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γ-Glutamyltranspeptidase from *Escherichia coli* K-12: Purification and Properties

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γ-Glutamyltranspeptidase (GGT) (EC 2.3.2.2) was purified from the periplasmic fraction of *Escherichia coli* K-12 to electrophoretic homogeneity. The final purification step, chromatofocusing, gave two protein peaks showing GGT activity (fractions A and B). The major heavy fraction (fraction A) consisted of two different subunits, with molecular weights of 39,200 and 22,000. The minor light fraction (fraction B) consisted of those with molecular weights of 38,600 and 22,000. Fraction A catalyzes the hydrolysis and transpeptidation of all γ-glutamyl compounds tested, but it prefers basic amino acids and aromatic amino acids as acceptors. The apparent K_m values for glutathione and γ-glutamyl-*p*-nitroanilide as γ-glutamyl donors in the transpeptidation reaction were both 35 μM, and those for glycylglycine and L-arginine as acceptors were 0.59 and 0.21 M, respectively. The enzyme was inhibited by some amino acids and by protease inhibitors and affinity-labeling reagents for GGT. The temperature stability of the purified GGT supports our hypothesis that *E. coli* GGT is synthesized only at lower temperature rather than that the synthesized GGT is degraded or inactivated at higher temperature.

γ-Glutamyltranspeptidase (GGT) catalyzes the hydrolysis of γ-glutamyl compounds (i.e., glutathione) and the transfer of their γ-glutamyl moieties to amino acids and peptides. GGTs have been purified from various mammalian cells, and extensive biochemical studies have been performed and reviewed (2, 12, 24, 25). Orlowski and Meister proposed that GGTs play a physiological role in the γ-glutamyl cycle which is involved in the amino acid uptake through the cell membrane into mammalian cells (18), but some results have not coincided with their hypothesis (7, 19–21). Various alternative functions of GGT have also been postulated, such as the sequential hydrolysis of glutathione (GSH) and its conjugates by GGT and a membrane-bound aminopeptidase (8, 26), detoxification through the synthesis of mercapturic acid (3, 16), and translocation of intracellular GSH (5, 6).

Although purification and enzymatic study of GGTs are important to elucidate their physiological role in bacterial cells, only Nakayama et al. in our laboratory have purified GGT from a bacterium to a homogeneous state and characterized it extensively (15). In the accompanying paper (22), we reported that the GGT activity of *Escherichia coli* K-12 is greatly affected by its growth temperature and is localized in the periplasmic space and that the periplasmic fraction we obtained on lysozyme treatment showed quite high specific GGT activity.

In this study, GGT was purified to a homogeneous state from *E. coli* K-12, and its properties were investigated to gain insight into the physiological role of bacterial GGTs and temperature-dependent GGT biosynthesis in *E. coli* K-12.

MATERIALS AND METHODS

Reagents. *N*-γ-Glutamyl-α-naphthylamide, *S*-methylglutathione, L-glutamic acid γ-monohydroxamate, *N*-(γ-L-glutamyl)-L-tyrosine, *N*-DL-γ-glutamylalanine, α-L-glutamyl-L-alanine, *N*-(γ-glutamyl)-L-phenylalanine, γ-L-glutamyl-L-histidine, L-glutamyl-γ-(3-carboxy-4-hydroxyanilide), L-glutamic acid γ-methyl ester, L-glutamic acid γ-monoethyl ester, L-2-methyl-dihydroxy-phenylalanine (L-2-methyl-DOPA), 3-*O*-methyl-DOPA, *O*-methyl-L-tyrosine, L-tryptophyl-L-alanine, L-tryptophyl-glycine, D-lysine, D-arginine, azaserine, fast garnet GBC salt, lysozyme, 6-diazo-5-oxo-norleucine, oxidized GSH, 1-chloro-3-tosylamido-7-amino-2-heptanone, and L-1-tosyl-amido-2-phenylethylchloromethyl ketone were purchased from Sigma Chemical Co. Tryptone was from Difco Laboratories, yeast extract was from Oriental Yeast Co., amido black 10B was from E. Merck AG, and phenylmethylsulfonyl fluoride was from Calbiochem-Behring. Phenyl-Sepharose CL-4B, Sephadex G-150, Sephadex G-100, blue dextran 2000, PBE 94, Polybuffer 74, and molecular weight markers were from Pharmacia. L-γ-glutamyl-*p*-nitroanilide (γ-GpNA), glycylglycine (gly-gly), glycyl-L-leucine, D-glutamine, and iodoacetate were from Wako Pure Chemical Co. Benzoyl-L-tyrosine ethyl ester, L-tyrosine ethyl ester, glycylglycylglycine, L-leucyl-glycine, L-DOPA, dithiothreitol, *p*-mercuribenzoate, *N*-ethylmaleimide, acrylamide, bisacrylamide, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, sodium dodecyl sulfate, Coomassie blue R-250, ammonium peroxide, D-cysteine, and D-tyrosine were from Nakarai Chemical Co. L-Theanine, α-(*N*-γ-DL-glutamyl)aminopropionitrile and *S*-carboxymethyl-L-cysteine were from Tokyo Kasei Co., and *S*-methyl-L-cysteine was from Aldrich Chemical Co. Reduced GSH was a generous gift from Kirin Brewery Co. DEAE-Cellulofine AH and Cellulofine GC-700-m were from Chisso Co., and L-(αS, 5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazole acetic acid (AT-125), a product of Upjohn Co., was given by Ajinomoto Co. γ-Glutamyl-L-DOPA was the same sample that Nakayama et al. in our laboratory previously described (14). L-γ-Glutamyl-methylamide was prepared enzymatically with bacterial glutamine synthetase, purified, and donated by Tachiki et al. in our laboratory (manuscript in preparation). Other chemicals were purchased from commercial sources.

