Mutational and Proteolytic Studies on a Flexible Loop in Glutathione Synthetase from *Escherichia coli* B: The Loop and Arginine 233 Are Critical for the Catalytic Reaction†

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ABSTRACT: The function of the flexible loop which is disordered in crystal structure analysis of glutathione synthetase from *Escherichia coli* B has been investigated by limited proteolysis and kinetic measurements for the wild-type and mutant enzymes. Proteolysis of the intact enzyme using arginyl endopeptidase or trypsin brought about a time-dependent decrease in the enzymatic activity and the production of protein fragments. SDS–polyacrylamide gel electrophoresis and peptide sequence analysis showed that only a peptide bond between arginine 233 and glycine 234 in the loop was cleaved. Further, native polyacrylamide gel electrophoresis revealed that the cleaved enzyme retained almost the same quaternary structure as that of the wild-type enzyme. Upon protease treatment, the presence of substrates, ATP and/or γ-L-glutamyl-L-cysteine (γ-Glu-Cys), protected the loop from cleavage, but the presence of glycine was not capable of protecting it. In addition, replacement of arginine 233 in the loop with lysine by site-directed mutagenesis increased the Michaelis constants for γ-Glu-Cys and glycine by factors of 28 and 213, respectively. The protection against cleavage on a similar protease incubation of this mutant enzyme was also observed in the presence of ATP and/or γ-Glu-Cys, but the effect in the presence of both substrates was half as large as that for the wild-type enzyme. These results suggest that the loop covers the active site while ATP and γ-Glu-Cys bind there and that it protects the unstable γ-Glu-Cys phosphate intermediate from decomposition by bulk water. Also, the role of arginine 233 is attributable to the fixing of the γ-Glu-Cys component. The present study illustrates an additional feature of the flexible loop that is one of the important factors for enzyme activity.

Glutathione synthetase [γ-L-glutamyl-L-cysteine:glycine ligase (ADP-forming), EC 6.3.2.3; GSHase][1] catalyzes the synthesis of glutathione from γ-L-glutamyl-L-cysteine (γ-Glu-Cys) and glycine in the presence of ATP. The substrate specificity of the enzyme from rat kidney is strict in its recognition of glycine but not in its recognition of the glutaminyl moiety of the dipeptide; no other amino acid than glycine was accepted as a substrate (Moore & Meister, 1987; Oppenheimer et al., 1979). Among GSHases of microbial origin, the enzyme from *Escherichia coli* B was purified and well characterized. From the analysis of the properties of the enzyme and the gene, it was proved that this enzyme is a tetramer of four identical subunits, each of which is composed of 316 amino acid residues (Gushima et al., 1983, 1984). By subcloning the gene into an overexpression plasmid, the gene was expressed at a level of over 50% of the total soluble proteins of *E. coli* cell (Kato et al., 1989a). In the catalytic mechanism, Meister (1974) has proposed that the reaction of GSHase from rat kidney proceeds through a formation of an active intermediate, γ-Glu-Cys phosphate, by an ordered mechanism. We have previously reported that GSHase from *E. coli* B was potently inhibited by methotrexate, an inhibitor of dihydrofolate reductase, and revealed that the binding of ATP and γ-Glu-Cys occurs in preference to that of glycine on the assumption that methotrexate binds to the ATP-binding site (Kato et al., 1987). Our laboratory has also studied the functional role of the cysteine residues of GSHase from *E. coli* B by chemical modification and site-directed mutagenesis. Consequently, it was proved that specific chemical modification of cysteine 289 inactivated the enzyme but that the replacement of cysteine 289 with alanine did not affect the catalytic activity (Kato et al., 1988). At present, no amino acid residue having a catalytic function has yet been identified.

We recently succeeded in the X-ray crystallographic analysis of GSHase from *E. coli* B at 2.7-Å resolution (Kato et al., 1989b; Yamaguchi et al., 1990). The electron density map was of such good quality that no main-chain break was found, except for the main chain from isoleucine 226 to glycine 242. The amino acid sequence of this invisible peptide, I^{226}-P-Q-G-G-E-T-R-G-N-L-A-A-G-G-R-G^{242}, contains six glycine residues. Further, this part of the peptide is not supposed to take a rigid conformation but a loop structure having flexibility and mobility even in a crystal state. Crystal structure analysis of the enzyme complexed with ATP suggested that the ATP-binding site was located under the loop structure. In proteins which require nucleotide triphosphate as a substrate, a glycine-rich structure, called a "phosphate loop", is commonly found, and a lysine residue in the loop recognizes the nucleotide as the substrate from the phosphate group (Pai et al., 1989; Reinstein et al., 1988, 1990; Saraste et al., 1990; Tagaya et

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[1] Abbreviations: DEAE, diethylaminoethyl; γ-Glu-Cys, γ-L-glutamyl-L-cysteine; GR, glutathione reductase; GSHase, glutathione synthetase; k_0^{app}, catalytic constant; K_m^{app}, Michaelis constant (an apparent dissociation constant); LB medium, Luria–Bertani medium; PAGE, polyacrylamide gel electrophoresis; PK–LDH, pyruvate kinase–lactate dehydrogenase; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TLCK, *p*-toluenesulfonyl-L-lysine chloromethyl ketone; TPCK, *p*-toluenesulfonyl-L-phenylalanine chloromethyl ketone. Mutant glutathione synthetase from *E. coli* B: R233A, arginine 233 → alanine exchange; R233K, arginine 233 → lysine exchange; R241A, arginine 241 → alanine exchange; R241K, arginine 241 → lysine exchange.

