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Flexible Loop That Is Novel Catalytic Machinery in a Ligase. Atomic Structure and Function of the Loopless Glutathione Synthetase[†,‡]

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ABSTRACT: The catalytic mechanism of glutathione synthetase is proposed to proceed via phosphorylation of the dipeptide substrate to yield an acyl phosphate intermediate; this intermediate is subsequently attacked by glycine, followed by loss of inorganic phosphate, leading to glutathione formation. A flexible loop (Ile226–Gly242) in *Escherichia coli* B glutathione synthetase is proposed to stabilize the acyl phosphate intermediate by preventing its decomposition by hydrolysis with water [Tanaka, T., Kato, H., Nishioka, T., & Oda, J. (1992) *Biochemistry 31*, 2259–2265; Tanaka, T., Yamaguchi, H., Kato, H., Nishioka, T., Katsube, Y., & Oda, J. (1993) *Biochemistry 32*, 12398–12404]. To investigate the function of the loop in the *E. coli* enzyme definitely, a loopless mutant in which the loop (Ile226–Arg241) was replaced with three residues of glycine was constructed. The crystal structure of the loopless mutant enzyme was essentially identical with that of the wild-type enzyme. Kinetic measurements showed that the replacement of the loop led to increases in the K_m values, especially for the glycine, and a 930-fold decrease in the k_0 value. Hence, the loopless mutant was 3×10^4 less active in terms of its specificity constant (k_0/K_m) for glycine than the wild-type enzyme. Moreover, the loopless mutant showed γ-L-glutamyl-L-cysteine-dependent ATP hydrolase activity to almost the same extent as its glutathione synthetase activity. These studies support the fact that the loop enhances the recognition of glycine as well as stabilizes the acyl phosphate intermediate so that the intermediate rapidly reacts with glycine.

A peptide synthetic enzyme, glutathione synthetase, catalyzes the ligation of γ-L-glutamyl-L-cysteine (γ-Glu-Cys)[1] and glycine in the presence of ATP and magnesium ion (Meister, 1974, 1985). The *Escherichia coli* B enzyme is a tetramer of identical subunits of 316 amino acid residues (M_r = 35 600) (Gushima et al., 1984). The crystal structure of the *E. coli* GSHase consists of three domains: the N-terminal, central, and C-terminal domains (Yamaguchi et al., 1993). The ATP molecule is located in the cleft between the central and C-terminal domains and is surrounded by two sets of the structural motif that belongs to those respective domains. Each motif consists of an antiparallel β-sheet and a glycine-rich loop. This motif appears to be common to the non-Rossmann-fold-type structure found in nucleotide-dependent enzymes (Cusack et al., 1990; Knighton et al., 1991; Ruff et al., 1991). Consequently, taking into account the ATP binding site facing the β-sheet structure, we have named the motif "the palmate fold". This motif is like an open right hand that grasps the

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‡ Crystallographic coordinates have been deposited in the Brookhaven Protein Data Bank under the file name 1GLV.

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[1] Abbreviations: GSHase, glutathione synthetase; γ-Glu-Cys, γ-L-glutamyl-L-cysteine; DELase, a loopless mutant enzyme of the *Escherichia coli* glutathione synthetase; rms, root mean square; Ant-ATP, 3′-*O*-anthraniloyl-ATP.