Bacterial strain and culture. Prototrophic *E. coli* K-12 MG1655 was the same as described in reference 22. Cells were picked up from an LB plate (13), inoculated into a 100-ml flask containing 25 ml of LB broth, and then grown at

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TABLE 1. Purification of GGT from *E. coli* K-12

Steps	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)
1. Lysozyme treatment	39,260	725	0.0185	100
2. Ammonium sulfate (60–80%)	8,321	643	0.0773	88.7
3. DEAE-Cellulofine AH	689	616	0.894	85.0
4. Cellulofine GC-700-m	214	205	0.954	28.3
5. Phenyl-Sepharose CL-4B	166	183	1.10	25.3
6. Sephadex G-100	95.1	101	1.06	13.9
7. Chromatofocusing				
Fraction A	60.2	84.2	1.40	11.6
Fraction B	5.62	7.92	1.41	1.1

37°C overnight with reciprocal shaking. A 5-ml amount of culture was transferred to a 2-liter Sakaguchi flask containing 500 ml of LB broth and then grown at 28°C overnight with reciprocal shaking. Two such subcultures were inoculated into a 30-liter jar fermentor (type MSJ-U 301, Marubishi Co.) containing 25 liters of LB broth. Cultivation was carried out at 20°C for 40 h with aeration (0.4 liter/liter of medium per min) and agitation (200 rpm). The grown cells were harvested with a refrigerated continuous-flow centrifuge (type GLE; Carl Padberg Gmbh). A total of 250 liters of culture was obtained.

Enzyme assay for GGT activity. GGT activity was determined spectrophotometrically by measuring the *p*-nitroaniline released from L-γ-glutamyl-*p*-nitroanilide as described previously (17), with slight modifications, and with an automatic amino acid analyzer (type K-101-AS; Kyowa Seimitsu Co.) with Kyowa Gel 62210-S.

(i) During the purification. The assay solution contained 1.25 μmol of L-γ-glutamyl-*p*-nitroanilide, 30 μmol of gly-gly, 25 μmol of Tris hydrochloride (pH 8.0), 37.5 μmol of NaCl, and the enzyme in a final volume of 0.5 ml. After incubation at 37°C, each reaction was terminated by the addition of 1 ml of 3.5 N acetic acid. The reaction mixtures were centrifuged at 10,000 × *g* (in a Kubota KM-152000 microfuge with an RA-150A rotor) for 5 min at 4°C. The difference in A_{410} between reaction mixtures with and without gly-gly was used to calculate the transferase activity. One unit of enzyme activity was defined as the amount of the enzyme which transferred 1 μmol of γ-glutamyl moiety per min. Specific activity is expressed in units per milligram of protein.

(ii) For the purified enzyme. The assay solution contained 1.25 μmole of γ-GpNA, 30 μmol of gly-gly (pH adjusted to 8.5), 25 μmol of Tris hydrochloride (pH 9.0 with gly-gly and pH 8.73 without gly-gly), and the enzyme in a final volume of 0.5 ml. After incubation at 37°C for 10 min (unless otherwise stated), the reaction was terminated and the A_{410} was determined as above. Hydrolyase activity of γ-glutamyl compounds was measured without a γ-glutamyl acceptor under the same conditions.

(iii) With an amino acid analyzer. When γ-glutamyl compounds other than γ-GpNA were used, each reaction was terminated by adding 50 μl of 0.1 N HCl or 60 μl of 20% trichloroacetic acid, and then the mixtures were injected into the amino acid analyzer. γ-Glutamyl-glycylglycine was measured to calculate the transferase activity, and glutamic acid was measured to calculate the hydrolyase activity.

Protein determination. Protein concentrations were determined by the method of Lowry et al. (11), with ovalbumin as a standard.