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al., 1987). Although GSHase is a member of the protein group requiring ATP as a substrate, no sequence similarity to the phosphate loop was detected and no lysine residue was found from isoleucine 226 to glycine 242. However, there are two arginine residues, argine 233 and arginine 241, in the loop instead of lysine. This difference brings up the question of whether or not one or both of the arginine residues have a role similar to that of the lysine residue on the phosphate loop.

As reported so far, limited proteolysis has provided a useful probe to study the structures of proteins. Under nondenaturing conditions, the protease selectively cleaves the peptide bond of proteins at exposed or protruded regions which have a loop or coiled structure. In tryptophan synthase from *E. coli* (Higgins et al., 1979; Miles & Higgins, 1978) and glutamine synthetase from *E. coli* (Dautry-Varsat et al., 1979; Lei et al., 1979; Monroe et al., 1984), proteolysis, under native conditions, cleaves their respective peptide chains at a loop structure, resulting in the loss of most of their catalytic activities. In these two enzymes, the loop digested by proteolysis takes part in their catalytic reactions.

In the present work, limited proteolysis under native conditions was applied to elucidate the functions of the loop in GSHase from *E. coli* B. In addition, mutant enzymes, in which arginine 233 or arginine 241 in the loop was replaced with lysine and alanine, were constructed, and their properties were investigated to compare the roles of the lysine residue in the phosphate loop.

Experimental Procedures

Bacterial Strains, Phage, and Plasmid. Plasmid pKGS00 is a pKK223-3 derivative containing a 1.2 kilobase pair fragment of the GSHase gene from *E. coli* B (Kato et al., 1988). Bacterial strain *E. coli* BW313 (*dut*, *ung*, *thi*-1, *rel* A, *spo*T1/F'*lys* A) was a gift from Dr. Kagamiyama, Medical College of Osaka. Bacteriophage M13 mp19 and *E. coli* JM109 were provided by Takara Shuzo Co., Ltd. (Kyoto, Japan).

Restriction Enzymes and Chemicals. Restriction enzymes were obtained from Toyobo Co., Ltd. (Osaka, Japan) and New England Biolabs, Inc. (Beverly, MA). Restriction enzymes, DNA-modifying enzymes, and arginyl endopeptidase (mouse submandibular protease, treated with TLCK and TPCK; 1.045 units/mg) (Levy et al., 1970) were purchased from Takara Shuzo Co., Ltd. Trypsin (12000 units/mg; treated with TPCK) from bovine pancreas was purchased from Sigma Chemical Co. (St. Louis, MO). Lactate dehydrogenase from pig heart, pyruvate kinase from rabbit muscle, and glutathione reductase from yeast were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). All the γ-Glu-Cys was a special gift from Kohjin Co., Ltd. (Tokyo, Japan). The Tris-HCl buffer used in the present study contains 5 mM $MgCl_2$, unless noted otherwise. Chemical used in these experiments were of the purest grade commercially available.

Measurement of GSHase Activity. Protein concentration was determined by the method of Lowry with bovine serum albumin as a standard (Lowry et al., 1951). The activity of GSHase was determined by two different methods. The first method (PK–LDH method) measures the formation of ADP with pyruvate kinase and lactate dehydrogenase as a coupling system (Brolin, 1983). Tris-HCl (pH 7.5, 50 mM), 50 μL, containing GSHase was added to an assay medium composed of 5 mM γ-Glu-Cys, 15 mM glycine, 10 mM ATP, 10 mM $MgSO_4$, 1 mM phosphoenolpyruvate, 0.24 mM NADH, 25 units of lactate dehydrogenase, 10 units of pyruvate kinase, 100 mM KCl, and 100 mM Tris-HCl (pH 7.5), in a total volume of 1 mL. Disappearance of NADH in the assay medium was monitored by absorbance measurements at 340 nm. The second method (GR method) measures the formation of glutathione (Tietze, 1969). Tris-HCl (pH 7.5, 50 mM), 10 μL, containing GSHase was added to an assay medium composed of 5 mM γ-Glu-Cys, 15 mM glycine, 10 mM ATP, 10 mM $MgSO_4$, and 100 mM Tris-HCl buffer (pH 7.5) in a total volume of 0.2 mL. After the incubation at 37 °C for 5–15 min, a test tube containing the reaction mixture was immersed in boiling water for 3 min to terminate the reaction. The glutathione produced was determined as the increasing rate of production of 2-nitro-5-thiobenzoate anion, which was formed by thiolysis of 5,5'-dithiobis(2-nitrobenzoic acid) by glutathione in the presence of NADPH and glutathione reductase. One unit of GSHase was defined as the amount that catalyzed the formation of 1 μmol of glutathione per minute at 37 °C and at pH 7.5. The above two methods gave consistent results within experimental error. For example, the activity of a purified preparation of the wild-type enzyme was 48.0 ± 1.5 units/mg determined by the PK–LDH method and 42.0 ± 5.4 units/mg determined by the GR method. In most experiments, activities were measured by the PK–LDH method. When the data for the steady-state kinetic parameters were collected, the concentration of the fixed substrates was set to over three times the K_m^{app} value of each substrate. The steady-state kinetic parameters K_m^{app} and k_0^{app} were determined by a nonlinear least-squares fit of the data using the method described by Sakoda and Hiromi (1976).

