutarate} + \text{L-glutamine} + \text{NADPH} \rightarrow 2\ \text{L-glutamate} + NADP^+ \quad \text{(ii)}$$

In this paper we report the purification of glutamate dehydrogenase from *E. coli* B/r and the characterization of some of its kinetic and molecular properties. We also have purified glutamate synthase and have obtained results that confirm those reported by Miller and Stadtman for *E. coli* W (13). Our interest in glutamate synthase derives from two earlier observations that suggested a possible association of glutamate dehydrogenase and glutamate synthase activities (14): glutamate formation in the presence of both ammonia and glutamine is not additive in a cell extract, and a partially purified preparation utilizes either ammonia or glutamine as an amino donor. Although we found that purified glutamate dehydrogenase and glutamate synthase catalyze reactions i and ii, respectively, we were unable to isolate a species having both activities. The explanation for the "nonadditivity" may be that glutamine, which for glutamate synthase is the specific amino donor, inhibits the activity of glutamate dehydrogenase competitively with respect to ammonia.

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MATERIALS AND METHODS

Bacterial growth and assays. The bacterial strain used for the enzyme purification was *E. coli* B/r. Cultures were grown aerobically in glucose-minimal medium (6) and were harvested by centrifugation in late exponential phase.

The ammonia- and glutamine-dependent activities were determined by measuring the rate of oxidation of NADPH, which was monitored continuously at 340 nm. The assay system was composed of the following: 50 mM potassium phosphate (pH 8.0), 10 mM 2-oxoglutarate, 70 μM NADPH, and 20 mM ammonium chloride for the ammonia-dependent activity or 20 mM L-glutamine for the glutamine-dependent activity. The activity of glutamate deami-

nation was assayed by monitoring NADPH formation at 340 nm in an assay mixture containing 50 mM potassium phosphate (pH 8.0), 10 mM L-glutamate, and 70 μM $NADP^+$. A unit of activity is equivalent to the oxidation or formation of 1 μmol of NADPH per min at 25 C.

Protein concentration was estimated by the colorimetric method of Lowry et al. (11) with bovine serum albumin as the standard.

Column chromatography. Diethylaminoethyl (DEAE)-Sephadex A-50, obtained from Pharmacia Fine Chemicals, Inc., was prepared and packed according to the manual of the manufacturer.

Bio-Gel A-5m (6% agarose), purchased from Bio-Rad Laboratories, was used for gel filtration. Molecular weight determinations were performed by the method of Andrews (1). The column was calibrated with standard proteins (5 mg in a 0.5- to 1.0-ml vol). Protein peaks were located spectrophotometrically or by enzymatic assay. The standard proteins were obtained from Sigma Chemical Co.

Electrophoresis in polyacrylamide gels. We followed the general method of Davis (7) for gel electrophoresis, except that spacer and sample gels were omitted. The samples (10 to 20 μg of protein) were applied to the surface of gels (5.5 by 0.5 cm; 5% acrylamide and 0.135% *N,N'*-methylenebisacrylamide) and were subjected to a current of 8 mA per gel in 50 mM sodium phosphate (pH 7.2) or 5 mM tris(hydroxymethyl)aminomethane-glycine (pH 8.2). In the case of gel electrophoresis in the presence of sodium dodecyl sulfate (18), 10-μg samples of protein were treated with 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol at 37 C for 2 h or longer and were applied to the gels (5.5 by 0.5 cm; 8% acrylamide and 0.2% *N,N'*-methylenebisacrylamide).

Gels were stained for 3 h with 0.25% Coomassie brilliant blue in 45% methanol-9% acetic acid and destained electrophoretically with a Canalco Quick-Destainer in 7% acetic acid. They were stored in 7% acetic acid. Enzymatic activity in the gels was detected by staining as described by Yarrison et al. (20).

Ultracentrifugal analysis. Ultracentrifugation experiments were done using a Spinco model E analytical ultracentrifuge equipped with an RTIC unit and electronic speed control. Sedimentation velocity was measured at 20 C with a schlieren optical system. Sedimentation equilibrium measurements were made at 20 C by meniscus depletion (21) with a Rayleigh interference optical system. In the latter measurements, the sample volume was 110 μl.

Zone centrifugation in sucrose density gradients. Zone centrifugation experiments were performed following the general method of Martin and Ames (12). Linear gradients from 5 to 20% sucrose, total volume 4.2 ml, were prepared. Samples of 0.1 or 0.2 ml were layered on the gradients and then were centrifuged at 4 C and 26,000 rpm for 16 h in a Spinco model L2-65B ultracentrifuge with an SW65 K rotor. About 40 fractions were collected from each tube.