Electrophoresis. Electrophoresis and protein staining were carried out according to the guidebook from Pharmacia (*Polyacrylamide Gel Electrophoresis–Laboratory Techniques*). Proteins of native gel were stained with amido black 10B and those of modified Laemmli gel were stained with Coomassie blue R-250. GGT activity staining was carried out by a modification of the method of Albert et al. (1). The gel was incubated at 37°C for 15 min in 50 ml of the staining mixture (25 ml of a 1.1-mg/ml concentration of γ-L-glutamyl-α-naphthylamide, 2 ml of 1 M K-phosphate buffer, pH 7.0, 10 ml of 0.3 M gly-gly, 25 mg of fast garnet GBC, and 13 ml

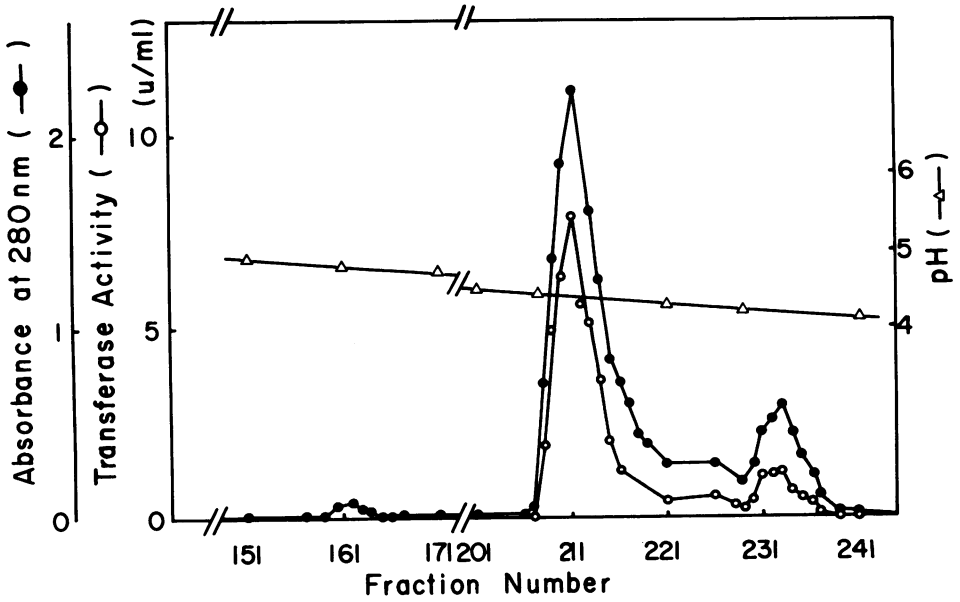

FIG. 1. Chromatofocusing pattern of *E. coli* GGT. Approximately 101 U of the enzyme was applied on a PBE 94 column (1.06 by 65 cm), equilibrated with 25 mM histidine-HCl (pH 6.2), and eluted with Polybuffer 74-HCl (pH 4.0) as described in Materials and Methods.

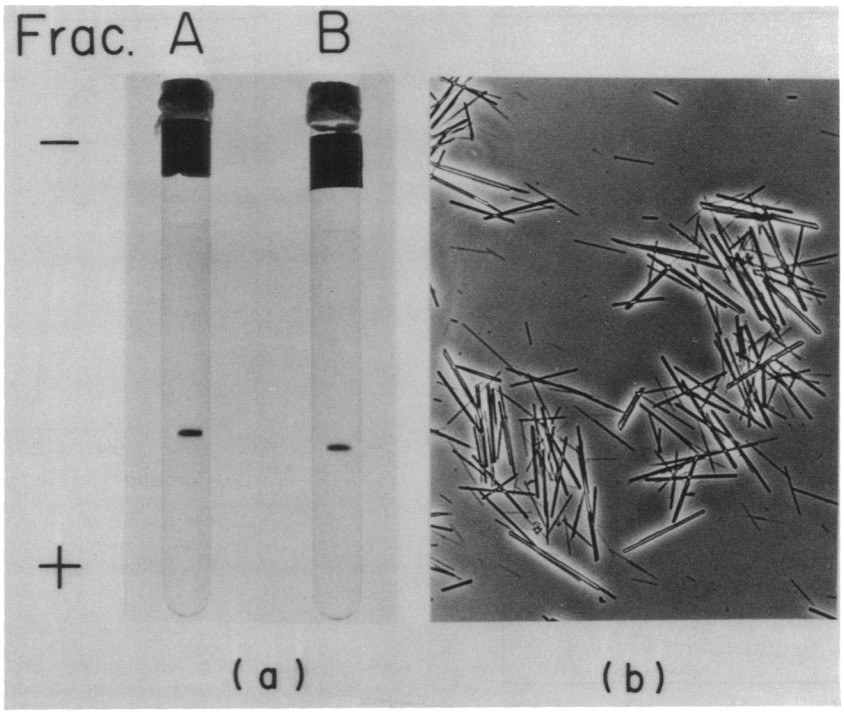

FIG. 2. Disk polyacrylamide gel electrophoresis of *E. coli* GGT. (a) Approximately 20 μg of each fraction (fractions A and B) was applied. Electrophoresis and protein staining were carried out as described in Materials and Methods. (b) Crystals of *E. coli* GGT fraction A. The photo was taken under a phase-contrast microscope.

of distilled water) and then washed once in 20 mM Tris hydrochloride (pH 8.0).

