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γ-Glutamyltransferase from the Outer Cell Envelope of *Treponema denticola* ATCC 35405

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The human oral spirochete *Treponema denticola* ATCC 35405 was shown to exhibit relatively high enzyme activity toward the γ-glutamyl amide bond present in *N*-γ-L-glutamyl-4-nitroaniline. The enzyme responsible for this catalysis (γ-glutamyltransferase [GGT]; EC 2.3.2.2) was purified by means of fast protein liquid chromatography to two sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)-pure forms from a mild (0.1%) Triton X-100 extract of washed cells. The GGT was studied primarily with regard to its hydrolytic activity by using *N*-γ-L-glutamyl-4-nitroaniline as a substrate, although the GGT was shown to catalyze transpeptidation reactions. The high-molecular-mass form of the GGT gave a value of about 213 kDa by SDS-PAGE when heat treatment was omitted and one of 26 kDa after heat treatment; mass spectrometry gave a value of 26.877. The larger form may represent an aggregate with nonprotein structures (possibly of a carbohydrate nature). The preliminary N-terminal sequence of the GGT is MKKPLIGITGSXLYETSQXXF. The enzyme was highly active on glutathione, transferring its Glu residue either to a water molecule or to the Gly-L-Leu dipeptide. The GGT stability was absolutely dependent on the presence of free thiol(s), while no evidence of metalloenzyme nature was obtained. The proposed location of the GGT in the outer cell envelope and its high activity on glutathione, a major nonprotein thiol present in virtually all cells, suggest that the GGT may play a role in the propagation of *T. denticola* within inflamed periodontal tissues.

Some members of the bacterial flora present in human subgingival dental plaque show high enzyme activity targeted at the γ-glutamyl amide bond of small synthetic and natural molecules. The specificity requirements of these enzymes dictate that for hydrolysis of the γ-amide bond, the α-amino group of the glutamyl residue must be free. For example, the cells of *Fusobacterium nucleatum* (26) and *Treponema denticola* (27) exhibited such enzyme activity when tested with glutathione (γ-glutamylcysteinylglycine [GSH]) (26) and with *N*-γ-L-glutamyl-2-naphthylamine (27), respectively. These organisms hydrolyzed the γ-glutamyl amide bond at a rate much higher than that of the amide bond involving the α-carboxyl group (26, 27), suggesting a specific role for enzymes responsible for this catalysis. Such enzymes may be called γ-glutamyltransferases (GGTs) or γ-glutamyl transpeptidases (EC 2.3.2.2). The relevance of this treponemal enzyme activity to infection can be envisaged as being associated with the following observations: (i) *T. denticola* has been shown to be associated with periodontal disease (17, 18, 37); (ii) GSH is a submajor component of all cells, its in vivo concentration ranging between 0.1 and 10 mmol/liter (15) (the present GGT is especially active on GSH); (iii) the thiol group and the γ-glutamyl residue are the most important structural features of GSH, which acts as an important producer and carrier of glutamic acid residues, its thiol group (in the form of recovered cysteine) contributing to the metabolism of volatile, malodorous compounds, and possibly also to the development of the chemically reducing conditions often prevailing in subgingival dental plaque and in the inflamed crevicular compartment; (iv) in mammalian and also in some bacterial cells, the GGTs (occasionally called ectoenzymes) are usually attached to the cell membranes or are present in the periplasmic space (9, 42, 43, 47). It is also possible that the GGT attacks small γ-glutamyl peptides liberated from host tissue structural proteins by other enzymes. There is no information about the properties of GGTs in the cells of *T. denticola* or other spirochetes.

GGT is involved in metabolite transport (9), playing a key role in the γ-glutamyl cycle which is one of the several mechanisms for the entry of amino acids into cells. The cycle is of major importance in the metabolism of GSH. Enzymes isolated from vertebrate cells are usually highly glycosylated (5, 42, 45). An intracellular GGT has been demonstrated in the periplasmic space of *Escherichia coli* K-12; this enzyme was not found to be glycosylated (42). Owing to the lack of information on treponemal GGTs, we investigated the properties of a GGT which is most likely associated with the outer cell membrane of *T. denticola* ATCC 35405.

MATERIALS AND METHODS

Source and cultivation of the organism and treatment of cells. Cells of *T. denticola* ATCC 35405 were grown anaerobically for 48 h in a tryptone-yeast extract-heart infusion broth containing 10% heat-inactivated rabbit serum (34). For the purification of the GGT, 1.2-liter aliquots of the growth medium in 1.5-liter screw-cap flasks were inoculated with 100-ml aliquots of cultures (24) and incubated anaerobically for 4 days at 37°C. The optical density at 660 nm was used to determine the number of cells; an optical density at 660 nm of 0.2, corresponding to 5×10^8 cells per ml (10), was used, although this value was not checked for the present study. The cells were harvested by centrifugation for 10 min at 16,500 × *g* and washed once with ice-cold water. This procedure and all subsequent steps of enzyme purification were carried out at 0 to 4°C, except for fast protein liquid chromatographic (FPLC) separations, which were carried out at 22°C.

Chemicals. Unless specifically mentioned, the chemicals used were obtained from Sigma. The water used in these studies was prepared with a Millipore Milli-Q system and had a resistance of 18 MΩ cm^{-1}.

Assay of enzyme activity. The activity of GGT in a reaction mixture containing, per liter, 0.1 mol of Tris (pH 8.0), 10 mmol of 2-mercaptoethanol (2-ME), and 0.2 mmol of *N*α-L-glutamyl-4-nitroaniline (GNA; dissolved in water) was routinely determined. This substrate was more convenient to use than the corresponding 2-naphthylamine derivative employed earlier (27). A 1- to 10-μl volume of enzyme was added to the reaction mixture (1.0 ml). The increase in

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TABLE 1. Purification of GGT from *T. denticola* ATCC 35405

Step	Vol (ml)	Amt of protein (mg/ml)	Amt of total protein (mg)	Activity (mmol/ml/min)	Sp act (mmol/min/mg)[a]	Total act (mmol/min)	Yield (%)
0.1% Triton extract	27.0	7.14	192.8	1.48	0.20 (1)	39.96	100.0
After isoelectric focusing	16.8	3.70	62.2	1.72	0.47 (2.3)	28.90	72.7
After hydroxylapatite FPLC	20.8	0.34	7.15	1.36	4.00 (20.0)	28.29	71.1
After phenyl-Sepharose FPLC	18.4	0.129	2.37	1.07	8.33 (41.6)	19.69	49.5
After Fractogel FPLC	22.3	0.041	0.92	0.61	14.70 (73.5)	13.60	33.9
After Superose 6 FPLC	3.0	0.15	0.45	4.00	26.7 (133.5)	12.00	30.1

[a] Determined with GNA under standard conditions described in Materials and Methods. Values in parentheses indicate purification factors.

absorption at 410 nm was monitored for 1 to 5 min in a thermostated cuvette at 30°C with a Shimadzu UV-265 recording spectrophotometer, and the value of 8,800 M^{-1} cm^{-1} was used for ε_{410}. Hydrolysis of GSH, its derivatives, and other peptides was tested in the above reaction mixture by replacing GNA with the peptide involved. Direct analysis of glutamic acid in these mixtures was carried out on a System 6300 high-performance amino acid analyzer (Beckman).

