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Tripeptide (Glutathione) Synthetase. Purification, Properties, and Mechanism of Action*

Elizabeth Dodd Mooz and Alton Meister

ABSTRACT: Tripeptide (glutathione, γ-glutamyl-α-aminobutyrylglycine) synthetase has been purified about 5000-fold from Baker's yeast; the enzyme is homogeneous by electrophoretic and ultracentrifugal criteria (sedimentation coefficient 6.1 S, mol wt 123,000). Various properties of the enzyme including its amino acid composition, kinetic behavior, and specificity have been studied. The ability of the enzyme to catalyze adenosine triphosphate–adenosine diphosphate (ATP–ADP) exchange decreases greatly during purification. Evidence has been obtained that the rate of formation of the enzyme-bound dipeptide intermediate (γ-glutamyl-α-aminobutyryl phosphate) in the absence of acceptor is of the same order as that of the over-all reaction, and that the ADP formed in this reaction dissociates relatively slowly from the enzyme. It is tentatively concluded there are about four active sites per molecule of enzyme.

Previous studies in this laboratory have provided evidence that the enzymatic synthesis of tripeptides [glutathione, ophthalmic acid (γ-glutamyl-α-aminobutyrylglycine)], which takes place according to eq 1,

$$\gamma\text{-glutamylamino acid} + \text{glycine} + \text{ATP}^{1} \underset{Mg^{2+}}{\overset{\text{synthetase}}{\rightleftharpoons}} \text{tripeptide} + \text{ADP} + P_i \quad (1)$$

involves the intermediate formation of an enzyme-bound carboxyl-activated dipeptide derivative, *i.e.*, γ-glutamylaminoacyl phosphate. Pulse-labeling experiments provided the initial evidence for such an intermediate; subsequently a compound with the properties of γ-glutamyl-α-aminobutyryl phosphate was isolated from reaction mixtures containing the yeast synthetase, γ-glutamyl-α-aminobutyrate, ATP, and magnesium ions (Nishimura *et al.*, 1963). It was later found that the enzyme could utilize chemically synthesized γ-glutamyl-α-aminobutyryl phosphate for both the synthesis of ATP and of γ-glutamyl-α-aminobutyrylglycine (Nishimura *et al.*, 1964).

The present studies were undertaken in an effort to purify the enzyme further and to obtain additional information about its properties and the mechanism of the reaction. In the course of this work a preparation of the enzyme from yeast has been achieved which is essentially homogeneous by electrophoretic and ultracentrifugal criteria. Various properties of the enzyme, including its molecular weight and amino acid composition, have been determined. Further

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[1] Abbreviations used: ADP and ATP, adenosine di- and triphosphates; DPNH, reduced diphosphopyridine nucleotide.

information concerning the enzyme-bound intermediate has been obtained and an estimate has been made of the number of binding sites per molecule of enzyme.

Experimental Section

Materials

Glutathione, bovine serum albumin, phosphoenolpyruvate (tricyclohexylammonium salt), pyruvate kinase, lactate dehydrogenase, DPNH, ATP, and ADP were obtained from the Sigma Chemical Co. ADP-8-^{14}C and ATP-8-^{14}C were purchased from Schwarz BioResearch, Inc. Glycine-1-^{14}C was obtained from New England Nuclear Corp. Sephadex G-200 and DEAE-cellulose (lot no. 1064) were obtained from Pharmacia and Brown Co., respectively. L-γ-Glutamyl-L-α-aminobutyric acid was prepared from the unlabeled amino acids or from uniformly labeled L-glutamate-^{14}C as previously described (Nishimura *et al.*, 1963, 1964) and also by enzymatic transpeptidation using purified hog kidney γ-glutamyl transpeptidase (Orlowski and Meister, 1965; Mooz, 1967). γ-L-Glutamylcysteine (Strumeyer and Bloch, 1962) and the γ-glutamyl peptides listed in Table III (Otani and Meister, 1957) were prepared as described.

Methods

Determination of Enzyme Activity. Enzymatic activity was determined by measuring the rate of formation of γ-glutamyl-α-aminobutyrylhydroxamate as follows. The reaction mixtures contained Tris-HCl buffer (50 μmoles, pH 8.3), potassium chloride (50 μmoles), magnesium sulfate (5 μmoles), ATP (2.5 μmoles), γ-L-glutamyl-L-α-aminobutyric acid (2.5 μmoles), salt-free hydroxylamine (225 μmoles), bovine serum albumin (0.5 mg), and enzyme in a final volume of 0.5 ml. After incubation at 37° for 30 min, 0.75 ml of ferric chloride reagent (0.37 M ferric chloride, 0.67 N HCl, and 0.20 M trichloroacetic acid) was added; the mixture was centrifuged and the absorbancy of the protein-free supernatant solution was read against a blank in a Bausch and Lomb Spectronic 20 or a Zeiss spectrophotometer at 535 mμ. Controls in which substrate and enzyme were separately omitted were employed and the values obtained (less than 0.05 absorbancy) were subtracted. The formation of inorganic phosphate (Fiske and Subbarow, 1925) was determined in parallel experiments and on the basis of these determinations a value of 0.305 was calculated for the absorbancy at 535 mμ for 1 μmole of γ-glutamyl-α-aminobutyrylhydroxamic acid. The formation of dipeptide hydroxamate under these conditions was linear with time and with enzyme concentration over the range 0.1–1.0/μmole of product formed. In this report a unit of tripeptide synthetase activity is defined as the amount of enzyme required to catalyze the synthesis of 1 μmole of γ-glutamyl-α-aminobutyrylhydroxamate in 30 min under the conditions described above. The corresponding values for synthesis of γ-glutamyl-α-aminobutyrylglycine were twice those obtained for hydroxamate synthesis. When γ-glutamylcysteine was substituted for γ-glutamyl-α-aminobutyric acid in the hydroxamate system, the values for activity were about 1.8 times greater.

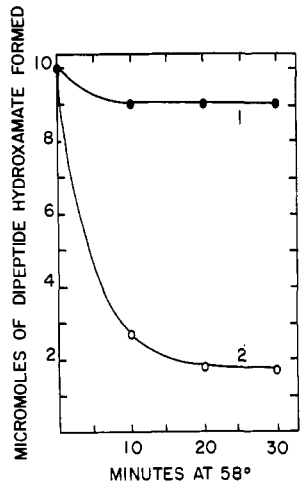

FIGURE 1: Protection of the enzyme against heat denaturation by glutathione. The enzyme (after step 4 of the purification procedure, Table I, pH 4.5) was heated at 58° in the presence (curve 1) and absence (curve 2) of 0.025 M glutathione; activity was determined as described in the text.

The assay described above was unsuitable for determination of activity during the early stages of enzyme purification due to relatively high blank values. Thus the first two steps of the purification procedure were followed by assays carried out as follows. The reaction mixtures contained Tris-HCl (50 μmoles, pH 8.3), potassium chloride (50 μmoles), magnesium sulfate (5 μmoles), ATP (2.5 μmoles), γ-L-glutamyl-L-α-aminobutyrate (2.5 μmoles), glycine-1-^{14}C (7.5 μmoles, 500,000 cpm), phosphoenolpyruvate (5 μmoles), bovine serum albumin (0.5 mg), pyruvate kinase (0.004 mg), and enzyme in a final volume of 0.5 ml. After incubation for 30 min at 37°, the reaction was stopped by the addition of 0.05 ml of 1.8 M perchloric acid. The denatured protein was removed by centrifugation and aliquots of the protein-free supernatant solution were subjected to paper electrophoresis for 115 min at 13 v/cm at 25° and pH 3.9 in a buffer consisting of glacial acetic acid–pyridine–water (25:7.5:1250). Under these conditions, ophthalmic acid moved 3–4 cm from the origin in the direction of the positive electrode, and glycine moved 1.5–2.5 cm from the origin in the opposite direction. After electrophoresis, the paper strips were cut into 1-cm sections and counted in an automatic gas-flow counter. Controls in which enzyme, ATP, and dipeptide were separately omitted were employed. The activity values obtained when moderately purified fractions were assayed by this procedure were about three times higher than those obtained by the hydroxamate assay method.