Molecular weight estimation. Molecular weights were determined by gel filtration on a Sephadex G-150 column (1.2 by 148 cm) equilibrated with 20 mM Tris hydrochloride (pH 8.0). Five standard proteins, cytochrome *c* (molecular weight, 12,400), RNase A (13,700), chymotrypsinogen A (25,000), ovalbumin (67,000), and bovine serum albumin

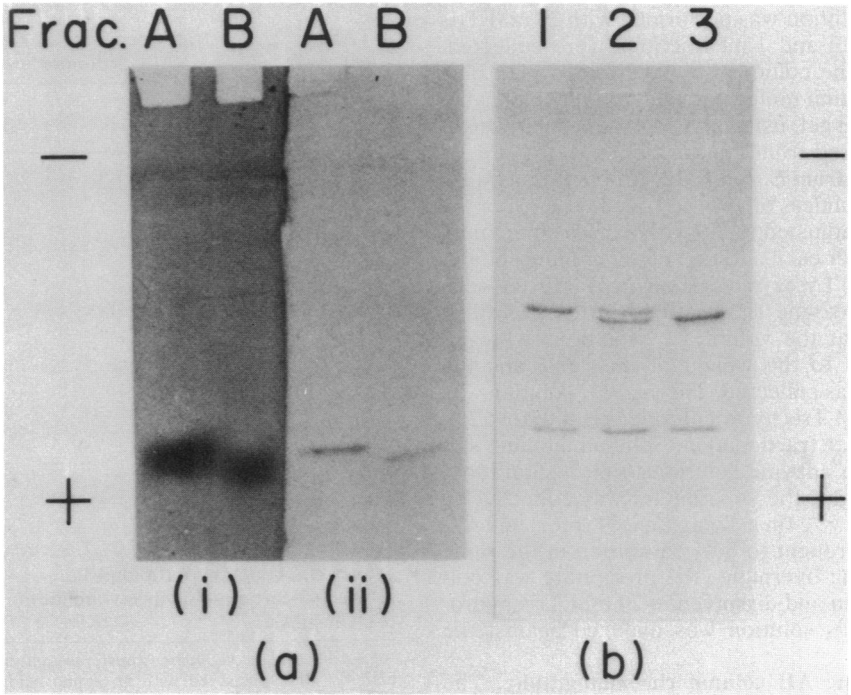

FIG. 3. Native slab polyacrylamide (a) and modified Laemmli (b) gel electrophoresis of *E. coli* GGT. (a) (i) GGT activity staining; (ii) protein staining. Approximately 15 μg of each fraction was applied. Electrophoresis and staining were carried out as described in Materials and Methods. (b) Lane 1 contained 10 μg of fraction A; lane 2, 10 μg of fraction A and 10 μg of fraction B; and lane 3, 10 μg of fraction B.

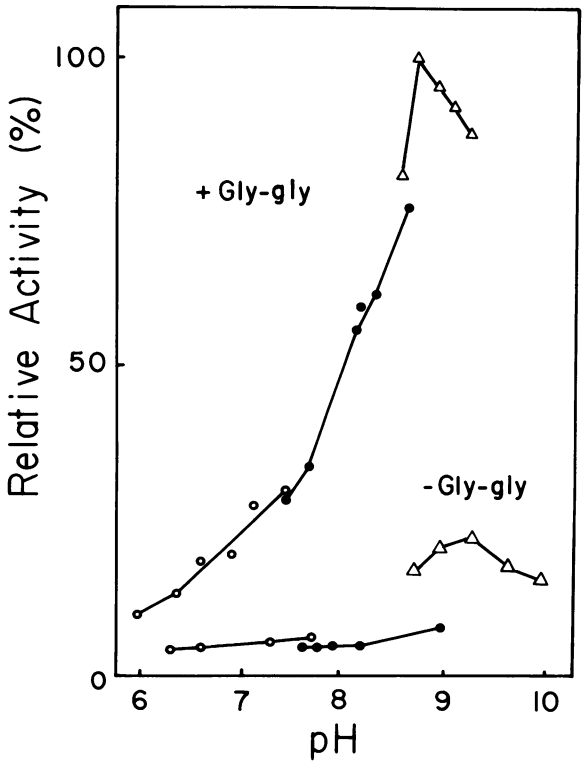

FIG. 4. pH profiles for *E. coli* GGT. *p*-Nitroaniline released with and without gly-gly was measured. The pH of the reaction mixture was measured with a pH meter and plotted. Symbols: NaAc-NaOH buffer (○); Tris hydrochloride buffer (●); boric acid-NaOH buffer (△).