Paper chromatography. Amino acids (i.e., glutamic acid and glutamine) in 2-μl samples were separated on Whatman filter paper no. 1 (20 by 20 cm) with a phenol-water (100:20, vol/vol) solvent system and were detected by ninhydrin.

RESULTS

Purification of glutamate dehydrogenase. (i) Procedure. Table 1 summarizes the purification of glutamate dehydrogenase from *E. coli* B/r. The related enzyme glutamate synthase also was purified in this process. All steps in the purification were carried out at 0 to 4 C with buffers containing 1 mM ethylenediaminetetraacetic acid and 10 mM 2-mercaptoethanol.

The harvested cells (30 g, wet weight) were suspended in 60 ml of 10 mM potassium phosphate (pH 7.2) and were disrupted by sonic oscillation with a Branson Sonifier (model W 140) at the highest intensity for three periods of 3 min each. The suspension of broken cells was centrifuged at 30,000 $\times$ *g* for 30 min. The supernatant fluid (crude extract) was dialyzed against 8 liters of 10 mM potassium phosphate (pH 7.2) for 16 h.

Nucleic acids were removed from the dialyzed crude extract by the slow addition, with stirring, of a neutralized 10% (wt/vol) solution of streptomycin sulfate. The volume of this addition was $^1/_{10}$ the volume of the extract. After 1 h of additional stirring, the suspension was centrifuged at 30,000 $\times$ *g* for 30 min. The supernatant fluid was dialyzed against 8 liters of 10 mM potassium phosphate (pH 7.2) for 16 h.

The dialyzed extract was treated with a saturated solution of ammonium sulfate adjusted to pH 7.2 with ammonium hydroxide. Proteins precipitating between 32.5 and 52.5% of saturation were dissolved in 9 ml of buffer A (20 mM potassium phosphate [pH 7.2] containing 0.1 M potassium chloride and 2 mM 2-oxoglutarate) and were dialyzed against 2 liters of the same buffer for 16 h.

The dialyzed fraction was applied to a column (50 by 2.5 cm) packed with DEAE-Sephadex A-50 and was equilibrated with buffer A. Proteins were eluted initially with 200 ml of buffer A and then with a linear gradient of potassium chloride concentrations from 0.1 to 0.7 M in 20 mM potassium phosphate (pH 7.2) containing 2 mM 2-oxoglutarate (total vol, 1 liter). The flow rate was 15 ml/h, and the fractions collected were 5.5 ml each.

The activities of interest were eluted in two regions of the gradient. Eighty percent of the glutamine-dependent activity (reaction ii) was found in peak I of the elution profile (Fig. 1) with the remainder appearing in peak II. The ammonia-dependent activity (reaction i), on the other hand, appeared predominantly in peak II and was barely detected in peak I. The fractions

TABLE 1. *Purification of glutamate dehydrogenase and glutamate synthase from E. coli B/r*

Fraction	Vol (ml)	Protein (mg)	Activity (U)		Sp act (U/mg of protein)	
			Ammonia dependent	Glutamine dependent	Ammonia dependent	Glutamine dependent
Crude extract	74	2,640	555	588	0.212	0.222
Streptomycin treatment	79	2,240	522	553	0.234	0.247
Ammonium sulfate (32.5–52.5% saturation)	20	1,090	492	518	0.451	0.475
DEAE-Sephadex						
Peak I[a]	2.0	39.0	2	234	0.011	6.01
Peak II	1.6	29.6	396	62.0	13.4	2.10
Heat treatment						
Peak I	1.5	14.2	1.5	171	0.106	12.1
Peak II	1.2	9.70	388	54.6	40.0	5.64
Bio-Gel A-5m						
GS[b]	1.3	6.21	0	138	0	22.2
GDH[c]	1.1	1.21	217	0	180	0

[a] The data in this line were obtained after ammonium sulfate precipitation (37.5 to 47.5% saturation) of the pooled fractions of peak I.
[b] GS, Glutamate synthase.
[c] GDH, Glutamate dehydrogenase.