Limited Proteolysis of GSHase. To 400 μL of 25 mM Tris-HCl (pH 7.3) containing the purified preparation (2 mg/mL) of the wild-type GSHase, arginyl endopeptidase (8 μg/20 μL) or trypsin (5 μg/20 μL) was added at 30 °C. A 40-μL aliquot of the reaction mixture was withdrawn at 0, 20, 30, 60, 120, 240, and 480 min after the beginning of the reaction, and 10 μL of 10% acetonitrile solution containing 5 mM phenylmethanesulfonyl fluoride (PMSF) was added immediately to stop the proteolysis. When the effects of the substrates of GSHase on the proteolysis were studied, the substrates were added to the GSHase solution. The aliquots of the reaction mixture were analyzed on SDS (Laemmli, 1970) and native PAGE (Davis, 1964), and their activities were determined by the PK–LDH method.

NH_2-terminal amino acid sequence analysis of the products of proteolysis was carried out to determine the digested site. The GSHase was treated with arginyl endopeptidase or trypsin under the conditions as mentioned above. After a 480-min reaction, the reaction mixture was separated on SDS–PAGE. Separated peptide fragments were blotted on a poly(vinylidene difluoride) membrane (Bauw et al., 1987; Matsudaira, 1987) by the electroblotting method (Vandekerckhove et al., 1985). Edman degradations were performed automatically by using a gas-phase peptide sequencer, Applied Biosystems model 477A/120A protein sequencer system. The 3-phenyl-2-thiohydantoin–amino acid derivatives were identified with high-performance liquid chromatography.

Limited proteolysis of R233K enzyme was performed under the same reaction conditions as that of the wild-type enzyme.

To examine the specificity of the proteolysis for GSHase, the wild-type GSHase was treated with arginyl endopeptidase under denaturing conditions. After GSHase was denatured at 65 °C for 10 min, the denatured GSHase was recovered by centrifugation at 12000*g*. This denatured preparation was treated with arginyl endopeptidase in 50 mM Tris-HCl buffer (pH 7.3) containing 1 M urea.

Site-Directed Mutagenesis. A *Kpn*I–*Bam*HI fragment (453 base pairs) encoding the partial GSHase gene was isolated

from pKGS00 and inserted into bacteriophage M13 mp19 to give *φKB453* for site-directed mutagenesis of arginine 233 and arginine 241. Oligonucleotide-directed mutagenesis was carried out by the method of Kunkel (1985). Oligonucleotide primers were designed to create a new recognition site of restriction enzyme. Mutants were screened with this created site. Oligonucleotide primers were as follows. (The underlined bases encode the new amino acid.)

R233A 5′-GGGCGAAACAGCTGGCAATCTGGC-3′

R233K 5′-GGCGAAACAAAAGGCAATCTGGC-3′

R241A 5′-GGCTGCCGGCGGTGCCGGTGAACC-3′

R241K
5′-GGCTGCCGGCGGTAAAGGTGAACCTCG-3′

A short DNA fragment containing the mutation site was excised from *φKB453* mutant by two appropriate restriction enzymes, *Kpn*I and *Eco*RV. After verifying DNA sequences by the chain termination method (Sanger et al., 1977), the fragment containing the desired mutations replaced the counterpart of pKGS00 to give expression vectors of the mutant enzyme.