Determination of Protein. The concentration of protein was determined by the turbidimetric procedure

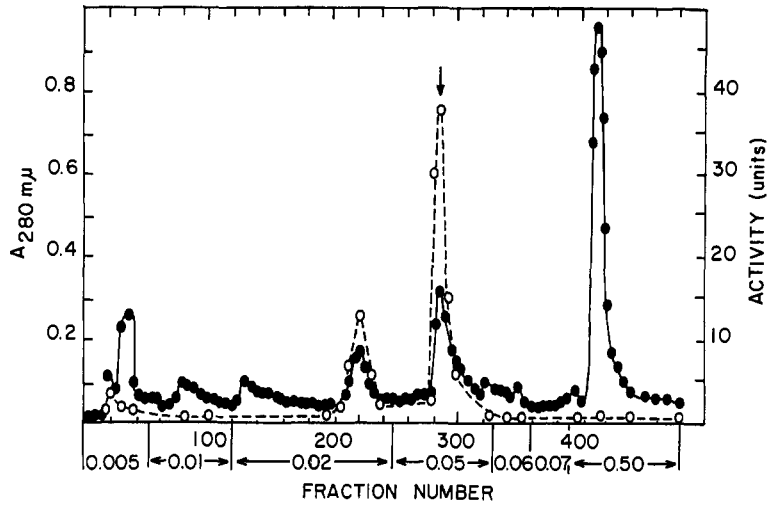

FIGURE 2: Purification of the enzyme on DEAE-cellulose. The dialyzed enzyme (30 ml, step 6; see the text) was added to the top of a column (6 × 16 cm, 5°) of DEAE-cellulose previously equilibrated with 0.005 M potassium phosphate buffer (pH 7.4) containing 0.001 M EDTA. Elution was carried out with 500–2000-ml portions of potassium phosphate buffers (concentrations indicated in the figure) at pH 7.4 containing 0.001 M EDTA. The left-hand ordinate gives the absorbancy of the eluate at 280 mμ and the right-hand ordinate gives the activity in enzyme units per milliliter; fractions of 16 ml were collected. (●) Protein and (○) activity.

of Bücher (1947) and also by the method of Lowry *et al.* (1951), using crystalline bovine serum albumin as a standard. The latter method gave values that were about 20% higher than those obtained by the former. Specific enzymatic activity is expressed in terms of units of activity (as determined by the hydroxamate assay) per milligram of protein (as determined by the method of Lowry *et al.*, 1951).

Separation and Determination of ADP and ATP. ADP and ATP were separated (for the determination of ATP–ADP-exchange activity) by chromatography on Dowex 1-formate columns (Pressman, 1960). After elution of nonadsorbable radioactivity with water, the column was treated with 45 ml of 0.05 M ammonium formate in 4 M formic acid to elute ADP. ATP was eluted with 30 ml of 0.5 M ammonium formate in 4 M formic acid. Aliquots of the eluates were dried on planchets and counted in an automatic gas-flow counter. ATP and ADP were separated (in the studies on the intermediate) by paper electrophoresis at pH 3.6 in a buffer consisting of 0.035 M citric acid and 0.0148 M sodium citrate by a procedure similar to that described by Sato *et al.* (1963); electrophoresis was carried out for 120 min at 0° at 40 v/cm.

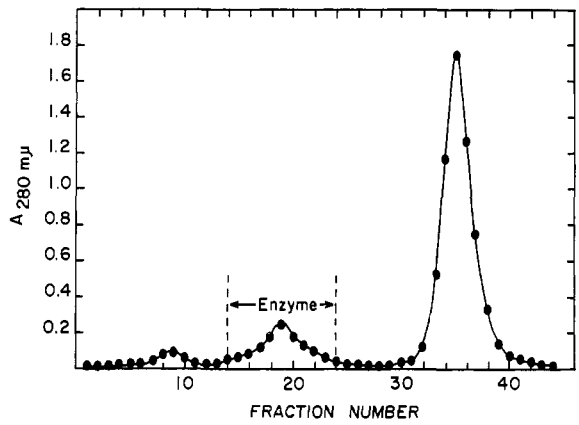

FIGURE 3: Purification of the enzyme on Sephadex G-200. The peak of activity indicated by the arrow in Figure 2 was concentrated, dialyzed, and added to a column of Sephadex G-200 (1.5 × 60 cm, 5°); elution was carried out with 0.005 M potassium phosphate buffer (pH 7.4) containing 0.001 M EDTA; fractions of 2.5 ml were collected.

Other Methods. γ-Glutamyl-α-aminobutyryl-^{14}C-hydroxamate was determined as previously described (Nishimura *et al.*, 1963). Phenol extraction of the enzyme was carried out as described by Bieber *et al.* (1964).

Purification of the Enzyme. The enzyme was isolated from a baker's strain of Budweiser yeast obtained from Anheuser-Busch, Inc. In early studies, the procedure of Snoke (1955), who isolated the enzyme from Brewer's yeast, was employed and preparations were obtained (through the nucleic acid fractionation step) that exhibited specific activity values in the range reported by Snoke. Exact comparisons of the activity values are not possible because different methods of enzyme assay and protein determination were employed. When attempts were made to scale-up the isolation it was found that the procedures following the heat denaturation step at 42° did not lead to significant purification. In the course of attempts to overcome

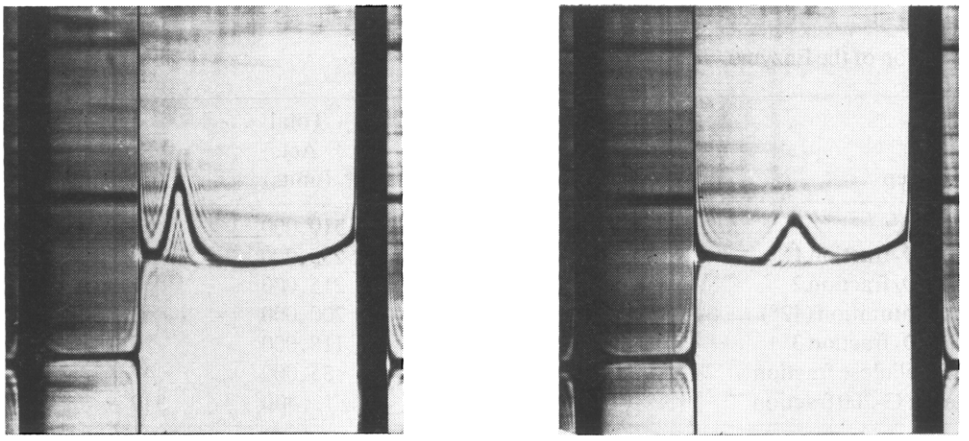

FIGURE 4: Sedimentation diagrams of the purified enzyme. Protein concentration (0.25%); 0.005 M potassium phosphate buffer (pH 7.4) containing 0.001 M EDTA and 0.1 M KCl; temperature, 7°; bar angle, 50°; speed, 59,780 rpm; the photographs were taken 24 (A) and 64 (B) min after final speed was reached; sedimentation is from left to right.

this difficulty, it was found that the enzyme was protected from denaturation at 58° by 0.025 M glutathione. Thus, as indicated in Figure 1, relatively little activity was lost when the enzyme solution (obtained after the 42° heat denaturation step) was heated at 58° for 30 min in the presence of glutathione; in its absence more than 80% of the initial activity was lost under these conditions. Application of this procedure after step 4 of Snoke's method gave about a threefold increase in specific activity at this point and the subsequent steps of his procedure then proceeded essentially as originally described. However, since neither the original nor the modified procedure gave a preparation that was homogeneous in the analytical ultracentrifuge, we have further purified the enzyme by column chromatography. For the sake of completeness and because certain modifications (including methods of assay and protein determination) were introduced, the entire method is given here. Unless otherwise stated, the temperature was maintained at 5°.

