Chemical and Physical Properties of *Escherichia coli* Glutamate Decarboxylase*

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ABSTRACT: This manuscript describes some chemical and physical properties of glutamate decarboxylase (EC 4.1.1.15) from *Escherichia coli* W. Attempts to determine the molecular weight of the native enzyme by measuring sedimentation and diffusion coefficients were unsuccessful because the $s:D$ ratio varied considerably with protein concentration ($s^0_{20,w}$ = 12.72 ± 0.35 and $D^0_{20,w}$ = 4.91 ● 0.37 × 10^{-7} cm^2 sec^{-1} at pH 4.5), indicating an associating–dissociating system. Molecular weight determinations by sedimentation equilibrium gave values of 310,000 at pH 4.5; lower molecular weight species were apparent at low protein concentrations and higher pH values.

Sedimentation equilibrium in Gu·HCl and acrylamide gel electrophoresis in sodium dodecyl sulfate gave a molecular weight of 50,000 for the enzyme subunit, confirming recent electron microscopic evidence by others that the native enzyme is a hexamer. Pyridoxal 5′-phosphate analyses, sulfhydryl group titrations, and peptide maps yielded no evidence for nonidentical subunits. The amino acid composition of glutamate decarboxylase is reported; the enzyme has an amino-terminal methionyl residue and a Lys-His-Thr·OH sequence at its carboxyl end.

Glutamate decarboxylase (L-glutamate 1-carboxy-lyase, EC 4.1.1.15) is one of several bacterial amino acid decarboxylases induced by their respective substrates in acidic growth media (Gale, 1940a–c). Partial purifications of *Escherichia coli* glutamate decarboxylase were described by Gale (1946), Umbreit and Gunsalus (1945), Najjar and Fisher (1954), and Shukuya and Schwert (1960a–c). The latter authors proposed that the enzyme was a dimer of mol wt 300,000 whose properties could be profoundly affected by protein concentration and temperature. Recent electron microscopic investigation (Tikhonenko *et al.*, 1968) has indicated that the enzyme has a hexameric structure that can be disrupted by dilution at low temperature.

The purification and crystallization of glutamate decarboxylase from *E. coli* W have been described (Strausbauch *et al.*, 1967). The present publication deals with some chemical and physical properties of the crystalline enzyme, including molecular weight determinations by sedimentation velocity and sedimentation equilibrium techniques, association–dissociation properties, and subunit structure. The amino acid composition of the enzyme and the nature of its end groups are also described. In the following publication, the amino acid sequence of the PLP[1] binding site is reported.

Materials and Methods

Partially purified glutamate decarboxylase was obtained as a by-product of the purification of pyruvate oxidase described by Williams and Hager (1966). The purified material was crystallized and assayed according to the method of Strausbauch *et al.* (1967). All enzyme preparations used were recrystallized three times and displayed specific activities of 110 ± 10 μ-moles of CO_2 per min per mg at pH 4.5, 38°; they exhibited only one band on polyacrylamide gel electrophoresis at pH 7.9 according to Davis (1964) even when as much as 50 μg of protein was applied.

Urea (J. T. Baker Chemical Corp.) was recrystallized from water, and iodoacetate (Eastman Organic Chemicals) from petroleum ether (bp 30–60°). Gu·HCl (Ultra-Pure grade) was obtained from Mann Research Laboratories; 5,5′-dithiobis-(2-nitrobenzoic acid), from Aldrich Chemical Co., Inc.; carboxypeptidase A, diisopropyl phosphorofluoridate treated to eliminate tryptic and chymotryptic activities, was supplied by Worthington Biochemical Corp.; and tosylamidophenylethyl chloromethyl ketone treated trypsin, from Gallard-Schlesinger Chemical Corp. All other reagents were of the highest grade available and used without further purification.

Initially, protein concentrations were determined by the biuret method of Weichselbaum (1946) using crystalline bovine serum albumin as a standard. Later, protein concentrations were determined spectrophotometrically at 280 mμ on the basis of an absorbance index, $A^{1\%}_{280}$, of 17.0 at pH 7.0, as determined on the crystalline enzyme. This value was obtained from several samples in which protein concentration was determined by amino acid analysis according to the method of Walsh and Brown (1962), or by counting the number of fringes obtained from Rayleigh interference patterns in the analytical ultracentrifuge according to the procedure of Perlmann and Longsworth (1948).

Ultracentrifugation experiments were performed in a Spinco Model E analytical ultracentrifuge equipped with electronic

* From the Department of Biochemistry, University of Washington, Seattle, Washington. *Received October 13, 1969.* This work was supported by grants from the National Science Foundation (GB-3249) and from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health (AM-7902). It was taken in part from a thesis presented to the Graduate Faculty of the University of Washington in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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[1] Abbreviations used are: PLP, pyridoxal 5′-phosphate; Gu·HCl, guanidine hydrochloride; $M_{n,r}$, $M_{w,r}$, and $M_{z,r}$, the number-, weight-, and z-average molecular weights, respectively, at individual radial distances r.

speed control and a schlieren optical system focused at the two-thirds plane of the cell (Svensson, 1954, 1956; Yphantis, 1964). The enzyme was diluted into, and exhaustively dialyzed against, the buffer chosen for a particular experiment.

