γ-Glutamyltranspeptidase is the key enzyme in glutathione metabolism, and we previously presented evidence suggesting that it belongs to the N-terminal nucleophile hydrolase superfamily. Enzymatically active γ-glutamyltranspeptidase, which consists of one large subunit and one small subunit, is generated from a common precursor through post-translational proteolytic processing. The processing mechanism for γ-glutamyltranspeptidase of Escherichia coli K-12 has been analyzed by means of in vitro studies using purified precursors. Here we show that the processing of a precursor of γ-glutamyltranspeptidase is an intramolecular autocatalytic event and that the catalytic nucleophile for the processing reaction is the oxygen atom of the side chain of Thr-391 (N-terminal residue of the small (β) subunit), which is also the nucleophile for the enzymatic reaction.

γ-Glutamyltranspeptidase (GGT; EC 2.3.2.2), which consists of one large subunit and one small subunit, is the key enzyme in glutathione metabolism and is widely distributed in living organisms (1–4). The molecular weight of the large subunit polypeptide is approximately 40,000, and that of the small subunit is about 20,000. GGT is clinically significant because elevation of its activity in serum is widely performed in blood testing. GGT is a soluble enzyme in glutathione metabolism and is widely distributed in mammalian tissues. First, the N terminus of E. coli GGT and mammalian GGTs. First, the N terminus of E. coli GGT and mammalian GGTs is the anchor domain in the plasma membrane (7, 8), i.e. E. coli GGT is a soluble periplasmic enzyme (9), whereas mammalian GGTs are membrane-bound enzymes. Second, E. coli GGT is a nonglycosylated enzyme, whereas mammalian GGTs are heterologously glycosylated (2, 10, 11). Taking advantage of its characteristics, we have studied E. coli GGT. It was known that both the large and small subunits of the mature GGT are generated from a common precursor (pro-GGT) through post-translational processing (12–20). cDNA and genomic DNA coding for GGT have been cloned from various organisms, and their nucleotide sequences indeed show that both the large and small subunits of GGT are coded in a single open reading frame (6, 21–30). Kuno et al. (31) suggested that the enzyme that cleaves pro-GGT into two subunits is a membrane-bound trypsin-like serine protease, but it has never been purified. GGT mutants of E. coli K-12 which are deficient in processing have been isolated by site-directed mutagenesis, and all nonprocessed mutants so far isolated have no enzymatic activity (20, 32). Some mutants that undergo slow processing were observed as both processed and nonprocessed molecules that can be separated by native PAGE. When the gel was subjected to activity staining, the processed form was stained, although the nonprocessed one was not (32). These findings indicate that this processing is essential for activation of the enzyme.

The enzymatic reaction catalyzed by GGT has been thought to proceed via a γ-glutamyl-enzyme intermediate (1, 2) followed by nucleophilic substitution by water, amino acids, or peptides. Recently, we determined that the catalytic residue of E. coli GGT is Thr-391, the N-terminal residue of the small subunit (33). In fact, this residue is invariably conserved in all GGTs and related enzymes whose amino acid sequences are known. In addition, GGT is an amidohydrolase, and it takes an amidohydrolase proposed by Brannigan et al. (35). They found that the elements of the catalytic centers of Ntn hydrolases are equivalent in structural alignment of the active sites, and they predicted that the post-translational processing of Ntn hydrolases is an autocatalytic event. Since then, some Ntn hydrolases, the β-subunit of 20 S proteasome (36–39), glycosylasparaginase (40–42), penicillin acylase (43, 44), and cephalosporin acylase (45–47), have been proved to arise from an inactive precursor through autocatalytic post-translational processing. Also, some of these studies showed that the side chains of the residues that are to be the new N-terminal residues after processing not only act as nucleophiles for the enzymatic reactions after maturation but also act as nucleophiles for the processing reactions. This may be another unique feature common to Ntn hydrolases. However, there are still some discrepancies among the detailed mechanisms of the processing reactions to be elucidated.

In this paper, we show that the processing of GGT is autocatalytic, and the characteristics of its processing were studied.
For clarity, the residues of E. coli GGT are numbered as a single chain (residues 1–580) comprising the signal peptide (residues 1–58), the large (α) subunit (residues 26–390), and the small (β) subunit (residues 391–580). For example, T391S means a mutant GGT (protein) whose Thr-391 was replaced by a Ser residue.