The pressed yeast cakes were crumbled into small particles and dried at 26° with occasional agitation for 4–5 days. The dried yeast was pulverized with a hammer mill and stored at 5° in air-tight plastic bags. The yeast (76 l.) was autolyzed by incubating with 228 l. of distilled water with moderate mechanical stirring in a jacketed glass-lined tank for 8–12 hr at 37°; the optimal time of autolysis was determined for each batch of yeast in a pilot experiment. Autolysis was terminated by the addition of 228 l. of distilled water (at 37°), after which the suspension was cooled and centrifuged in a DeLaval centrifuge.[2] About 379 l. of supernatant solution was obtained and treated with 332 g of solid ammonium sulfate/l.; after standing for 16 hr, the precipitate was collected by centrifugation with a series of 12 refrigerated Sharples centrifuges, and suspended in water to give a final volume of 68 l. After determination of the concentration of ammonium sulfate by direct Nesslerization, solid ammonium sulfate was added to yield a final concentration of 260 g/l. The pH was brought to 8.0 by addition of 3 N ammonium hydroxide and the mixture was allowed to stand for 16 hr and then centrifuged in the Sharples. The supernatant solution was treated with 82 g of solid ammonium sulfate/l. and was allowed to stand for 16 hr. This precipitate was collected by centrifugation in the Sharples, dissolved in the minimal amount (about 3 l.) of water, and then dialyzed for 16 hr against distilled water. The large-scale portion of the preparation was terminated at this point; 800-ml portions of the dialyzed solution (total volume about 4 l.) were stored frozen at −15° until used.

The pH of the dialyzed solution (about 800 ml) was adjusted to 4.5 by addition of 0.7 N sulfuric acid and the solution was rapidly heated to 42° by placing it in a beaker surrounded by a 60° water bath. The solution was maintained at 42° for 30 min and then rapidly cooled, after which the precipitate was removed by centrifugation in a Servall centrifuge. The supernatant solution (about 600 ml) was treated with 280 g of solid ammonium sulfate/l. and the mixture was allowed to stand for 1 hr after which the precipitate was collected by centrifugation and dialyzed for 16 hr against 0.005 M potassium phosphate buffer (pH 7.4) containing 0.001 M EDTA. Portions (15–60 ml) of this solution were chromatographed on columns of DEAE-cellulose

[2] The autolysis and first two ammonium sulfate fractionation steps were carried out at the New England Enzyme Center at Tufts University School of Medicine. We thank Dr. Stanley E. Charm, Director of the Center, and his colleagues for their valuable contribution to this work.

TABLE I: Purification of the Enzyme.

Step	Vol. (ml)	Protein Concn (mg/ml)	Total Act. (units)	Sp. Act. (units/mg)	Yield (%)
1. Autolysate[a]	384,000	20	510,000	0.066	(100)
2. $(NH_4)_2SO_4$ fraction 1	75,000	16	235,000	0.20	46
3. $(NH_4)_2SO_4$ fraction 2	4,800	44	215,000	1.0	42
4. Heat denaturation (42°)	3,600	18	200,000	3.1	39
5. $(NH_4)_2SO_4$ fraction 3	390	45	118,000	6.7	23
6. DEAE-cellulose fraction	78	9.5	35,000	47	6.9
7. Sephadex G-200 fraction	44	0.9	13,400	340	2.6

[a] From 76 l. of dried yeast; experimental details are given in the text.

TABLE II: Amino Acid Analysis of Tripeptide Synthetase.

Amino Acid	Moles of Amino Acid per Mole of Enzyme (123,000 g)			
	24-hr Hydrolysis	48-hr Hydrolysis	72-hr Hydrolysis	Estimated Values[a]
Lysine	78	80	81	80
Histidine	22	23	23	23
Arginine	39	40	43	41
Aspartic acid	114	112	114	114
Threonine	47	43	48	46
Serine	71	70	70	70
Glutamic acid	119	122	122	121
Proline	53	52	52	52
Glycine	76	77	78	77
Alanine	87	88	88	88
Cysteic acid[b]	8	—	—	8
Valine	52	62	68	68
Methionine	—	12	11	12
Isoleucine	44	46	55	55
Leucine	98	104	105	102
Tyrosine	28	33	—	30
Phenylalanine	35	36	—	36
Tryptophan[c]	—	—	—	8

[a] The values were averaged except for valine and isoleucine where the value for the 72-hr hydrolysis was taken. The recovery of amino acid residues was 94%. [b] Value obtained by oxidation with performic acid prior to hydrolysis with HCl (Schram *et al.*, 1954). [c] Value obtained from absorbancy data (Beaven and Holiday, 1952).

equilibrated with the same buffer. A representative elution pattern is given in Figure 2. The effluent representing the peak containing most of the activity (eluted with 0.05 M phosphate)[3] was concentrated by vacuum dialysis to a volume of 2–3 ml, and this solution was dialyzed against 0.005 M potassium phosphate buffer (pH 7.4) containing 0.001 M EDTA, and then added to the top of a column of Sephadex G-200 previously equilibrated with the same buffer; a representative elution pattern is given in Figure 3. In several preparations the separation on Sephadex G-200 was incomplete and, therefore, this step was repeated.

Table I describes a typical purification of the enzyme in which the first three steps were scaled up and carried out in the Enzyme Center. The over-all purification

[3] Attempts to purify the active fraction eluted with 0.02 M buffer by passage through Sephadex were not usually successful.