Sedimentation velocity experiments were performed at 52,640 rpm in Kel-F 2° double-sector cells with 12-mm optical paths. Displacement of sedimenting boundaries was recorded on Kodak metallographic plates and measured with a Nikon two-dimensional microcomparator. For concentration dependence studies, sedimentation coefficients were evaluated at initial solute concentrations, and the best fit was determined by the method of least squares.

Diffusion coefficients were also determined in the analytical ultracentrifuge employing a double-sector, capillary-type synthetic boundary cell. Experiments were performed at 3000 rpm, where sedimentation of the protein and convectional disturbances were negligible. The diffusion coefficient was determined by the inflection point method; the concentration dependence was evaluated at one-half the initial solute concentration and the best fit was obtained by the method of least squares.

Sedimentation equilibrium experiments were performed by the high-speed method of Yphantis (1964), employing six-channel Kel-F cells with 3-mm columns. A small amount of fluorocarbon FC-43 was added to each channel so that as much as possible of the protein solution was visible. The speed of centrifugation was chosen so that the concentration of material near the meniscus approached zero. Most experiments were performed with 0.4–0.8 mg/ml of protein and reached equilibrium in 18–24 hr. Interference patterns were analyzed on a modified Nikon microcomparator according to the method of Teller (1967) until fringes could no longer be resolved. Data reduction was performed on the IBM 7090–7094 ISYS system using a computer program developed by Teller *et al.* (1969); point-average molecular weight moments as a function of protein concentration are given directly by this program.

For ultracentrifuge experiments under denaturing conditions glutamate decarboxylase was dissolved in 6 M Gu·HCl containing 0.05 M Tris·HCl and 2.5×10^{-3} M EDTA (pH 7.5) and equilibrated by dialysis against this solvent for 1 week; dilutions were made with the same solvent. Prior to the ultracentrifugations, β-mercaptoethanol (0.1 M final) was added to all solutions.

Densities were determined in a pycnometer; a partial specific volume of 0.735 cc/g was calculated from the amino acid composition according to the method of McMeekin and Marshall (1952).

Polyacrylamide gel electrophoresis of the enzyme was carried out in 6% gels in the buffer system of Davis (1964); the gels were stained with aniline blue black in 7.5% acetic acid. For a study of the subunit structure of the enzyme, electrophoresis was also carried out in 0.1% sodium dodecyl sulfate according to Shapiro *et al.* (1967). The sodium dodecyl sulfate treated gels were stained with coomassie brilliant blue, then stored in 7.5% acetic acid.

Pyridoxal 5'-phosphate content of protein solutions was determined by the phenylhydrazone method of Wada and Snell (1961) after deproteinization with 0.3 N perchloric acid. A fresh PLP standard solution was prepared daily, based on a molar absorbance index of 6600 at 388 mμ in 0.1 N NaOH (Peterson and Sober, 1954).

Amino Acid Analyses. Samples from two separate preparations of three-times-recrystallized glutamate decarboxylase were dialyzed for 16 hr against 0.1 M NaCl, then exhaustively against twice-distilled water. Duplicate protein samples from each preparation were hydrolyzed in oxygen-free sealed tubes in 5.7 M HCl at 107 ± 1° for periods of 20, 42, 65, and 94 hr according to the procedure of Moore and Stein (1963); norleucine was added as an internal standard (Walsh and Brown, 1962). Analyses were performed on a Spinco Model 120C amino acid analyzer according to the method of Spackman *et al.* (1958).

For supporting analyses, total cysteine and cystine content was determined following performic acid oxidation according to the procedure of Moore (1963), as modified by Hirs (1967). Determination of sulfhydryl groups was performed according to Ellman (1959) on both native and sodium dodecyl sulfate denatured enzyme following dialysis of the protein as described above. Final concentrations were 0.1–0.3 mg/ml of decarboxylase, 5×10^{-4} M 5,5'-dithiobis(2-nitrobenzoic acid), 0.1 M sodium phosphate, 10^{-4} M EDTA buffer (pH 8.0), and 0.2% sodium dodecyl sulfate when added. Absorbance measurements were made at 412 mμ at 2-min intervals until the values remained constant. Tryptophan content was determined spectrophotometrically by the method of Bencze and Schmid (1957), and by the colorimetric procedure of Spies and Chambers (1949).

Peptide maps of tryptic digests of the enzyme were made according to the method of Katz *et al.* (1959) following carboxymethylation of the sulfhydryl groups in the dark and at pH 8.0 according to the procedure of Canfield and Anfinsen (1963). Descending chromatography was carried out in 1-butanol–pyridine–acetic acid–water (45:30:9:36) employing phenol red as a marker. Since no peptide migrated faster than the marker, chromatography was continued until the phenol red was within 1 in. of the bottom of the paper. Chromatography was followed by high-voltage electrophoresis for 70 min at 50 V/cm in pyridine:acetic acid:water, 1:10:389, pH 3.6 (Michl, 1951). Papers were stained with both ninhydrin and Pauly's histidine reagent.

End-Group Analyses. The carboxyl-terminal sequence of glutamate decarboxylase was determined by carboxypeptidase A digestion of carboxymethylated protein in the presence of 0.1% sodium dodecyl sulfate (Guidotti, 1960). At various time intervals, aliquots containing 0.1 μmole of decarboxylase were removed and precipitated with 25% trichloroacetic acid; the supernatant was extracted three times with an equal volume of ether to remove excess trichloroacetic acid and dried in a vacuum desiccator. Quantitative analysis of the amino acids released was performed on the amino acid analyzer.