Protein determination. Protein concentrations in chromatographic separations were determined spectrophotometrically at 280 nm. Pierce's (Pierce Chemical Company, Rockford, Ill.) bicinchoninic acid procedure was used to determine the protein concentrations used in the purification table (Table 1).

Experiments on whole cells. Washed whole cells in 1.0-ml reaction mixtures containing 1.0 mmol of GNA per liter in buffer (pH 8.0) and 2.5×10^9 cells (10) were studied. Small aliquots of chemical affectors were added, and the mixtures were incubated at 30°C. The following tests were performed: effect of 2-ME, chlorhexidine acetate, metronidazole, bacitracin, and Zn(II) and involvement of transferase activity (with both water and Gly-L-Leu as acceptors and GNA or GSH as glutamyl donors). In all experiments, the cells were first preincubated at 30°C in buffer for 12.5 min before addition of other reagents.

Purification of the enzyme. The cells were harvested for enzyme purification after 4 days of growth. The cells were stored at −20°C and thawed for the present purpose. An 8-ml volume of cell mass was suspended in 5.0 mmol of phosphate buffer (pH 6.8) per liter which contained 0.1 mmol of EDTA and 10 mmol of 2-ME per liter (the phosphate buffer with the EDTA and 2-ME additions will be called phosphate buffer below; unless specified, the buffer concentration was 20 mmol/liter). Small volumes of Triton X-100 (Pierce) were added in an iced water bath to a final concentration of 0.1%. Sixty minutes later, the suspension was centrifuged for 15 min at $27{,}000 \times g$. The supernatant fluid (27 ml) was recovered.

(i) Isoelectric focusing with Rotofor. The above 27-ml supernatant fluid was mixed with 3.0 ml of 40% Pharmacia Ampholine ampholytes (pH range, 3.5 to 5.0) and 25 ml of water. The resulting 55-ml sample was loaded into a Bio-Rad Rotofor cell. Isoelectric focusing in the cell required 3 h at a constant power of 12 W at 4°C. At equilibrium, the conditions were 500 V and 12 mA. Twenty fractions were collected, and their pH values were immediately adjusted to 6.8. Fractions 14 to 20 were combined, and the resulting enzyme was passed through a 0.45-μm-pore-size Millipore membrane. The final volume was 16.8 ml. The primary purpose of the Rotofor procedure (not shown) was to combine a separation step with one which also concentrated the enzyme.

(ii) Hydroxyapatite FPLC. The enzyme from the previous step was separated on a Calbiochem high-resolution hydroxyapatite column (1 by 12 cm) with a linear phosphate gradient. Eluent A was phosphate buffer. Eluent B was 1.0 mol of phosphate buffer per liter. The GGT was eluted in fractions 55 to 75 (Fig. 1A). The fractions were combined, and the enzyme was concentrated (Amicon Centriprep-30) to 2.0 ml.

(iii) Phenyl-Sepharose 4B FPLC. The enzyme from the previous step was made 1.0 M with regard to NH_4Cl (by mixing 1 volume of 3.0-mol/liter NH_4Cl and 2 volumes of enzyme) and was loaded onto a column (1 by 12 cm). Eluent A was 1.0-mol/liter NH_4Cl in phosphate buffer. Eluent B was a mixture of EDTA and 2-ME in water. The gradient was formed as follows: 0 to 15 min, 0% B; 15 to 45 min, 0 to 90% B; and 45 to 80 min, 100% B. A 15-μl volume of 1.0-mol/liter phosphate buffer was added onto the bottom of the collection tubes before chromatography. The flow rate was 0.6 ml/min. Fractions 58 to 72 were combined (Fig. 1B).

(iv) Anion-exchange FPLC. The enzyme from the previous step was applied on a Fractogel (1 by 25 cm) EMD TMAE-650 column. Eluent A was phosphate buffer, while eluent B was 1.0-mol/liter NaCl in the same buffer. The gradient was formed as follows: 0 to 25 min, 0% B; 25 to 55 min, 0 to 20% B; 55 to 65 min, 20 to 100% B; and 65 to 70 min, 100 to 0% B. The loading of the sample took place manually and the gradient program was started at 11 min. Fractions 53 to 59 were combined (Fig. 1C), and the enzyme was concentrated to 0.95 ml with a Centricon-30 membrane.

(v) Gel filtration FPLC. Aliquots of ca. 0.35 ml of the enzyme from the previous step were passed through a Superose 12 column (not shown). The active fractions were combined (2.0 ml), and the enzyme was finally rechromatographed on a Superose 6 column (Fig. 1D). The elutions were carried out in the phosphate buffer containing 0.17-mol/liter NaCl. The purification is summarized in Table 1. The GGT was stored in the final elution buffer at 4°C. 2-ME was necessary to maintain full activity of the enzyme during purification. Regardless of the presence of 10 mmol of 2-ME per liter, the purified enzyme gradually lost its activity during storage.

SDS-PAGE. The homogeneity of the purified protein (containing 10 mmol of 2-ME per liter) was studied by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Pharmacia's PhastSystem, 8 to 25% Phastgel gradient, and silver staining. The experiments were carried out with an enzyme which had not been heat treated and after the standard heat treatment (5 min at 100°C). Sigma's molecular weight markers MW-SDS-200 were used as standard proteins.

Determination of kinetic constants. K_m and maximum rate of hydrolysis (V_{max}) values were calculated with the Enzpack 3 program (Biosoft, Ferguson, Mo.). Because the hydrolysis of the GNA and GSH followed the normal Michaelis-Menten kinetics, Lineweaver-Burk, Hanes-Wolf, and Eadie-Hofstee plots, as well as the direct linear method and the Wilkinson method, gave essentially similar numerical values. The results are given as mean values obtained from the above five procedures.