**EXPERIMENTAL PROCEDURES**

**Medium and Reagents**—LB broth (Miller) was purchased from Difeo Laboratories. All antibiotics, 6-diazo-5-oxo-norleucine (DON), dithiothreitol, isopropyl-β-D-thiogalactopyranoside, penicillin G, and chloramphenicol (pCM), were purchased from Sigma Chemical Co. (St. Louis, MO). Bacteriological peptone and beef extract were purchased from Becton Dickinson Co. (Cockeysville, MD). 

**Bacterial Strains, Plasmids, and Oligonucleotides**—The bacterial strains, plasmids, and oligonucleotides used in this study are listed in Table I. Strain SH1220 harboring pMAL-p2-ggt in GGT-deficient strain SH641 (25, 50) overproduces enzymatically active MBP-GGT, which consists of the large subunit (365 amino acids) fused to a maltose-binding protein consisting of 386 amino acids (including linker) at its N terminus, and the small subunit (180 amino acids). pMAL-p2-ggt was designed so that the GCG codon of the N-terminal amino acid residue of the large subunit of the ggt gene of E. coli K-12 comes just after the factor Xa cleavage site (at XmnI) of pMAL-p2 and was constructed as follows. pMAL-p2 was cleaved with XmnI and BamHI and then ligated with the 1-kb EcoRV-BamHI fragment of pSH253 to obtain strain SH641. Strain SH154 was transformed with pMAL-p2-T391A to obtain strain SH1220. Strain SH641 was transformed with pMAL-p2-T391A to obtain strain SH1354 and was used to synthesize MBP-GGT in the periplasm.

Table I. Strain SH1220 harboring pMAL-p2-ggt in GGT-deficient strain SH641 (25, 50) overproduces enzymatically active MBP-GGT, which consists of the large subunit (365 amino acids) fused to a maltose-binding protein consisting of 386 amino acids (including linker) at its N terminus, and the small subunit (180 amino acids). pMAL-p2-ggt was designed so that the GCG codon of the N-terminal amino acid residue of the large subunit of the ggt gene of E. coli K-12 comes just after the factor Xa cleavage site (at XmnI) of pMAL-p2 and was constructed as follows. pMAL-p2 was cleaved with XmnI and BamHI and then ligated with the 1-kb EcoRV-BamHI fragment of pSH253 to obtain strain SH641. Strain SH154 was transformed with pMAL-p2-T391A to obtain strain SH1220. Strain SH641 was transformed with pMAL-p2-T391A to obtain strain SH1354 and was used to synthesize MBP-GGT in the periplasm.

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strain SH1227, a E. coli GGT antisemirum, this protein was purified as follows. Strain SH1227 was inoculated into a 100-ml flask containing 5 ml of LB broth with 100 μg/ml ampicillin and 0.2% glucose and grown at 37 °C overnight with reciprocal shaking. Four ml of the culture was transferred to a 1-liter flask containing 400 ml of the same medium and grown at 37 °C with reciprocal shaking. The culture to induce overexpression of the 100,000 protein. After incubation for 4 h, the cells were harvested by centrifugation, suspended in 20 ml of 50 mM Tris-HCl (pH 8), and then subjected to ultrasonication. The precipitate obtained on centrifugation at 10,000 × g for 15 min at 4 °C was dissolved in 1 ml of 6 × guanidine HCl and 1 mM EDTA in 50 mM Tris-HCl (pH 8). The mixture was kept at room temperature for 1 h and then centrifuged at 10,000 × g for 15 min at 4 °C. The supernatant was subjected to gel filtration on a Sephacryl S-300 column (0.7 × 115 cm; Amersham Biosciences) previously equilibrated with the same buffer. The fractions were eluted in 1-ml fractions. The fractions containing only the 100,000 protein were examined by SDS-PAGE and then stored at 4 °C until used. Strain SH1220 was grown at 20 °C in 200 ml of LB medium with 100 μg/ml ampicillin. MBP-GGT was induced by the addition of isopropyl-β-D-thiogalactopyranoside (final 0.3 mM) and purified from the periplasmic fraction of a cell-free extract of strain SH1227 by procedures quite different from those we used to purify the wild-type GGT from E. coli, it was confirmed to be pro-MBP-GGT, as follows. The molecular weights of pro-MBP-GGT, the large subunit of GGT fused with MBP, and the small subunit were estimated from their amino acid sequences to be 100,000, 80,000, and 20,000, respectively. The 100,000, 80,000, and 20,000 proteins migrated the same on SDS-PAGE as pro-MBP-T391A (the processing-deficient mutant GGT fused with MBP), and the large and small subunits of MBP-GGT (the wild-type GGT fused with MBP), respectively (data not shown). Both the 100,000 and 80,000 proteins were reactive with rabbit anti-E. coli GGT antiserum and also anti-MBP antiserum (New England Biolabs) (data not shown). Moreover, the N-terminal amino acid sequence of the 20,000 protein was determined to be TTHYS. After incubation, the sample showed GGT activity with γ-GpNA as a substrate. The correlation of the extent of the processing and the increase in the enzyme activity in the course of processing of pro-MBP-GGT was determined. A part of the processing reaction mixture withdrawn was mixed with the loading buffer, and the extent of processing was analyzed by SDS-PAGE. Another part was incubated with 0.5 mM γ-GpNA and 50 mM Tris-HCl (pH 8.75) at 37 °C in the cuvette of a spectrometer (model UV-1600PC; Shimadzu, Kyoto, Japan). Increasing rate of absorbance at 410 nm was measured to determine the amount of γ-GpNA released/min, and the enzymatic activity was calculated. As shown in Fig. 1D, the extent of the processing and the increase in the enzyme activity correlated well. Therefore, the 100,000 protein was assigned as pro-MBP-GGT and was autocatalytically processed into the 80,000 protein (the large subunit fused with MBP) and the 20,000 protein (the small subunit). Similarly, the 60,000 protein isolated from strain HW430 was reactive with anti-GGT antiserum. After removal of ammonium sulfate, the protein was incubated at 37 °C. The