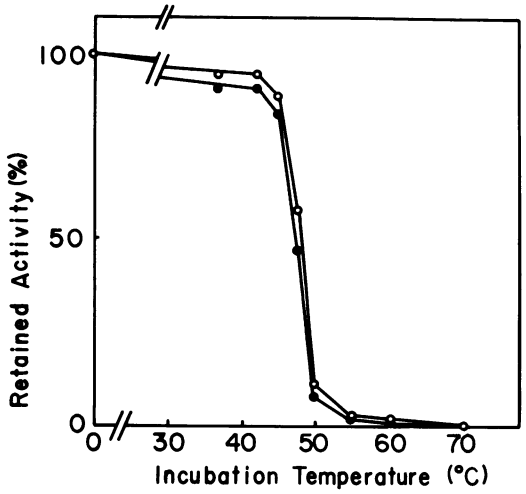

FIG. 5. Temperature stability of *E. coli* GGT. A 0.5-ml portion of the enzyme solution was incubated at various temperatures for 15 min in 20 mM Tris hydrochloride (pH 8.0), and then the transferase (●) and hydrolyase (○) activities were measured.

(67,000), were used; elution was performed with 20 mM Tris hydrochloride (pH 8.0) and 1-ml fractions were collected. The void volume of the column was determined with blue dextran 2000. The subunit molecular weights were estimated on a modified Laemmli gel, using an LMW molecular weight calibration kit purchased from Pharmacia.

Purification of GGT from *E. coli* K-12. All operations were carried out at 0 to 5°C unless otherwise stated. The pH of the enzyme solution was adjusted to 7.0 with a 2.5% ammonium hydroxide solution after each addition of ammonium sulfate.

Purification steps. (i) Lysozyme treatment. Harvested cells were treated with lysozyme as described in the preceding paper (22), except that the volumes of solutions were increased in proportion to the weight of the cells, and the periplasmic fraction was collected. The enzyme solution was dialyzed against 20 mM Tris hydrochloride (pH 8.0) for 12 h.

(ii) Ammonium sulfate fractionation. Solid ammonium sulfate was added to the enzyme solution to 60% saturation. After standing overnight, the supernatant was collected by centrifugation at 6,000 × *g* (in a Tomy no. 9N rotor) and 4°C for 20 min and then brought to 80% saturation in the same manner. After standing overnight, the precipitate was collected by centrifugation and dissolved in 20 mM Tris hydrochloride (pH 8.0). This solution was dialyzed against the same buffer.

(iii) DEAE-Cellulofine AH column chromatography. The dialyzed enzyme solution was applied to a DEAE-Cellulofine AH column (6.1 by 75 cm) previously equilibrated with 20 mM Tris hydrochloride (pH 8.0). The column was washed with the same buffer, and then the enzyme was eluted in 17.5-ml fractions with 50, 100, and 150 mM NaCl in the buffer. The active fractions (2.99 liters) were combined and concentrated by the addition of ammonium sulfate to 90% saturation. After standing overnight, the precipitate was collected by centrifugation and then dissolved in 50 mM Tris hydrochloride (pH 8.0).

(iv) Cellulofine GC-700-m column chromatography. The enzyme solution was applied to a Cellulofine GC-700-m column (4 by 91 cm) previously equilibrated with 50 mM Tris hydrochloride (pH 8.0). The enzyme was eluted with the same buffer in 20-ml fractions. The active fractions (80 ml)

TABLE 2. Substrate specificity of *E. coli* GGT for γ-glutamyl donors[a]

Substrate	Relative rate (%)	
	Transferase	Hydrolyase
γ-GpNA	100	100
D,L-γ-Glu-Ala	107	330
L-Theanine	105	131
S-Methyl-glutathione	99.2	161
L-Glu-γ-monohydroxamate	97.1	330
γ-L-Glu-L-Tyr	95.2	334
L-γ-Glu-methylamide	94.7	236
GSH	90.0	137
γ-L-Glu-L-His	81.1	218
L-Glu-γ-monomethyl ester	80.2	10.2
γ-L-Glu-L-DOPA	79.3	181
L-Glu-γ-monoethyl ester	79.0	11.7
Oxidized GSH	72.7	220
L-Gln	71.1	33.1
D-Gln	71.1	347
γ-L-Glu-α-naphthylamide	68.1	122
γ-L-Glu-L-Phe	67.3	234
α-(*N*-γ-D,L-Glu)aminopropionitrile	66.7	117
L-Glu-γ-(3-carboxy-4-hydroxy-anilide)	29.8	216
α-L-Glu-L-Ala	0.0	0.0

[a] γ-Glutamylglycylglycine (transferase activity) and glutamate (hydrolyase activity) were measured with an amino acid analyzer as described under Materials and Methods, with replacement of γ-GpNA by its analogs under the same conditions. Concentrations of 2.5 and 60 mM for donors and gly-gly, respectively, were used. Activity is expressed relative to that found with γ-GpNA (100%).

were combined and concentrated by the addition of ammonium sulfate to 90% saturation. After standing overnight, the precipitate was collected by centrifugation and then suspended in 10 mM K-phosphate buffer (pH 7.4). Ammonium sulfate was added to the enzyme solution to 0.8 M, and then the solution was dialyzed against 0.8 M ammonium sulfate solution in 10 mM K-phosphate buffer (pH 7.4).

