Flexibility Impaired by Mutations Revealed the Multifunctional Roles of the Loop in Glutathione Synthetase†

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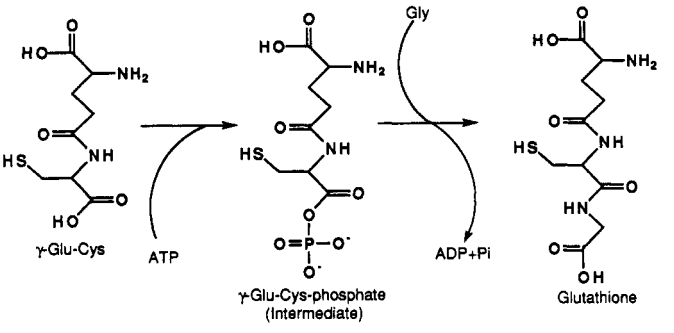
ABSTRACT: The loop from Ile-226 to Arg-241 in the glutathione synthetase (GSHase) from *Escherichia coli* B is rich in glycine and alanine and too flexible to take a fixed conformation [Yamaguchi, H., Kato, H., Hata, Y., Nishioka, T., Kimura, A., Oda, J., & Katsube, Y. (1993) *J. Mol. Biol. 229*, 1083–1100; Tanaka, T., Kato, H., Nishioka, T., & Oda, J. (1992) *Biochemistry 31*, 2259–2265]. To restrict the flexibility, three residues in the loop, Pro-227, Gly-229, and Gly-240, were replaced with alanine and valine residues. Variability in conformations of the mutant loops and shifts in the distribution of conformers between the open and closed states were assessed by steady-state kinetics, X-ray crystallographic structure analysis, and proteolysis with arginyl endopeptidase. Mutant enzymes replaced with a valine residue at the basal positions of the loop (P227V, G240V, and P227V/G240V) were identical with the wild-type enzyme in their crystal structures, except the loop region. The mutant loops retained apparent conformational variability, so as to take the open and closed states and to protect the acyl phosphate intermediate from the decomposition uncoupled from glutathione synthesis, but lost the catalytic activity; K_m^{app} values for glycine and γ-Glu-Cys were sensitive to the mutations and drastically increased, and the k_0^{app} value was fatally reduced in the P227V/G240V mutant enzyme. The present results suggest that adjustability of the loop to the closed state is required for the recognition of the substrates, γ-Glu-Cys and glycine, and for the chemical interactions with the bound substrates.

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 amino acid other than glycine was accepted as a substrate (Moore & Meister, 1987; Oppenheimer et al., 1979). On the catalytic mechanism, Meister proposed that the reaction catalyzed by GSHase from rat kidney proceeds by an ordered mechanism through the formation of an active intermediate, γ-L-glutamyl-L-cysteinyl phosphate (Meister, 1974) (Scheme I).

GSHase from *Escherichia coli* (*E. coli*) B is a tetramer of four identical subunits, each of which is composed of 316 amino acid residues (Gushima et al., 1983, 1984). We have studied the GSHase on the inhibition by methotrexate (Kato et al., 1987), the chemical modification and site-directed mutagenesis of cysteine residues (Kato et al., 1988), the affinity labeling of the lysine residues at the ATP-binding site (Hibi et al., 1993), and the crystallization (Kato et al., 1989).

Scheme I: Proposed Reaction Catalyzed by GSHase



We have recently determined the three-dimensional structure of the GSHase at 2.0-Å resolution by X-ray crystallographic analysis (Yamaguchi et al., 1993). The ATP-binding site of the enzyme is located in the cleft formed by two β-sheets along the boundary of two domains (Figure 1). The structural topology of the site is quite different from that of the nucleotide-binding motif typically found in adenylate kinase (Dreusicke & Schulz, 1988) and the Ha-ras p21 protein (Pai et al., 1989). It has neither a Rossmann-fold (Rossmann et al., 1975) nor a P-loop motif (Saraste et al., 1990), although two peptide regions from Gly-164 to Gly-167 and from Ile-226 to Arg-241 are assigned as a glycine-rich loop. The ATP-binding site of the GSHase, named the palmate fold, is rather similar to those of the cyclic AMP-dependent protein kinase *c* subunit

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[1] Abbreviations: DEAE, diethylaminoethyl; GSHase, glutathione synthetase; γ-Glu-Cys, γ-L-glutamyl-L-cysteine; GR, glutathione reductase; k_0^{app}, a first-order rate constant; K_m^{app}, Michaelis constant; LB medium, Luria–Bertani medium; LDH, lactate dehydrogenase; PK, pyruvate kinase; R_F, mean change in structure amplitude between the wild-type and mutant GSHase data sets, $R_F = 2\sum||F_{mutant}| - |F_{wild\text{-}type}||/\sum(|F_{mutant}| + |F_{wild\text{-}type}|)$; $R_{merge(F)} = \sum_h\sum_i|F(h)_i - \bar{F}(h)|/\sum_h\bar{F}(h)$; $R_{merge(I)} = \sum_h\sum_i|I(h)_i - \bar{I}(h)|/\sum_h\bar{I}(h)$; SDS, sodium dodecyl sulfate; TLCK, *p*-tolylsulfonyl-L-lysine chloromethyl ketone; TPCK, *p*-toluenesulfonyl-L-phenylalanine chloromethyl ketone. Mutant glutathione synthetase from *Escherichia coli* B: G229A, glycine 229 → alanine exchange; G229V, glycine 229 → valine exchange; G240A, glycine 240 → alanine exchange; G240V, glycine 240 → valine exchange; P227A, proline 227 → alanine exchange; P227V, proline 227 → valine exchange; P227V/G240V, both proline 227 → alanine and glycine 240 → valine exchanges.