Overexpression and Purification of Wild-Type and Mutants of GSHase. The expression plasmid was used to transform *E. coli* JM109 by the method of Hanahan (1983). Transformants were grown in 500 mL of LB medium (1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl; pH 7.5) at 37 °C, with vigorous shaking. At log phase (OD_{600} = 0.6–0.7), isopropyl β-D-thiogalactopyranoside was added to yield a final concentration of 1 mM. After being cultured for 10 h, the cells were harvested by centrifugation at 8000*g* for 10 min at 0 °C (Kato et al., 1989a). The cells were suspended in 30 mL of 20 mM Tris-HCl buffer, pH 7.5, and were sonicated. The suspension was centrifuged at 15000*g* for 20 min at 0 °C to give a cell-free extract (0.9 g of protein). In our previous study, the wild-type GSHase was purified on DEAE-Cellulofine A-800 column chromatography (Kato et al., 1989b). In the present study, salting-out without any chromatography was successful. A potassium phosphate buffer (pH 6.8, 50 mM) containing 5 mM $MgCl_2$ (buffer I), 16 mL, was saturated with ammonium sulfate and then added to the cell-free extract at 0 °C to produce a 35% ammonium sulfate saturation. It was left standing for 12 h at 0 °C and then centrifuged at 20000*g* for 20 min at 0 °C. The ammonium sulfate precipitant was resuspended with 20 mL of buffer I. After being dialyzed overnight against buffer I, the suspension was centrifuged at 15000*g* for 20 min to remove the insoluble fraction. The supernatant, 20 mL, was reprecipitated out by adding 6.8 mL of buffer I saturated with ammonium sulfate and allowed to settle overnight at 0 °C. The precipitate was recovered by centrifugation and dialyzed overnight against several changes of buffer I. They were stored at 0 °C until they were used.

The present purification procedure gave a purified preparation of mutant enzymes higher than 95% in purity based on SDS–PAGE. The average yield of mutant protein was about 0.15 g from a 500-mL culture. To examine contamination by proteases, purified enzyme preparations were incubated at 37 °C for 1 h in 25 mM Tris-HCl, pH 7.5. On SDS–PAGE, they showed no other peptide fragment due to proteolytic digestion, which proved that these preparations were free from protease contamination.

Results

Limited Proteolysis of the Wild-Type GSHase. GSHase from *E. coli* B was incubated with arginyl endopeptidase under native conditions at 30 °C. The reaction mixture of the proteolysis by arginyl endopeptidase were analyzed on SDS–PAGE (Figure 1a). As shown in Figure 1a, the peptide of the intact GSHase at 35.6 kDa decreased as proteolysis proceeded, while the two peptide fragments due to the proteolytic cleavage appeared at 26 and 10 kDa and increased in their intensities. No other peptide fragment was detected even after prolonged proteolysis. NH_2-terminal amino acid sequence analysis of the 10-kDa peptide fragment gave a sequence of G-N-L-A-A-G-G-R-G, which is matched to the sequence from glycine 234 to glycine 242. The calculated molecular mass of the peptide from glycine 234 to the COOH-terminal residue and the peptide from the NH_2 terminal to arginine 233 is 10599.9 and 24979.4 Da, respectively. Therefore, it was clear that GSHase was hydrolyzed at only one site between arginine 233 and glycine 234 by arginyl endopeptidase. Trypsin digestion gave the same results as the arginyl endopeptidase digestion.

When GSHase denatured at 65 °C for 10 min was incubated with arginyl endopeptidase at 30 °C for 15 min, more than 15 peptide fragments were visible on SDS–PAGE. Therefore, the proteolysis at arginine 233 was specific to the

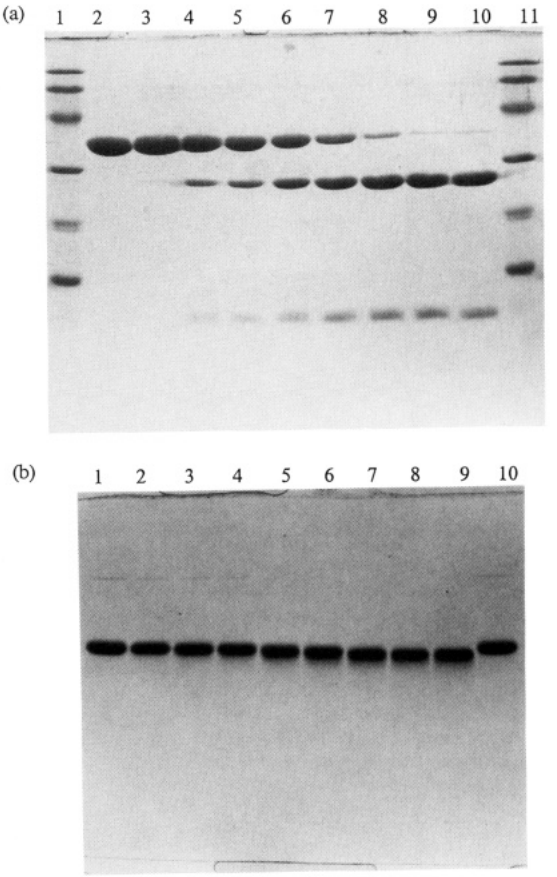

FIGURE 1: Limited proteolysis of the wild-type glutathione synthetase with arginyl endopeptidase. The glutathione synthetase (2 mg/mL) was treated with arginyl endopeptidase (8 μg/mL) in 400 μL of 25 mM Tris-HCl (pH 7.3) buffer. Aliquots were removed from the reaction mixture at the times mentioned below, and 5 mM PMSF (0.25 vol) was added to terminate the reaction. The aliquots were analyzed on 12% SDS–PAGE (a) and 10% native PAGE (b). (a) Samples are molecular mass markers (lanes 1 and 11: 94, 67, 43, 30, 20, and 14 kDa), purified glutathione synthetase (lane 2), products after 0-, 20-, 30-, 60-, 120-, 240-, and 480-min reactions (lanes 3–9), and products after a 480-min reaction in which the reaction was terminated not with PMSF addition but with SDS treatment at 60 °C for 10 min (lane 10). (b) Samples are purified enzymes (lanes 1 and 10), products after 0-, 20-, 30-, 60-, 120-, 240-, and 480-min reactions (lanes 2–8), and products after a 480-min reaction with no PMSF addition (lane 9).