Amino acid sequencing. The enzyme was dialyzed against 1,000 volumes of water with a Centricon 30 membrane and subjected to reverse-phase high-performance liquid chromatography (HPLC), amino acid analysis, and N-terminal sequencing. The reverse-phase HPLC was carried out on a Vydac (Hesperia, Calif.) analytical C_{18} column (no. 218TP52; 0.21 by 25 cm; particle size, 5 μm), with acetonitrile gradient (20 to 70% B) containing 0.1% trifluoroacetic acid. Amino acid analysis was carried out with an Applied Biosystems (Foster City, Calif.) model 420H amino acid analyzer and by standard procedures (see Table 2) (52). The N-terminal sequencing was performed on an Applied Biosystems model 473 protein sequencer and a 420H amino acid analyzer, which use automated Edman degradation and phenylhydantoin-amino acid analysis (2, 12).

Laser desorption mass spectrometry. The masses of the low-molecular-weight forms of the GGT were studied by means of matrix-assisted laser desorption ionization time-of-flight mass analysis. The analyses were performed on a Vestec-2000 Lasertec Research (Houston, Tex.) laser desorption linear time-of-flight mass spectrometer, equipped with a 337-nm VSL-337ND nitrogen laser (Laser Science, Inc.) with a 3-ns pulse width and a 1.2-m flight tube, operated at an ion accelerating voltage of 23 kV and a multiplier voltage of 3 kV. The matrix solutions were saturated with sinapinic acid in 1:1 acetonitrile and 0.1% aqueous trifluoroacetic acid; 0.5 pmol of myoglobin was loaded as an internal standard. The sample mixtures consisted of 1 μl of matrix, 1 μl of analyte, and 1 μl of internal standard. A 1-μl aliquot was loaded onto the probe.

Analysis of carbohydrates. Carbohydrate analysis of the enzyme was carried out on a Dionex BioLC instrument by means of high-performance anion exchange-pulsed amperometric detection. A Dionex CarboPac PA1 column (4 by 250 mm) was used. The procedure is described in the Dionex Workbook and Manual (4a).

Chemical modification of GGT and effect of chemical affectors. The purpose of these studies was to elucidate the nature of the active amino acid residues which possibly are involved in GGT activity by limiting the tests to include typical, widely employed chemical modification reactions and enzyme affectors used under standard conditions described in the literature. Consequently, modification with 2,3-butanedione and phenylglyoxal (to study the possible involvement of active arginyl residues) was carried out under protection from light (25). Diethylpyrocarbonate (which normally reacts with histidyl or tyrosyl residues) (30), diisopropylfluorophosphate (DFP) (which is known to inhibit serine enzymes), tetranitromethane (tested primarily for tyrosyl residues) (36, 43), and *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ; highly specific for active carboxyl groups) (35, 38), were studied as described in cited references. Other chemical reagents (potential enzyme inhibitors and activators) were studied in standard reaction mixtures with GNA as the substrate by adding small volumes (5 to 20 μl) of affector solutions to the standard reaction mixtures (vide supra).

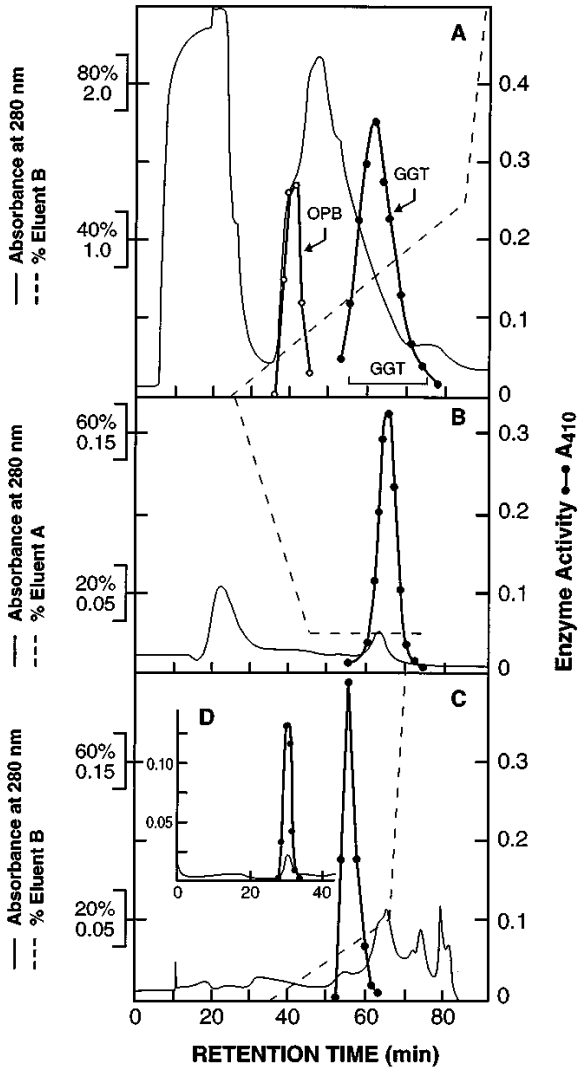

FIG. 1. Purification of GGT. (A) FPLC of the enzyme on a hydroxyapatite gel. The gradient shown was applied after application of the 16.8-ml sample was complete. The fraction volume was 1.0 ml, and the flow rate was 1.0 ml/min. The separation of a reference enzyme (oligopeptidase B [OPB] [22, 23]) is indicated. (B) Separation of the enzyme from the previous step on a phenyl-Sepharose 4B column. The fraction volume was 0.6 ml, and the flow rate was 0.6 ml/min. (C) Separation of the enzyme from the previous step on a Fractogel column. The fraction volume was 1.5 ml, and the flow rate was 1.5 ml/min. (D) Final separation of the enzyme on a Superose 6 gel packed in a Pharmacia 10/30 column. The fraction volume was 0.5 ml, and the flow rate was 0.5 ml/min. The protein (solid lines) is shown as A_{280} (the scale on the left relates to both enzyme activity and protein; the absorption units [indicated in the full scale] used in protein monitoring are indicated). Dashed lines, gradients.