The amino-terminal group was determined according to the isocyanate procedure of Stark and Smyth (1963). Glutamate decarboxylase (0.2–0.5 μmole) was dissolved in 5 ml of 8 M urea containing *N*-ethylmorpholine–acetate buffer (pH 8.0) and carbamylation was carried out with KCNO for 18 hr at 50°. A control sample was subjected to the same treatment except that no KCNO was added. Correction for losses was made as described by Stark and Smyth (1963).

Results

Molecular Weight Determination. Sedimentation velocity experiments were performed on the three-times-crystallized

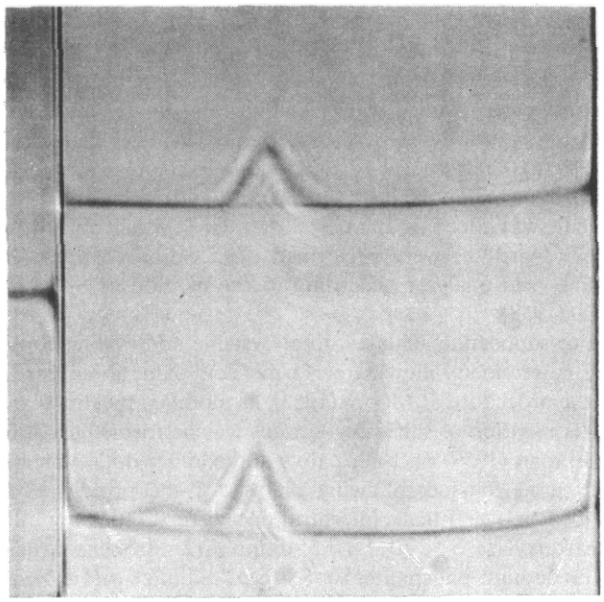

FIGURE 1: Sedimentation velocity patterns of glutamate decarboxylase in 0.1 M pyridine·HCl buffer (pH 4.5) (upper pattern) and in 0.1 M sodium phosphate buffer (pH 7.0) (lower pattern). Experiments were run at 2.0 and 2.5 mg per ml of enzyme, respectively, and at 52,640 rpm and 20°. Pictures were taken after 24 min.

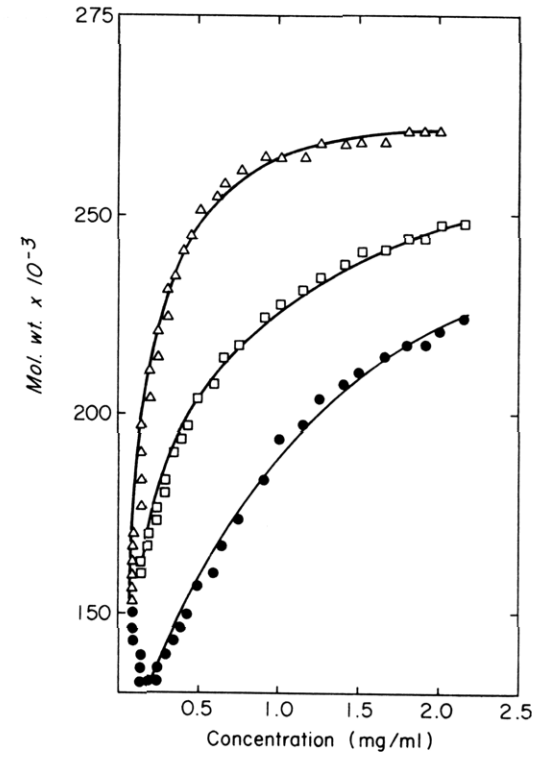

FIGURE 3: High-speed ultracentrifuge equilibrium of glutamate decarboxylase in 0.1 M sodium phosphate buffer, pH 7.0, 20°. Representative distribution of molecular weight moments as obtained from the computer program of Teller (1967). □ represents $M_{n,r}$; △ represents $M_{w,r}$; and ● represents $2M_{n,r} - M_{w,r}$. Experiments were run for 32 hr at 12,000 rpm.

enzyme at both pH 4.5 and 7.0 (Figure 1, upper and lower patterns, respectively) and exclusively at 20.0° since previous work had indicated that the enzyme undergoes inactivation (Shukuya and Schwert, 1960c) and disaggregation (Tikhonenko *et al.*, 1968) at low temperature. At pH 7.0, the enzyme showed two sedimenting peaks, one of 11.5 S representing 80–90% of the material, and a second of 6.0 S (10–20%). The same material showed a single symmetrical peak when experiments were carried out at pH 4.5 (Figure 1, upper pattern), with a sedimentation constant of 12.3 S similar to that of the major component observed at pH 7.0. Clearly, the double peak seen at the higher pH value results from dissociation of the enzyme; therefore, molecular weight determinations by sedimentation and diffusion coefficient measurements were carried out exclusively at the lower pH value.

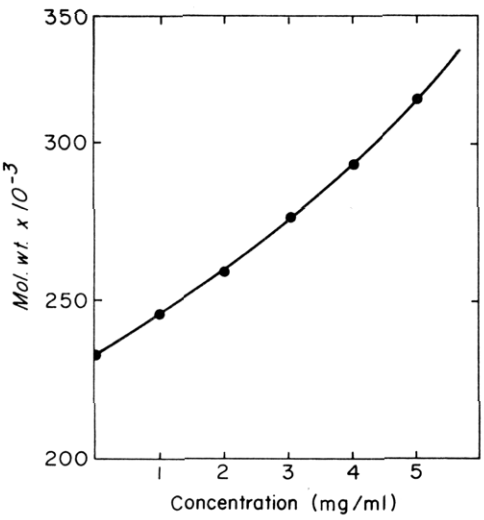

FIGURE 2: Variation of molecular weight as a function of protein concentration as computed from s and D values obtained from a series of measurements in 0.1 M pyridine·HCl buffer, pH 4.5, 20°.