RESULTS

Purification of the Precursors of GGT and Confirmation of Autocatalytic Processing of GGT in Vitro—The 100,000 protein was isolated from strain SH1227 as described under “Experimental Procedures.” After guanidine HCl had been removed, the protein was incubated at 37 °C and then subjected to SDS-PAGE followed by Coomassie Blue staining or Western blot analysis. As the incubation time was increased, the 100,000 band decreased, and 80,000 and 20,000 bands increased (Fig. 1A). Because the 100,000 protein was isolated from the insoluble fraction of a cell-free extract of strain SH1227 by procedures quite different from those we used to purify the wild-type GGT from E. coli, it was confirmed to be pro-MBP-GGT, as follows. The molecular weights of pro-MBP-GGT, the large subunit of GGT fused with MBP, and the small subunit were estimated from their amino acid sequences to be 100,000, 80,000, and 20,000, respectively. The 100,000, 80,000, and 20,000 proteins migrated the same on SDS-PAGE as pro-MBP-T391A (the processing-deficient mutant GGT fused with MBP), and the large and small subunits of MBP-GGT (the wild-type GGT fused with MBP), respectively (data not shown). Both the 100,000 and 80,000 proteins were reactive with rabbit anti-E. coli GGT antiserum and also anti-MBP antiserum (New England Biolabs) (data not shown). Moreover, the N-terminal amino acid sequence of the 20,000 protein was determined to be TTHYS. After incubation, the sample showed GGT activity with γ-GpNA as a substrate. The correlation of the extent of the processing and the increase in the enzyme activity in the course of processing of pro-MBP-GGT was determined. A part of the processing reaction mixture withdrawn was mixed with the loading buffer, and the extent of processing was analyzed by SDS-PAGE. Another part was incubated with 0.5 mM γ-GpNA and 50 mM Tris-HCl (pH 8.75) at 37 °C in the cuvette of a spectrometer (model UV-1600PC; Shimadzu, Kyoto, Japan). Increasing rate of absorbance at 410 nm was measured to determine the amount of γ-GpNA released/min, and the enzymatic activity was calculated. As shown in Fig. 1D, the extent of the processing and the increase in the enzyme activity correlated well. Therefore, the 100,000 protein was assigned as pro-MBP-GGT and was autocatalytically processed into the 80,000 protein (the large subunit fused with MBP) and the 20,000 protein (the small subunit). Similarly, the 60,000 protein isolated from strain HW430 was reactive with anti-GGT antiserum. After removal of ammonium sulfate, the protein was incubated at 37 °C. The

Measurement of Mass of the Large Subunit Processed from Pro-T391S—Ammonium sulfate was removed from the stored pro-T391S sample as described above. After incubation at 37 °C for 48 h, the large subunit was isolated by the reverse phase high performance liquid chromatography, and its mass was measured by ion spray mass spectrometry (model API-3000; PE Sciex) as described previously (33, 55).