RESULTS

Experiments on whole cells: hydrolysis of GSH. The results are summarized as follows. Triton X-100 (at 0.1%) readily dissolved the GGT from washed cells; the enzyme was not released from the cells when it was incubated in buffer (without added detergent). The whole cells did not liberate any free glutamic acid when they were incubated in buffer only but hydrolyzed GNA and GSH at a high rate and catalyzed the transferase reactions described below with purified GGT. Washed whole cells rapidly hydrolyzed GSH by liberating free glutamic acid into the medium. Bacitracin and metronidazole had no appreciable effect on the ability of the cells to hydrolyze GNA. NaCl and chlorhexidine acetate did not release the enzyme from the cells. The latter, however, slightly activated the enzyme: 0.24- and 1.92-mmol/liter chlorhexidine activated hydrolysis of GNA (water as acceptor) by 23 and 28%, respectively. When Gly-L-Leu was used as an acceptor, chlorhexidine acetate increased the rate of liberation of glutamic acid from GSH and GNA but slightly inhibited the appearance of glycine in the medium (it is understandable that the substrates used and their hydrolysis products may be attacked by enzymes other than GGT). Experiments with 10 mmol of 2-ME per liter showed that inactivated GGT present in washed whole cells can be fully reactivated. The reactivated cells hydrolyzed GSH and GNA rapidly. Although Zn(II) was a strong inhibitor of purified GGT, a Zn(II) concentration of 0.1 mmol/liter was required to totally inhibit GGT in the cell mixture (with GNA and GSH as substrates).

Purification of GGT. The sequence of purification steps was designed and tested to minimize the number of procedures requiring dialysis and concentration. The largest percentage increase in specific activity was achieved by hydroxyapatite FPLC (Fig. 1 and Table 1). Evaluation of the purification factor (129-fold purification was achieved) should consider the fact that the starting material represented a mild detergent extract of low protein concentration, obtained from the cells' outer envelope rather than from the whole cell mass. Owing to the lability of the GGT, the presence of 2-ME at a relatively high concentration (10 mmol/liter) in elution buffers was a prerequisite for successful purification; without 2-ME, the GGT activity was normally destroyed in a few hours. However, even when totally inactivated GGT was present in washed, thawed cells, it could be fully reactivated upon addition of 2-ME. This reactivation process was slow and could last for several hours. Purified GGT, inactivated by removing the 2-ME from the enzyme, was reactivated at a higher rate, i.e., in 10 to 20 min.

Purity of GGT. The enzyme was homogeneous by SDS-PAGE. GGT which was not heat treated gave only one stained band corresponding to an approximate molecular mass of 213 kDa. SDS-PAGE carried out after the standard heat treatment gave only one band corresponding to a mass of 26 kDa (Fig. 2). 2-ME did not affect the rate of migration of the protein. The homogeneity of the enzyme protein was verified independently

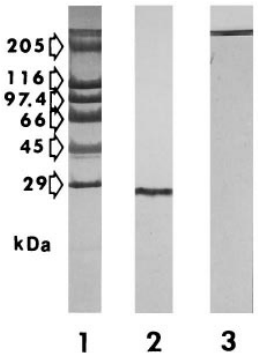

FIG. 2. SDS-PAGE study of the purified enzyme after (lane 2) and before (lane 3) standard heat treatment. Lane 1, separation of the molecular mass standards.

TABLE 2. Preliminary partial amino acid content of *T. denticola* ATCC 35405 GGT

Amino acid	No. of residues per molecule[a]
Aspartic acid/asparagine	17
Serine	12
Histidine	5
Threonine	7
Proline	12
Valine	20
Leucine	33
Lysine	13
Glutamic acid/glutamine	24
Glycine	26
Arginine	7
Alanine	29
Tyrosine	7
Methionine	8
Isoleucine	23
Phenylalanine	10
Tryptophan	ND
Cysteine	ND

[a] Hydrolysis of the enzyme protein (10 pmol) was carried out for 1 h at 200°C in 6 mol of HCl per liter. The values were not corrected for possible loss of amino acids during hydrolysis. ND, not determined.

at the University of Michigan Medical School Protein Structure and Sequencing Facility by means of microbore-HPLC on a Vydac C_{18} reverse-phase column.

Physical and molecular characteristics. The isoelectric point of the GGT (determined with the Rotofor cell) was 5.2 to 5.4. A mass spectrometric assay of the small-molecule form gave a value of 26,877. This analysis also revealed the presence of a minor form whose mass was almost half of the above value, i.e., 13,481. This form was not detected by SDS-PAGE. The equipment used did not allow study of the mass of the larger 213-kDa form. Amino acid analysis suggested an average minimum molecular mass of 26 kDa for the smaller form. The length of this peptide was at least 253 residues. The preliminary amino acid composition of the GGT is shown in Table 2. The N-terminal sequence of the GGT was MKKPLIGITGSX LYETSQXXF (X represents unknown residues). At this stage, this sequence was not shown to display significant homology with known sequences. Carbohydrate analysis of the 213-kDa form showed the presence of glucosamine, galactose, mannose, and glucose in a ratio of 0.5:1.0:1.8:5.4. Analysis of a 371-pmol sample of the GGT suggested the presence of the following amounts of these carbohydrates: glucosamine, 55 pmol; galactose, 113; mannose, 206; and glucose, 613. The analysis did not show any traces of fucose, 2-deoxyglucose, or galactosamine.

Evidence of transferase activity. Experiments were carried out to demonstrate the ability of the GGT to catalyze a transferase reaction. The enzyme was incubated in a mixture containing 25 mmol of Tris (pH 8.0), 1 mmol of GSH (dissolved in above buffer), and 10 mmol of Gly-L-Leu per liter at 30°C. Control mixtures were made without the dipeptide acceptor. After 30 min of reaction, it was observed that when Gly-L-Leu was used as an acceptor, the amount of glutamic acid present in the reaction mixture was only one-half of the amount when water was used as an acceptor. Because Gly-L-Leu did not inhibit the GGT-catalyzed reactions (tested with GNA as the substrate), it was concluded that upon hydrolysis of the Glu-Cys bond of GSH, the liberated Glu residue was transferred to the Gly-L-Leu molecule.

Effect of pH on GGT activity. When the enzyme activity was tested in bis-Tris-propane (pH range, 6.5 to 9.8) and Tris (pH range, 7 to 9) buffers, the maximum rate of hydrolysis of GNA was observed between pH values of 7.5 and 8.0 (not shown). The rate versus pH curves were bell shaped.

TABLE 3. Relative hydrolytic activities of GGT on GSH and some of its derivatives compared with the rate of hydrolysis of GNA and some peptides[a]

Substrate	Relative activity (mean of three experiments)[b]
GSH	100
S-(*p*-Nitrobenzyl)-GSH	67
Mono-(des-glu)-GSH (oxidized)	32.5
S-(*p*-Azidophenacyl)-GSH	28
Coenzyme A GSH disulfide	26
GSSG (oxidized GSH)	23
GNA	11.5
γ-Glu-Leu	7
γ-Glu-Cys	3
γ-Glu-Gly	0

[a] The reactions were carried out in 25 mmol of Tris (pH 8.0) per liter, with 0.1-mmol/liter initial substrate concentrations at 30°C in the presence of 10 mmol of 2-ME per liter. The amount of GGT added was 0.18 μg.