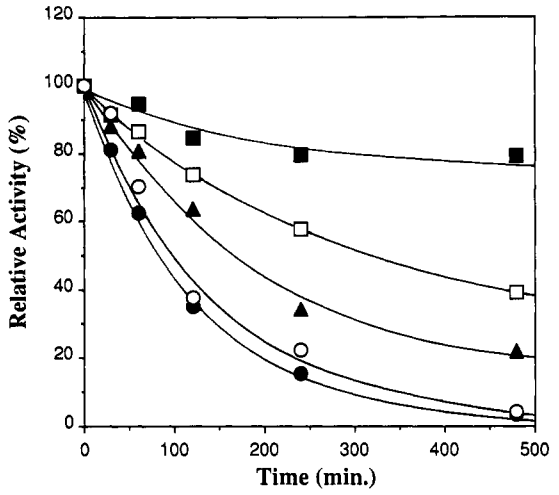

FIGURE 2: Kinetics of proteolytic inactivation of the glutathione synthetase. The glutathione synthetase (2 mg/mL) was treated with arginyl endopeptidase (8 μg/mL) in 400 μL of 25 mM Tris-HCl (pH 7.3) buffer. The reactions were carried out in the presence of 5 mM γ-Glu-Cys (▲), 10 mM ATP and 20 mM Mg^{2+} (□), 15 mM glycine (○), 5 mM γ-Glu-Cys, 10 mM ATP, and 20 mM Mg^{2+} (■), and in the absence of the substrates (●). Aliquots were withdrawn from the reaction mixture at the times indicated, and 5 mM PMSF (0.25 vol) was added to terminate the reaction. The activity of each aliquot was determined by the PK–LDH method. The results are expressed as the percentage of the activity of the aliquot removed at 0 min.

folding of GSHase under the native conditions.

In spite of the cleavage into two peptide fragments, only one protein band appeared on native PAGE, and its migration was equal to that of the intact enzyme (Figure 1b). Such a result obviously indicates that the tetrameric structure of GSHase was hardly changed by the proteolysis.

Furthermore, it was also observed that the activity of GSHase decreased with the duration of the treatment by arginyl endopeptidase. Figure 2 shows a plot of the activity of the digested GSHase relative to the intact enzyme against the duration of the arginyl endopeptidase treatment in the presence or absence of substrates. After the reaction in the absence of the substrates for 480 min, only 3.6% of the activity remained, as shown by the curve with solid circles. The decrease in the activity showed a good correspondence to the decrease in the amount of the intact enzyme on SDS–PAGE in Figure 1a. Consequently, in the following experiments, the degree of the proteolysis was measured by the remaining activity.

In order to get further insight into the role of the flexible loop, proteolysis with arginyl endopeptidase placed intentionally in the presence of substrates was examined. When one or two of the substrates was added to the reaction mixture, the activity of GSHase was significantly retained: as shown in Figure 2, 22.4% activity in the presence of 5 mM γ-Glu-Cys and 39.3% activity in the presence of 10 mM ATP after 480 min. In the presence of both of these two substrates at 5 mM γ-Glu-Cys and 10 mM ATP, the remaining activity was up to 79.1%. Glycine is the substrate that had a slight effect on the proteolysis. These results showed that γ-Glu-Cys and ATP protected GSHase from proteolysis by arginyl endopeptidase.

Limited Proteolysis of R233K Mutant GSHase. To analyze the functional role of arginine 233 in the loop, the expression plasmid for R233K mutant gene in which codon for arginine 233 was replaced with that of lysine was constructed and expressed in *E. coli* JM109. The specific activity of the purified preparation of R233K enzyme was 3.2% of that of the wild-type enzyme. For the incubation of this mutant enzyme

Table I: Activities of Mutant Enzymes[a]

enzyme	sp act. (units/mg)	rel act.[b] (%)
R233A	3.12	6.5
R233K	1.54	3.2
R241A	43.4	90.4
R241K	58.6	122
wild-type	48.0	100

[a] Assayed by the PK–LDH method. One unit of enzyme was defined as the amount producing 1 μmol of glutathione per minute under standard assay conditions (10 mM ATP, 5 mM γ-Glu-Cys, 15 mM glycine at 37 °C, pH 7.5). Background activity was subtracted from these values. [b] Relative activity is the percentage of the specific activity of the wild-type enzyme.

with arginyl endopeptidase under native conditions for 480 min at 30 °C, no peptide fragment other than the intact R233K enzyme was detected on SDS–PAGE. In contrast, when the mutant enzyme was treated with trypsin at 30 °C, two peptide fragments of 26 and 10 kDa appeared and increased for the duration of the proteolysis. The NH_2-terminal sequence of the 10-kDa fragment was determined to be G-N-L-A-A-G-G, which matched the sequence from glycine 234 to glycine 240 of GSHase. This result shows that the amide bond between lysine 233 and glycine 234 was cleaved by trypsin under the native conditions. In other words, the results of the proteolysis of the R233K mutant enzyme with trypsin cogently supported the proposition that this enzyme has a structure similar to that of the wild-type enzyme under conditions where they are free from their substrates.