(v) Phenyl-Sepharose CL-4B column chromatography. The enzyme solution was applied to a Phenyl-Sepharose CL-4B column (1.9 by 47 cm) equilibrated with 0.8 M ammonium sulfate in 10 mM K-phosphate buffer (pH 7.4). The enzyme was eluted in 10-ml fractions by elution with a gradient formed between 0.8 M ammonium sulfate in 10 mM K-phosphate buffer (pH 7.4) and 40% ethyleneglycol in 10 mM K-phosphate buffer (pH 7.4). The active fractions (210 ml) were combined and concentrated by the addition of ammonium sulfate to 90% saturation. After standing overnight, the precipitate was collected by centrifugation and then suspended in 50 mM Tris hydrochloride (pH 8.0).

(vi) Sephadex G-100 column chromatography. The enzyme solution was applied to a Sephadex G-100 column (2.2 by 106 cm) equilibrated with 50 mM Tris hydrochloride (pH 8.0). The enzyme was eluted with the same buffer in 2-ml fractions. The active fractions (22 ml) were combined and concentrated by the addition of ammonium sulfate to 90% saturation. After standing overnight, the precipitate was collected by centrifugation, suspended in 25 mM histidine-HCl (pH 6.2), and then dialyzed against the same buffer.

(vii) Chromatofocusing. The enzyme solution was applied on a PBE 94 column (1.06 by 65 cm) equilibrated with 25 mM histidine-HCl (pH 6.2). The enzyme was eluted in 2-ml fractions with a descending linear gradient of Polybuffer 74-HCl (ninefold dilution, pH 4.0), from pH 6 to 4. The active fractions were eluted at pH 4.4 to 4.3 (16 ml) and 4.2 to 4.1 (6 ml). Each of them was concentrated by the addition of ammonium sulfate to 90% saturation.

Crystallization. Ammonium sulfate was added to the homogeneous enzyme solution in 20 mM Tris hydrochloride (pH 8.0) (14.2 mg of protein per ml; 0.1 ml) carefully until it became slightly turbid, and then the mixture was placed in a refrigerator. Crystallization of the enzyme as fine needles was observed from the next day and was completed within 1 week.

TABLE 3. Substrate specificity of *E. coli* GGT for γ-glutamyl acceptors[a]

Substrate	Concn (mM)	Relative rate (%)
Gly-gly	60	100
	20	29.4
L-Ala	60	0.0
L-Arg	60	141
D-Arg	60	18.1
L-Asn	60	31.8
L-Asp	60	4.2
L-α-Amino-*n*-butyrate	60	12.7
γ-Amino-*n*-butyrate	60	22.3
L-Cys	20	13.5
D-Cys	20	0.0
S-Methyl-L-Cys	20	13.5
S-Carboxymethyl-L-Cys	20	1.9
L-Cysteic acid	20	0.0
L-Cyn	20	0.6
L-Glu	60	10.2
Gly	60	20.8
L-His	60	49.5
L-Hse	60	0.0
L-Ile	60	13.8
L-Leu	20	2.5
L-Lys	60	121
D-Lys	60	16.5
L-Met	60	91.8
L-Phe	60	42.9
L-Phe-methyl ester	20	28.3
L-Pro	60	2.4
L-Ser	60	9.6
L-Thr	60	22.9
L-Trp	20	29.8
L-Tyr	20	2.8
D-Tyr	20	1.1
L-DOPA	20	63.9
L-2-Methyl-DOPA	20	25.6
3-Methyl-L-Tyr (3-*O*-Methyl-DOPA)	20	13.3
O-Methyl-L-Tyr	20	5.6
L-Tyr ethyl ester	20	18.9
Benzoyl-L-Tyr ethyl ester	20	0.0
L-Val	60	10.8
Gly-L-Leu	60	12.5
L-Leu-gly	60	12.5
L-Trp-L-Ala	60	11.0
L-Trp-gly	60	23.6
Gly-gly-gly	60	14.2

[a] Transferase activity was measured by a spectrophotometric method as described under Materials and Methods. Activity is expressed relative to that found with 60 mM gly-gly (100%).

TABLE 4. K_m values[a]

Parameter	K_m (μM)
Transferase activity	
Donor[b]	
GSH	35
γ-GpNA	35
Acceptor[c]	
Gly-gly	0.59 M
L-Arg	0.21 M
Hydrolyase activity	
Donor	
GSH	29
γ-GpNA	68

[a] Calculated by Lineweaver-Burk plotting.
[b] 60 mM gly-gly was used as an acceptor. These values are apparent K_m values.
[c] 2.5 mM γ-GpNA was used as a donor.

RESULTS

Enzyme purification. The purification scheme (Table 1), involving ammonium sulfate fractionation and five column chromatographic fractionations, led to a 76-fold purification with a recovery of 12.7%. The final step of chromatofocusing gave three protein peaks (Fig. 1). The proteins eluted at pH 4.4 to 4.3 and pH 4.2 to 4.1 showed GGT activity. Each active peak was collected separately and named fraction A

TABLE 5. Inhibition of *E. coli* GGT by amino acids

Amino acid[a]	Relative rate (%)	
	Transferase	Hydrolyase
None	100	100
L-Ala	43.0	91.6
L-Gln	11.0	19.8
D-Gln	96.1	100

[a] A 10 mM concentration of each amino acid was added to the reaction mixture.