[b] The released glutamic acid was analyzed with an amino acid analyzer. Under the above conditions, the rate of hydrolysis of GSH by the GGT was 42.2 μmol/min/mg of protein.

Substrate specificity. GSH and its derivatives were hydrolyzed most rapidly, much faster than GNA (Table 3). Results from studies employing di- and tripeptides with a free α- or a γ-glutamyl residue in the N-terminal position were as follows. γ-Glu-Leu and γ-Glu-Cys were hydrolyzed at a low rate (Table 3). The following molecules were not hydrolyzed: γ-Glu-Gly, α-Glu-Glu, γ-Glu-Gln, γ-Glu-Gly-Phe, γ-Glu-His, α-Glu-Glu, α-Glu-Lys, and γ-D-Glu-Gly (unless otherwise specified, all amino acids were in L form).

Kinetic constants. K_m and V_{max} values were determined for GNA and GSH (Table 4). Determination of these constants in the hydrolysis of GSH was based on analysis of released glutamic acid. Water was used as an acceptor in both reactions.

Effect of thiol reagents. In experiments on the effect of thiol reagents, the involvement of 2-ME, present in the GGT, was nullified by diluting the enzyme immediately prior to tests. Iodoacetamide (1.0 mmol/liter) irreversibly inhibited the enzyme by forming a stable enzyme-inhibitor complex. However, addition of 5-mmol/liter 2-ME fully restored enzyme activity, indicating that the complex could be chemically broken down and that a cysteine residue was involved. Mere dilution of the reaction mixture did not reactivate the enzyme as often happens in typical reversible inhibition. Zn(II), tested at a low 1.0-μmol/liter level, caused a 52% inhibition which was fully reversible upon addition of 10 mmol of 2-ME per liter (the enzyme activity was very sensitive to zinc ion; concentrations of

TABLE 4. Apparent catalytic constants for reactions catalyzed by the GGT[a]

Substrate	K_m (mM)	V_{max} (mM min^{-1})	V_{max}/K_m
GNA	0.98	58.8×10^{-3}	0.06
GSH	0.38	55.1×10^{-3}	0.145

[a] The reactions were carried out in 0.1-mol/liter Tris (pH 8.0 containing 10 mmol of 2-ME per liter) at 30°C. Values are the means of those obtained with five different kinetic procedures as described in the text. The hydrolysis of GNA at 410 nm was determined, while that of GSH was based on direct analysis of glutamic acid with an amino acid analyzer (regarding the water molecule as a γ-glutamyl acceptor in both cases).

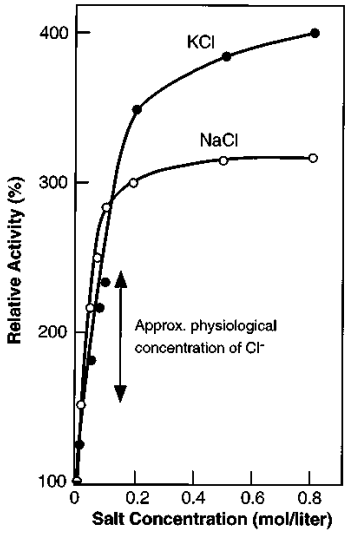

FIG. 3. Effect of NaCl and KCl on the rate of hydrolysis of GNA by purified GGT tested in standard reaction mixture. Approx, approximately.

zinc higher than those described above completely inactivated the GGT). E-64, i.e., *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane, used at a 0.2-mmol/liter concentration and *p*-hyroxymercuribenzoic acid (at 0.5 μmol/liter) inhibited by more than 50 and 90%, respectively (E-64 is specific for active site cysteine residues in proteinases; thiols do not interfere with this reaction). These results suggest that the activity of the *T. denticola* GTT depends on an active cysteinyl residue.

Effect of thiols. The stability of the GGT was absolutely dependent on the presence of a thiol such as 2-ME. However, dithioerythritol was an even stronger activator; dithiothreitol and 2-ME were almost equally effective. Because of the absolute dependence of the enzyme on free SH groups in its environment, it was not always possible to quantitatively measure the exact degree of activation; the activation was rather an all-or-nothing type of reaction. The final degree of activation depended on the state and purity of the enzyme. 2-ME effectively protected the enzyme against inhibition caused by hydroxylamine and also restored the activity of the hydroxylamine-inactivated GGT (both compounds were tested at 10 mmol/liter).

Effect of metal chelators. None of the metal chelators tested [8-hydroxyquinoline 5-sulfonic acid, ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, and EDTA; all tested at 0.05- to 0.10-mmol/liter concentrations] had any remarkable effect on the GGT-catalyzed hydrolysis of GNA. However, EDTA was added (to a final concentration of 0.1 mmol/liter) to the enzyme during purification owing to the sensitivity of the GGT to heavy-metal cations [such as Zn(II) and Hg(II)].

Effect of Cl^-. Previous studies have shown that chloride ions act selectively on the activity of treponemal outer membrane peptidases, some enzymes being strongly activated, while some are inactivated (21, 28). The activity of GGT was increased by as much as threefold in the presence of Cl^- (Fig. 3). Significant activation was observed at physiologic [Cl^-]. However, it appears that KCl was a stronger activator than NaCl, indicating that the cations involved also play a role in this activation.

Other reagents. Benzalkonium chloride (an anion-active detergent inhibiting several proteinases) (unpublished observations from this laboratory), bacitracin (inhibiting various proteinases) (20), bestatin (inhibiting primarily aminopeptidases and other exopeptidases) (4), and metronidazole (an agent used against certain pathogens [19]) had no effect on GGT-catalyzed reactions. A 1-mmol/liter concentration of captopril, i.e., [2S]-1-(3-mercapto-2-methylpropionyl]-L-proline, an agent which inhibits the angiotensin-converting enzyme (39), activated the GGT by ca. 50%, probably because this reagent contains a free thiol group.

