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γ-GLUTAMYLGLUTAMINE LACTAMASE IN MAMMALIAN TISSUES

YASUO KAKIMOTO, AKIRA KANAZAWA AND ISAMU SANO
The Department of Neuropsychiatry, Osaka University Medical School, Fukushima-ku, Osaka (Japan)

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SUMMARY

An enzyme that catalyzes the degradation of γ-L-glutamyl-L-glutamine to pyrrolidone carboxylic acid and glutamic acid has been purified 14-fold from an extract of acetone powder of rat liver.

The substrate specificity of the enzyme preparation was examined on 9 γ-glutamyl peptides, 3 α-glutamyl peptides, 4 dipeptides containing a glutamine residue in the carboxy terminal position, and 15 other compounds. Other properties of the enzyme are also described. This enzyme seems to be different from previously known peptidases and from γ-glutamyl lactamase, and is tentatively referred to as γ-glutamylglutamine lactamase.

The distribution of the enzyme in various organs of the rat and guinea pig has been examined.

INTRODUCTION

Various γ-glutamyl peptides have been isolated from bovine brain and identified in this laboratory[1-4]. Of these peptides γ-L-glutamyl-L-glutamine was found in the highest concentration. The purpose of the present experiment was to explore an enzyme reaction which may participate in the degradation of the peptide in tissue. An enzyme which degrades γ-glutamylglutamine to pyrrolidone carboxylic acid and glutamine was found in various mammalian tissues. The nature of the enzyme reaction is similar to that of γ-glutamyl lactamase described by CONNELL AND HANES[5] and CLIFFE AND WALEY[6]. These authors did not characterize the enzyme for its substrate specificity and other properties, and the distribution of the enzyme in nature was not examined. During the course of the present study, evidence suggesting that this enzyme is different from the enzyme which catalyzes the breakdown of γ-glutamylglutamine was obtained, and the latter enzyme was tentatively designated γ-glutamylglutamine lactamase. This paper reports the partial purification of the enzyme, its properties and its distribution in mammalian tissues.

METHODS AND MATERIALS

Chemical compounds. Most of the peptides used in the present study were prepared in this laboratory, and the methods of synthesis have been published[1,2,4] or are to be published. Glycylglutamine, alanylglutamine, valylglutamine and prolylglutamine were kindly supplied by Dr. S. SAKAKIBARA, The Institute for Protein Research, Osaka University. Glycylleucine, leucylglycylglycine, leucylglycine and carbobenzoxyglycylphenylalanine were purchased from The Peptide Center, The Institute for Protein Research, Osaka University.

Assay of enzyme activity. The amount of glutamine formed from γ-glutamylglutamine was measured spectrophotometrically with the ninhydrin procedure. In a typical experiment, a mixture of 0.2 ml of enzyme solution, 0.1 ml of 0.25 M γ-glutamylglutamine, which had been neutralized to pH 8.0±0.1 with sodium bicarbonate and 0.1 ml of 0.05 M potassium phosphate buffer (pH 8.0) were incubated at 25° for 30 min. The reaction was stopped by heating the reaction mixture in a water bath maintained at 80° for 1 min. This did not cause a detectable change in the amount of glutamine which may be cyclized to pyrrolidone carboxylic acid upon heating. A 10-μl aliquot was spotted on a paper, along with 10 μl of 10 mM of glutamine solution, and subjected to paper electrophoresis at a gradient of 100 V/cm for 10 min. A mixture of pyridine, acetic acid and water (1:10:189) (pH 3.6) was used as a buffer for the electrophoresis. Each paper was dried at 90°, immersed in a mixture of 0.2% ninhydrin in acetone, pyridine and acetic acid (18:1:1), and heated at 90° for 3 min. An area containing glutamine was eluted with 3 ml of 50% ethanol, and the absorbance was measured at 570 mμ. If the enzyme reaction proceeded to more than 50% completion, the original enzyme solution was diluted so that more than 60% of the substrate remained unreacted after incubation, and its activity was measured again. When a crude enzyme preparation was used, the substrate solution was replaced by water in the reaction mixture, incubated, and the solution was used as a blank. The absorbance of the chromophores derived from interfering materials was subtracted from the value obtained in the presence of γ-glutamylglutamine. 1 unit of enzyme is defined as the amount of enzyme which catalyzes the formation of 1 μmole of glutamine in 1 min at 25°.