TABLE 6. Inhibition of *E. coli* GGT by various inhibitors

Inhibitor	Final concn (mM)	Relative rate (%)	
		Transferase[a]	Hydrolyase[b]
None		100	100
AT-125	1	0.0	0.0
DON[c]	1	0.0	2.9
Azaserine	1	0.0	3.8
L-Ser + borate	1	97.8	98.1
	10	14.4	58.1
D-Ser + borate	1	104	100
	10	110	100
Iodoacetate	1	56.7	27.3
$HgCl_2$	1	102	60.2

[a] 0.25 ml of the enzyme solution was incubated at 37°C for 15 min with 30 μmol of gly-gly, 25 μmol of Tris hydrochloride (pH 9.0), and 0.5 or 5 μmol of an inhibitor, and the enzyme reaction was started by adding 1.25 μmol of γ-GpNA (0.25 ml).

[b] 0.25 ml of the enzyme solution was incubated at 37°C for 15 min with 25 μmol of Tris hydrochloride (pH 8.73) and 0.5 or 5 μmol of an inhibitor, and the enzyme reaction was started by adding 1.25 μmol of γ-GpNA (0.25 ml).

[c] DON, 6-Diazo-5-oxo-norleucine.

and fraction B, respectively. Both fractions were subjected to disk polyacrylamide gel electrophoresis (Fig. 2a), and each gave a single band. Fraction A, the major fraction, was crystallized by the addition of solid ammonium sulfate to the enzyme solution (Fig. 2b). Both fractions were subjected to native slab polyacrylamide gel electrophoresis, and then GGT activity staining and protein staining were performed (Fig. 3a); Fig. 3 clearly shows that both fractions exhibit GGT activity. Both fractions were subjected to modified Laemmli gel electrophoresis separately and as a mixture (Fig. 3b). The light subunits of both fractions were found to have the same molecular weight, but the heavy subunit of fraction A was heavier than that of fraction B.

Molecular weight and subunit structure. The molecular weights of the two fractions were determined to be ca. 58,000 (fraction A) and 57,000 (fraction B) by Sephadex G-150 gel filtration. Modified Laemmli gel electrophoresis of the two fractions gave two bands of 39,200 and 22,000 (fraction A) and 38,600 and 22,000 (fraction B), indicating that the native enzyme contains one each of these two subunits.

Catalytic properties. The purified enzyme (both fractions) exhibits both transferase and hydrolyase activities. Other properties were investigated only for the major fraction, fraction A. Under standard conditions, fraction A showed transferase activity of 6.2 U/mg and hydrolyase activity of 1.1 U/mg. The optimum pH for the transferase activity from γ-GpNA to gly-gly and that of hydrolyase activity of γ-GpNA were 8.73 and 9.25, respectively (Fig. 4). The optimum temperature for both transferase and hydrolyase activities was 50°C under standard conditions, except with incubation for 15 min. The temperature stability of the enzyme was investigated by incubation of the enzyme at various temperatures (Fig. 5). The enzyme retained 84% of the initial transferase activity on incubation at 45°C for 15 min and lost 92% of the activity on incubation at 50°C.

Substrate specificity. Hydrolysis and transpeptidation to gly-gly from various γ-glutamyl compounds were measured at pH 8.7 (Table 2). The reaction rate for hydrolysis of γ-GpNA, 0.420 nmol/min, and that for the γ-glutamyl transfer from γ-GpNA to gly-gly, 0.475 nmol/min, were taken as 100%, respectively. α-Glutamyl-L-alanine was not used as a substrate. Various amino acids and peptides could serve as γ-glutamyl acceptors, from γ-GpNA (Table 3). Basic amino acids, L-Arg, L-Lys, and L-His, aromatic amino acids, L-DOPA and L-Phe, and L-Met were good acceptors besides gly-gly. α-Amino-*n*-butyric acid, which is not an α- but an ω-amino acid, was also used as an acceptor. The K_m values for typical γ-glutamyl donors and acceptors were determined and are shown in Table 4. The results show that GGT has low K_m values for γ-glutamyl donors, but extremely high ones for acceptors.

Inhibition by amino acids and other inhibitors. The transferase and hydrolyase activities of the GGT were strongly inhibited by L-Ala and L-Gln (Table 5), but not significantly by L-Glu, L-Ser, L-Asp, and L-α-amino butyrate. Inhibition by affinity labeling reagents (9, 23) and other inhibitors, including sulfhydryl reagents, was also investigated (Table 6). Other sulfhydryl compounds and reagents, dithiothreitol, β-mercaptoethanol, *p*-mercuribenzoate, and *N*-ethylmaleimid, did not have any significant effect on the enzyme activity. Protease inhibitors, phenylmethylsulfonyl fluoride, 1-chloro-3-tosylamido-7-amino-2-heptanone, and L-1-tosylamido-2-phenylethylchloromethyl ketone, inhibited the enzyme moderately.