In Vitro Processing of Pro-GGTs—The buffer in Micro Bio-Spin 6 centrifuged at 1,000 × g to obtain pSH11248. The ACT codon for Thr-391 was mutated by the manufacturer. To remove guanidine HCl from the stored sample, 75 mM EDTA in 50 mM Tris-HCl (pH 8) according to the protocol recommended by the manufacturer (Biolabs) was exchanged with 50 mM Tris-HCl with appropriate pH (pH 8 or 7) according to the protocol recommended by the manufacturer (Pure Chemicals). The protein band profiles on the gels were scanned with a scanner (model GT-8700F; Seiko-Epson, Suwa, Japan).

N-terminal Analysis of Proteins—The proteins (separated on SDS-PAGE) were electrophoresed onto a polyvinylidene difluoride membrane (Millipore). The membrane was stained with 0.025% Coomassie Blue R-250 or a silver staining kit (Wako Pure Chemicals). The protein band profiles on the gels were scanned with a scanner (model GT-8700F; Seiko-Epson, Suwa, Japan). Increasing rate of absorbance at 410 nm was measured to determine the amount of γ-GpNA released/min, and the enzymatic activity was calculated. As shown in Fig. 1D, the extent of the processing and the increase in the enzyme activity correlated well. Therefore, the 100,000 protein was assigned as pro-MBP-GGT and was autocatalytically processed into the 80,000 protein (the large subunit fused with MBP) and the 20,000 protein (the small subunit). Similarly, the 60,000 protein isolated from strain HW430 was reactive with anti-GGT antiserum. After removal of ammonium sulfate, the protein was incubated at 37 °C. The
60,000 band decreased, and the 40,000 and 20,000 bands increased in time-dependent manners (Fig. 1B). The N-terminal amino acid sequence of the 60,000 protein was AP-PAP, and that of the 20,000 protein was STHYS. The mass of the large subunit generated from pro-T391S by in vitro processing was 39,223 Da, which is well matched with the mass of the large subunit of wild-type GGT which we determined previously (33, 55). Considering the N-terminal amino acid sequence of pro-T391S, the mass of the large subunit generated from pro-T391S, and the amino acid sequence of E. coli GGT deduced from the DNA sequence of the ggt gene (6), we concluded that the C-terminal of the large subunit generated from pro-T391S by in vitro processing was Gln-390. The incubated sample also showed GGT activity. These results indicate that the 60,000 protein was pro-T391S without the signal peptide and that it was processed autocatalytically
Processing was very slow (Fig. 1). CFirmed to be processed autocatalytically, albeit that the rate of Pro-T391S was incubated for 0, 6.5, 13, 25, and 48 h (0.25 mM, 2 mM TPCK, was purchased from Wako Pure Chemicals and ture of AEBSF, bestatin, E-64 protease inhibitor, EDTA, and Protease Inhibitor Mixture for Bacterial Cell Extracts, a mix- mmm, 20 h (lanes 1–7 in A). Pro-T391S was incubated for 0, 2, 4, 6, 12.5, 18.5, and 24.5 h (lanes 1–7 in B). C and D, pro-MBP-GGT (C) and pro-T391S (D) were incubated at 37 °C without (a) and with (b) protease inhibitor mixture for bacterial cell extracts (0.25 mM AEBSF, 21 μM bestatin, 2.5 μM E-64 protease inhibitor, 1.06 mM EDTA, and 25 μM pepstatin A). Pro-MBP-GGT was incubated for 0, 0.25, 0.75, 1.5, 3, 6, and 20 h (lanes 1–7 in C). Pro-T391S was incubated for 0, 6.5, 13, 25, and 48 h (lanes 1–5 in D). The small subunits are not shown in this figure.

into the large subunit and the small subunit of T391S. Pro-T391C was also isolated from SH3107 and was con- firmed to be processed autocatalytically, albeit that the rate of processing was very slow (Fig. 1C).