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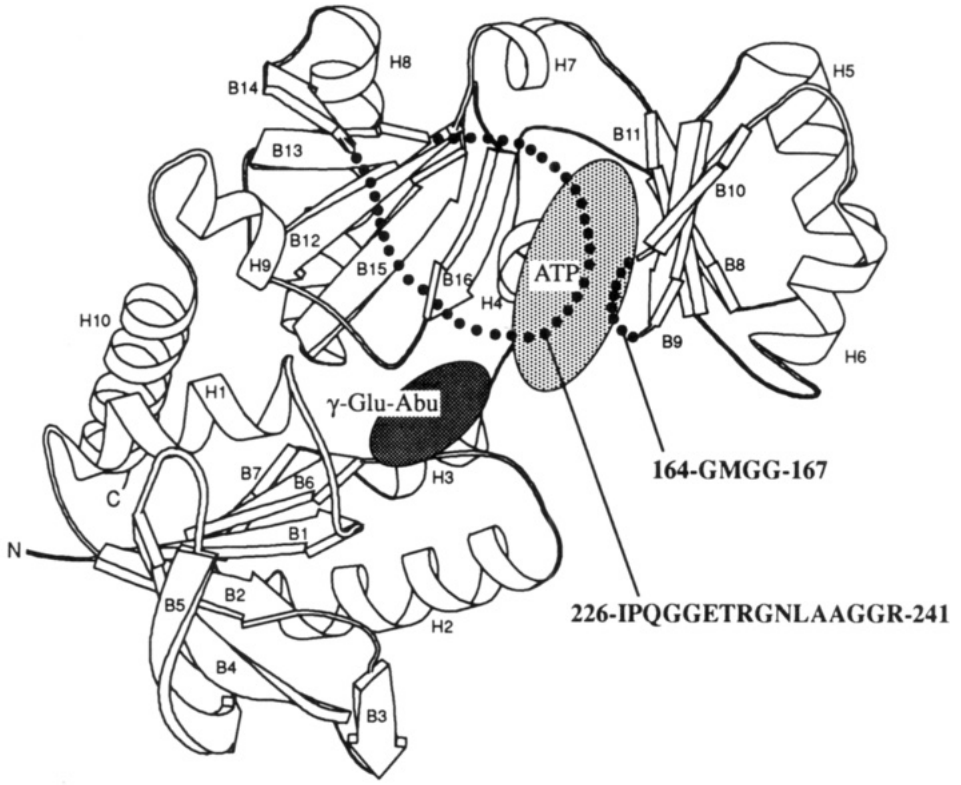

FIGURE 1: Schematic drawing of a subunit of GSHase. A subunit of GSHase is composed of an N-terminal domain (containing β-strands B1–B7 and helices H1–H3), a central domain (containing B8–B11 and H5–H6), and a C-terminal domain (containing B12–B16 and H4 and H7–H10). ATP binds at the cleft between the central and C-terminal domains. γ-Glu-Abu, which is an analog of γ-Glu-Cys, is supposed to bind between the N-terminal and C-terminal domains. The invisible loop structure from Ile-226 to Arg-241 is supposed to extend over the substrate-binding sites.

(Knighton et al., 1991) and seryl- and aspartyl-tRNA synthetases (Cusack et al., 1990; Ruff et al., 1991).

Limited proteolysis of the GSHase with arginyl endopeptidase has revealed that the loop from Ile-226 to Arg-241 is an indispensable structural unit for the catalytic activity of the GSHase (Tanaka et al., 1992). The peptidase cleaved the peptide only at the C-terminal side of Arg-233 in the loop. Although the tetrameric structure of the enzyme was retained after the cleavage of the peptide, the proteolysis resulted in the complete loss of catalytic activity. The functional role of the loop suggested from the results of the limited proteolysis is to cover the acyl phosphate intermediate so as to protect it from spontaneous decomposition by water. Replacement of Arg-233 with a lysine residue, however, resulted in the fatal loss of the activity as evidenced by the large increase in the K_m^{app} values for glycine and γ-Glu-Cys and prompted us to study some additional, complex functional roles of the loop.

The loop from Ile-226 to Arg-241 contains five glycine, two alanine, and one proline residue and is so flexible that previous trials to determine the conformation of the loop by crystallographic analysis have failed, even with the crystals of GSHase in complex with the substrates (Yamaguchi et al., 1993). In the present study, Pro-227, Gly-229, and Gly-240 in the loop were replaced with alanine and more bulky valine residues in order to perturb or reduce the flexibility of the loop and to analyze how the catalytic function of the GSHase is affected by modifying the flexibility. Pro-227 and Gly-240 are located at the base region, where the loop is supposed to hang on the remaining part of the enzyme, and Gly-229 is at the middle part of the loop. Variability in conformations of the mutant loops and shifts in conformer distributions between the open and closed states were assessed by steady-state kinetics, X-ray crystallographic structure analysis, and proteolysis with arginyl endopeptidase.