The determination of pyrrolidone carboxylic acid. The enzymic reaction mixture was passed through a 0.3 cm × 0.5 cm column of Amberlite IR-120, H^+ form, 100–200 mesh, to adsorb glutamine, γ-glutamylglutamine and enzymic protein, and the column was washed with 1 ml of water. To the total effluent was added 1/6 volume of concentrated hydrochloric acid; the solution was then sealed in a tube, and heated in boiling water for 4 h. The solution was evaporated to dryness *in vacuo*, and the residue was dissolved in 0.3 ml of water. A 10-μl aliquot was applied to paper for electrophoresis, and the amount of glutamic acid was determined in the same manner as for glutamine. The recovery of pyrrolidone carboxylic acid as glutamic acid in this procedure was quantitative (100±3%).

Purification of γ-glutamylglutamine lactamase

Step 1. Preparation of the extract of acetone powder. 34 g of rat liver were homogenized at 0°, mixed with 500 ml of cold acetone (−20°) and filtered. The cake was washed with 500 ml of the cold acetone, and dried in air for 30 min to yield 10.4 g of

powder. The powder was stirred in 208 ml of 0.05 M potassium phosphate buffer (pH 8.0) for 1 h at 0°, centrifuged at 12 000 × *g* for 1 h, and 189 ml of the supernatant solution was obtained.

Step 2. Acid treatment. The supernatant solution was chilled to −3°, brought to pH 5 by the addition of cold 1 M acetic acid, and centrifuged at 12 000 × *g* for 20 min. The pH of the supernatant solution was brought back to 8.0 by the addition of cold 1 M potassium hydroxide.

Step 3. Fractionation with ammonium sulfate. To the above solution solid ammonium sulfate was added to 50% saturation at 0°. The mixture was stirred for 1 h and centrifuged at 10 000 × *g* for 20 min at 0°. The supernatant solution was removed, and solid ammonium sulfate was added to 90% saturation, and after being stirred for 1 h, the suspension was centrifuged at 10 000 × *g* for 30 min. The sediment was taken up in 10 ml of 0.05 M potassium phosphate buffer (pH 8.0) and desalted by passing the solution through a 3 cm × 20 cm column of Sephadex G-25 equilibrated with the above buffer to obtain 42 ml of a red solution.

Step 4. Heat treatment. The temperature of the solution was rapidly raised to 55° in a water bath, kept at 55° for 3 min, then the solution was cooled to 5° in an ice bath, and centrifuged at 5000 × *g* for 20 min.

Step 5. Fractionation with ammonium sulfate. The solution was brought to 60% saturation of ammonium sulfate by the addition of the saturated solution, and after standing for 1 h at 0° centrifuged at 12 000 × *g* for 20 min. Solid ammonium sulfate was added to 85% saturation, and after 1 h at 0°, the mixture was centrifuged at 12 000 × *g* for 30 min at 0°. The sediment was taken up in 10 ml of 0.01 M potassium phosphate buffer and desalted by passing the solution through a 3 cm × 20 cm column of Sephadex G-25 equilibrated with the same buffer to obtain 30 ml of the desalted solution.