Effects of Protease Inhibitors on the Autocatalytic Processing of GGT—The effects of protease inhibitors on the processing of GGT were determined. TLCK, TPCK, and phenylmethylsulfonyl fluoride were added to processing reaction mixtures (final, 2 mM), and then their effects were examined (Fig. 2, A and B). Protease Inhibitor Mixture for Bacterial Cell Extracts, a mixture of AEBSF, bestatin, E-64 protease inhibitor, EDTA, and pepstatin, was purchased from Wako Pure Chemicals and added to the processing reaction mixture (final concentrations 0.25 mM, 21 μM, 2.5 μM, 1.06 mM, and 25 μM, respectively), and then its effects were examined (Fig. 2, C and D). No obvious inhibition of processing of GGT was observed on the addition of these protease inhibitors.

Effects of SH Reagents on the Autocatalytic Processing of GGT—When pro-T391C was incubated with 0.2 mM 5,5’-dithio- bis(2-nitrobenzoic acid), 5 mM sodium iodoacetate, or 0.1 mM pCMB, the processing of the precursor was affected (data not shown). Because the effect of pCMB was the most obvious, its effects on the processing of pro-MBP-GGT and pro-T391S were also examined. No inhibition of the processing of pro-MBP- GGT or pro-T391S was found, whereas the processing of pro- T391C was abolished completely (Fig. 3). Similar results were obtained when the precursors were preincubated overnight with the SH reagents at 4 °C before the processing experiment (data not shown).

Effect of NH₂OH on the Autocatalytic Processing of GGT—To 90 μl of the processing reaction mixture of pro-T391S and pro- T391C, 10 μl of 2.5 mM NH₂OH, which was dissolved in 1 mM BisTris-propane and adjusted pH to 7.0, was added, and then its ef- fect was observed. As shown in Fig. 4, the processing reaction of pro-T391C was very much accelerated by NH₂OH, whereas the acceleration of that of pro-T391S was rather moderate.

Effects of Protease Inhibitors on the Autocatalytic Processing of GGT—The amount of pro-T391S after various incubation times in Fig. 1B was measured and plotted against the incubation time (Fig. 1E). The effects of the initial concentration of pro-T391S on its processing were compared using three different initial concentrations of pro-T391S (Fig. 6C). At each sampling time, the ratios of processed large subunit to nonprocessed precursor were almost the same for the three concentrations tested. These findings indicate that the processing reaction obeys first-order kinetics. Because pro-T391S was confirmed to exist as a monomer on native gradient PAGE (data not shown), these results indicate that the processing is an intramolecular reaction. This was also the case of the processing reaction of pro-T391C in Fig. 1C.
The processing of the β-subunit of 20 S proteasome, glycosyl-
asparaginase, penicillin acylase, and cephalosporin acylase, four enzymes belonging to the Ntn hydrolase superfamily, were experimentally shown to be autocalytic, as originally predicted by Brannigan et al. (35) in structural studies. However, it has not been shown that this feature is common to all members of this superfamily. Various evidence strongly suggests that GGT is an Ntn hydrolase, although the three-dimensional structure of its catalytic site has not been determined. Supposing that GGT is an Ntn hydrolase, it is possible that its processing is an autocalytic event. To clarify this, pro-MBP-GGT, pro-T391S, and pro-T391C were purified to electrophoretic homogeneity, and then in vitro processing experiments were performed. Because nonprocessing or slow processing precursors of GGT, that is, pro-T391A, pro-T391C, and pro-T391S as well as mature form MBP-GGT expressed from pMAL-p2-ggt localized in the periplasm (data not shown), we purified them from the plasmid fractions. However, we cannot observe the precursor of the wild-type GGT (pro-GGT) or that of MBP-GGT (pro-MBP-GGT) expressed from pMAL-p2-ggt, maybe because they do not form the mature forms so fast that we cannot detect them. On the other hand, pro-MBP-GGT that was expressed in the buffer (pH 6–8.5), they were processed into the large subunit and the small subunit in the absence of another protein (pro-

**DISCUSSION**

The processing of the β-subunit of 20 S proteasome, glycosyl-asparaginase, penicillin acylase, and cephalosporin acylase, four enzymes belonging to the Ntn hydrolase superfamily, were experimentally shown to be autocalytic, as originally predicted by Brannigan et al. (35) in structural studies. However, it has not been shown that this feature is common to all members of this superfamily. Various evidence strongly suggests that GGT is an Ntn hydrolase, although the three-dimensional structure of its catalytic site has not been determined. Supposing that GGT is an Ntn hydrolase, it is possible that its processing is an autocalytic event. To clarify this, pro-MBP-GGT, pro-T391S, and pro-T391C were purified to electrophoretic homogeneity, and then in vitro processing experiments were performed. Because nonprocessing or slow processing precursors of GGT, that is, pro-T391A, pro-T391C, and pro-T391S as well as mature form MBP-GGT expressed from pMAL-p2-ggt localized in the periplasm (data not shown), we purified them from the plasmid fractions. However, we cannot observe the precursor of the wild-type GGT (pro-GGT) or that of MBP-GGT (pro-MBP-GGT) expressed from pMAL-p2-ggt, maybe because they process to the mature forms so fast that we cannot detect them. On the other hand, pro-MBP-GGT that was expressed in the buffer (pH 6–8.5), they were processed into the large subunit and the small subunit in the absence of another protein (pro-