Determination of sedimentation and diffusion as a function of enzyme concentration showed that both these parameters exhibit a rather large concentration dependence. Each determination was carried out at six concentrations of enzyme ranging from 1.5 to 5.4 mg per ml and rectilinear extrapolation to zero concentration was performed using a least-squares equation of the form $s_{20} = s^0_{20}(1 - KC)$, where K is an expression of the concentration dependence (slope). Values for $s^0_{20,w}$ of 12.72 ± 0.35 S and for $D^0_{20,w}$ of 4.91 ± 0.37 × 10^{-7} cm^2 sec^{-1} were obtained. A strong concentration dependence was revealed by the steepness of the slopes obtained for both sedimentation (−0.082 ml/mg) and diffusion (−0.280 ml/mg); this is indicative of an associating–dissociating system. Since s and D were affected to different extents by changes in protein concentration, the s:D ratio varied considerably as a function of protein concentration, and the calculated molecular weight values (using a partial specific volume of 0.735 ml/g, as computed from the amino acid analysis) varied widely with enzyme concentration (Figure 2). Obviously, this approach was unsuited for the determination of the molecular weight of the native enzyme.

In order to obtain this value, sedimentation equilibrium experiments were carried out at both pH 7.0 and 4.5. Representative distributions of molecular weight moments as a function

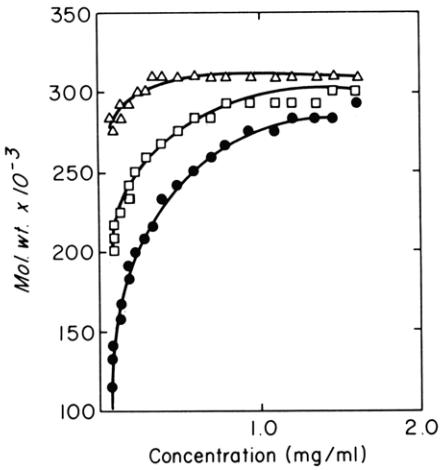

FIGURE 4: High-speed ultracentrifuge equilibrium run of glutamate decarboxylase in 0.1 *M* pyridine·HCl buffer, pH 4.5, 20°. Representative distribution of molecular weight moments as obtained from the computer program of Teller (1967). □ represents $M_{n,r}$; △ represents $M_{w,r}$; and ● represents $2M_{n,r} - M_{w,r}$. Experiments were run for 32 hr at 12,000 rpm.

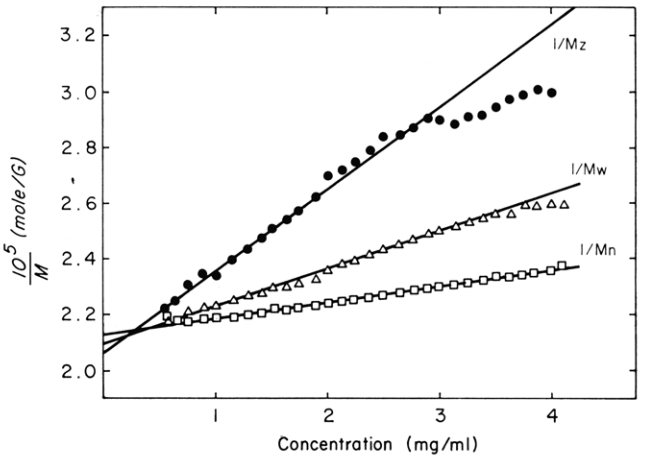

FIGURE 5: Reciprocal number-average, weight-average, and *z*-average molecular weight moments as a function of glutamate decarboxylase concentration. □ represents $M_{n,r}$; △ represents $M_{w,r}$; and ● represents $M_{z,r}$. Experiments were performed in 0.05 M Tris·HCl–0.1 M β-mercaptoethanol buffer (pH 7.0) in the presence of 6 M guanidine·HCl, 20°. Experiments were run for 31 hr at 34,000 rpm.

of protein concentration at pH 7.0 and 20° are illustrated in Figure 3. At all concentrations considered, the molecular weight distribution falls drastically on dilution of the protein, indicating that considerable heterogeneity exists with respect to the size of the molecular species present. Data reduction of the point-average molecular weight moments according to Teller *et al.* (1969) indicates the presence of 63% heavy component (whole cell average) with number-average $M_{n,r}$ 338,760 and weight-average $M_{w,r}$ 334,475. Similar data were obtained in 0.10 M pyridine·HCl buffer (pH 4.5) (Figure 4). Again, data reduction indicates a strong tendency for the enzyme to dissociate with decreasing protein concentration; at this pH the equilibrium between the different molecular species favors the formation of the heavy component and a good correlation between the number-average (295,000) and weight-average (310,000) molecular weights can be seen. No change in the distribution of molecular weight was observed upon addition of 10^{-4} M PLP, 0.01 M β-mercaptoethanol, or 10^{-3} M EDTA. On the basis of a subunit molecular weight of *ca.* 50,000, as described below, it would appear that the heavy component observed in the ultracentrifuge is a hexamer, as has been seen by electron microscopy (Tikhonenko *et al.*, 1968). Preliminary results indicate that the smaller molecular structures observed at lower protein concentrations may be dimers and tetramers.