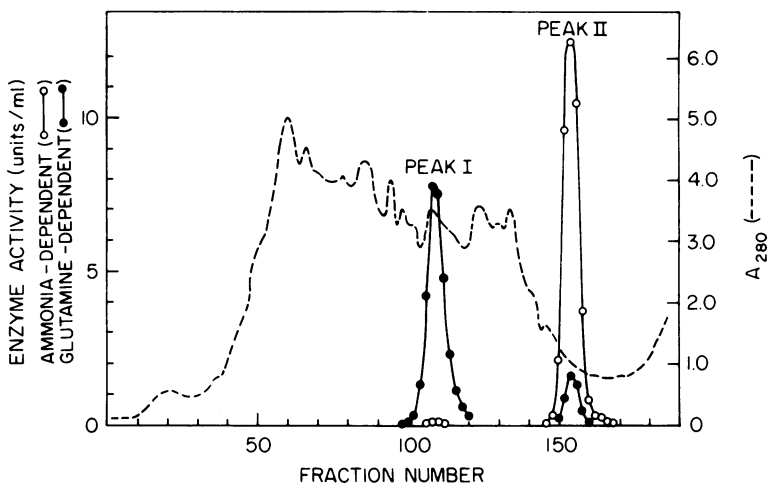

FIG. 1. *Elution profile of glutamate dehydrogenase and glutamate synthase from a DEAE-Sephadex column. The preparation of samples and the elution procedure are described in the text. Protein is represented by absorbance at 280 nm; ammonia- and glutamine-dependent activities were assayed as described in the text.*

corresponding to peak I were pooled and treated for the further purification of glutamate synthase. The pooled fractions of peak II were used for the purification of glutamate dehydrogenase.

The pooled fractions of peak I were treated with ammonium sulfate. Proteins precipitating between 37.5 and 47.5% of saturation were dissolved in 2 ml of buffer B (20 mM sodium phosphate [pH 7.2] containing 2 mM 2-oxoglutarate). The solution was placed in a water bath at 60 C for 10 min with shaking, and then centrifuged at 16,000 × *g* for 15 min. The resulting supernatant fluid was applied to a column (90 by 1.5 cm) that had been packed with Bio-Gel A-5m, 200 to 400 mesh, and was washed with buffer B. Proteins were eluted with buffer B at a flow rate of 3.5 ml/h. Fractions (2.7 ml) were

collected. Those having specific activity of glutamate synthase greater than 18 U/mg of protein were pooled and concentrated in an Amicon Diaflo cell equipped with a PM-10 membrane. The final preparation (glutamate synthase) was stored at 0 to 4 C.

The pooled fractions of peak II were concentrated with an Amicon Diaflo cell and were subjected to heat treatment and gel filtration as described for the fractions of peak I. Fractions having specific activity of glutamate dehydrogenase greater than 150 U/mg of protein were pooled and concentrated. The final preparation (glutamate dehydrogenase) was stored at 0 to 4 C.

(ii) Purity of enzyme preparations. Purity of the two enzyme preparations was established by polyacrylamide gel electrophoresis, ultracentrifugal analysis, and gel filtration. Samples containing 20 μg of protein were applied to polyacrylamide gels as described above. Electrophoresis at pH 7.2 revealed a single protein band for each preparation. The protein bands were shown to be glutamate dehydrogenase and glutamate synthase by the appropriate activity stain (20) and by the assay of the enzymes eluted from the sliced gels. Electrophoresis at pH 8.2 yielded a diffuse major band plus minor bands for glutamate dehydrogenase and a diffuse major band for glutamate synthase; however, the protein and activity stains corresponded exactly, indicating no contaminating proteins.

Sedimentation equilibrium measurements also supported the purity of both enzyme preparations. Yphantis plots, plots of the logarithm of fringe displacement versus the square of the distance from the axis of rotation, were linear. The purity of the enzyme preparations was further suggested by the results from gel filtration on Bio-Gel A-5m; a single protein peak contained enzyme with constant specific activity throughout.

Kinetic properties and stability. (i) Substrate specificity and Michaelis constants. Kinetic studies with the purified enzymes established that glutamate dehydrogenase and glutamate synthase catalyze reactions i and ii, respectively. An analysis of the reaction products by paper chromatography also indicated that glutamate dehydrogenase cannot utilize glutamine as the amino donor. No radioactive glutamic acid was found when glutamate dehydrogenase was incubated in the assay mixture containing radioactive glutamine and samples from the assay mixture were chromatographed. Both enzymes are specific for NADPH; NADH cannot serve as the coenzyme for reaction i or ii.

Table 2 summarizes the Michaelis constants

TABLE 2. *Michaelis constants of glutamate dehydrogenase and glutamate synthase from E. coli B/r*

Substrate[a]	K_m (M)	
	Glutamate dehydrogenase	Glutamate synthase
2-Oxoglutarate	6.4×10^{-4}	3.6×10^{-5}
Ammonia	1.1×10^{-3}	
Glutamine		3.0×10^{-4}
Glutamic acid	1.3×10^{-3}	—[b]
NADPH	4.0×10^{-5}	8.5×10^{-6}
$NADP^+$	4.2×10^{-5}	—[b]

[a] All other assay conditions are described in Materials and Methods.

[b] The constants for these substrates were not measured since the aminating reaction of glutamate synthase is virtually irreversible.