**FIG. 2. Effect of an SH reagent (pCMB) on the autocalytic processing of pro-MBP-GGT (A), pro-T391S (B), and pro-T391C (C).** The precursors in 50 mM Tris-HCl (pH 7) were incubated at 37°C without (a) and with (b) 0.1 mM pCMB. Pro-MBP-GGT was incubated for 0, 0.25, 0.75, 1.5, and 3 h (lanes 1–5 in A); pro-T391S was incubated for 0, 6, 12, 24, and 48 h (lanes 1–5 in B); and pro-T391C was incubated for 0, 2, 4.5, 6, and 8 days (lanes 1–5 in C). The small subunits are not shown in this figure.

**FIG. 4. Effect of NH₄OH on the autocalytic processing of pro-T391S (A) and pro-T391C (B).** The precursors in 50 mM Tris-HCl (pH 7) were incubated at 37°C without (a) and with (b) 0.25 mM NH₄OH. Pro-T391S was incubated for 0, 6, 12, 24, and 48 h (lanes 1–5 in A), and pro-T391C was incubated for 0, 3, 6, 12, 18, 24, 36, and 48 h (lanes 1–8 in B). The small subunits are not shown in this figure.

**FIG. 5. Effects of potent GGT inhibitors and a substrate, γ-GpNA, on the autocalytic processing.** A and B, pro-MBP-GGT (A) and pro-T391S (B) in 50 mM Tris-HCl (pH 8) were incubated at 37°C without an inhibitor (a) and with 2 mM AT-125 (b) and 2 mM DON (c). Pro-MBP-GGT was incubated for 0, 0.25, 0.75, 1.5, 3, 6, and 20 h (lanes 1–7 in A), and pro-T391S was incubated for 0, 2, 4, 6, 12, 18, and 24 h (lanes 1–7 in B). C, pro-T391S in 50 mM Tris-HCl (pH 8) was incubated at 37°C without (a) and with (b) 0.5 mM γ-GpNA. The mixtures were incubated for 0, 6, 12, 24, and 48 h (lanes 1–5 in C). The small subunits are not shown in this figure.

**FIG. 6. Proof of intramolecular processing.** A, Western blot analysis of whole cells expressing both pro-MBP-GGT and pro-T391A. Lane 1, strain SH1220 (pMAL-p2-ggt/SH641); lane 2, SH1444 (pMAL-p2-ggt and pT391A/SH641); lane 3, SH1443 (pT391A/SH641); and lane 4, SH642 (pSH101/SH641). The strains were grown in LB medium with appropriate antibiotics at 20°C for 2 days. Cells obtained from 1-ml aliquots of these cultures were denatured by boiling with 100 μl of the loading buffer containing SDS and dithiothreitol, and then 5 μl of the resulting supernatants was subjected to SDS-PAGE followed by Western blot analysis with anti-E. coli GGT antiserum. B, in vitro processing of coexisting pro-MBP-GGT and pro-T391A. Lanes 1 and 2, pro-MBP-GGT in 50 mM Tris-HCl (pH 8) was incubated at 37°C for 0 and 4 h, respectively. Lanes 3 and 4, pro-MBP-GGT and pro-T391A were incubated together for 0 and 4 h, respectively. Lanes 5 and 6, pro-T391A was incubated for 0 and 4 h, respectively. Lane 7, purified wild-type GGT. C, effect of the initial pro-T391S concentration on the processing rate. Pro-T391S in 50 mM Tris-HCl (pH 8) was incubated at 37°C for 0, 6, 12, and 18 h (lanes 1–4) at the concentrations of 2.0 mg/ml (a), 0.2 mg/ml (b), and 0.02 mg/ml (c). After the reaction had been terminated, samples from a and b were diluted 100-fold and 10-fold with 1× loading buffer, respectively. The same volumes of the samples (5 μl) were subjected to SDS-PAGE, and the gel was subjected to silver staining. The small subunits are not shown in this figure.
Autocatalytic Processing of GGT