Step 6. Purification with DEAE-cellulose. The solution was poured on to a 3 cm × 20 cm column of DEAE-cellulose which had been buffered with 0.01 M potassium phosphate (pH 8.0) and 120 ml of the same buffer was then passed through the column. The enzyme was eluted with 0.1 M potassium phosphate buffer (pH 8.0). A

TABLE I

PARTIAL PURIFICATION OF γ-GLUTAMYLGLUTAMINE LACTAMASE FROM RAT LIVER

Fraction	*Total units**	*Total protein (mg)***	*Specific activity****	*Recovery (%)*
1	380	3170	0.12	100
2	259	1990	0.13	69
3	180	655	0.28	48
4	154	575	0.27	41
5	172	408	0.42	45
6	112	67	1.67	30

* μmoles glutamine formed per min. The enzyme solutions were diluted with 0.05 M potassium phosphate buffer (pH 8.0) so that an 0.2-ml aliquot of the diluted solution formed about 8 μmoles of glutamine when incubated with 25 μmoles of γ-glutamylglutamine with a final volume of 0.4 ml for 30 min at 25°.

** Protein concentration was determined according to the method of WARBURG AND CHRISTIAN[7].

*** μmoles glutamine formed per min per mg protein.

fraction of the eluate between 108 and 230 ml was collected, and stored in a deep freezer at −20°.

This enzyme preparation could be stored at −20° for at least 2 weeks without detectable loss of activity. The data at various stages of purification are summarized in Table I. The above procedures were reproducible with similar results in four separate batches.

RESULTS

The extracts of rat liver and brain were found to catalyze a rapid formation of glutamine from γ-glutamylglutamine in a preliminary study, but neither glutamic acid nor γ-glutamylglutamic acid were formed in the reaction mixture. A boiled tissue extract caused no degradation of the peptide. The enzyme was partially purified to determine whether it is different from the known peptidases, and to clarify its properties.

Products of the enzyme reaction

A mixture of 0.4 ml of 0.25 M γ-glutamylglutamine and 0.8 ml of the enzyme solution purified to Step 6 were incubated at 25° for 2 h, when the mixture was found to contain no detectable amount of the original peptide. 10-μl aliquots of the reaction mixture were subjected to paper electrophoresis at pH 3.6 and to paper chromatography with three different solvent systems[1], and the ninhydrin reagent was sprayed. The mixture contained a single ninhydrin-positive compound, and its migration distance and R_F values coincided with that of glutamine. A half portion of the reaction mixture was acidified to pH 1 by the addition of hydrochloric acid, extracted with 5 portions of 10 ml of ethyl acetate, and the combined extracts were evaporated to dryness *in vacuo*. The dried residue was dissolved in 0.5 ml of water and 10-μl portions were subjected to paper electrophoresis at pH 3.6 and to paper chromatography with three different systems[8]. The papers were subjected to the aniline xylose reaction[8], and a compound was found whose migration distance and R_F values were the same as those of pyrrolidone carboxylic acid. Upon acid hydrolysis of the extract, and of an extract of an area of the paper chromatogram corresponding to pyrrolidone carboxylic acid, glutamic acid was formed.

Stoichiometry of the reaction

The amounts of the glutamine and pyrrolidone carboxylic acid formed and of the γ-glutamylglutamine degraded were determined to establish the stoichiometric relation in the enzyme reaction. The results of the experiments are summarized in Table II. An amount of glutamine was formed which is equimolar to the amount of γ-glutamylglutamine that disappeared. The amounts of pyrrolidone carboxylic acid were a little less than the theoretical value on the assumption that γ-glutamylglutamine splits into equimolar amounts of glutamine and pyrrolidone carboxylic acid. Although a proper explanation is not possible for the small divergence from stoichiometry, it may be reasonable to conclude that the enzyme splits the substrate to pyrrolidone carboxylic acid and glutamine nearly quantitatively, since no other probable products could be found in the reaction mixture. No trace amount of glutamic acid was detected in the original enzyme reaction mixture, and this indicates

TABLE II

STOICHIOMETRIC RELATION OF THE ENZYMIC BREAKDOWN OF γ-GLUTAMYLGLUTAMINE

Reaction mixtures in Expts. 1, 2 and 3 consisted of 0.2 ml of Step-6 enzyme preparation (0.1 mg protein), 0.1 ml of 0.05 M potassium phosphate buffer (pH 8.0) and 0.1 ml of 0.1 M neutralized γ-glutamylglutamine. In Expt. 4, 0.05 M neutralized γ-glutamylglutamine was used. Reaction time for Expts. 2, 3 and 4 was 60 min, and 30 min for Expt. 1. Incubation was performed at 25°. The reaction was stopped by heating the mixture at 80° for 1 min.