Subunit Structure. Sedimentation equilibrium experiments were carried out in 0.05 M Tris·HCl–0.1 M β-mercaptoethanol buffer (pH 7.0) and 6 M Gu·HCl. In Figure 5, reciprocal weight-average, number-average, and *z*-average molecular weight moments as a function of enzyme concentrations are illustrated for a typical run. Treatment of the data in this fashion assumes homogeneity of the protein and eliminates thermodynamic nonideality by extrapolation to infinite dilution. The plots yield straight lines with slopes proportional to the virial coefficients and intercepts determined by the reciprocal molecular weight of the material. From three runs performed at different enzyme concentrations, values of M_n = 47,050 ± 200 g/mole, M_w = 50,070 ± 200 g/mole, and M_z =

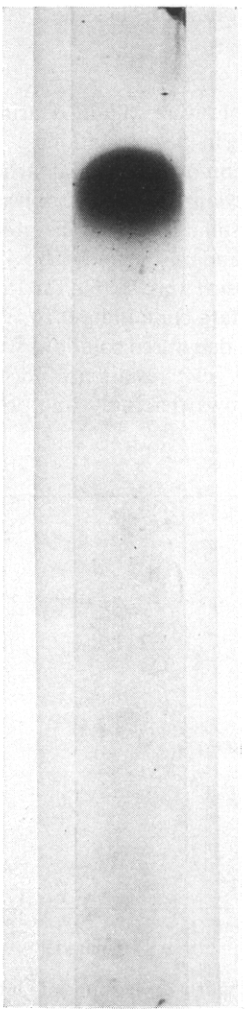

FIGURE 6: Polyacrylamide gel electrophoresis of sodium dodecyl sulfate denatured glutamate decarboxylase. Conditions are described in text; 7 μg of protein was applied to 5% gel and electrophoresis was run for 5 hr at 8 V/cm.

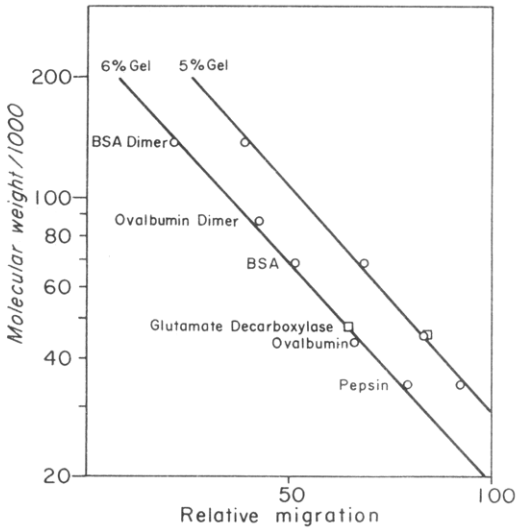

FIGURE 7: Molecular weight determination of glutamate decarboxylase subunit from polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Semilog plot of polypeptide molecular weight against distance of migration relative to the front in 0.1% sodium dodecyl sulfate polyacrylamide gels. □ represents glutamate decarboxylase, and ○ various protein standards of known molecular weight. Conditions are described in text.

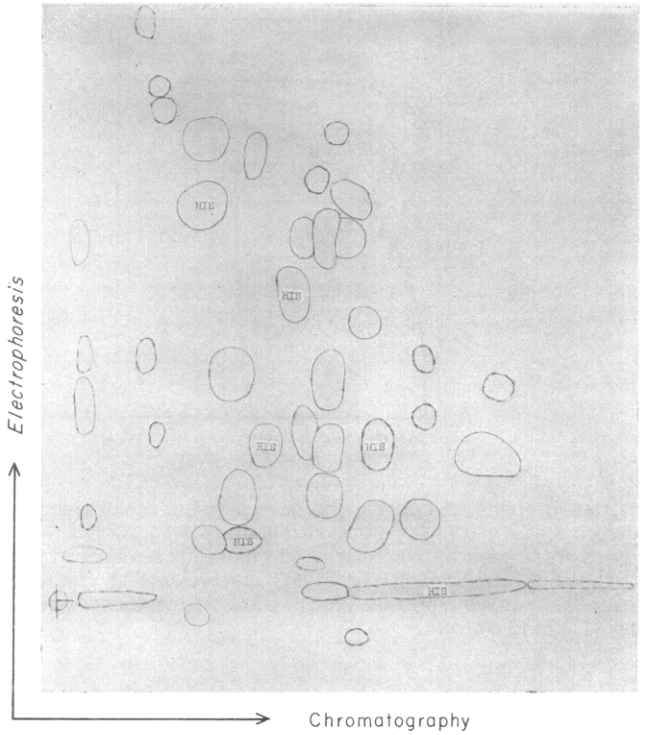

FIGURE 9: Peptide map of trypsin-digested carboxymethylated glutamate decarboxylase. Ninhydrin positive spots are outlined; Pauly positive, histidine-containing peptides are marked (His). Conditions are described in text.

49,090 ± 300 g/mole were obtained using a partial specific volume of 0.735 ml/g in the calculations.