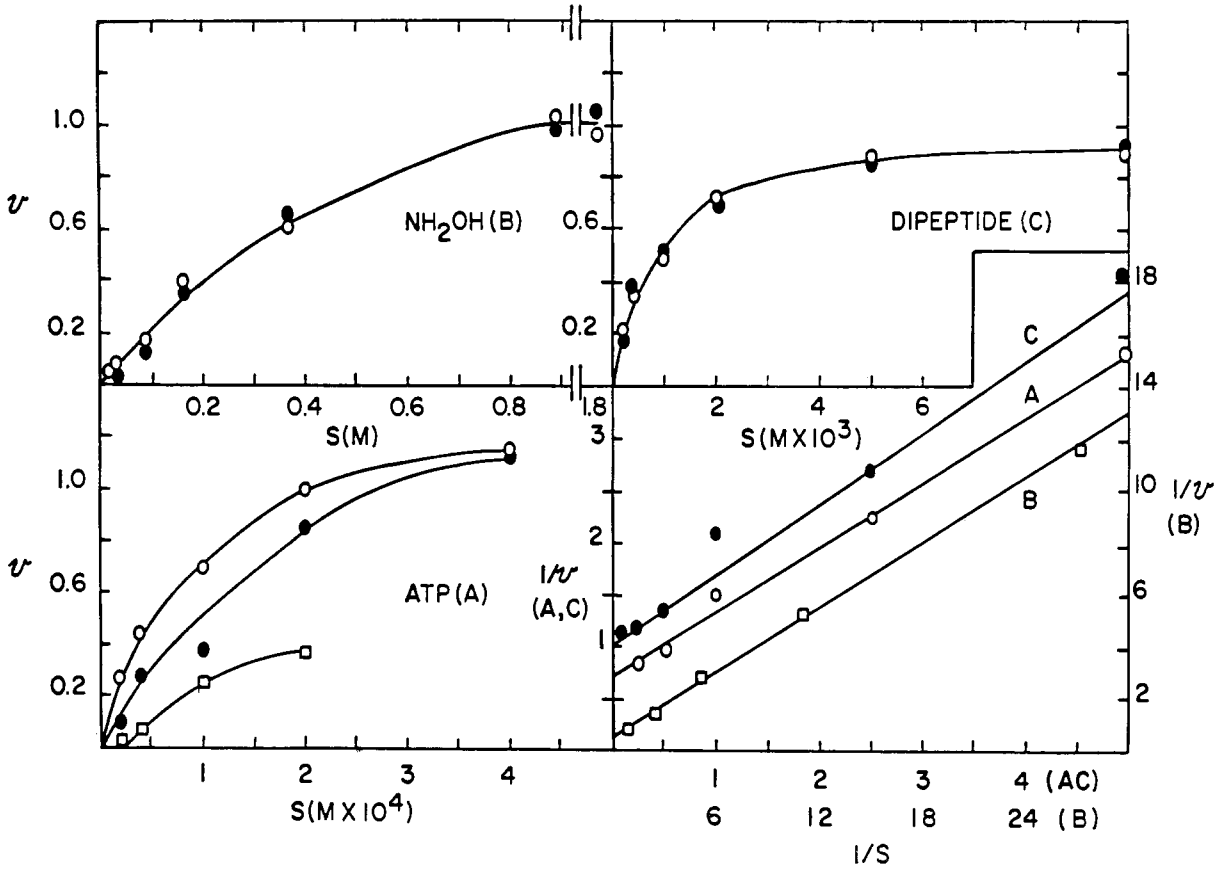

FIGURE 5: Effect of varying concentrations of dipeptide, NH_2OH, and ATP on dipeptide hydroxamate formation. The reaction mixtures consisted of Tris-HCl buffer (pH 8.4, 50 μmoles), KCl (50 μmoles), $MgSO_4$ (5 μmoles), ATP (2.5 μmoles or varied), bovine serum albumin (0.5 mg), salt-free hydroxylamine (225 μmoles or varied), γ-glutamyl-α-aminobutyrate (2.5 μmoles or varied), and enzyme (0.5–2 units) in a final volume of 0.5 ml. The formation of dipeptide hydroxamate was determined as described in the text. Values of S are given as moles per liter. In the experiments represented by open circles the reaction mixtures also contained pyruvate kinase (100 μg) and phosphoenolpyruvate (5 μmoles). The curve described by values represented as squares was obtained in experiments in which ADP (1 μmole) was added.

achieved was approximately 5000-fold. The enzyme proved to be relatively stable at virtually all steps of purification and could be stored frozen at −15° for weeks without significant loss of activity. The relative stability of the enzyme is indicated by the observation that heating of the preparation obtained at step 4 of the purification procedure at 48° for 15 min did not result in significant loss of activity; under these conditions about 30 and 80%, respectively, of the activity was destroyed at 54 and 58°.

Physical Properties of the Enzyme. The purification of the enzyme was followed by acrylamide gel disc electrophoresis; the gel was prepared by the method of Ornstein and Davis[4] as modified by Hjerten *et al.* (1965), and electrophoresis was carried out in an analytical acrylamide gel electrophoresis unit with 0.37 M Tris-glycine buffer (pH 9.5). The protein samples together with an equal volume of 20% sucrose were applied to the gel and electrophoresis was carried out at 25° at 3–5 ma/tube for 60 min. Staining was carried out with naphthol blue black (Amido Black) in 15% acetic acid for 1 hr; destaining was carried out in 7% acetic acid for 16 hr or by horizontal electrolytic destaining in an apparatus designed by Mr. William W. Lewis of this laboratory. Under these conditions, the purified enzyme (25 μg) moved in a single sharp band. Several smaller bands seen in the preparation obtained after step 6 of the purification procedure were not present in the purified enzyme.

The enzyme sedimented in the analytical ultracentrifuge (Spinco Model E) as a single homogeneous component. The sedimentation coefficient, determined under the conditions described in Figure 4, was 6.1 S. Molecular weight determinations by the method of Yphantis (1964) gave a value of 123,000 (three determinations), assuming a value of 0.74 for the partial

[4] Ornstein, L., and Davis, B. J., Special Publication, Rochester, N. Y., Distillation Products Industries.

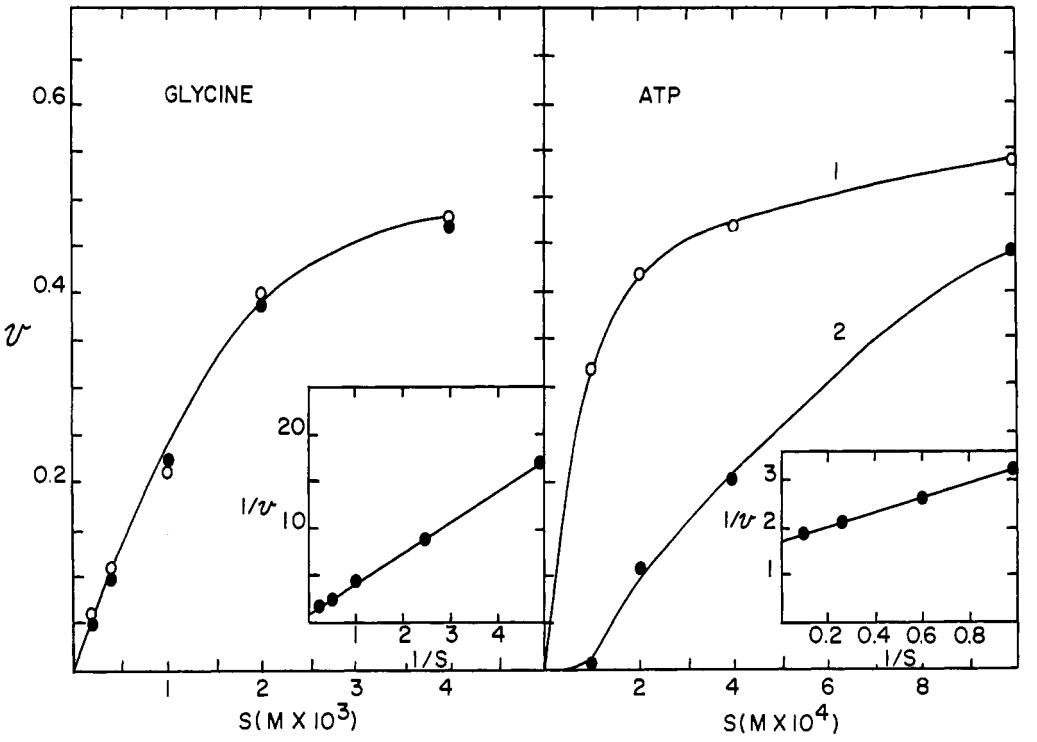

FIGURE 6: Effect of varying concentrations of glycine and ATP on tripeptide formation. The reaction mixtures were the same as given in Figure 5 except that glycine (5.0 μmoles or varied) was substituted for NH_2OH; open circles represent experiments in which pyruvate kinase and phosphoenolpyruvate were added. The reaction was followed by determinations of inorganic phosphate.

specific volume.[5] The purified enzyme exhibited a single absorbancy maximum at 280 mμ; the absorbancy at 280 mμ was 1.5 for a solution containing 1 g of protein/l. The ratio of the absorbancy at 280 mμ to that at 260 mμ was 1.60.