EXPERIMENTAL PROCEDURES

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

Restriction Enzymes, DNA-Modification 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 (Levy et al., 1970) (mouse submandibular protease, treated with TLCK and TPCK, 1.045 units/mg) were purchased from Takara Shuzo Co., Ltd. Trypsin (12 000 units/mg, treated with TPCK) from bovine pancreas was purchased from Sigma Chemical Co. (St. Louis, MO). Lactate dehydrogenase (LDH) from pig heart, pyruvate kinase (PK) from rabbit muscle, and glutathione reductase from yeast were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). γ-Glu-Cys was a special gift from Kohjin Co., Ltd. (Tokyo, Japan). The Tris-HCl buffer used in the present study contained 5 mM $MgCl_2$, unless noted otherwise. Chemicals used in the present work were of the purest grade commercially available.

Site-Directed Mutagenesis. An *Eco*RI–*Pst*I fragment (1207 base pairs (bp)) encoding the GSHase gene was isolated

from pKGS00 and inserted into bacteriophage M13 mp19 to give φEP1207 for site-directed mutagenesis. Site-directed mutagenesis was carried out by the method of Kunkel (1985). Oligonucleotide primers used for the mutations were designed to create a new recognition site of the restriction enzyme. Mutants were screened with this created site. The oligonucleotide primers used in the present studies were as follows (the underlined bases encode the new amino acid):

P227A 5′-CCTGGCGCGCATTGCGCAGGGG-3′

P227V 5′-CCTGGCGCGCATTGTGCAGGGG-3′

G229A 5′-CCGCAGGCGGGCGAAACGCGTGGC-AATC-3′

G229V 5′-CGCGTATACCGCAGGTGGGCGAAA-CC-3′

G240A 5′-GCTGCCGGCGCTCGCGGTG-3′

G240V 5′-GCTGCCGGCGTTCGCGGTG-3′

After the DNA sequences were verified by the dideoxy method (Sanger et al., 1977), a short DNA fragment containing a mutation site was excised from the φEP1207 mutant with two appropriate restriction enzymes, *Kpn*I and *Eco*RV. This short fragment replaced its counterpart in pKGS00 to give an expression plasmid of the mutant GSHase.

Overexpression and Purification of the Wild-Type and Mutant GSHases. The expression plasmid was used to transform *E. coli* JM109 by the method of Hanahan (1983). From the transformant, mutant GSHase was isolated and purified by the method described in a previous study (Tanaka et al., 1992).

Protein concentration was calculated from the absorption coefficient, $A_{cm}^{1\%} = 9.02 \pm 0.44$, that was experimentally determined at 280 nm in 0.05 M potassium phosphate buffer (pH 7.0) by the dry-weight method (Kato et al., 1989; Kupke & Dorrier, 1978). The average yield of mutant protein was about 0.15 g from a 500-mL culture.

The purified preparations of the mutant GSHases were stored at 4 °C until they were used. To examine contamination from proteases, purified preparations were incubated at 37 °C for 1 h in 25 mM Tris-HCl (pH 7.5). On SDS–polyacrylamide gel electrophoresis, they showed no other peptide fragment due to proteolytic digestion. This proved that the present preparations were free from protease contamination.

Measurement of Activities of GSHases. The amount of glutathione synthesized was determined by the "GR method" (Tietze, 1969). The amount of ATP consumed was measured by the "PK-LDH method" (Brolin, 1983).

When ATP consumption (ATP hydrolysis) is coupled to the glutathione synthesis, the PK-LDH method gives the same result as the GR method. To estimate the rate of ATP hydrolysis uncoupled from glutathione synthesis, ATP consumption was measured in the presence of ATP and γ-Glu-Cys, but not glycine. Incubation without γ-Glu-Cys served as a control. The rate of the coupled ATP hydrolysis was determined by subtracting the uncoupled ATP consumption from the total ATP consumption, which was measured in the presence of all three substrates. For the wild-type GSHase, the coupled ATP consumption was consistent with the amount of glutathione synthesized, within experimental errors (Tanaka et al., 1992).

Data for the steady-state kinetic parameters were measured by determining the coupled ATP consumption using the PK-LDH method. The concentration range of the fixed substrate was set to range over 3 times its K_m^{app} value. 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).

Crystallization of P227V, G240V, and P227V/G240V Mutant GSHases. Two mutant GSHases, G240V and P227V/G240V, were crystallized by a microdialysis method with seeding of the wild-type GSHase crystal. The crystallization solution was seeded by adding 10 μL of a suspension to a microdialysis cell (100 μL) that contained 1% (w/v) mutant GSHase, 10% saturated ammonium sulfate, 5 mM $MgCl_2$, and 50 mM potassium phosphate buffer (pH 6.8). The inner solution was dialyzed against 20 mL of 50 mM potassium phosphate buffer (pH 6.8) containing 25% saturated ammonium sulfate, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 0.02% NaN_3 at 20 °C. In the case of P227V, the buffer system was changed to 50 mM Tris-HCl (pH 7.5), without seeding of the wild-type GSHase.