Further information on the subunit structure of the enzyme was obtained by acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Crystalline glutamate decarboxylase (and protein standards, namely, bovine serum albumin, ovalbumin, and pepsin) were heated for 15 min at 60° in 0.1% sodium dodecyl sulfate containing 0.14 M β-mercaptoethanol (pH 7.2). The clear, denatured solutions were applied to 5 and 6% polyacrylamide gels; results are illustrated in Figures 6 and 7. Figure 6 shows that only one protein band appears after electrophoresis of denatured glutamate decarboxylase and supports once more the conclusion that the two peaks seen during ultracentrifugation at pH 7.0 represent two (or more) aggregation states of the same molecule rather than contamination by foreign material. Figure 7 indicates that the sodium dodecyl sulfate denatured enzyme has a molecular weight between 45,500 and 48,000.

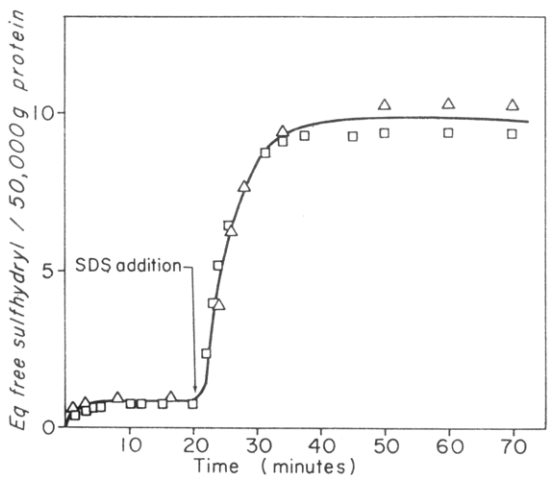

FIGURE 8: Titration of sulfhydryl groups of glutamate decarboxylase in the absence and presence of 0.2% sodium dodecyl sulfate (SDS). The determinations were performed at a protein concentration of 0.11 mg/ml (□-□) and 0.24 mg/ml (△-△) in 0.1 M sodium phosphate buffer, pH 8.0, 20° containing 10^{-4} M EDTA and 5×10^{-4} M 5,5′-dithiobis(2-nitrobenzoic acid).

Early analyses for PLP in various crystalline samples gave results varying from 1 mole of cofactor/50,000–70,000 g of protein. These preparations were extensively dialyzed at 4° prior to analysis in order to remove possible unspecifically bound PLP; the concentration of enzyme was kept at 15 mg/ml or more to minimize disaggregation. It was found, however, that when these dialyses were conducted at low pH values (*e.g.*, in 0.05 M pyridine·HCl buffer, pH 5.0 or below) a precipitate formed in those samples which later displayed a low PLP content. Analyses of the precipitated material and mapping of the tryptic peptides indicated that it consisted of apparently unstable, resolved enzyme. Elimination of this inactive precipitate produced more consistent PLP analyses, with values falling between 1 mole of PLP/50,000–55,000 g of protein. This agrees well with the minimum subunit molecular weight obtained above.

Amino Acid Composition. The amino acid analysis was carried out on two samples obtained from separate preparations of the enzyme. For simplicity, the values presented in Table I are the averages obtained from four times of hydrolysis; also presented are integral number of residues per 50,000 g of protein, the closest estimate for the molecular weight of the enzyme subunit.

TABLE I: The Amino Acid Composition of *E. coli* Glutamate Decarboxylase.

Amino Acid	g of Amino Acid Residues/100 g			Residue	
	Run 1	Run 2	Average[a]	Per 50,000 g	Nearest Integer
Lysine	5.61	5.77	5.69 ± 0.11	22.2	22
Histidine	2.12	2.26	2.19 ± 0.10	8.0	8
Ammonia[b]	1.29		1.29	40.3	40
Arginine	7.52	7.60	7.56 ± 0.06	24.2	24
Aspartic acid[c]	11.43	11.46	11.44 ± 0.05	49.7	50
Threonine[b]	3.48	3.52	3.50 ± 0.08	17.3	17
Serine[b]	2.95	2.98	2.97 ± 0.03	17.0	17
Glutamic acid[c]	12.17	11.80	11.99 ± 0.26	46.4	46
Proline	4.70	5.12	4.89 ± 0.20	25.2	25
Glycine	3.98	3.86	3.92 ± 0.09	34.3	34
Alanine	5.66	5.50	5.58 ± 0.11	39.2	39
Valine[d]	4.64	4.45	4.55 ± 0.13	22.9	23
Methionine	3.58	3.74	3.66 ± 0.11	13.9	14
Isoleucine[d]	5.68	5.73	5.70 ± 0.04	25.2	25
Leucine[d]	8.97	8.99	8.98 ± 0.01	39.6	40
Tyrosine	4.95	4.94	4.94 ± 0.01	15.1	15
Phenylalanine	6.42	6.34	6.38 ± 0.06	21.6	22
Half-cystine[e]	2.09		2.09 ± 0.15	10.2	10
Tryptophan[f]	3.98		3.98 ± 0.19	10.6	11
Total[g]			100.00		442

[a] Given with standard deviation. [b] Determined by extrapolation to zero time of hydrolysis. [c] Includes both amide and free acid. [d] Determined at maximum time of hydrolysis in order to allow for complete release. [e] Determined both with Ellman's reagent and as cysteic acid (see text). [f] Determined spectrophotometrically and colorimetrically (see text). [g] Total does not include ammonia.