Expt. No.	*Glutamine formed (μmoles)*	*Pyrrolidone carboxylic acid formed (μmoles)*	*γ-Glutamyl-glutamine degraded (μmoles)*
1	3.5	3.0	2.6
2	5.8	4.3	5.3
3	5.8	4.2	5.2
4	4.0		4.0

that the reaction is not of hydrolytic nature. The enzyme preparation did not catalyze the formation of pyrrolidone carboxylic acid from glutamine or glutamic acid.

Substrate specificity

The relative activities of various peptides against the Step-6 enzyme preparation to the activity of γ-glutamylglutamine are shown in Table III. Of eight γ-glutamyl peptides, no detectable activity was found except with γ-glutamylserine and γ-glutamylalanine. α-Glutamyl peptides, dipeptides containing glutamine at the carboxy terminals, showed no activities. Of various other peptides, glycylleucine, leucylglycylglycine and arginylleucine were hydrolyzed by the preparation at a much slower rate than γ-glutamylglutamine.

γ-Glutamyl lactamase and γ-glutamylglutamine lactamase

Rat liver was homogenized with 0.05 M potassium phosphate buffer (pH 8.0) and centrifuged for 30 min at 14 000 × *g*. A fraction of the supernatant solution which precipitated between 60 and 85% saturation of ammonium sulfate was dissolved in a small volume of water, and the solution was desalted by treatment with Sephadex G-25 (Preparation 1). This preparation corresponds roughly with the preparation used by CONNELL AND HANES[5] who studied γ-glutamyl lactamase. A portion of the preparation was subjected to heat treatment at 58° for 3 min followed by precipitation with 85% saturation with ammonium sulfate. The precipitate was dissolved in a small volume of water, and desalted with Sephadex G-25 (Preparation 2). The substrates that were metabolized by the preparation of γ-glutamylglutamine lactamase were examined with the above two preparations for their relative activities to γ-glutamylglutamine. The result is shown in Table IV. A comparison of Tables III and IV makes it clear that γ-glutamyl lactamase is different from γ-glutamylglutamine lactamase which did not metabolize γ-glutamylglycine. Different relative activities with glycylleucine and leucylglycylglycine to γ-glutamylglutamine were also shown, indicating that the activity of the Step-6 enzyme preparation against the first two peptides is due to contaminating peptidases in the preparations.

TABLE III

SUBSTRATE SPECIFICITY OF γ-GLUTAMYLGLUTAMINE LACTAMASE

Amino acid residues of the peptides in this table were L-form. Relative activities of the peptides are expressed by the ratio (%) of the activity of a given compound to that of γ-glutamylglutamine. 5 μmoles of each compound which had been neutralized to pH 8 with the addition of potassium bicarbonate were incubated with 0.1 ml the Step-6 enzyme preparation in a final volume of 0.15 ml at 25° for 30 min. The enzymic activity against peptides not containing glutamine was assayed by measuring the amount of the liberated amino acid from the peptides in the same manner as glutamine. The enzymic activity for glutamic acid and glutamine was assayed by measuring the amount of the substrates before and after incubation.