Fig. 7. Proposed mechanism for autocatalytic processing of GGT. A, abstraction of the proton of the hydroxyl group of Thr-391 by an unidentified base results in the reactive oxygen atom. The addition of Thr-391 O\(^{\cdot}\) to the carbonyl carbon of Gln-390 is followed by the formation of a transitional tetrahedral intermediate. B, cleavage of the C-N bond through protonation of the amino group of Thr-391 yields an ester intermediate (N-O acyl shift). C, hydrolysis of the ester by water yields a carbonyl group on Gln-390 and a hydroxyl group on Thr-391.

They use the same oxygen atom as a nucleophile. In an Ntn hydrolase, the amino group of the newly generated N terminus on processing plays a part in the catalytic reaction as a general base in the hydroxyl/a-amine dyad and activates the hydroxyl (or thiol) group of the side chain of the N-terminal amino acid residue, which is the nucleophile of the enzymatic reaction of an Ntn hydrolase (61). This mechanism may be applicable to the enzymatic reaction of GGT, although the three-dimensional structure of the active center of GGT has not been determined. Different from the case of the enzymatic reaction, the a-amine group of Thr-391 is involved in the peptide linkage in the precursor and is not available as a base in the processing reaction. Then, what acts as a base in the processing reaction? It has been suggested that a His residue near the processing site of glycosylasparaginase is essential for its processing and acts as a base in this reaction, from the results of biochemical studies (40, 41). However, x-ray crystallographical results suggested that the \(\beta\)-carboxyl group of the Asp residue just before the Thr residue, whose side chain is the nucleophile of this enzyme, acts as the base (42). In the case of the \(\beta\)-subunit of 20 S proteasome, the residue just before the catalytic Thr residue is the invariably conserved Gly, which has no side chain that can act as a base like Asp in glycosylasparaginase. Schmidtke et al. proposed that Lys-33 of the \(\beta\)-subunit of human 20 S proteasome acts as the base in its autocatalytic processing (36). On the contrary, Ditzel et al. (39) showed that there is no amino acid base but a water molecule that can act as the base at the active center for processing, in a crystallographical study of the

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β-subunit precursor of yeast 20 S proteasome. Similarly, in the cases of precursors of penicillin acylase (44) and class I cephalosporin acylase (56), the residues just before the catalytic Ser residues are Gly, and no nearby amino acid base is found in the crystals of the precursors. Also, a water molecule was suggested to act as the base in cephalosporin acylase (56). For the time being, it seems that the mechanisms of autocatalytic processing exhibit some variation among the members of this superfamily, especially regarding the base. In the case of E. coli GGT, no big difference was found in the processability even when Gln-390, which precedes nucleophile Thr-391, was replaced by Ala (32). In fact, the C termini of the large (α) subunits of all mammalian GGTs and related enzymes so far isolated are Gly. When His-393 of E. coli GGT, which is invariably conserved in GGTs, was replaced by a Gly residue, the mutant strain only expressed pro-GGT, no mature GGT being observed (20). On the contrary, the corresponding human GGT mutant enzyme, H383A, was expressed as a heterodimer in insect cells (62). Therefore, the possibility that His-393 acts as the base is ruled out. A crystallographical study of precursor GGT is essential to elucidate this problem.

Seemüller et al. (37) suggested that the autocatalytic processing of the β-subunit of archaeabacterial 20 S proteasome is an intramolecular event, whereas Guan et al. (40) and Kasche et al. (43) clearly proved that the processing of glycosylaspartase and penicillin acylase, respectively, is an intramolecular event. Besides these studies, on the autocatalytic processing of other Ntn hydrolases including the β-subunit of human 20 S proteasome (36), cephalosporin acylase (45, 46), and glutamin phosphoribosylpyrophosphate amidotransferase from Bacillus subtilis (63) suggested that it was an intramolecular event. The results we reported in this paper show that the autocatalytic processing of GGT is an intramolecular event, the proposed mechanism underlying intramolecular autocatalytic processing of GGT being presented in Fig. 7. Because of overall and processing site sequence similarity among GGT, GGT-related enzyme (64, 65), γ-glutamyl leukotriene (66), and Dep of Bacillus anthracis (67), the processing mechanisms of all these enzymes might be similar.

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