DISCUSSION

GGTs have been purified from various mammalian cells, and their properties and functions in living cells have been studied extensively (2, 12, 24, 25), whereas for bacterial cells, only Nakayama et al. (15) purified a GGT to a homogeneous state from *P. mirabilis* and studied its properties. In the accompanying paper (22), we reported that the GGT activity of *E. coli* is strongly affected by its growth temperature, and cells grown at 20°C exhibit much higher activity than *P. mirabilis* cells. Nakayama et al. (15) purified GGT from a completely disrupted cell homogenate of *P. mirabilis*, but tremendously high purification (15,200-fold) was needed to obtain a homogeneous preparation; they also reported that they could obtain a GGT solution with 7.6-fold-higher specific activity when the cells were treated gently with ultrasonic. In the accompanying paper (22), we reported that *E. coli* GGT is localized in the periplasmic space and that the periplasmic fraction obtained on lysozyme treatment showed 28.8-fold-higher specific activity than the cell homogenate of *P. mirabilis* obtained by Nakayama et al. with a Dyno-Mill. Because of this, we used the periplasmic fraction of *E. coli* as a starting material in this study. A 76-fold purification gave a homogeneous GGT preparations with a specific activity of 1.4 U/mg.

The final step of the purification, chromatofocusing, gave two protein peaks showing GGT activity, a major peak, fraction A, and a minor peak, fraction B. Both fractions were homogeneous, as judged by disk gel electrophoresis, and GGT activity was associated with both protein bands. Nakayama et al. (15) also found a minor band on disk gel electrophoresis of their fraction 2 preparation of *P. mirabilis* GGT, but they could not isolate it. Since we used a much bigger chromatofocusing column than they did, we could isolate the minor peak as a homogeneous protein. Fraction B might be an isozyme or a protein modified by proteolysis. To elucidate the physiological significance of this minor peak, further study is needed.

Highly purified preparations of renal GGTs have been obtained from various mammalian species. The enzymes have light subunits of about 22,000 molecular weight, and they can be separated into two groups with respect to the size of their heavy subunit: one group with molecular weights of 46,000 to 50,000 (rat and rabbit) and one group

with molecular weights of about 64,000 (human, bovine, sheep, and hog) (24). *P. mirabilis* GGT has subunits of 47,000 and 28,000 (15). The light subunit of *E. coli* has a molecular weight of 22,000, which is the same as those of mammalian renal GGTs, while the heavy subunit has a molecular weight of 39,000, which is much smaller than those of mammalian renal GGTs and *P. mirabilis* GGT.

E. coli GGT uses various γ-glutamyl compounds as donors for the transpeptidation reaction and as substrates for the hydrolysis reaction, and it uses various amino acids and peptidases as acceptors of the γ-glutamyl moiety, as reported for mammalian GGTs (24) and *P. mirabilis* GGT (15). Mammalian GGT prefers neutral amino acids such as L-Cys, L-Glu, L-Met, L-Ala, and L-Ser as acceptors. Branched-chain amino acids are relatively poor acceptors, while D-amino acids and L-Pro are inactive. *P. mirabilis* GGT prefers aromatic amino acids such as L-Phe and L-Trp and basic amino acids such as L-His and L-Arg. *E. coli* GGT prefers basic amino acids, especially L-Arg and L-Lys, aromatic amino acids such as L-Phe and L-DOPA, and L-Met. D-Amino acids are relatively poor acceptors. L-Ala, L-Cys, L-Glu and L-Ser, which are good acceptors for mammalian GGTs, are poor acceptors or cannot act as acceptors. γ-Glutamyl donors which *E. coli* GGT prefers for the transpeptidation reaction and those for the hydrolysis reaction are not always the same. The K_m values for γ-glutamyl donors are quite low, while those for γ-glutamyl acceptors are extraordinarily high. This suggests that transpeptidation is not the major function of this enzyme and that this enzyme does not participate in amino acid transport.

Several amino acids were checked as to whether or not they inhibit GGT activity. Compared with *P. mirabilis* GGT, *E. coli* GGT is not inhibited as strongly by amino acids. L-Ala and L-Glu, however, inhibit GGT activity fairly strongly.

GGT affinity labeling reagents, AT-125, azaserine, and 6-diazo-5-oxo-nor-leucine, strongly inhibit GGT activity. L-Ser with borate inhibits *E. coli* GGT, while D-Ser with borate, which inhibits mammalian GGTs (24), does not inhibit *E. coli* GGT. That protease inhibitors, phenylmethylsulfonylfluoride, 1-chloro-3-tosylamido-7-amino-2-heptanone, and L-1-tosylamido-2-phenylethylchloromethyl ketone, moderately inhibit GGT activity suggests that *E. coli* GGT might possess a latent proteinase activity, as Gardell and Tate described (4).

The purified *E. coli* GGT is stable on incubation at 45°C for 15 min. This also supports our hypothesis that *E. coli* GGT is synthesized only at lower temperature, rather than that the synthesized GGT is degraded or inactivated at higher temperature.

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