(K_m) we obtained for purified glutamate dehydrogenase from *E. coli* B/r. Values for glutamate synthase, similar to those for the enzyme from *E. coli* W (13), also are shown for comparison. The values for glutamate synthase are from 4 to 20 times lower than the corresponding values for glutamate dehydrogenase, which is consistent with the proposed dominant role for glutamate synthase in vivo (16).

(ii) Inhibition by homoserine. L-Homoserine has been observed to inhibit both ammonia- and glutamine-dependent activities in cell extracts of *E. coli* B/r (14). This inhibition also is reported for glutamate synthase from *E. coli* W (13). These findings were confirmed for purified glutamate dehydrogenase and glutamate synthase from *E. coli* B/r. L-Homoserine is a competitive inhibitor with respect to both ammonia and glutamine. The corresponding values of K_i are 25 mM for glutamate dehydrogenase and 13 mM for glutamate synthase.

(iii) Inhibition by glutamine. Glutamine, which for glutamate synthase is the specific amino donor, was found to inhibit the activity of glutamate dehydrogenase. The inhibition of the aminating reaction by glutamine was competitive with respect to ammonia, K_i of 60 mM, whereas the inhibition of the deaminating reaction was noncompetitive with respect to glutamate. On the other hand, ammonia, which for glutamate dehydrogenase is the specific amino donor, had no effect on the activity of glutamate synthase.

(iv) Stability. Glutamate dehydrogenase is quite stable at 4 C and at room temperature in water, 20 mM potassium phosphate, or sodium phosphate (pH 7.2). The enzyme remains fully active at room temperature for at least 3 months. Storage at 4 C preserves its activity even longer. On the other hand, the enzyme is

inactivated by freezing-thawing. The enzyme stability in four solvent systems was examined at different temperatures, and the results are summarized in Table 3.

Glutamate dehydrogenase is stable when heat treated. Almost total activity was recovered after treatment at 60 C for 10 min or at 37 C for 5 h; the 60 C treatment was then used as a step in purification.

(v) **Denaturation and renaturation.** Glutamate dehydrogenase was treated at 37 C for 2 h with guanidine hydrochloride of various concentrations (0 to 6.0 M) and 10 mM dithiothreitol in 20 mM sodium phosphate (pH 7.2). The remaining activity was assayed in a reaction mixture supplemented with guanidine hydrochloride at the same concentration used for the treatment. Activity of the treated enzyme also was measured after diluting guanidine hydrochloride with the reaction mixture (600-fold dilution) (Fig. 2A).

Glutamate dehydrogenase was completely inactivated by guanidine hydrochloride at concentrations higher than 1.0 M. After the dilution of guanidine hydrochloride, the enzyme treated at concentrations lower than 2.0 M recovered almost total activity. On the other hand, no recovery of activity was noted when the enzyme had been treated at concentrations higher than 4.0 M. The incubation of the enzyme in its appropriate assay mixture for16 h had no effect on recovery. Renaturation of the enzyme treated with 6.0 M guanidine hydrochloride was attempted by the removal of the denaturant with gel filtration on Bio-Gel A-5m; the eluted protein, however, had no enzymatic activity. Similar results, but at lower concentrations of guanidine hydrochloride, were obtained with glutamate synthase (Fig. 2B).

Molecular properties of glutamate dehydrogenase. (i) **Molecular weight.** Sedimentation equilibrium measurements were performed in 50 mM sodium phosphate (pH 7.2) containing 2 mM 2-oxoglutarate, 1 mM ethylenediaminetetraacetic acid, and 5 mM 2-mercaptoethanol, against which the sample had been previously dialyzed at 4 C for 16 h. The protein concentrations used were 0.2 to 0.4 mg/ml. Photographs were taken after 24 h of centrifugation at 20 C and 12,000 rpm. The Yphantis plots were linear for all protein concentrations used (Fig. 3). The molecular weight was calculated to be 300,000 ± 20,000, with a value of 0.74 ml/g as the partial specific volume since it is an average value for globular proteins (15). Molecular weights determined at various protein concentrations fluctuated randomly within experimental error.

From gel filtration on Bio-Gel A-5m, 200 to 400 mesh, the molecular weight of glutamate dehydrogenase was estimated to be 300,000 ± 30,000. The column was calibrated by using phosphorylase a (molecular weight, 370,000), catalase (molecular weight, 230,000), and yeast alcohol dehydrogenase (molecular weight, 150,000).