X-ray Diffraction Data Collection. Crystals of G240V were isomorphous to the wild-type GSHase (Yamaguchi et al., 1993), with unit cell dimensions of $a = b = 164.3$ Å and $c = 88.0$ Å. With four crystals, 16 309 reflections were collected to a resolution of 2.7 Å on a Rigaku C-5 four-circle diffractometer. The number of independent reflections was 10 898. The $R_{merge(F)}$ value was 0.035. Crystals of P227V/G240V were isomorphous to the wild-type enzyme, with unit cell dimensions of $a = b = 164.5$ Å and $c = 87.9$ Å. Diffraction data of the crystals were collected on a Rigaku R-AXIS IIc (Sato et al., 1992). The number of reflections collected up to 2.2-Å resolution was 17 863. A total of 7565 independent reflections collected to 2.7-Å resolution was used for the structure refinement. The $R_{merge(I)}$ value was 0.081. The stability of the crystals against X-ray irradiation was equivalent to that of the wild-type GSHase crystals. A full data set was obtained by scaling and merging individual data sets according to the method of Hamilton (Hamilton et al., 1965). Crystals of P227V were isomorphous to the wild-type enzyme, with unit cell dimensions of $a = b = 170.0$ Å and $c = 87.8$ Å. Diffraction data of the crystals were collected on a Rigaku R-AXIS IIc (Sato et al., 1992). The number of reflections collected up to 2.2-Å resolution was 15 177. A total of 8588 independent reflections collected to 2.7-Å resolution was used for the structure refinement. The $R_{merge(I)}$ value was 0.075. The stability of the crystals against X-ray irradiation was equivalent to that of the wild-type GSHase crystals. A full data set was obtained by scaling and merging individual data sets according to the method of Hamilton (Hamilton et al., 1965).

Limited Proteolysis of Mutant GSHases. Limited proteolysis was carried out as described in a previous study (Tanaka et al., 1992). The enzyme preparation (400 μg in 400 μL of Tris-HCl, pH 7.5) was incubated with arginyl endopeptidase (8 μg in 20 μL of Tris-HCl, pH 7.5) under native conditions at 30 °C. After 0, 30, 60, 120, and 240 min from the beginning of the reaction, the GSHase activity of the solution was determined by the GR method. When the effects of the existence of the substrates on proteolysis were examined, proteolysis was carried out in the presence of the substrates. The concentrations of the substrates were as follows: 10 mM ATP, 15 mM glycine and/or 0.1 and 5 mM γ-Glu-Cys for

the wild-type GSHase; 10 mM ATP, 15 mM glycine and/or 5 mM γ-Glu-Cys for P227V enzyme; 10 mM ATP, 15 mM glycine, and/or 5 or 30 mM γ-Glu-Cys for G240V enzyme; 10 mM ATP, 15 mM glycine, and/or 5 or 30 mM γ-Glu-Cys for P227V/G240V enzyme.

RESULTS

Steady-State Kinetics for Mutant GSHases. To disturb the flexibility of the loop (Ile-226 to Arg-241) of the GSHase, glycine and proline residues at three positions (227, 229, and 240) in the loop were replaced with alanine and valine residues by site-directed mutagenesis. Seven mutant genes were constructed and expressed in *E. coli* to give P227A, P227V, G229A, G229V, G240A, G240V, and P227V/G240V mutant GSHases.

If the loop impaired by mutations did not completely protect the labile acyl phosphate intermediate (Scheme I) from hydrolytic decomposition by water, part of the intermediate would decompose without forming glutathione. To estimate how complete the protection was, the amount of ATP consumption (the ATP hydrolytic activity uncoupled from glutathione synthesis) was measured for each mutant GSHase under conditions where glycine was absent from an otherwise complete mixture. Without glycine as a substrate, the intermediate formed is not utilized for glutathione synthesis, but is left to decompose if water is accessible. The uncoupled ATP hydrolytic activity was less than 5% of the glutathione synthetic activity. In another experiment, the amounts of glutathione synthesized and ATP consumed (the ATP hydrolytic activity coupled to glutathione synthesis) for each mutant GSHase were measured separately in the complete mixture using the GR and PK-LDH methods, respectively. At fixed substrate concentrations of 5 mM γ-Glu-Cys, 10 mM ATP, and 15 mM glycine, the amount of ATP consumed was equal to that of glutathione synthesized within experimental error. For the wild-type GSHase, the glutathione synthetic activity, the coupled ATP hydrolytic activity, and the uncoupled ATP hydrolytic activity were 42.0 ± 5.4, 48.0 ± 1.5, and 2.0 ± 0.1 units/mg, respectively. Therefore, none of the mutant loops was impaired so much as to fail to protect the intermediate from decomposition uncoupled from glutathione synthesis. In the following steady-state kinetic measurement, glutathione synthetic activities of GSHase were measured with the PK-LDH method instead of the GR method.

The effects of the loop mutations on the catalytic activity were measured by determining kinetic parameters: the Michaelis constants for the three substrates, K_m^{app}, and the catalytic rate constants, k_0^{app} (Table I). Replacement with alanine residues at the three positions in the loop did not affect their catalytic activities as much; changes in the K_m^{app} and k_0^{app} values were in the range of less than one-quarter of those of the wild-type enzyme. These replacements did slightly disturb the conformations of the loop.