Amino Acid Composition. The amino acid composition of the enzyme was determined by the procedure of Spackman *et al.* (1958); the values obtained are given in Table II.

Kinetic Studies. The effects of varying the concentrations of γ-glutamyl-α-aminobutyrate, ATP, and hydroxylamine on the formation of the dipeptide hydroxamate were determined (Figure 5). Addition of an ATP-regenerating system (pyruvate kinase plus phosphoenolpyruvate) did not affect the results except at low concentrations of ATP (Figure 5C). The K_m values for dipeptide, hydroxylamine, and ATP were, respectively, 5×10^{-4}, 0.8, and 0.83×10^{-4} M. Studies on the effects of varying the concentrations of ATP and glycine on tripeptide synthesis (Figure 6) gave K_m values of 1×10^{-4} and 2×10^{-3} M, respectively.

Substrate Specificity. A number of γ-glutamyl peptides were tested in the standard hydroxamate assay system; the formation of hydroxamate and inorganic phosphate was determined. Of the dipeptides tested, only γ-L-glutamyl-L-α-aminobutyrate, γ-L-glutamyl-L-cysteine, and γ-L-glutamyl-L-alanine were significantly active under the conditions employed (Table III).

The specificity of the enzyme with respect to the acceptor amino acid was also examined. The synthesis reaction was studied in a system containing radioactive β-alanine,[6] L-aspartate, or L-alanine in place of glycine-^{14}C; under these conditions, no evidence was obtained for significant synthesis of ^{14}C-tripeptide. These amino acids were also tested as replacements for glycine-^{14}C in the glutathione–glycine-exchange reaction catalyzed by the enzyme (Snoke and Bloch, 1955); no exchange was detected under conditions in which 42% of the added glycine-^{14}C was incorporated into the tripeptide (Mooz, 1967).

Studies on the Intermediate. Experiments of the pulse-labeling type were reported previously (Nishimura *et al.*, 1963) in which relatively large amounts of the enzyme were incubated with ^{14}C-dipeptide and ATP; after brief incubation a mixture of hydroxylamine and an excess of unlabeled dipeptide were added; under these conditions, there was a much greater formation of γ-glutamyl-α-aminobutyryl-^{14}C-hydroxamate than would be expected if the labeled and unlabeled dipep-

[5] We are indebted to Dr. Rudy H. Haschemeyer for carrying out the ultracentrifugal studies.

[6] The isolation of γ-glutamylcysteinyl-β-alanine from bean seedlings has been reported (Carnegie, 1963).

TABLE III: Specificity of the Enzyme with Respect to Dipeptide.[a]

Dipeptide	Formation of	
	Hydroxamate (μmoles)	P_i (μmoles)
γ-L-Glutamyl-L-α-aminobutyrate	1.00	0.94
γ-L-Glutamyl-L-cysteine	1.77	1.85
γ-L-Glutamyl-L-alanine	0.39	0.32
γ-L-Glutamylglycine	0.01	<0.01
γ-L-Glutamyl-L-leucine	<0.01	<0.01
γ-L-Glutamyl-β-alanine	<0.01	<0.01
γ-D-Glutamylglycine	<0.01	<0.01
α-L-Glutamyl-L-leucine	<0.01	<0.01
α-L-Glutamyl-L-alanine	<0.01	<0.01
L-Aspartyl-L-alanine[b]	<0.01	<0.01

[a] The standard assay system for hydroxamate formation was used containing 2.5 μmoles of dipeptide and 1.0 unit of enzyme (see the text). [b] Contained about equal amounts of the α- and β-aspartyl peptides (5.0 μmoles of this dipeptide was used).

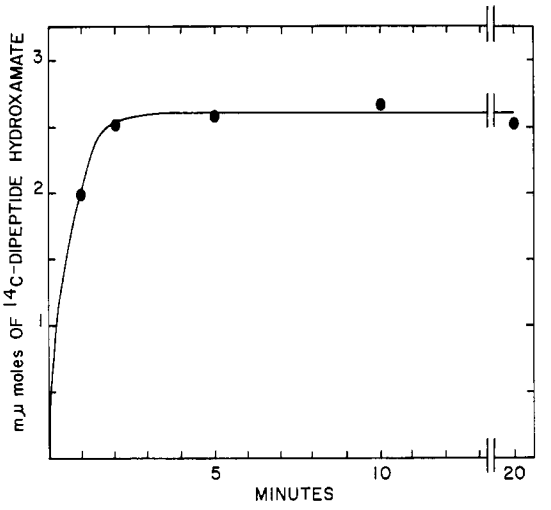

FIGURE 7: Effect of variation of initial incubation period on the pulse-labeling experiment. The reaction mixtures contained Tris-HCl (20 μmoles, pH 8.4), KCl (10 μmoles), $MgCl_2$ (1.0 μmole), ATP (0.2 μmole), γ-glutamyl-α-aminobutyric-^{14}C acid (52 mμmoles, 90,000 cpm), and enzyme (46 units) in a final volume of 0.08 ml. The enzyme was added last (all reactants were initially at 0°) and the mixture was then placed at 37° for the time intervals indicated; then, the mixtures were cooled in ice and treated with 0.03 ml containing γ-glutamyl-α-aminobutyrate-^{12}C (5 μmoles) and NH_2OH (80 μmoles). After 1 min, 0.01 ml of 60% $HClO_4$ was added and the precipitated protein was removed by centrifugation. The formation of γ-glutamyl-α-aminobutyryl-^{14}C hydroxamate was determined.

tides had equilibrated. In controls in which both labeled and unlabeled dipeptides were added initially together there was, as expected, relatively little formation of ^{14}C-dipeptide hydroxamate (Nishimura *et al.*, 1963). Subsequently, a compound was isolated from reaction mixtures containing enzyme, ATP, and γ-glutamyl-α-aminobutyrate, that exhibited the properties expected of γ-glutamyl-α-aminobutyryl phosphate (Nishimura *et al.*, 1964). These observations led to the conclusion that enzyme-bound γ-glutamyl-α-aminobutyryl phosphate was formed under these conditions, and experiments with chemically synthesized γ-glutamyl-α-aminobutyryl phosphate have supported this interpretation (Nishimura *et al.*, 1964).

Additional information about the intermediate has been obtained in other experiments of the pulse-labeling type. Thus, as indicated in the experiment described in Figure 7, in which the initial incubation period (enzyme + ATP + ^{14}C-dipeptide) was varied, the intermediate is formed rapidly, and under the conditions employed there is no appreciable increase in ^{14}C-dipeptide hydroxamate after the initial "burst" of its formation. In this experiment (and also those described in Figure 8 and Table IV), the assumption is made that the additional radioactivity found in the experiment (over that of the control in which the initial incubation mixture contained both the ^{14}C- and ^{12}C-dipeptides) represents the amount of ^{14}C-dipeptide that becomes bound to the enzyme in the initial period of incubation; this value can, therefore, be obtained from that of the radioactivity of the product and the initial specific activity of the ^{14}C-dipeptide added. A similar approach is used in the analogous experiments with ATP-^{14}C (Table IV).

In another type of experiment, after initial incubation of enzyme with ATP and labeled dipeptide (for 2 or 20 min), an excess of unlabeled dipeptide was added alone, and at various intervals thereafter hydroxylamine was added (Figure 8). Under these conditions about 70% of the label was lost within 10 sec, and there was no significant difference between the experiment and the control after 60 sec. The results were similar whether initial incubation periods of 2 or 20 min were employed. These findings indicate that the formation of the intermediate is rapid and that the labeled intermediate exchanges rapidly with unlabeled dipeptide in the absence of acceptor.