Chemical modification of GGT. Experiments for the chemical modification of GGT were performed in the presence of 10-mmol/liter 2-ME and are summarized as follows. Diethylpyrocarbonate (3.3 mmol/liter) caused a 41% inactivation of the enzyme in 5 min in 90-mmol/liter Mes (pH 6). No reactivation of GGT activity was observed after addition of 20-mmol/liter hydroxylamine (instead, further inactivation of the enzyme was observed). These observations suggest that the activity of the GGT may not depend on an active histidyl residue. DFP, tested at 2.7 mmol/liter, irreversibly inactivated the enzyme by 90%. Since the inactivated enzyme was fully reactivated upon addition of 10 mmol of 2-ME per liter, it is more likely that the modification of an active cysteinyl, and not of a seryl residue, was involved in this reaction. 2,3-Butanedione (dissolved in methanol; tested at 3.0 mmol/liter) inactivated the enzyme in 5 min by ca. 50% in 0.1-mol/liter Tris (pH 8.0). This inactivation was fully reversed by 2-ME. Phenylglyoxal (dissolved in methanol; tested at 2.0 mmol/liter) inactivated the GGT in 5 min by ca. 75% in 50-mmol/liter phosphate buffer (pH 8.0). This inactivation was also reversed by 2-ME, but only to a degree of ca. 50%. Experiments with the diketone and the ketonealdehyde, carried out under protection from light, suggest that the activity of the GGT may depend on an active arginyl group. Tetranitromethane, tested at 1.0- to 6.0-mmol/liter concentrations (in 50-mmol/liter phosphate buffer [pH 8.0]), inactivated the GGT in a time- and concentration-dependent manner: 3.0- and 1.0-mmol/liter reagent caused 50 and 35% inactivation, respectively, in 30 min. The degree of inactivation observed may not be considered quite typical of enzymes whose activity depends on active tyrosyl residues. EEDQ (tested at a 2.5-mmol/liter concentration at 30°C in 0.1-mol/liter bis-Tris propane buffer [pH 6.0]) caused only a 20% inactivation in 30 min. These results do not at this stage unequivocally prove the dependence of GGT activity on an active carboxyl group.

DISCUSSION

The primary rationale behind this study was to investigate the hydrolysis of GNA (when water was the acceptor of the glutamyl residue) by washed whole cells of *T. denticola* and by an enzyme (GGT) most likely present in the outer cell envelope of that organism. Whole cells did readily liberate free glutamic acid from GNA, although it became apparent that GSH was by far a better substrate. In the absence of any added acceptors (other than water), the liberated glutamic acid remained unmetabolized in the cell suspension and in the standard reaction mixtures, allowing its direct chemical assaying by means of amino acid analysis. The enzyme was also shown to catalyze the transfer of the glutamyl residue to a peptide acceptor, entitling one to regard this enzyme as a true transferase. Further studies of the peptide transfer proper remain to be undertaken. Although GSH may normally be located at high concentrations only in intracellular compartments, it may get liberated in relatively large quantities in connection with the damage caused by other (ecto)enzymes present in perio-

dontopathic organisms. However, the best natural substrate(s) of the GGT remains to be investigated.

The present GGT activity most likely corresponds to the similar activity previously detected in the cells of *T. denticola* when *N*-γ-L-glutamyl-2-naphthylamine was used as a substrate (27). It is possible that this GGT does not at all participate in the peptidolytic processing of larger peptide and protein molecules (owing to the absence of γ-glutamyl linkages in most peptides). The GGT may simply contribute to the establishment of the chemically reducing conditions necessary for the operation of several other thiol-dependent enzymes present in the outer cell wall structures of the treponemes. The enzyme may also help create the necessary chemically reducing environment that the cells' propagation itself requires.

Most of the available chemical information regarding GGTs has been obtained from vertebrate enzymes. Reports on mammalian (40, 42, 45) and chick embryo (5) GGTs have almost systematically regarded these enzymes as membrane-bound (sialo)glycoproteins, although it is evident that some organisms may produce GGTs not associated with carbohydrate structures. Several microorganisms have been reported to exhibit GGT activity (3, 11, 29, 31–33, 46–51). Although the *E. coli* GGT displayed relatively low activity, this microbial GGT has been studied in great detail (46, 47). Unlike the mammalian enzymes, the *E. coli* GGT has not been found to be glycosylated (42, 46, 47). The carbohydrate analyses carried out in this study do not unequivocally claim that the *T. denticola* GGT is a glycoenzyme. Amphiphilic, aggregated forms of mammalian GGTs can be solubilized in the presence of detergents (such as Triton X-100); the high-molecular-mass form of those enzymes results from the association of the enzyme's hydrophobic domain with other molecules. Whether the present high-molecular-mass 213-kDa form represents such an aggregate or a true polymer of the 26-kDa form must be investigated in future studies.

The amino acid analyses carried out on the 26-kDa form suggest that the length of the GGT peptide chain is about 253 residues. Consequently, the 26-kDa form may represent the true hydrophilic treponemal GGT. However, it is possible that the full in vivo activity of this enzyme presumes its close structural association with nonprotein structures. Other bacterial GGTs may differ from the *E. coli* GGT with regard to the presence of carbohydrates. Earlier studies suggested that the prolyl oligopeptidase of *T. denticola* resembles the mammalian more than the bacterial enzyme (28). It is possible that the present GGT is a glycoprotein resembling the vertebrate GGTs. The cells of *T. denticola* are confined to certain human inflammatory habitats. It may thus be logical that some of the pathogen's ectoenzymes, such as the present GGT, resemble the host's enzymes, perhaps to avoid immunochemical detection. The presence of carbohydrates as structural components of the GGT molecule may still be considered unusual in a bacterial protein. It is possible that the carbohydrates found are derived from structures that are associated only with the GGT enzyme and that they do not constitute an integral component of the enzyme molecule proper.

The aim of the chemical modification tests was to elucidate their conformity with previous, more-thorough modification studies of mammalian GGTs. The information so far published suggests the importance of an enzyme amino group (6), a histidine residue (1), a carboxyl group (6, 48), a cysteine group (6, 48), and an arginine and/or a lysine residue (7, 13, 41, 45) in the catalytic mechanism. The present tests are in congruence with the possible importance of an arginine residue and perhaps of a histidine moiety in treponemal GGT activity. Although no strong evidence for the involvement of an active carboxyl group was obtained, it has been suggested that an active glutamyl residue operates in human GGT (13). An active tyrosyl residue was suggested to be associated with the binding of the GSH thiol by human placenta GGT (14), an observation which may also concern the treponemal GGT. Although the above preliminary modification studies must be completed by more detailed experiments, it is virtually certain that the present GGT is a thiol-dependent enzyme. Because GSH per se is a thiol, it is possible that this substrate also acts as an effective in vivo activator of the enzyme.