	Relative activity
γ-Glutamyl peptides	
γ-Glutamylglutamine	100
γ-Glutamylglutamic acid	0
γ-Glutamylglycine	0
γ-Glutamylalanine	8
γ-Glutamylserine	40
γ-Glutamyl-γ-aminobutyric acid	0
γ-Glutamyllysine	0
γ-Glutamylaniline	0
Glutathione	0
α-Glutamyl peptides	
α-Glutamylglutamic acid	0
α-Glutamylglycine	0
α-Glutamylserine	0
Peptides containing glutamine at carboxy-terminal residues	
Glycylglutamine	0
Alanylglutamine	0
Valylglutamine	0
Prolylglutamine	0
Other derivatives of glutamic acid	
Glutamic acid	0
Glutamine	0
Carbobenzoxyglutamic acid	0
Other peptides	
Glycylglycine	0
Glycylleucine	7
Leucylglycylglycine	15
Leucylglycine	0
Carbobenzoxyglycylphenylalanine	0
γ-Aminobutyrylhistidine	0
Alanyllysine	0
Arginylleucine	20
Arginylphenylalanine	0
Serylarginine	0
Carbobenzoxyalanine	0
Carbobenzoxy-γ-aminobutyric acid	0

Effect of substrate concentration

Fig. 1 shows the relationship between the reaction velocity and the concentration of γ-glutamylglutamine at pH 8.0 and 25°. Various concentrations of the substrate were incubated for 30 min with the Step-6 enzyme preparation.

TABLE IV

RELATIVE ACTIVITY OF SUBSTRATES TO γ-GLUTAMYLGLUTAMINE FOR CRUDE ENZYME PREPARATIONS

Substrate	*Preparation 1**	*Preparation 2**
γ-Glutamylglutamine	100	100
γ-Glutamylglycine	50	174
γ-Glutamylglutamic acid	22	31
Glycylleucine	138	67
Leucylglycylglycine	148	174

* The preparations are described in the text.

Effect of pH

The activity exhibited no sharp optimum in the range of pH from 7 to 9, but fell off above pH 9 and below 7.

Effects of metals, inhibitors and coenzymes

The addition of $1 \cdot 10^{-3}$ M magnesium chloride and manganese chloride showed no effect on the enzyme activity, while the same concentration of cobalt chloride inhibited the enzyme 20% and that of zinc sulfate 96%. Potassium cyanide (10^{-3} M), sodium azide (10^{-3} M), *p*-chloromercuribenzoate (10^{-4} M), ethylenediaminetetraacetate (10^{-3} M), citric acid (10^{-2} M), cysteine (10^{-3} M) and glutathione (10^{-2} M), showed no effect on the enzyme activity. The addition of 1 M hydroxylamine did not change the rate or nature of the product of the enzymic reaction. Sodium fluoride inhibited the enzyme about 40% at a concentration of $1 \cdot 10^{-3}$ M. The enzyme activity was not influenced by the addition of AMP, ATP, NAD, NADP, FAD or pyridoxal phosphate at $5 \cdot 10^{-3}$ M.

Distribution of the enzyme in tissues of rat and guinea pig

Results of the assay of the enzyme activity in various tissues are shown in

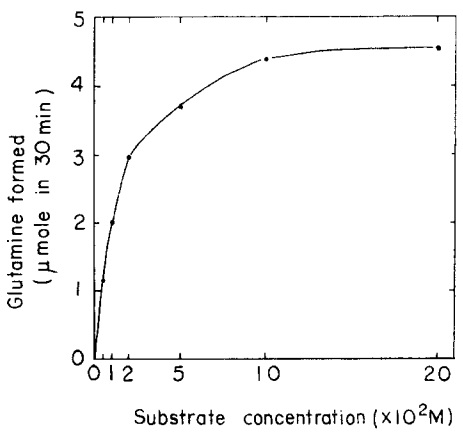

Fig. 1. Relationship between reaction rate and substrate concentration. The reaction mixture consisted of 0.2 ml of the enzyme solution, pH 8.0 (Step-6 enzyme), and neutralized substrate solution. The final volume was adjusted to 0.4 ml with the addition of water. The mixture was incubated at 25° for 30 min, and the glutamine formed was measured as described in the text.