Although these results are consistent with those obtained in earlier experiments, it has become apparent that the formation of labeled dipeptide hydroxamate under these conditions does not reflect the maximum formation of enzyme-bound intermediate. Thus, as indicated by the experiments described in Table IV, the formation of ADP-^{14}C in parallel experiments was somewhat greater than that of ^{14}C-dipeptide hydroxamate. It would appear that the reaction of the

TABLE IV: Formation of ^{14}C-Dipeptide Hydroxamate and ADP-^{14}C in Pulse-Labeling Experiments.

Expt	Conditions[a]	^{14}C-Dipeptide Hydroxamate (mμmoles)	ADP-^{14}C (mμmoles)
1	(Enzyme + ^{14}C-dipeptide + ATP-^{12}C) + (^{12}C dipeptide + ATP-^{12}C + NH_2OH)	1.5, 1.7; 1.4[b]	
2	(Enzyme + ^{12}C-dipeptide + ATP-^{14}C) + (^{12}C-dipeptide + ATP-^{12}C + NH_2OH)		2.6, 2.3; 2.7[b]

[a] The components given in the first set of parentheses (enzyme, 30 units; γ-glutamyl-α-aminobutyrate, 50 mμmoles (59,000 cpm, when labeled); and ATP, 200 mμmoles (1.2×10^6 cpm, when labeled)) were incubated in a final volume of 0.09 ml containing Tris-HCl (10 μmoles, pH 8.4), KCl (10 μmoles), and $MgCl_2$ (1.0 μmole). After incubation at 37° for 2 min, the components listed in the next set of parentheses (γ-glutamyl-α-aminobutyrate, 2.5 μmoles; ATP, 10 μmoles; and NH_2OH, 90 μmoles; volume 0.03 ml) were added. After 1 min at 0°, 0.01 ml of 60% $HClO_4$ was added and the formation of ^{14}C-dipeptide hydroxamate and ADP-^{14}C was determined as described in the text. [b] After incubation of the components given in the first set of parentheses, 0.01 ml of 60% $HClO_3$ was added to precipitate the protein; then the components given in the second set of parentheses were added.

intermediate with hydroxylamine is not complete under these conditions; it should be noted that the concentration of hydroxylamine used in these studies is somewhat less than the K_m value for hydroxylamine in the over-all reaction. In experiments in which higher concentrations of hydroxylamine were employed substantially more ^{14}C-dipeptide was formed. Increased ^{14}C-dipeptide formation was also observed when phosphoenolpyruvate and pyruvate kinase were added after the initial incubation, followed by further incubation for 1 min prior to simultaneous addition of unlabeled dipeptide and hydroxylamine.

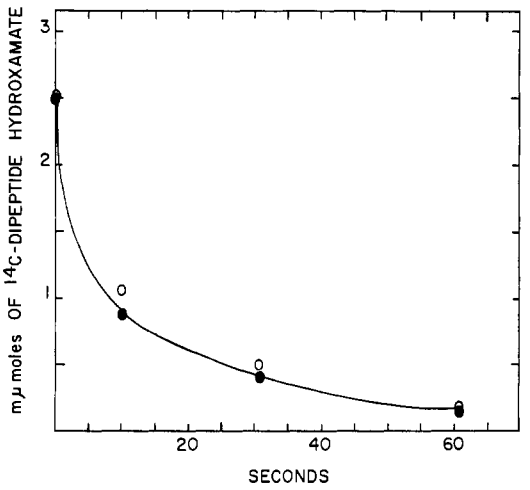

FIGURE 8: Stability of enzymatically formed dipeptide intermediate (modified pulse-labeling experiment). The reaction mixtures were the same as given for the initial incubation in Figure 7. After 2 (circles) or 20 (squares) min, the mixtures were treated with 0.02 ml containing γ-glutamyl-α-aminobutyrate-^{14}C (5 μmoles) and allowed to stand at 0° for the intervals indicated, at which time 0.01 ml of a solution containing NH_2OH (80 μmoles) was added. After standing for 1 min at 0°, 0.01 ml of 60% $HClO_4$ was added and the ^{14}C-dipeptide hydroxamate was determined.

In the experiments described in Figure 9, conditions were used under which the ADP formed was rapidly removed; thus, the reaction between the synthetase, ATP, and dipeptide was studied in the presence of pyruvate kinase, phosphoenolpyruvate, lactic dehydrogenase, and DPNH. Under these conditions added ADP was utilized extremely rapidly, so rapidly that the course of this reaction could not be followed. As indicated in Figure 9, when the enzyme was added to a reaction mixture containing ATP, phosphoenolpyruvate, pyruvate kinase, lactic dehydrogenase, and DPNH there was a very slow decrease in absorbancy indicating the presence of a slight ATPase activity; addition of heat-inactivated tripeptide synthetase did not affect the absorbancy indicating the absence of ADP in the enzyme preparation. When dipeptide was then added there was a rapid formation of ADP followed by a much slower formation of ADP whose rate was 2.7 times that of the ATPase. When additional dipeptide was added to this reaction mixture there was no change in rate. On the other hand, when more enzyme was added a similar rapid formation of ADP occurred. The immediate formation of ADP under these conditions (corrected for the ATPase and dipeptide-stimulated ATPase) was proportional to the amount of enzyme added. Addition of heat-inactivated synthetase at this point did not affect the absorbancy indicating the absence of an acceptor (*e.g.*, glycine) in the enzyme solution itself. A number of experiments of this type were carried out using different amounts of enzyme. The relationship between the amount of enzyme and the formation of ADP in a number of experiments is summarized in Figure 10. The ratio

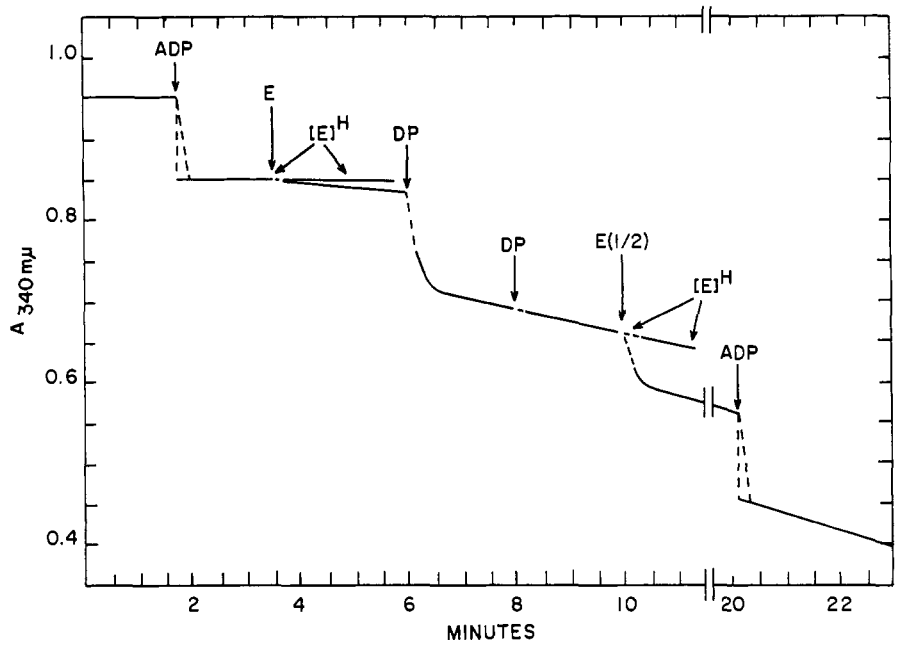

FIGURE 9: Formation of ADP on incubation of the enzyme with dipeptide and ATP. The reaction mixtures contained initially Tris-HCl (100 μmoles, pH 8.5), KCl (50 μmoles), $MgCl_2$ (5 μmoles), ATP (1 μmole), phosphoenolpyruvate (5 μmoles), pyruvate kinase (100 μg), lactic dehydrogenase (100 μg), and DPNH, in a volume of 0.6 ml. The absorbancy values (corrected for volume changes) were followed in a Cary Model 14 recording spectrophotometer. Additions of ADP (10 mμmoles), tripeptide synthetase (E 104 units, or E ($^1/_2$) 52 units), tripeptide synthetase (heated at 100° for 3 min; $[E]^H$ 104 units), and dipeptide (DP, 0.25 μmole) were made as indicated. Solid lines represent tracings recorded; the dashed lines were extrapolated. The changes observed after addition of ADP were complete before the first observation after mixing was made (10–15 sec).