Sulfhydryl Group. Titrations using Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid)) are illustrated in Figure 8. The enzyme was dissolved in 0.1 M sodium phosphate and 10^{-4} M EDTA (pH 8.0) and titration was carried out in the absence and the presence of 0.2% sodium dodecyl sulfate. The calculations were based on a molar absorbance index of 13,600 at 412 mμ (Ellman, 1959). One sulfhydryl group per 50,000 g is titrated in the absence of sodium dodecyl sulfate and nine additional groups become available after denaturation. The value of ten sulfhydryl groups titrated in sodium dodecyl sulfate agrees with that obtained by performic acid oxidation, indicating that the enzyme contains no disulfide bond.

Peptide Maps. Glutamic decarboxylase was carboxymethylated as described in Methods and the substituted protein was digested with tosylamidophenylethyl chloromethyl ketone treated trypsin at a molar ratio of 1:40, pH 8.3 and 37°. Base uptake was very rapid for the first 10 min and then leveled off; little difference was observed between peptide maps obtained from periods of digestion between 1 and 9 hr. A map from a 3-hr digest is illustrated in Figure 9. The sample applied was clear, with no evidence for the presence of an undigested "core." From the amino acid analysis presented in Table I, the appearance of 47 peptides can be predicted if the subunits consist of identical peptide chains of mol wt 50,000; 43–46 peptide spots were indeed counted. Likewise, 8 histidine-containing peptides would be predicted and 5–7 Pauly-positive spots were observed.

End-Group Analysis. Carboxypeptidase A digestion conditions were found under which attack of the carboxymethylated enzyme could proceed with the release of only a limited number of residues, and at a rate such that analysis of successive samples would provide evidence on the structure of the carboxyl end of the protein. A total of 28 mg of carboxymethylated protein was attacked by carboxypeptidase A at a molar ratio of 1:34 in the presence of 0.1% sodium dodecyl sulfate in *N*-ethylmorpholine–acetate buffer, pH 8.0, 31°. Aliquots were withdrawn and analyzed at various times; the data are presented in Figure 10. Blanks containing either carboxypeptidase alone or enzyme alone showed no release of amino acid. From these results, the carboxyl-terminal sequence (. . . Phe,Glu)Lys-His-Thr · OH can be inferred. On the basis of a molecular weight of 50,000 for the enzyme monomer, 0.81 equiv of threonine was liberated.

It was hoped that further confirmation of subunit size could be obtained from the cyanate procedure of Stark and Smyth (1963) for determining the amino-terminal residue of the enzyme. Since the carbamylated protein was insoluble following removal of urea and excess reagents, quantitative analysis was based on amino acid analysis of an aliquot removed after cyclization of the carbamyl protein. Some of the fractions

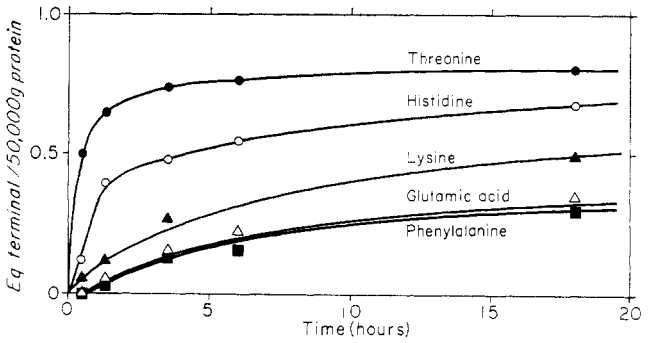

FIGURE 10: Carboxyl-terminal determination of glutamate decarboxylase by carboxypeptidase A. Conditions are described in text.

were subjected to an additional hydrolysis in 3 M HCl to destroy pyrrolidonecarboxylic acid. When such a treatment was included, 0.36 mole of NH_2-terminal methionine/50,000 g of protein was obtained and no other amino acid residue was detected. Without this additional step, the yield of methionine was increased to 0.44 mole/50,000 of g protein, but significant amounts of glutamic acid and glycine appeared in the analysis. Analyses were also performed in the presence of serotonin to check for an amino-terminal tryptophanyl residue, but none was detected. Further attempts to determine the amino-terminal groups of the enzyme using leucine aminopeptidase were unsuccessful.

Discussion

The data presented herein show that glutamate decarboxylase is an oligomeric protein composed of identical or nearly identical subunits. In that sense, it is similar to other PLP-containing decarboxylases and, for that matter, to PLP-containing enzymes in general (see Boeker and Snell, 1968). Determinations of the molecular weight of the native enzyme from sedimentation and diffusion coefficient measurements were ambiguous because the enzyme behaves as an associating–dissociating system; the calculated values increased from 240,000 to over 350,000 with increasing enzyme concentration. This behavior led to an erroneous preliminary assignment of a molecular weight of 365,000 to the crystalline enzyme (Strausbauch *et al.*, 1967). Sedimentation equilibrium experiments, however, indicate that the native decarboxylase has a molecular weight of 300,000 ± 10,000; but even here, a degree of uncertainty is introduced by the associating–dissociating properties of the protein. The molecular weight of the subunits of glutamate decarboxylase was therefore determined as accurately as possible, using a number of physical and chemical approaches. Both ultracentrifugal analysis in Gu·HCl and polyacrylamide gel electrophoresis in sodium dodecyl sulfate gave values of 50,000 for the subunit molecular weight with no evidence for heterogeneity with respect to size.