On the other hand, replacement with valine residues at positions 227 and 240 significantly impaired the enzymatic activity, k_0^{app}/K_m^{app}. Pro-227 and Gly-240 in the loop are necessary for the catalytic function of the GSHase. In the P227V, G240V, and P227V/G240V mutant GSHases, the $1/K_m^{app}$ values for glycine decreased to 8, 0.2, and 0.7% that of the wild-type enzyme, respectively. Those for γ-Glu-Cys also decreased, to 13, 2, and 3%, respectively. The value of K_m^{app} for ATP, however, was not affected as much by these replacements. Additionally, the substitutions with a valine residue greatly reduced the rate constant. In particular, when both of these positions were simultaneously replaced with a valine residue (P227V/G240V mutant GSHase), the rate constant decreased to only 1/500 that of the wild-type enzyme. Substitution at position 229 with a valine residue affected neither of the two kinetic parameters.

Table I: Apparent Kinetic Constants for the Mutant and Wild-Type Enzymes[a]

enzymes	substrates	K_m^{app} (mM)	k_0^{app} (s^{-1})	k_0/K_m
P227A[b]	γ-Glu-Cys	0.89 ± 0.11	112.7 ± 4.7	127
	ATP	0.46 ± 0.09	104.9 ± 4.7	228
	glycine	1.18 ± 0.14	96.2 ± 3.2	81.5
P227V[c]	γ-Glu-Cys	1.81 ± 0.48	19.2 ± 2.2	10.6
	ATP	0.99 ± 0.30	29.1 ± 3.2	29.4
	glycine	11.5 ± 1.6	26.9 ± 1.6	2.34
G229A[b]	γ-Glu-Cys	0.11 ± 0.03	64 ± 3.5	581
	ATP	0.22 ± 0.05	55 ± 2.7	250
	glycine	0.44 ± 0.06	63 ± 2.3	143
G229V[b]	γ-Glu-Cys	0.78 ± 0.05	77 ± 1.7	98.7
	ATP	0.12 ± 0.02	69 ± 1.7	575
	glycine	0.72 ± 0.12	72 ± 2.9	100
G240A[b]	γ-Glu-Cys	0.69 ± 0.12	81 ± 4.5	117
	ATP	0.30 ± 0.13	80 ± 7.1	267
	glycine	0.89 ± 0.18	78 ± 4.0	87.6
G240V[d]	γ-Glu-Cys	9.81 ± 1.02	6.15 ± 0.4	0.627
	ATP	1.41 ± 0.33	9.0 ± 0.6	6.38
	glycine	547 ± 225	13.2 ± 2.9	0.024
P227V/G240V[e]	γ-Glu-Cys	7.19 ± 1.60	0.30 ± 0.03	0.042
	ATP	0.66 ± 0.35	0.25 ± 0.03	0.379
	glycine	132.8 ± 88.0	0.28 ± 0.06	0.0021
wild-type[b]	γ-Glu-Cys	0.24 ± 0.01	160 ± 1.1	667
	ATP	0.24 ± 0.01	143 ± 1.2	596
	glycine	0.91 ± 0.04	150 ± 2.2	165

[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 are added as the fixed substrate in the reaction mixture of each enzyme. [b] 5 mM γ-Glu-Cys, 10 mM ATP, 15 mM Gly. [c] 5 mM γ-Glu-Cys, 10 mM ATP, 30 mM Gly. [d] 15 mM γ-Glu-Cys, 10 mM ATP, 800 mM Gly. [e] 15 mM γ-Glu-Cys, 10 mM ATP, 400 mM Gly.

Crystal Structure of P227V, P227V/G240V, and G240V GSHases. Fourier maps with coefficients ($|F_{o(mutant)}| - |F_{o(wild\text{-}type)}|$) and ($2|F_{o(mutant)}| - |F_{o(wild\text{-}type)}|$) showed no significant additional peaks. This strongly suggested that the mutations to valine at Pro-227 and Gly-240 in the loop caused no serious ill effect on the structure of the wild-type GSHase.

In order to confirm this suggestion, a detailed structure analysis of the mutant GSHases was carried out. A structural refinement of G240V was carried out with the Hendrickson & Konnert restrained least-squares program, PROLSQ (Konnert & Hendrickson, 1980), using the structure of the wild-type GSHase as an initial model. After 18 cycles, the final *R* factor converged to 20.2% at 2.7 Å. The structure of P227V/G240V was refined first with X-PLOR (Brünger et al., 1989) and then with PROLSQ. The final *R* factor after PROLSQ refinement converged to 17.3% at 2.7 Å. The structure of P227V was refined with X-PLOR. The final *R* factor converged to 17.7% at 2.7 Å.

No clear electron density corresponding to the flexible loop was detected, which disabled us from tracing the chain in the electron density maps ($2|F_{obs}| - |F_{calc}|$). The flexibility of the mutant loops then was retained at the same level as that of the wild-type loop. The structure of the loop region of the mutant enzymes remained undetermined. The discrepancies between the structure of the wild-type GSHase and the refined models of the P227V, G240V, and P227V/G240V mutant GSHases were 0.42, 0.32, and 0.19 Å rms of the corresponding C_α atoms, respectively. Except for the loop, the structures of the three mutant GSHases were concluded to be identical with that of the wild-type enzyme.