(ii) **Subunit structure.** Polyacrylamide gel

TABLE 3. *Stability of glutamate dehydrogenase from E. coli B/r* [a]

Solvent system	Activity retained (%)			
	25 C	4 C	−17 C	−70 C
i	100	100	57	87
ii	100	100	70	82
iii	100	100	4	48
iv	100	100	7	47

[a] The activity of the enzyme was assayed after it had been left overnight at room temperature (25 C), 4 C, −17 C, and −70 C in the following solvent systems: (i) water, (ii) 20 mM potassium phosphate (pH 7.2), (iii) 20 mM sodium phosphate (pH 7.2), and (iv) 20 mM sodium phosphate (pH 7.2) containing 2 mM 2-oxoglutarate, 1 mM ethylenediaminetetraacetic acid, and 5 mM 2-mercaptoethanol.

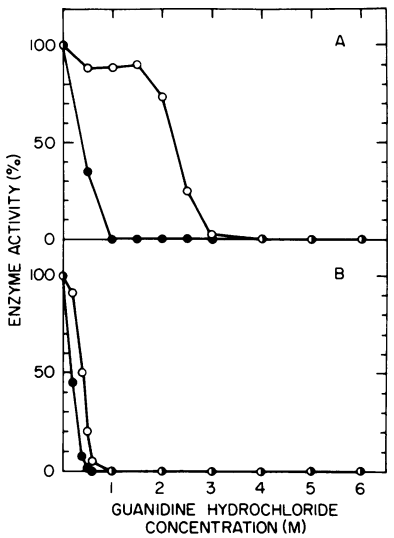

FIG. 2. *Effects of guanidine hydrochloride on the activities of glutamate dehydrogenase and glutamate synthase. The enzymes were treated at 37 C for 2 h with the denaturant and 10 mM dithiothreitol in 20 mM sodium phosphate, pH 7.2. Activity was assayed in the presence and absence of the denaturant. The denaturant was removed by 30- to 600-fold dilution with the assay mixture. Relative activity in the presence (●) and after the removal (○) of the denaturant is plotted against the concentration of the denaturant. (A) Glutamate dehydrogenase; (B) glutamate synthase.*

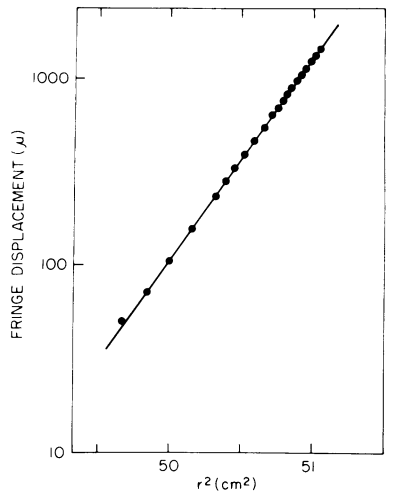

FIG. 3. *Sedimentation equilibrium measurement for glutamate dehydrogenase. A sample, 0.3 mg/ml, previously dialyzed against 50 mM sodium phosphate (pH 7.2) containing 2 mM 2-oxoglutarate, 1 mM ethylenediaminetetraacetic acid, and 5 mM 2-mercaptoethanol, was centrifuged at 12,000 rpm and 20 C. The logarithm of fringe displacement is plotted with respect to the square of the radial distance.*

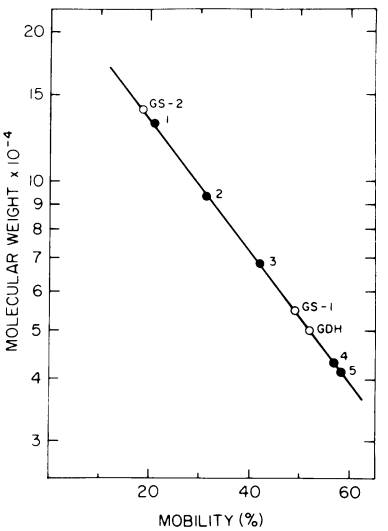

FIG. 4. *Molecular weight estimation of subunits of glutamate dehydrogenase and glutamate synthase by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. The estimated molecular weights for the subunit of glutamate dehydrogenase (GDH) and for the small (GS-1) and the large (GS-2) subunits of glutamate synthase are 50,000 ± 5,000, 55,000 ± 5,000, and 140,000 ± 15,000, respectively. Standard proteins are: (1) β-galactosidase (molecular weight, 130,000), (2) phosphorylase a (molecular weight, 94,000), (3) bovine serum albumin (molecular weight, 68,000), (4) ovalbumin (molecular weight, 43,000), and (5) liver alcohol dehydrogenase (molecular weight, 41,000).*

electrophoresis in the presence of sodium dodecyl sulfate yielded a single band for glutamate dehydrogenase. The molecular weight of the subunit was estimated to be 50,000 ± 5,000 (Fig. 4). By comparison, purified glutamate synthase yielded two distinct bands, and the molecular weights of these subunits were estimated to be 55,000 ± 5,000 and 140,000 ± 10,000.