Several lines of evidence support the conclusion that the subunits of glutamate decarboxylase are identical. A single sulfhydryl group is titrated by 5,5′-dithiobis(2-nitrobenzoic acid) per enzyme monomer. Carboxypeptidase A digestion produces sequential release of threonyl, histidyl, and lysyl residues in amounts commensurate with a subunit molecular weight of *ca.* 50,000. Peptide maps have approximately the number of spots predicted for identical subunits of this molecular weight. PLP analyses show that 1 mole of coenzyme is bound per 50,000 g. Finally, as discussed in the following manuscript, sequence analysis indicates that there is a single PLP binding site of unique structure.

An element of uncertainty is introduced by the results of amino-terminal analyses. Only 0.44 equiv of methionine/50,000 g of protein was obtained, to the exclusion of any other amino acid. Whether this low value results from an exceptionally poor yield or from a partially blocked NH_2-terminal residue (Marcker and Sanger, 1964) is not known. Attempts to eliminate this uncertainty using leucine aminopeptidase were unsuccessful.

The ultracentrifuge experiments have shown that the enzyme tends to dissociate at low concentrations in the absence of substrate. Despite these results and the fact that a detailed study of the correlation between the quaternary structure and the activity of this enzyme has not been carried out, several observations indicate that it is the associated species (mol wt 300,000) which is enzymatically active: (a) while dissociation increases with pH and becomes prominent near neutrality, the optimum activity of the enzyme is at pH 3.8; (b) conditions such as low temperature which favor dissociation also lead to inactivation (Shukuya and Schwert, 1960c; Tikhonenko *et al.*, 1968); and (c) there is no decrease of activity at enzyme concentrations up to 0.1 mg/ml as one would expect if the associated form were inactive. The dissociation of glutamate decarboxylase at high pH values is similar to that observed by Boeker and Snell (1968) for *E. coli* arginine decarboxylase. Further comparisons of the chemical, physical, and immunological properties of *E. coli* decarboxylases are being pursued.

Acknowledgments

The authors wish to express their deep appreciation to Dr. Lowell Hager, University of Illinois, for generous supplies of the starting material. They also wish to acknowledge the excellent help of Mrs. Terry Duewer in connection with the ultracentrifuge experiments, and of Mr. Richard B. Olsgaard for the amino acid analyses. Their thanks also go to Dr. David C. Teller for his invaluable advice and help in interpreting the ultracentrifuge data.

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Structure of the Binding Site of Pyridoxal 5′-Phosphate to *Escherichia coli* Glutamate Decarboxylase*

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ABSTRACT: Pyridoxal 5′-phosphate is bound to *Escherichia coli* glutamate decarboxy-lyase (EC 4.1.1.15) in two spectral forms with absorption maxima at 340 and 415 nm. The second species which is formed at low pH and is attributed to a Schiff base was reduced with $NaBH_4$ to produce an inactive enzyme in which pyridoxal 5′-phosphate was found to be covalently bound to an ε-amino group of a lysyl residue. This derivative was digested with trypsin and the peptide containing the 5′-phosphopyridoxyl residue was isolated by a combination of gel filtration and ion exchange chromatography. Its amino acid sequence was determined from a sequential Edman degradation coupled with end group determinations using Dansyl·Cl and from a carboxypeptidase A digestion; the following structure was obtained: Ser-Ile-Ser-Ala-Ser-Gly-His-(PylP)Lys-Phe. An improved procedure for the isolation of 5′-phosphopyridoxyl containing peptides, based on the "diagonal" procedure of Brown and Hartley (Brown, J. R., and Hartley, B. S. (1963), *Biochem. J. 89*, 59P), is described; it takes advantage of the altered elution of these peptides from Dowex after digestion with alkaline phosphatase.

The spectral properties of PLP[1] in glutamate decarboxylase were studied in detail by Shukuya and Schwert (1960b). They found that at pH values below 5, the enzyme has an absorption maximum at 415 nm which diminishes as the pH is increased, with the concomitant appearance of a new peak at 340 nm. This spectral shift was similar to those described earlier for other PLP-containing enzymes (Kent *et al.*, 1958; Jenkins and Sizer, 1959) and was attributed to the formation of a substitution aldimine from a Schiff base. Anderson and Chang (1965) were able to bind PLP covalently to an ε-amino group of a lysyl residue of glutamate decarboxylase by reducing the "415 nm" form of the enzyme with $NaBH_4$ according to the procedure of Fischer *et al.* (1958). Since, in the case of glycogen phosphorylase, it was found that $NaBH_4$ reduction caused no transfer of PLP to other sites on the protein, and no gross change in the architecture of the enzyme (Fischer *et al.*, 1958), a unique tool appeared to be available for deter-

* From the Department of Biochemistry, University of Washington, Seattle, Washington. *Received October 3, 1969.* This work was supported by grants from the National Science Foundation (GB-3249) and from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health (AM-7902). It was taken in part from a thesis presented to the Graduate Faculty of the University of Washington in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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[1] The abbreviations used for pyridoxal 5′-phosphate and its derivatives are those tentatively accepted by the Commission on Biochemical Nomenclature, IUB, namely, PLP, pyridoxal 5′-phosphate; Pxy, pyridoxyl; 5′-*P*-Pxy, pyridoxyl 5′-phosphate. Dansyl is dimethylaminonaphthalenesulfonyl.