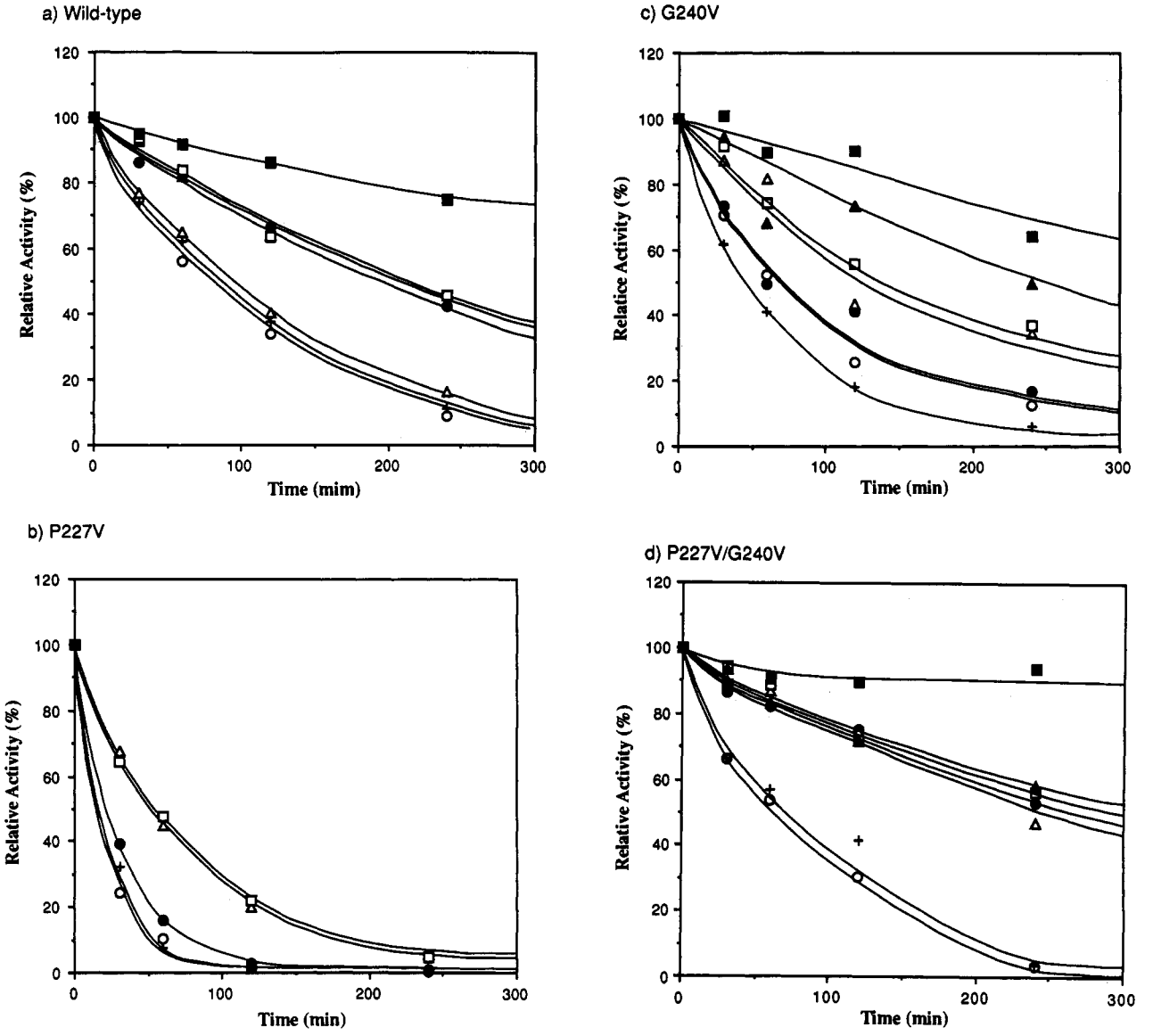

FIGURE 2: Kinetics of proteolytic inactivation of mutant GSHases. Effects of substrates on the proteolysis of GSHase. GSHase was incubated with arginyl endopeptidase in the presence of the substrates. The remaining activity at the time indicated was measured and expressed as the percentage of the activity at 0 min: (a) wild-type GSHase; (b) P227V; (c) G240V; (d) P227V/G240V. The concentrations of the substrates are as follows (numbers in parentheses are the concentrations of γ-Glu-Cys, ATP, and Gly in mM, respectively: (a) ■ (5, 10, 0), □ (0.1, 10, 0), ● (0, 10, 0), ▲ (5, 0, 0), △ (0.1, 0, 0), + (0, 0, 15), and ○ (0, 0, 0); (b) □ (5, 10, 0), ● (0, 10, 0), △ (5, 0, 0), + (0, 0, 15), and ○ (0, 0, 0); (c and d) ■ (30, 10, 0), □ (5, 10, 0), ● (0, 10, 0), ▲ (30, 0, 0), △ (5, 0, 0), + (0, 0, 15), and ○ (0, 0, 0).

Limited Proteolysis of Mutant Enzymes with Arginyl Endopeptidase. The mutant enzymes were treated with arginyl endopeptidase under native conditions. Three mutant GSHases, P227V, G240V, and P227V/G240V, were digested with arginyl endopeptidase at 30 °C. SDS–polyacrylamide gel electrophoresis of the digested GSHases showed that two peptide fragments, 26 and 10 kDa, appeared and increased with the duration of the treatment, while the amount of the intact peptide (36 kDa) decreased (data not shown). No other peptide fragment was observed. Increment of the two fragments and decrement of the intact enzyme were almost parallel to the loss of the GSHase activity.