The molecular weight of the glutamate dehydrogenase subunit was also determined by sedimentation equilibrium measurements in 6.0 M guanidine hydrochloride. The enzyme solutions (0.2 to 0.4 mg/ml) had been previously dialyzed against 6.0 M guanidine hydrochloride and 0.1 M 2-mercaptoethanol in 50 mM sodium phosphate (pH 7.2). The Yphantis plots permitted the calculation of a molecular weight of 48,000 ± 3,000, by using a value of 0.73 ml/g for the partial specific volume of the unfolded polypeptide chain (Fig. 5).

The molecular weights of the native enzyme and the subunit lead us to postulate that glutamate dehydrogenase (molecular weight, 300,000) is a hexamer composed of identical subunits (molecular weight, 50,000).

(iii) Sedimentation studies. Although each of the purified enzymes has only one activity and no species having both ammonia- and glutamine-dependent activities was found, a bifunctional complex of glutamate dehydrogenase and glutamate synthase might have been

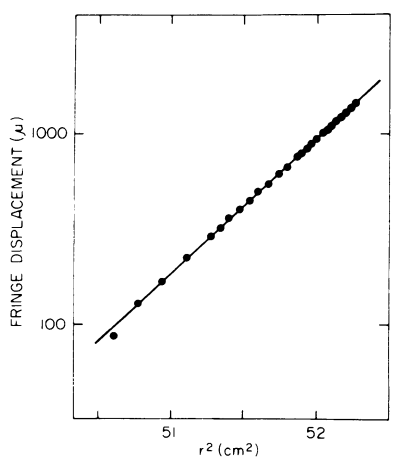

FIG. 5. *Sedimentation equilibrium measurement for glutamate dehydrogenase in 6.0 M guanidine hydrochloride. A sample, 0.3 mg/ml, previously dialyzed against 6.0 M guanidine hydrochloride and 0.1 M 2-mercaptoethanol in 50 mM sodium phosphate (pH 7.2), was centrifuged at 30,000 rpm and 20 C. The logarithm of fringe displacement is plotted with respect to the square of the radial distance.*

lost during purification. Such a complex was suggested for the protein contained in peak II from a DEAE-Sephadex column (Fig. 1) since the peaks of ammonia- and glutamine-dependent activities were superimposable (14). Two active forms of glutamate synthase also have been suggested (13, 14). To explore these possibilities further, we examined the sedimentation behavior of the two enzymes at each step in the purification.

Sedimentation coefficients for the two enzymes were first estimated by sedimentation velocity experiments. Schlieren patterns revealed a single symmetrical peak for each of the purified enzymes throughout the course of the experiment. Glutamate dehydrogenase and glutamate synthase have sedimentation coefficients of 13.3S (at a protein concentration of 2.8 mg/ml) and 19.5S (at a protein concentration of 4.2 mg/ml), respectively, at 20 C in 50 mM sodium phosphate (pH 7.2) containing 1 mM ethylenediaminetetraacetic acid and 10 mM 2-mercaptoethanol.

Purified glutamate dehydrogenase and glutamate synthase, when subjected to ultracentrifugation in sucrose density gradients, yielded single peaks, which on the basis of the above studies were labeled "13S" and "20S," respectively (Fig. 6). The sedimentation pattern of a mixture of the purified enzymes was the same as that obtained when the sedimentation pattern of one purified enzyme was superimposed on that of the other. These sedimentation patterns were used as standards to follow the two enzymes at each step in the purification procedure.

Different sedimentation patterns (Fig. 7a through e') were observed with samples taken at various steps in the purification of the enzymes (Table 1). (a) For the crude extract, ammonia-dependent activity appeared at the 13S position, whereas glutamine-dependent activity appeared at both the 13S and 20S positions. (b) The same pattern was obtained after streptomycin treatment. (c) For the ammonium sulfate fraction (32.5 to 52.5% of saturation) both ammonia- and glutamine-dependent activities were found only at the 13S position. (d) For the fractions of peak I from a DEAE-Sephadex column, glutamine-dependent activity was located only at the 20S position, corresponding to purified glutamate synthase. (d') For the fractions of peak II, on the other hand, a profile similar to that for the ammonium sulfate fraction was obtained but with lower glutamine-dependent activity. (e) Heat treatment of the fractions from peak I did not change the profile seen in Fig. 7d. (e') Similarly, heat treatment of the fractions from peak II did not affect the profile seen in Fig. 7d'.