TABLE V: Ability of the Enzyme to Catalyze the ADP–ATP-Exchange Reaction.[a]

Reaction Components	Enzyme Preparation: Purifcn Stage (step)	Act. (units)	Protein (μg)	Exchange Act. (%)[b]
Enzyme + ATP + ADP-^{14}C	5	1.7	240	49.0
Enzyme + ATP + ADP-^{14}C + DP	5	1.7	240	49.8
Enzyme + ATP + ADP-^{14}C + DP + glycine	5	1.7	240	47.1
Enzyme + ADP-^{14}C	5	1.7	240	7.5
Enzyme + ATP + ADP-^{14}C	6	1.7	33	12.4
Enzyme + ATP + ADP-^{14}C + DP	6	1.7	33	16.0
Enzyme + ATP + ADP-^{14}C + DP + glycine	6	1.7	33	14.0
Enzyme + ADP-^{14}C	6	1.7	33	2.1
Enzyme + ATP + ADP-^{14}C	7 (final)	1.7	5.0	6.2
Enzyme + ATP + ADP-^{14}C + DP	7	1.7	5.0	5.1
Enzyme + ATP + ADP-^{14}C + DP + glycine	7	1.7	5.0	5.8
Enzyme + ADP-^{14}C	7	1.7	5.0	3.9

[a] The reaction mixtures consisted of Tris-HCl buffer (50 μmoles, pH 8.4), KCl (50 μmoles), $MgSO_4$ (5 μmoles), bovine serum albumin (0.5 mg), ADP-8-^{14}C (5 μmoles, 224,00 cpm), ATP (5 μmoles), glycine (5 μmoles), γ-glutamyl-α-aminobutyric acid (DP, 2.5 μmoles), and enzyme as indicated. After incubation at 37° for 60 min, the solutions were deproteinized by addition of 1.0 ml of ethanol and centrifugation. ADP and ATP were separated and determined as described in the text. [b] Per cent of total counts found in ATP.

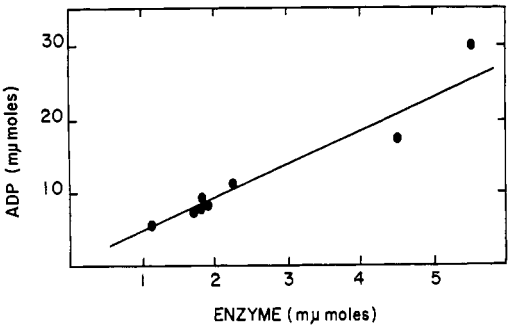

FIGURE 10: Relationship between ADP formation and enzyme. The immediate formation of ADP observed after addition of enzyme or dipeptide in experiments carried out as described in Figure 9 is plotted against the amount of enzyme used.

of the values for ADP formation to enzyme in these studies was in the range 3.7–5.5 moles/mole of enzyme.

Ability of the Enzyme to Catalyze the ADP–ATP-Exchange Reaction. In agreement with earlier studies (Snoke and Bloch, 1955) preparations of the synthetase were found to catalyze exchange between ADP and ATP; the reaction is dependent only on the presence of the enzyme, nucleotides, and magnesium ions. As indicated in Table V the enyzme at step 5 of the purification procedure (ammonium sulfate fraction 3) catalyzes substantial exchange.[7] However, an equivalent quantity of enzyme (as judged by synthesis activity) at step 6 of the purification (DEAE-cellulose fraction) catalyzed much less exchange, and the final purified preparation (step 7, Sephadex G-200 fraction) catalyzed even less exchange. It is clear from the data given in Table V that the ability of the enzyme to catalyze ATP–ADP exchange decreases as the synthetase activity is purified and therefore that the ratio of exchange activity to synthetase activity decreases markedly during purification. The findings also indicate that addition of dipeptide, or dipeptide plus glycine did not significantly affect the results obtained with any of the enzyme fractions examined.

Attempt to Detect Phosphorylation of the Enzyme. Experiments were undertaken to determine whether the synthetase becomes phosphorylated in a manner similar to succinyl thiokinase (Bieber *et al.*, 1964). In these studies, relatively large amounts of enzyme, glutathione, magnesium ions, radioactive inorganic phosphate, and ADP were incubated after which the reaction mixture was extracted with phenol. In similar studies on succinyl thiokinase, significant amounts of radioactive phosphate were incorporated into a phenol-soluble form, which has been shown to be associated with formation of an *N*-phosphorylimidazole derivative of the enzyme. In the experiments with the tripeptide synthetase there was no significant incorporation of inorganic phosphate into the phenol-soluble phase. If one assumes that 1 mole of phosphate can be bound/active site and that there are four sites per mole of enzyme (mol wt 123,000), then the observed incorporation of ^{32}P was less than 0.025% of theoretical (Mooz, 1967).

Discussion

The present studies provide additional information concerning the intermediate formed in the reaction catalyzed by tripeptide synthetase. The double-pulse labeling experiment (Figure 8) indicates that the dipeptide moiety of the intermediate exchanges rapidly with unlabeled peptide. The initial rate of such exchange can be roughly estimated from the data of Figure 8 as of the order of 0.5–0.8 mμmole/sec at 0°; this corresponds to about 90 mμmoles/30 min at 37° (assuming a temperature coefficient of about 3) or about the same order of magnitude as the rate of the over-all reaction. These considerations are consistent with the belief that the intermediate is on the major catalytic pathway.

The findings are also in accord with the previous conclusion that the acyl phosphate intermediate is bound to the enzyme and that little if any free acyl phosphate exists. The slow linear formation of ADP after addition of dipeptide observed in the experiment described in Figure 9 suggests that the enzyme-bound acyl phosphate breaks down slowly. This phenomenon seems to be analogous to the slow formation of pyrrolidonecarboxylic acid and inorganic phosphate from glutamate and ATP catalyzed by glutamine synthetase in the absence of ammonia (Krishnaswamy *et al.*, 1962), and to the carbon dioxide dependent cleavage of ATP to ADP catalyzed by carbamyl phosphate synthetase (Anderson and Meister, 1966).

The present preparation of the enzyme appears to be the most highly purified thus far obtained. Although it is homogeneous by electrophoretic and ultracentrifugal criteria, one cannot exclude the possibility that trace amounts of other activities are present. The ATP–ADP exchange catalyzed by the purified enzyme preparation does not seem to represent a catalytic activity of the synthetase itself. The fact that the ability of the enzyme preparation to catalyze this exchange reaction decreases markedly as the synthetase activity is purified suggests that the exchange activity is not a function of the synthetase itself but is probably catalyzed by a contaminating protein. It may be noted that although partially purified preparations of glutamine synthetase can catalyze the ATP–ADP-exchange reaction, the purified enzyme does not (Meister, 1962). It is of interest that the ADP–ATP exchange catalyzed by the most purified enzyme was not significantly increased by addition of dipeptide and glycine (Table V), suggesting that the rate of the reversal of tripeptide synthesis is very low under the conditions used in these experiments.