The substrates of GSHase also prevented trypsin from cleaving the R233K enzyme. The activity of the mutant enzyme remaining after 120 min of proteolysis was 32.3% of the initial activity, while the remaining activities were 47.2, 48.1, and 75.7% in the presence of 30 mM γ-Glu-Cys, 10 mM ATP, and both 30 mM γ-Glu-Cys and 10 mM ATP, respectively. In these experiments, the substrates were added to the proteolysis reaction mixture at a concentration over three times their K_m^{app} values of the mutant enzymes, in order to saturate the active site. To compare the effects of the substrates on the proteolysis of the wild-type enzyme with that of the R233K enzyme, the degree of protection by γ-Glu-Cys and ATP was estimated from the ratio of the activity after 120 min of proteolysis in the presence of both substrates to that in the absence of the substrates. The presence of both γ-Glu-Cys and ATP increased the protection of the R233K enzyme from the tryptic digestion 2.3-fold, while their presence increased the protection of the wild-type enzyme from the arginyl endopeptidase digestion 5.2-fold. Thus, it becomes clear that the effects of γ-Glu-Cys and ATP on the proteolysis of R233K enzyme is about the half of that observed in the wild-type enzyme.

Catalytic Activities and Kinetic Constants of Mutant Enzymes. The specific activities of R233A, R233K, R241A, R241K, and wild-type enzymes, which were measured by the PK–LDH method, are summarized in Table I. Although R233A and R233K enzymes were nearly inactive, R241A and R241K enzymes retained the activity of the wild-type enzyme. The synthesis of glutathione with these enzymes was confirmed by the GR method, and the results agreed with those obtained by the PK–LDH method.

The apparent K_m^{app} and k_0^{app} values of these enzymes were also measured to determine which substrate, and how much the binding property of the substrate, was affected by the replacement of the arginine (Table II). As is obvious from the tabulated data, the replacement of arginine 233, R233A and R233K, resulted in a drastic increase in the K_m^{app} values for glycine and γ-Glu-Cys. In particular, the K_m^{app} value for

Table II: Apparent Kinetic Constants of the Mutant and Wild-Type Glutathione Synthetases[a]

enzymes	substrates	K_m^{app} (mM)	k_0^{app} (s^{-1})
R233A[c]	γ-Glu-Cys	3.01 ± 0.50	31 ± 1.8
	ATP	0.89 ± 0.24	31 ± 2.1
	glycine	14.8 ± 1.36	30 ± 0.8
R233K[d]	γ-Glu-Cys	6.72 ± 1.08	25 ± 1.9
	ATP	1.17 ± 0.15	22 ± 0.1
	glycine	194 ± 2.7	45 ± 3.0
R241A[b]	γ-Glu-Cys	0.75 ± 0.05	121 ± 2.6
	ATP	0.22 ± 0.02	117 ± 2.7
	glycine	1.1 ± 0.10	113 ± 4.0
R241K[b]	γ-Glu-Cys	0.33 ± 0.03	149 ± 3.1
	ATP	0.35 ± 0.03	158 ± 3.0
	glycine	0.80 ± 0.10	140 ± 3.9
wild-type[b]	γ-Glu-Cys	0.24 ± 0.01	160 ± 1.1
	ATP	0.24 ± 0.01	143 ± 1.2
	glycine	0.91 ± 0.04	150 ± 2.2

[a] Assayed by the PK–LDH method. The steady-state kinetic parameters K_m^{app} and k_0^{app} were determined by a nonlinear least-squares fit of the data using the method described by Sakoda and Hiromi (1976). The following concentrations of substrates were adopted as the fixed substrate in the reaction mixture: [b] 5 mM γ-Glu-Cys, 10 mM ATP, and 15 mM glycine; [c] 15 mM γ-Glu-Cys, 10 mM ATP, and 150 mM glycine; [d] 15 mM γ-Glu-Cys, 6 mM ATP, and 800 mM glycine.

glycine of R233K enzyme is 213 times greater than that of the wild-type enzyme, wherease the K_m^{app} values for ATP of these two mutants did not increase so significantly. k_0^{app} values of R233A and R233K mutant enzymes decreased by a factor of 5–6, compared with R241A and R241K, which had almost the same catalytic rate as that of the wild-type enzyme. Therefore, the substitution of arginine 233 produced a drastic change in the kinetic constants.