TABLE V

DISTRIBUTION OF γ-GLUTAMYLGLUTAMINE LACTAMASE ACTIVITY IN VARIOUS ORGANS OF RAT AND GUINEA-PIG

Tissues weighing about 1 g were homogenized with 9 vol. of cold 0.05 M potassium phosphate buffer (pH 8.0) and centrifuged at 13 000 × *g* for 30 min at 0°. A 0.2-ml aliquot of the supernatant solution was incubated with 25 μmoles of neutralized γ-glutamylglutamine in a final volume of 0.4 ml for 30 min at 25°. For rat kidney 0.1 ml of the supernatant solution was used because the activity of the enzyme was excessively high. For the blank, substrate solution was replaced by water. Each number represents the result of a single estimation.

Animal	*Organ*	*Enzyme activity (units/g wet tissue)*	
		Animal 1	*Animal 2*
Rat	kidney	49.7	43.3
	liver	20.4	16.0
	spleen	3.3	2.0
	heart	2.0	2.0
	brain	1.7	2.3
	lung	1.7	3.7
	skeletal muscle	1.0	1.3
	intestine	0.0	0.0
Guinea-pig	kidney	11.7	14.7
	liver	11.0	10.3
	pancreas	5.0	4.0
	spleen	3.7	7.7
	heart	1.3	2.0
	brain	2.0	2.7
	lung	4.7	6.7
	skeletal muscle	1.3	2.3
	intestine	3.0	4.0

Table V. Enzyme activity was highest in the rat kidney among the organs examined. The liver of rat and guinea pig, and kidney of guinea pig also contained a high enzyme activity. The low enzyme activity was distributed evenly in other tissues.

DISCUSSION

γ-Glutamylglutamine lactamase seems to be a different enzyme from the enzymes that are known to degrade oligopeptides. Although the enzyme was not purified extensively, the preparation obtained showed a relatively high substrate specificity on γ-glutamylglutamine. The present enzyme preparation did not hydrolyze leucylglycine, glycylglycine, γ-aminobutyrylhistidine or carbobenzoxyglycylphenylalanine, substrates of leucine aminopeptidase[9], glycylglycine dipeptidase[10], carnosinase[11] and carboxypeptidase[12] respectively. The enzyme preparation hydrolyzed glycylleucine and leucylglycylglycine, but glycylleucine dipeptidase[13] and amino tripeptidase[14] seem to be different enzymes from γ-glutamylglutamine lactamase as described above. γ-Glutamyltransferase which may transfer the γ-glutamyl residue to water is known to catalyze the transfer of the glutamyl residue from various γ-glutamyl peptides[15], while the present enzyme acts on only a few γ-glutamyl peptides among many substrates of γ-glutamyltransferase. In order to establish the identity of the present enzyme it may be necessary to examine the actions of the various purified enzymes

against γ-glutamyl peptides including γ-glutamylglutamine. The facts explained above and the effects of metals and inhibitors on the γ-glutamylglutamine lactamase, which are different from their effects on the other known peptidases, support the postulation that γ-glutamylglutamine lactamase is a separate enzyme which has not previously been reported.

Glutamine formed from γ-glutamylglutamine appears to be derived from the carboxy terminal glutamine residue of the peptide. This postulation was supported by the finding that γ-glutamylserine was cleaved into pyrrolidone carboxylic acid and serine by the same enzyme preparation. Because free glutamic acid was not detected in the reaction mixture, and because glutamic acid and glutamine are resistant to the enzyme reaction, intramolecular rearrangement of γ-glutamylglutamine with a concomitant formation of pyrrolidone carboxylic acid is considered to be effected on the enzyme surface. The over-all reaction is not hydrolytic in nature. The enzyme reaction was not reversible. Heating of the aqueous solution of γ-glutamyl peptides was found to lead to non-enzymic formation of pyrrolidone carboxylic acid[15]. Although the mechanism of this non-enzymic reaction is not known, the reaction seems to be characteristic of the chemical nature of γ-glutamyl peptides, and a similar mechanism of the reaction is likely to be catalyzed by γ-glutamyl lactamase[5] and by the present enzyme.

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