The cleavage site for limited proteolysis was determined by amino-terminal amino acid sequence analysis of the 10-kDa fragment of the proteolysis of G240V mutant GSHase. The amino-terminal sequence of the fragment was Gly-Asn-Leu-Ala-Ala, consisting of the sequence starting from Gly-234. It was confirmed that the mutant GSHase was hydrolyzed only at the peptide bond between Arg-233 and Gly-234 as the wild-type enzyme; the site of the cleavage was not affected by the mutations in the loop. Figure 2a–d shows the time course of the proteolysis monitored by measuring the activity of the digested GSHase. The activity remaining decreased with the duration of the protease treatment.

G240V and P227V/G240V mutant GSHases were inactivated by the proteolysis at a rate similar to that of the wild-type enzyme; the activity that remained after the 120-min digestion was 25.8, 30.3, and 34.2% activity of the intact G240V, P227V/G240V, and the wild-type enzymes, respectively. In contrast, P227V mutant GSHase was more susceptible to proteolysis; the activity that remained after the 120-min proteolysis was only 1.4% that of the intact enzyme. These results indicate that G240V and P227V/G240V mutant loops are in conformational distributions similar to that of the wild-type loop, while the loop with valine substituted at Pro-227 was more stabilized in a conformational state susceptible to proteolysis.

Limited Proteolysis in the Presence of Substrates. Figure 2a–d shows the remaining activity of the wild-type, P227V, G240V, and P227V/G240V GSHases after the arginyl

Table II: Observed Protective Effects of the Substrates on Proteolysis of GSHases[a]

	[γ-Glu-Cys]	[ATP]	wild-type	P227V	G240V	P227V/G240V
no substrate	no	no	–	–	–	–
single substrate	low	no	–	ND	+	++
	high	no	++	+	++	++
	no	high	++	–	–	++
two substrates	low	high	++ (sum)	ND	+ (sum)	++ (less)
	high	high	++++ (sum)	+ (sum)	+++ (more)	++++ (sum)
	K_m^{ATP}		0.24	0.99	1.41	0.66
	$K_m^{\gamma\text{-Glu-Cys}}$		0.24	1.81	9.81	7.19

[a] In this table, – indicates that proteolysis of GSHases in the presence of the substrate(s) proceeded to the same degree as that in the absence of the substrate; no protective effect by the substrates was observed. + shows that some degree of the protective effect was observed; the number of +'s corresponds to the degree of the effect. In parentheses: sum indicates that the observed effect is almost the sum of the effects observed in the presence of each single substrate; less indicates less than the sum; more indicates more than the sum. High and low in the concentration of the substrates columns were defined on the basis of their K_m^{app} values determined with each mutant GSHase.

endopeptidase treatment in the presence of the substrates of GSHase. The remaining activity of GSHases was higher after proteolysis in the presence of ATP or/and γ-Glu-Cys than in their absence. In other words, the two substrates protected the GSHases from proteolysis at the loop. No protective effect was observed by adding glycine. Neither ATP nor γ-Glu-Cys inhibited the proteolytic activity of arginyl endopeptidase under the present experimental conditions.

The protective effects of ATP and γ-Glu-Cys are summarized in Table II. In the wild-type GSHase, the protective effect increased with increasing substrate concentration. In the presence of both substrates, the effect was a sum of the protective effects that were observed in the presence of either ATP or γ-Glu-Cys. Therefore, the protective effects of ATP and γ-Glu-Cys were concentration-dependent and additive. These two natures of the protective effect were retained in the proteolysis of the mutant GSHases. Slight deviations from additivity were observed in the G240V (at high concentrations of the two substrates) and P227V/G240V (at low γ-Glu-Cys and high ATP concentrations) mutant GSHases. These deviations reflect some defects in conformational adaptivity to the bound substrates of the mutant loops.

DISCUSSION

Crystallographic analysis of the wild-type GSHase suggested that the loop is flexible enough to take diverse conformations and to fluctuate among them rapidly (Yamaguchi et al., 1993). Proteolysis of the GSHase at Arg-233 in the loop revealed that, among the various possible conformations, the loop takes two limiting states: a protruded "open" state and an underexposed "closed" state (Tanaka et al., 1992). These two states are in equilibrium. In the open state, the loop protrudes into the bulk water phase and is accessible to arginyl endopeptidase. Upon binding of a substrate, the equilibrium shifts to the closed state in which the loop is not accessible to the protease. The proteolysis then is a good indication of how much of the loop is in the open state. Because G240V and P227V/G240V mutant loops were as susceptible to proteolysis as the wild-type loop, the equilibrium between the two states was not significantly disturbed by the replacement. The P227V mutant loop was more susceptible to proteolysis. The substitution of Pro-227 with a valine residue might shift the equilibrium to the open state or expose Arg-233 to the bulk water phase even in the closed state.

The reaction catalyzed by GSHase has been supposed to proceed via an intermediate labile acyl phosphate, γ-Glu-Cys phosphate. In the course of the reaction, the loop in the closed state protects the intermediate from attack by bulk water. If a mutant loop were unable to shield the reaction intermediate from the approach of water, the intermediate would decompose to γ-Glu-Cys and uncouple from glutathione synthesis. The loss of intermediate due to decomposition was estimated as less than 5% of the intermediate consumed for the present mutant GSHases. The mutant loops take a state closed tightly enough to couple the intermediate formation (ATP consumption) to glutathione synthesis. The X-ray crystallographic analysis of the P227V, G240V, and P227V/G240V mutant GSHases showed that the mutations in the loop neither induced substantial loss of flexibility in the crystalline state nor disturbed the other part of the structure of GSHase.