Discussion

Limited Proteolysis of the Loop. Our X-ray crystallographic studies on GSHase from *E. coli* B have also revealed that each subunit is composed of three domains: N-terminal (residues 1–121), central (residues 134–201), and C-terminal (residues 202–316) domains. A difference Fourier map of the crystals of the enzyme–ATP complex furthermore showed that ATP binds in the cleft between the central and C-terminal domains. Furthermore, as referred to in the introduction, a disordered loop consisting of 17 amino acid residues from isoleucine 226 to glycine 242 exists. The fact that no electron density is observed in the above-mentioned region is interpretable in two ways: (1) the loop moves rapidly and takes no fixed position during the collection of X-ray diffraction data; and/or (2) the loop is fixed, but its internal conformation is different from molecule to molecule in the crystal. These observations of the crystals of the free and substrate-complexed enzymes suggested that the loop structure would be deeply involved in the catalytic action of GSHase. One function of such a loop was supposed to be an extension over the cleft to cover the substrate-binding site when the substrates bind and to facilitate the subsequent transformation of substrates into product. To probe the role of the loop, we have used limited proteolysis along with kinetic measurements.

When the wild-type GSHase was treated with arginyl endopeptidase under native conditions, GSHase was hydrolyzed only at the COOH side of arginine 233 to yield two peptide fragments on SDS–PAGE and was almost completely inactivated by the proteolysis. On native PAGE, however, GSHase cleaved at arginine 233 gave a single band, the same as the intact enzyme, suggesting that the two peptide fragments associated to keep the quaternary structure the same as that of the intact enzyme, in spite of the cleavage of the main chain. Thus, the proteolytic digestion locally affected the enzyme structure only in the loop region. Accordingly, it is reasonable to consider that the loss of the activity of GSHase by proteolysis was induced not by a global deformation of the tetrameric structure but by the local destruction of the loop structure. This means that the loop is one of the structural units required for GSHase catalysis.

In the present study, we assumed two causes of the inactivation by proteolysis at arginine 233: (1) the loop structure itself is indispensable for the catalytic activity. If the loop is just a structure to support and carry the residue which is essential for enzymatic activity, then the cleavage of the loop results in the failure of the catalysis because of the incorrect position of the essential residue; (2) arginine 233 in the loop participates in the catalytic action of GSHase. The cleavage of the loop induced a critical loss of the interactions of arginine 233 with the substrates or with some catalytic residues required for the catalysis.

In each subunit of GSHase, there are 19 arginine residues. When inspected on a graphic display, most of the arginine residues are distributed on the solvent-accessible surface of GSHase in the tetrameric structure. In spite of their surface distributions, the fact that arginyl endopeptidase was able to approach only arginine 233 under native conditions indicates that arginine 233 is highly exposed or protrudes from the protein surface to the bulk solvent phase. The proteolysis with arginyl endopeptidase at arginine 233, however, was suppressed when either one or both of the two substrates, ATP and γ-Glu-Cys, was present. Such protection effects can be accounted for as follows: that is, upon binding to GSHase, these two substrates bring arginine 233 from a highly exposed to less exposed state. Since the binding sites of these two substrates are in the cleft beneath the loop, the binding of the substrates to the sites probably switches the internal conformation or mobility of the loop from a state in motion to that of one trapped on the inside of the enzyme.

Triosephosphate isomerase from yeast (Joseph et al., 1990; Pompiliano et al., 1990) and lipases from human (Winkler et al., 1990) and *Rhizomuchor miehei* (Brady et al., 1990; Brzozowski et al., 1991) have a loop which works as a lid for the active site. Their loops open or close over the active site when their substrate or inhibitor associates the site. They move as a rigid body, in other words, they have a strikingly similar internal structure in the open and closed forms. In contrast to the loops in these enzymes, the loop of GSHase was not detectable on electron density maps even in the presence of the substrates. Consequently, it can be safely concluded that the loop of GSHase retains high mobility in the underexposed state.

The reaction catalyzed by GSHase has been supposed to proceed intermediately via an enzyme-bound acyl phosphate, γ-glutamylcysteinyl phosphate, which is labile and easily hydrolyzed by water (Meister, 1974). In the course of the reaction, the loop works as a lid which shields the reaction intermediate from the solvent water and prevents it from being decomposed before forming glutathione. Should this interpretation by correct, the cleavage of the loop at arginine 233 would not protect the intermediate from decomposition. Actually, the activity loss of the enzyme was found to be large, as expected.