The present and previously reported data support the view that the synthesis of tripeptide catalyzed by

[7] At this stage of purification the exchange activity was close to the theoretical maximum (50%); the data given in Table V, therefore, represent minimal values.

this enzyme involves intermediate formation of an acyl phosphate, *i.e.*, dipeptidyl phosphate. (It is, nevertheless, not excluded that phosphate is bound to the enzyme at some stage during the reaction.) Strumeyer (1959) found that the synthesis of glutathione from γ-glutamylcysteine labeled with ^{18}O is accompanied by the formation of inorganic phosphate containing isotopic oxygen. Although this observation is consistent with the acyl phosphate hypothesis, Strumeyer's data indicate that about two atoms of isotopic oxygen are incorporated per mole of glutathione synthesized. This observation may be explained in terms of a mechanism of the following type:

$$E + RCOO^- + ATP \rightleftharpoons E[RCOOPO_3^{2-}][ADP] \quad (I)$$

$$E[RCOOPO_3^{2-}][ADP] + R'\text{-}NH_2 \rightleftharpoons E[RCONHR'] + [HPO_4^{2-}] + ADP \quad (II)$$

$$E[RCONHR'] \rightleftharpoons E + RCONHR' \quad (III)$$

According to this scheme, reaction of enzyme-bound acyl phosphate with acceptor leads initially to the formation of an enzyme–product complex which then dissociates (reaction III). If the dissociation of enzyme–product complex to enzyme and free product occurs relatively slowly compared to the other reactions, more than one atom of isotopic oxygen could be transferred from the carboxyl group of the dipeptide to inorganic phosphate.

It was previously postulated that nucleotide functions in a catalytic role and thus represents a portion of the enzyme-bound activated substrate complex; a tentative scheme for the reaction catalyzed by tripeptide synthetase was presented (Figure 4; Nishimura *et al.*, 1964), which is analogous to that proposed for glutamine synthetase (Krishnaswamy *et al.*, 1962). The present observations support the belief that the ADP formed in the reaction between enzyme, ATP, and dipeptide in the absence of acceptor remains attached to the enzyme. Thus, in the experiment described in Figure 9, the immediate decrease in absorbancy is considerably slower than expected (and observed) for free ADP added to the system under comparable conditions. The rate of formation of free ADP under these conditions is considerably less than that observed for acyl phosphate formation (Figure 8). The findings, therefore, indicate that in the absence of glycine, dissociation of ADP from the enzyme occurs relatively slowly; presumably, ADP dissociates much more rapidly from the enzyme in the presence of glycine and ATP. The inability of the enzyme to catalyze significant ADP–ATP exchange in the presence of dipeptide (Table V) provides further evidence that ADP remains attached to the enzyme in the absence of acceptor.

The findings do not exclude the possibility that the formation of the acyl phosphate is preceded by phosphorylation of the enzyme by ATP. Such a mechanism appears plausible for the reaction catalyzed by succinyl thiokinase, in which there is also evidence for formation of an enzyme-bound acyl phosphate intermediate (Nishimura and Meister, 1965); thus, Nishimura (1967) has recently demonstrated the formation of succinyl phosphate from phosphorylated succinyl thiokinase and succinate. Although the present studies provide no evidence for a phosphorylated form of tripeptide synthetase, other approaches may be required for demonstration of such an intermediate form of this enzyme and others that catalyze analogous reactions (*e.g.*, glutamine synthetase).

Acknowledgment

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The Mechanism of the Reaction of β-Hydroxyaspartate with L-Aspartate β-Decarboxylase. A New Type of Pyridoxal 5′-Phosphate–Enzyme Inhibition*

Edith Wilson Miles and Alton Meister

ABSTRACT: L-Aspartate β-decarboxylase from *Alcaligenes faecalis* is strongly and competitively inhibited by both *threo-* and *erythro*-β-hydroxyaspartate. The enzyme decarboxylates these amino acids at very slow rates to yield serine; however, the rate of CO_2 liberation exceeds that of serine formation. Incubation of the enzyme with β-hydroxyaspartate leads to a shift of the maximum absorbancy from 360 to 325 mμ; subsequent addition of α-keto acids does not affect the absorbancy suggesting that this form of the enzyme does not contain pyridoxamine 5′-phosphate. Sodium borohydride reduced the holoenzyme in the absence but not in the presence of β-hydroxyaspartate, suggesting that the β-hydroxyaspartate derivative of the enzyme is not a Schiff base. The form of the enzyme that absorbs maximally at 325 mμ (E-325) is inactive in the decarboxylation of L-aspartate and in the desulfination of cysteine sulfinate. Studies in which the enzyme was incubated with [^{14}C]β-hydroxyaspartate and then subjected to gel filtration show that E-325 contains a moiety derived from carbon atoms 1 to 3 of β-hydroxyaspartate; this is converted to serine on further incubation. Treatment of E-325 with 2,4-dinitrophenylhydrazine in hot acid solution yields a compound that appears to be the osazone of glycolaldehyde. The findings indicate that β-hydroxyaspartate is decarboxylated to yield an enzyme–partial substrate intermediate, which is in equilibrium with an inactive enzyme complex.

Aspartate β-decarboxylase has been isolated in apparently homogeneous form from *Alcaligenes faecalis* (Novogrodsky *et al.*, 1963; Novogrodsky and Meister, 1964; Wilson and Meister, 1966) and from *Achromobacter* (Wilson, 1963; Wilson and Kornberg, 1963). This enzyme is subject to at least two different types of inhibition by amino acids. Novogrodsky and Meister (1964) found that L-aspartate and a variety of other L-amino acids inactivate the enzyme by transaminating with the enzyme-bound pyridoxal 5′-phosphate to form pyridoxamine 5′-phosphate. α-Keto acids reactivate the enzyme by reversing the transamination reaction. Conversion of the pyridoxal 5′-phosphate–enzyme (λ_{max} 360 mμ) to the pyridoxamine 5′-phosphate–enzyme (λ_{max} 325 mμ) and the reversal of this transformation were demonstrated spectrophotometrically. Wilson and Kornberg (1963) reported that *threo-* and *erythro*-β-hydroxyaspartate are strong competitive inhibitors of L-aspartate β-decarboxylase from *Achromobacter* and produce a change in the maximum absorbancy of the enzyme from 360 to 325 mμ. However, subsequent treatment of the enzyme with α-keto acids does not affect the absorbancy, suggesting that this form of the enzyme does not contain pyridoxamine 5′-phosphate. Sodium borohydride reduced the holoenzyme in the absence but not in the presence of β-hydroxyaspartate, suggesting that the β-hydroxyaspartate derivative is not a Schiff base.

We now report that *threo-* and *erythro*-β-hydroxyaspartate inhibit L-aspartate β-decarboxylase from *A. faecalis* in a similar manner and that both L isomers of β-hydroxyaspartate are slowly decarboxylated to yield L-serine. Kinetic studies of these reactions and of the inhibition produced by β-hydroxyaspartate have been carried out. These investigations and experiments on the chemical nature of the intermediate form of the enzyme that exhibits maximum absorbancy at 325 mμ indicate that a new type of pyridoxal 5′-phosphate–enzyme inhibition is involved.

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