In spite of this apparent normality in the open/closed states, K_m^{app} values drastically increased with the valine replacements in the loop (Table I). ATP and γ-Glu-Cys bound to GSHase could form interactions with the loop and with the remaining part of the enzyme. The latter interactions are supposed to be almost the same as those in the wild-type enzyme because the crystal structures of the remaining part of the three mutant GSHases were almost identical with that of the wild-type enzyme. Therefore, the observed loss of catalytic activity could be ascribed to some disruptive interactions between the bound substrates and the mutant loops in the closed state.

We have constructed a loopless GSHase in which the loop composed of 17 residues was deleted and replaced by four glycine residues (H. Kato et al., manuscript in preparation). The binding affinity of the loopless GSHase to the substrates is a good indication of the net interactions of the substrates with the remaining part of the enzyme. K_m^{app} values of the loopless GSHase measured in a preliminary experiment were about 1.5, 0.7, and 30 mM for ATP, γ-Glu-Cys, and glycine, respectively. These values are almost the same as or even less than the values in Table I for the mutants with the valine residue substitutions. Because the K_m^{app} values for ATP increased with the loop mutations, but did not exceed the value for loopless GSHase, the remaining part of the GSHase structure would be responsible for the binding of ATP. On the other hand, the K_m^{app} values for γ-Glu-Cys and glycine were sensitive to the loop mutations. Some of them were much larger than the corresponding K_m^{app} values for the loopless enzyme.

Kinetic parameters measured suggest that binding of glycine and γ-Glu-Cys mostly depends on the interactions with the loop in the closed state rather than with the other part of the enzyme. The mutant loops lost most of their interactions with the bound substrates or even inhibited substrate binding.

Proteolysis at Arg-233 in the presence of substrates is another parameter monitoring the interactions of the bound substrates with the loop in the closed state. The protective effect of ATP and γ-Glu-Cys reflects the stabilization of the loop in the closed state. Upon binding of the substrate to the enzyme, the equilibrium between the open and closed states shifts to the closed state. GSHase has a binding site for ATP and γ-Glu-Cys each (Figure 1), although the binding site for glycine still remains unknown. The additive and concentration-dependent nature of the protective effect suggests that ATP and γ-Glu-Cys bound at their binding sites independently interact with the loop in the closed state. The loop composed of 17 residues could be enough long to cover both of the

substrate-binding sites. Since the site of the proteolysis, Arg-233, is located at the middle of the loop, each of the two regions of the loop separated by Arg-233, Ile-226 to Thr-232 and Gly-234 to Arg-241, might make contact with either of the two substrates, independently.

Glycine was sensitive to the mutations on the loop at residues 233, 227, and 240, which increased the K_m^{app} values for glycine. Replacement of Gly-240 with valine, resulting in the G240V and P227V/G240V mutant enzymes, greatly increased the K_m^{app} values for glycine and reduced the apparent rate constant, k_0^{app}. Glycine showed no effect on the proteolysis of the wild-type and mutant GSHases, except for the G240V mutant enzyme, in which the presence of glycine accelerated the proteolysis of the loop. In other words, the binding of glycine hindered the G240V mutant loop from taking the closed state or stabilized it in the open state.

The catalytic rate constant, k_0^{app}, was much more sensitive to the loop mutations, and it drastically decreased with the simultaneous mutations with valine residues at Pro-227 and Gly-240. Although the rate-limiting step is still not identified in the reaction catalyzed by the wild-type GSHase, the decrease in the rate constant could be interpreted in one of two ways: (1) The mutations might shift the rate-limiting step to the rate of the loop changing its conformation with the progress of the catalytic reaction. The loop has to take the closed state as the substrates bind to the enzyme and take the open state after the production of glutathione for product release. The substitutions with valine residues might make the rate of the loop in a conformation in the closed state changing to another in the open state (or vice versa) slower and rate-limiting. (2) The rate-limiting step in the mutants is the same as that in the wild-type enzyme. The mutations might hinder the loop and cause it to take conformations in which γ-Glu-Cys phosphate and glycine take their proper, mutual orientations required for the formation of glutathione or in which the side-chain group on the loop acts as a catalyst; the mutations hinder the adoption of the closed conformation necessary for efficient catalysis.

Substitutions of Pro-227 and Gly-240 with valine residues affected the equilibrium and the catalytic role of the loop, while those of Gly-229 resulted in trivial effects. Pro-227 and Gly-240 are located at the basal part of the loop and probably work as a hinge of the loop. Mutations to valine at the hinge might limit the conformations of the loop, resulting in a fatal functional effect. Gly-229 is at about the middle of the loop. Steric strain at this position could be freed without affecting the mobility and conformation of the loop.

Deduced from the present experimental results, a conformational allowance of the loop might be required for the following, possible functions: the loop might (1) align the substrates into their proper orientations so that the carboxyl group of γ-Glu-Cys, the γ-phosphate group of ATP, and the amino group of glycine could react to result in the synthesis of glutathione; (2) carry some catalytic residues that accelerate the reaction; and (3) recognize γ-Glu-Cys and glycine as substrates. The mutations on the loop in the present study might restrict conformational distribution between the open and closed states and conformational adjustability to the closed state, which is necessary for chemical interactions with the bound substrates to recognize them as substrates and to accelerate chemical reactions.

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