The Roles of Arginine 233. Among the 20 amino acids, arginine is the one that was examined and found to work for the recognition and stabilization of a negatively charged substrate at the active site of enzymes. In the crystal structures

of enzymes such as lactate dehydrogenase from *Bacillus stearothermophilus* (Grau et al., 1981), carboxypeptidase A1 from bovine pancreas (Christianson & Lipscomb, 1989), thymidylate synthase from *Lactobacillus casei* (Hardy et al., 1987) and *E. coli* (Matthews et al., 1990; Montfort et al., 1990), phosphofructokinase from *B. stearothermophilus* (Schirmer & Evans, 1990), and ribulose bisphosphate carboxylase from *Rhodospirillum rubrum* (Andersson et al., 1989), it was observed that one or more arginine residues located at their substrate-binding sites. The guanidyl side chain of the arginine residue is found near the carboxyl and phosphate groups of the bound substrates and is supposed to have the role of substrate recognition. Arginine residues in the loop were studied by chemical and enzymatic modification (Colanduoni & Villafranca, 1985; Moss et al., 1988, 1990; Murgola & Yanofsky, 1974; Yanofsky, 1967) and by replacement with other amino acids (Kawasaki et al., 1987).

All of the three substrates of GSHase have a negatively charged carboxylate or phosphate group. Arginine 233 consequently appears to play an important role in the recognition of a certain substrate, stabilization of the bound substrate through charge–charge interactions, and/or fixing of the loop for shielding the unstable reaction intermediate from the solvent.

In the case of treating the R233K enzyme with arginyl endopeptidase and trypsin, arginyl endopeptidase did not digest the R233K enzyme at all, as expected from the lack of arginine residue at 233, but trypsin digested R233K enzyme at the COOH-terminal side of lysine 233 to give only two peptide fragments. Therefore, the loop of R233K enzyme is also exposed to the solvent phase as was the case of the wild-type enzyme. ATP and γ-Glu-Cys protected the R233K enzyme from proteolysis by trypsin, though the protection was about a half of that observed for the wild-type enzyme. Such proteolysis of the R233K mutant enzyme by trypsin indicates that the replacement of arginine with lysine at residue 233 did not affect the conformation and mobility of the loop. Therefore, the loss of activity of R233K enzyme can be attributed to an intrinsic difference in the physicochemical nature between lysine and arginine side chains. On the other hand, replacement of arginine 241 in the loop with lysine or alanine did not strikingly affect the enzyme activity. This result supports the idea that arginine 233 is essential for the catalytic activity of GSHase.

Analysis of steady-state kinetics of mutant enzymes, R233A and R233K, showed that the replacement of arginine 233 affected the binding property of the substrates rather than the rate-limiting step. The mutations resulted in lowering of the binding property of γ-Glu-Cys and significant lowering of that of glycine: 12.5–28-fold and 16.3–213-fold increases in K_m^{app} values, respectively. On the other hand, the effect on the binding property of ATP was not significant: a 3.7–4.9-fold increase in K_m^{app} value. The existence of arginine 233 in a glycine-rich region is similar to that of the lysine residue on the phosphate loop which participates in the binding of ATP. Arginine 233 participates slightly in the binding of ATP because the replacement of arginine 233 with lysine in the R233K enzyme did not improve the ATP-binding property. These K_m^{app} values of GSHases showed that arginine 233 mostly participates in the recognition and binding of glycine.

The rate of glutathione synthesis by the mutant enzymes, R233A or R233K, is 5 times slower than that for the wild-type enzyme. At present, it has not been determined which reaction step is rate-limiting: either formation of γ-Glu-Cys phosphate intermediate or that of glutathione. We suppose that as a result of the low affinity of γ-Glu-Cys and glycine, these substrates take their proper orientations to form a new chemical bond which results in low rate constants.

Reaction Mechanism Deduced from the Results of Limited Proteolysis and Mutation. From the experimental results described above, when taken together, it was realized that the loop is essential for the catalysis of GSHase. In the case of proteolysis where the substrates ATP and γ-Glu-Cys are present, the protection was most effective when both of the two substrates were present. On the basis of these results, it is proposed that the loop closes the active site when they bind to their binding sites and contributes to the formation and stabilization of the γ-Glu-Cys phosphate intermediate. Moreover, arginine 233 in the loop seems to be responsible for the recognition and binding of γ-Glu-Cys and glycine. This participation of arginine 233 is proposed to be the cause of the decrease in the specific activity of GSHase, when glycine methyl ester instead of glycine was added to reaction mixture (Tanaka, Nishioka, and Oda, unpublished data). Therefore, what is important is that the COOH of glycine must necessarily be free when it binds the enzyme. In doing so, the guanidyl side chain of arginine 233 probably interacts with the COOH groups of γ-Glu-Cys and glycine. Despite the extremely low concentration of glycine in the solvent phase, the high selectivity for glycine can be looked upon as a result of the protection of γ-Glu-Cys phosphate intermediate from water and the strict distinction to glycine from water by the loop. From the data obtained here, the functional roles of the loop and arginine 233 in GSHase are different from that of the phosphate loop reported so far.

In order to further elaborate the functional role of the flexible loop in GSHase, several mutant enzymes in which the glycine residues in the loop are replaced with other amino acid residues are being constructed, so as to differ from the wild-type enzyme in the mobility and conformation of the loop. Details about functions of the loop will be revealed through the analysis of steady-state kinetics and substrate specificities of these mutant enzymes.

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