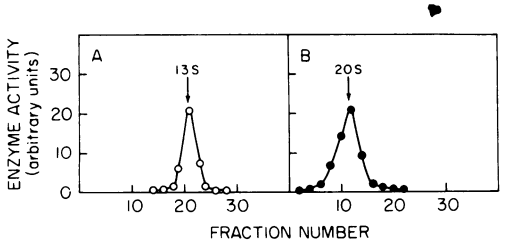

FIG. 6. *Sedimentation in sucrose density gradients of purified glutamate dehydrogenase and glutamate synthase. Gradient preparation, centrifugation, and assays for activity are described in the text. Sedimentation is from right to left. (A) Glutamate dehydrogenase; (B) glutamate synthase.*

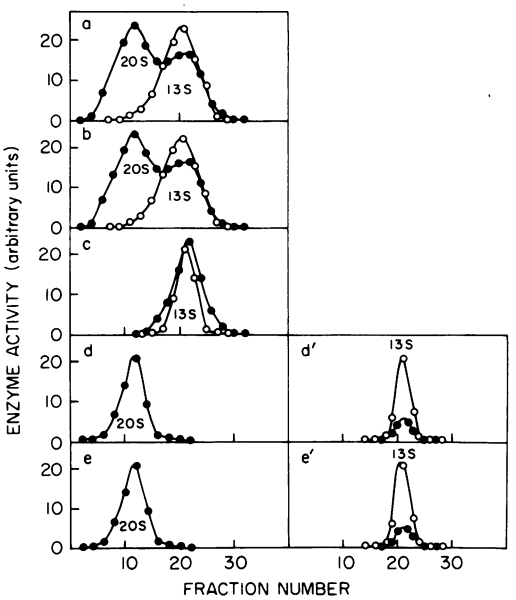

FIG. 7. *Sedimentation in sucrose density gradients of samples from each step in the purification of glutamate dehydrogenase and glutamate synthase. Gradient preparation, centrifugation, and assays for activity are described in the text. Sedimentation is from right to left. Symbols:* (○), *ammonia-dependent activity;* (●), *glutamine-dependent activity.* (a) *Crude extract;* (b) *streptomycin-treated extract;* (c) *ammonium sulfate fraction (32.5 to 52.5% of saturation);* (d *and* d') *fractions of peaks I and II, respectively, from a DEAE-Sephadex column;* (e *and* e') *heat-treated fractions of peaks I and II, respectively, from a DEAE-Sephadex column.*

At each stage in the purification, the peak of ammonia-dependent activity always occurred at 13S, the position of activity for purified glutamate dehydrogenase. On the other hand, the glutamine-dependent activity corresponded to a peak at 13S, 20S, or in some cases at both.

The possible formation of a complex between glutamate dehydrogenase and glutamate syn-

thase was examined by gel filtration and polyacrylamide gel electrophoresis, in addition to the sedimentation experiments reported above. When a crude extract was applied to a column (90 by 1.5 cm) of Bio-Gel A-5m, a peak of ammonia-dependent activity and two peaks of glutamine-dependent activity were eluted, each corresponding to known forms of the enzymes. No peaks having both activities were found at positions corresponding to molecular weights of possible complexes. Gel electrophoresis at pH 7.2 of a mixture of the purified enzymes revealed no new bands corresponding to complexes. In addition, sodium dodecyl sulfate electrophoresis of a mixture amidinated with dimethyl suberimidate, by the method of Davies and Stark (5), yielded no bands other than those corresponding to the components of the two enzymes.

All the foregoing results indicate that the protein in peak II was not a complex but a mixture of the two individual enzymes and that there exists no species having both ammonia- and glutamine-dependent activities.

(iv) Two active forms of glutamate synthase. Although purified glutamate synthase was shown to consist of a single molecular species with nonidentical subunits, under certain conditions this enzyme was converted to a second active form similar to glutamate dehydrogenase in sedimentation coefficient and chromatographic behavior on DEAE-Sephadex. Two active forms of glutamate synthase were suggested by the results of DEAE-Sephadex column chromatography (Fig. 1). When the fractions in peak II were reapplied to a DEAE-Sephadex column, the elution profile of the activity reproduced exactly that of the first DEAE-Sephadex column; glutamine-dependent activity was found in both peak I and peak II. When the single 13*S* glutamate synthase species resulting from ammonium sulfate treatment (Fig. 7c) was chromatographed on a DEAE-Sephadex column, glutamine-dependent activity occurred in both peaks I and II. When the fractions corresponding to either one of the peaks were rechromatographed on a DEAE-Sephadex column, glutamine-dependent activity again appeared in both peaks I and II. Furthermore, sedimentation experiments showed that purified glutamate synthase (20*S*), when treated with ammonium sulfate, is converted to a 13*S* species.