Compared with the mammalian enzyme, the *E. coli* GGT has been reported to possess relatively low activity which may be associated with a change or changes in the amino acid sequence (42) and not with the noninvolvement of carbohydrates in the enzyme structure. A possible candidate for a single-amino-acid-residue change might be Cys-453 in rat GGT (16), which corresponds to Ser-465 in *E. coli* GGT (46). Assuming that these residues are located in or near the active site, the *T. denticola* GGT resembles more closely the rat GGT (Cys-453) than the *E. coli* GGT (Ser-465). The present modification experiments showed that the inactivation of the present GGT by DFP could be fully reversed by the addition of 2-ME, indicating that a cysteinyl residue and not a seryl residue was affected by DFP. The rat kidney GGT contains Thr-523, another hydroxy amino acid, which interacts with the substrate analog acivicin (8, 45). Provided that more detailed studies of the present GGT support these preliminary observations, the treponemal GGT may turn out to be catalytically and structurally relatively similar to mammalian GGTs studied previously and to differ from the *E. coli* GGT with regard to association with a carbohydrate moiety.

REFERENCES

1. **Allison, R. D.** 1985. γ-Glutamyl transpeptidase: kinetics and mechanism. Methods Enzymol. **113:**419–437.
2. **Andrews, P. C.** 1994. Neuropeptide structure determination. Methods Neurosci. **5:**5–15.
3. **Aumayr, A., T. Hara, and S. Ueda.** 1981. Transformation of *Bacillus subtilis* in polyglutamate production by deoxyribonucleic acid from *B. natto*. J. Gen. Appl. Microbiol. **27:**115–123.
4. **Beynon, R. J., and G. Salvesen.** 1989. Commercially available protease inhibitors, p. 241–249. *In* R. J. Beynon and J. S. Bond (ed.), Proteolytic enzymes: a practical approach. IRL Press, Oxford, United Kingdom.

4a. **Dionex Corporation.** 1989. Dionex workshop and manual. Glycoprotein workshop. Dionex Corp., Sunnyvale, Calif.

5. **Dvorakova, L., V. Lisy, and F. Stastny.** 1992. Developmental changes in the activity of membrane-bound γ-glutamyl transpeptidase and the sialylation of synaptosomal membranes from the chick embryonic brain. Eur. J. Biochem. **203:**669–672.
6. **Elce, J. S.** 1980. Active-site amino acid residues in γ-glutamyltransferase and the nature of the γ-glutamyl-enzyme bond. Biochem. J. **185:**473–481.
7. **Fushiki, T., K. Iwami, K. Yasumoto, and K. Iwai.** 1983. Evidence for an essential arginyl residue in bovine milk γ-glutamyltransferase. Biochemistry (Tokyo) **93:**795–800.
8. **Gololobov, M. Y., and R. C. Bateman.** 1994. γ-Glutamyl-transpeptidase-catalysed acyl-transfer to the added acceptor does not proceed via the Ping-Pong mechanism. Biochem. J. **304:**869–876.
9. **Graber, R., and G. A. Losa.** 1995. Changes in the activities of signal transduction and transport membrane enzymes in CEM lymphoblastoid cells by glucocorticoid-induced apoptosis. Anal. Cell. Path. **8:**159–176.
10. **Haapasalo, M., V. Singh, B. C. McBride, and V.-J. Uitto.** 1991. Sulfydryl-dependent attachment of *Treponema denticola* to laminin and other proteins. Infect. Immun. **59:**4230–4237.
11. **Hasegawa, M., and I. Matsubara.** 1978. γ-Glutamylpeptide formative activity of *Corynebacterium glutamicum* by the reverse reaction of the γ-glutamylpeptide hydrolytic enzyme. Agric. Biol. Chem. **42:**371–381.
12. **Hunkapiller, M. W., R. M. Hewick, W. J. Dreyer, and L. E. Hood.** 1983. High-sequencing with a gas-phase sequenator. Methods Enzymol. **91:**339–413.
13. **Ikeda, Y., J. Fujii, and N. Taniguchi.** 1993. Significance of arg-107 and glu-108 in the catalytic mechanism of human γ-glutamyl transpeptidase. J. Biol. Chem. **268:**3980–3985.
14. **Karshikoff, A., P. Reinemer, R. Huber, and R. Ladenstein.** 1993. Electrostatic evidence for the activation of the glutathione thiol by Tyr7 in pi-class