Gel filtration of a crude extract on Bio Gel A-5m yielded two peaks of activity corresponding to apparent molecular weights of 800,000 and 200,000. Since purified glutamate synthase (20*S*) has a molecular weight of 800,000, we inferred that the apparent molecular weight of 200,000 corresponds to the 13*S* species. Gel filtration of the fractions corresponding to peak II from a DEAE-Sephadex column resulted in the separation of the ammonia-dependent activity from the glutamine-dependent activity that was eluted at the position corresponding to an apparent molecular weight of 200,000. These results lead us to propose that glutamate synthase has two interconvertible active forms of different molecular sizes. One form has molecular weight 800,000 and a sedimentation coefficient of 20*S*, and the other has apparent molecular weight 200,000 and a sedimentation coefficient of 13*S*.

DISCUSSION

We have isolated glutamate dehydrogenase from *E. coli* B/r. The purity of the preparation has been established by polyacrylamide gel electrophoresis, analytical ultracentrifugation, and gel filtration. The molecular weight of purified glutamate dehydrogenase is calculated to be 300,000 ± 20,000 from sedimentation equilibrium data (Fig. 3). This method has the least experimental error among those available for the determination of molecular weight. Nevertheless, the molecular weight estimated from gel filtration on Bio-Gel A-5m is in close agreement with that given above. Glutamate dehydrogenase from *E. coli* B/r, then, has a molecular weight similar to that of the homologous enzymes from bovine liver (molecular weight, 320,000) (8), *Neurospora* ($NADP^+$-specific form; molecular weight, 288,400) (2), *Salmonella typhimurium* (molecular weight, 280,000) (3), *Clostridium* (molecular weight, 275,000) (19), *Peptococcus aerogenes* (molecular weight, 266,000) (9), and *Mycoplasma* (molecular weight, 250,000) (20). However, its value is quite different from that for the enzymes of *Thiobacillus novellus* ($NADP^+$- and NAD^+-specific forms; molecular weight 120,000) (10) and *Neurospora* (NAD^+-specific form; molecular weight 480,000) (17).

The molecular weight of the glutamate dehydrogenase subunit is 50,000 ± 5,000, as obtained from polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Fig. 4). This value is in close agreement with that determined from sedimentation equilibrium measurements in 6.0 M guanidine hydrochloride (Fig. 5). From the molecular weights of the native enzyme and its subunit, we propose that glutamate dehydrogenase is a hexamer composed of subunits with identical molecular weight. A similar subunit structure has been reported for this enzyme from bovine liver (8) and for the $NADP^+$-specific form of this enzyme from *Neurospora* (2).

It has been suggested that glutamate syn-

thase from *E. coli* W has two active forms (13). We have observed two active species for the enzyme from *E. coli* B/r. Since these forms appeared in the crude extract, it is possible that two active forms normally exist in vivo. A partial characterization of the two forms has revealed that the larger one has an apparent molecular weight of 800,000 and a sedimentation coefficient of 20S whereas the smaller one has an apparent molecular weight of 200,000 and a sedimentation coefficient of 13S. The sedimentation coefficient of 13S is high for a molecular weight of 200,000, whereas a value of 20S is low for a molecular weight of 800,000, suggesting a substantial deviation from spherical shape for these species.

In contrast to the relatively small number of microbial species from which glutamate dehydrogenase has been purified to homogeneity, there are many species for which the Michaelis constants of this enzyme have been determined in cell-free extracts or partially purified preparations. The K_m values for NADPH and $NADP^+$ and the relative K_m values for glutamate and 2-oxoglutarate are generally similar among species, whereas there is considerable variation with respect to the K_m for ammonia (16-fold) and the K_m for 2-oxoglutarate (10-fold) (4). It is interesting that our K_m values for glutamate dehydrogenase from *E. coli* appear to differ significantly from those of the closely related species *S. typhimurium*. Our K_m values for NADPH, $NADP^+$, and ammonia are approximately 2, 4, and 4 times higher, respectively, than the corresponding K_m values reported for the enzyme from *S. typhimurium*, whereas our K_m values for glutamate and 2-oxoglutarate, respectively, are about 40 and 6 times lower (4). In *S. typhimurium*, the K_m for glutamate is 12.5 times that for 2-oxoglutarate and Coulton and Kapoor (4) have concluded from this that in vivo the glutamate dehydrogenase reaction is essentially unidirectional in favor of glutamate biosynthesis. In *E. coli*, these K_m values only differ by twofold and, thus, if these differences are real, the reaction in this organism may not be entirely unidirectional.

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