glutathione transferase. Eur. J. Biochem. **215:**663–670.
15. **Kosower, E. M.** 1976. Chemical properties of glutathione, p. 1–35. *In* I. M. Arias and W. B. Jacoby (ed.), Glutathione: metabolism and function. Raven Press, New York, N.Y.
16. **Laperche, Y., F. Bulle, T. Aissani, M.-N. Chobert, M. Aggerbeck, J. Hanoune, and G. Guellaen.** 1986. Molecular cloning of rat kidney γ-glutamyl transpeptidase cDNA. Proc. Natl. Acad. Sci. USA **83:**937–941.
17. **Loesche, W. J.** 1988. The role of spirochetes in periodontal disease. Adv. Dent. Res. **2:**275–283.
18. **Loesche, W. J.** 1993. Bacterial mediators in periodontal disease. Clin. Infect. Dis. **16**(Suppl. 4)**:**S203–S210.
19. **Loesche, W. J., J. R. Giordano, P. P. Hujoel, J. Schwarz, and B. A. Smith.** 1992. Metronidazole in periodontitis (III): reduced need for surgery. J. Clin. Periodontol. **19:**103–112.
20. **Mäkinen, K. K.** 1972. Inhibition by bacitracin of some hydrolytic enzymes. Int. J. Prot. Res. **4:**21–28.
21. **Mäkinen, K. K., C.-Y. Chen, and P.-L. Mäkinen.** 1996. Proline iminopeptidase from the outer cell envelope of the human oral spirochete *Treponema denticola* ATCC 35405. Infect. Immun. **64:**702–708.
22. **Mäkinen, K. K., and P.-L. Mäkinen.** 1996. The peptidolytic capacity of the spirochete system. Med. Microbiol. Immunol. **185:**1–10.
23. **Mäkinen, K. K., P.-L. Mäkinen, W. J. Loesche, and S. A. Syed.** 1995. Purification and general properties of an oligopeptidase from *Treponema denticola* ATCC 35405—a human oral spirochete. Arch. Biochem. Biophys. **316:** 689–698.
24. **Mäkinen, K. K., P.-L. Mäkinen, and S. A. Syed.** 1992. Purification and substrate specificity of an endopeptidase from the human oral spirochete *Treponema denticola* ATCC 35405, active on furylacryloyl-Leu-Gly-Pro-Ala and bradykinin. J. Biol. Chem. **267:**14285–14293.
25. **Mäkinen, K. K., P.-L. Mäkinen, S. H. Wilkes, M. E. Bayliss, and J. M. Prescott.** 1982. Photochemical inactivation of *Aeromonas* aminopeptidase by 2,3-butanedione. J. Biol. Chem. **257:**1765–1772.
26. **Mäkinen, K. K., E. Söderling, S. A. Syed, and P.-L. Mäkinen.** 1990. Hydrolysis of γ-glutamyl linkages by *Fusobacterium nucleatum*. Curr. Microbiol. **20:**5–11.
27. **Mäkinen, K. K., S. A. Syed, P.-L. Mäkinen, and W. J. Loesche.** 1987. Dominance of iminopeptidase activity in the human oral bacterium *Treponema denticola* ATCC 35405. Curr. Microbiol. **14:**341–346.
28. **Mäkinen, P.-L., K. K. Mäkinen, and S. A. Syed.** 1994. An endo-acting proline-specific oligopeptidase from *Treponema denticola* ATCC 35405: evidence of hydrolysis of human bioactive peptides. Infect. Immun. **62:**4938–4947.
29. **Milbauer, R., and N. Grossowicz.** 1965. γ-Glutamyl transfer reactions in bacteria. J. Gen. Microbiol. **41:**185–194.
30. **Miles, E. W.** 1977. Modification of histidyl residues in proteins by diethylpyrocarbonate. Methods Enzymol. **47:**431–442.
31. **Nakayama, R., H. Kumagai, and T. Tochikura.** 1984. Leakage of glutathione from bacterial cells caused by inhibition of γ-glutamyltranspeptidase. Appl. Environ. Microbiol. **47:**653–657.
32. **Nakayama, R., H. Kumagai, and T. Tochikura.** 1984. Purification and properties of γ-glutamyltranspeptidase from *Proteus mirabilis*. J. Bacteriol. **160:** 341–346.
33. **Nakayama, R., H. Kumagai, and T. Tochikura.** 1984. γ-Glutamyltranspeptidase from *Proteus mirabilis*: localization and activation by phospholipids. J. Bacteriol. **160:**1031–1036.
34. **Ohta, K., K. K. Mäkinen, and W. J. Loesche.** 1986. Purification and characterization of an enzyme produced by *Treponema denticola* capable of hydrolyzing synthetic trypsin substrates. Infect. Immun. **53:**213–220.
35. **Pougeois, R., M. Satre, and P. V. Vignais.** 1978. *N*-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, a new inhibitor of the mitochondrial F_1-ATPase. Biochemistry **17:**3018–3023.
36. **Riordan, J. F., and B. L. Vallee.** 1972. Nitration with tetranitromethane. Methods Enzymol. **25:**515–521.
37. **Riviere, G. R., K. S. Elliot, D. F. Adams, L. G. Simonson, L. B. Forgas, A. M. Nilius, and S. A. Lukehart.** 1992. Relative proportions of pathogen-related oral spirochetes (PROS) and *Treponema denticola* in supragingival and subgingival plaque from patients with periodontitis. J. Periodontol. **63:**131–136.
38. **Saccomani, G., M. L. Barcellonas, and G. Sachs.** 1981. Reactivity of gastric ($H^+ + K^+$)-ATPase to *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline. J. Biol. Chem. **256:**12405–12410.
39. **Salvesen, G., and H. Nagase.** 1981. Inhibition of proteolytic enzymes, p. 83–104. *In* R. J. Beynon and J. S. Bond (ed.), Proteolytic enzymes: a practical approach. IRL Press, Oxford, United Kingdom.
40. **Sanderink, G.-J., Y. Artur, and G. Siest.** 1988. Human aminopeptidases: a review of the literature. J. Clin. Chem. Clin. Biochem. **26:**795–807.
41. **Schasteen, C. S., N. P. Curthoys, and D. J. Reed.** 1983. The binding mechanism of glutathione and the anti-tumor drug L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (AT-125;NSC-163501) to γ-glutamyltransferase. Biochem. Biophys. Res. Commum. **112:**564–570.
42. **Smith, T. K., and A. Meister.** 1994. Active deglycosylated mammalian γ-glutamyl transpeptidase. FASEB J. **8:**661–664.
43. **Sokolovsky, M., J. F. Riordan, and B. L. Vallee.** 1966. Tetranitromethane. A reagent for the nitration of tyrosyl residues in proteins. Biochemistry **5:**3582–3589.
44. **Song, L. J., M. Q. Ye, M. Troyanovskaya, E. Wilk, S. Wilk, and D. P. Healy.** 1994. Rat kidney glutamyl aminopeptidase (aminopeptidase A): molecular identity and cellular localization. Am. J. Physiol. **36:**F546–F557.
45. **Stole, E., and A. Meister.** 1991. Interaction of γ-glutamyl transpeptidase with glutathione involves specific arginine and lysine residues of the heavy subunit. J. Biol. Chem. **266:**17850–17857.
46. **Suzuki, H., H. Kumagai, T. Echigo, and T. Tochikura.** 1989. DNA sequence of the *Escherichia coli* K-12 γ-glutamyltranspeptidase gene, *ggt*. J. Bacteriol. **171:**5169–5172.
47. **Suzuki, H., H. Kumagai, and T. Tochikura.** 1986. γ-Glutamyltranspeptidase from *Escherichia coli* K-12: formation and localization. J. Bacteriol. **168:** 1332–1335.
48. **Szewczuk, A., and G. E. Connell.** 1965. The reaction of iodoacetamide with the active center of γ-glutamyl transpeptidase. Biochim. Biophys. Acta **105:** 352–367.
49. **Szewczuk, A., and M. Mulczyk.** 1967. γ-Glutamyl transpeptidase in bacteria. Arch. Immunol. Ther. Exp. **15:**395–397.
50. **Szewczuk, A., and M. Mulczyk.** 1970. γ-Glutamyl transpeptidase from *Pseudomonas aeruginosa*. Arch. Immunol. Ther. Exp. **18:**515–526.
51. **Talalay, P. S.** 1954. Glutathione breakdown and transpeptidation reactions in *Proteus vulgaris*. Nature (London) **174:**516–517.
52. **Tarr, G. E.** 1986. Manual Edman sequencing system, p. 155–194. *In* J. E. Shively (ed.), Methods of protein microcharacterization. Humana Press, Clifton, N.J.

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