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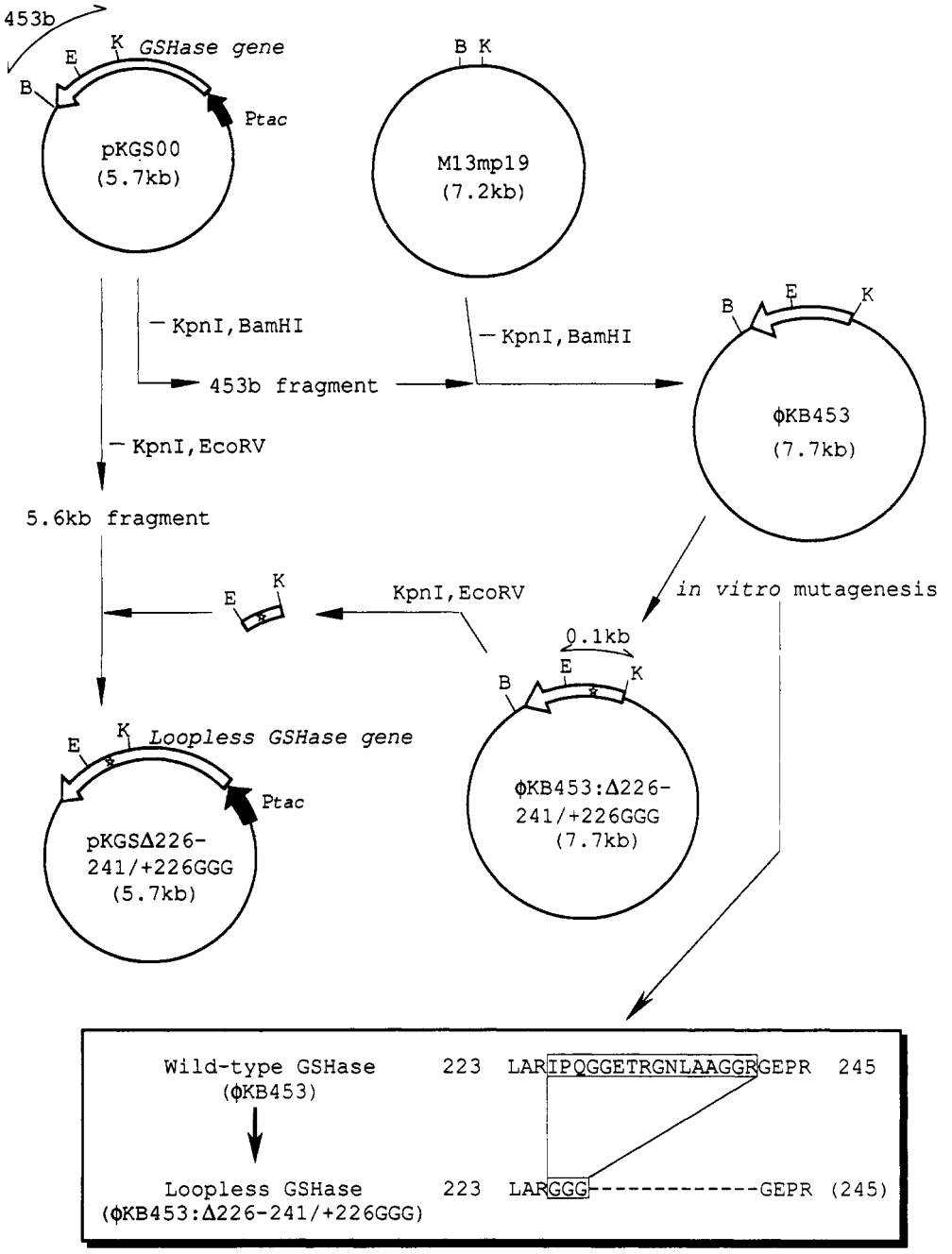


FIGURE 1: Schematic representation of the strategy for the construction of a loopless mutant of *E. coli* glutathione synthetase. The loop from Ile226 to Arg241 was replaced with three Gly residues to give a sequence of four Gly residues. Symbols are *Bam*HI (B), *Eco*RV (E), *Kpn*I (K), and the mutation site (☆).

ATP molecule in its palm with the thumb being the glycine-rich loop. One of the glycine-rich loops, residues Ile226 to Gly242, has ill-defined electron densities and appears to be highly disordered in the native enzyme crystals (Yamaguchi et al., 1993). It has been hypothesized that the disorder may result from the flexibility or mobility of this loop structure. The flexible loop seems to extend over the bound substrates in the active site to move from an "open" position in the unliganded enzyme into a "closed" position in the case of the enzyme with substrates bound at the active site (Tanaka et al., 1992, 1993). The two glycylglycine sequences, positioned near both of the termini of the loop, seem to act as hinges in the conformational change of the flexible loop structure. Such loop closure is an essential step in the catalytic mechanisms of lactate dehydrogenase (Rossmann et al., 1972) and triosephosphate isomerase (Knowles, 1991; Pompliano et al., 1990).

To elucidate the role of the flexible loop, we designed a loopless mutant (DELase) in which the loop from Ile226 to Arg241 was replaced with three Gly residues to give a sequence of four Gly residues (Figure 1). As residues 225 and 243 are located on the termini of two different β-strands that form a double-stranded antiparallel sheet structure, the replacement of the loop with three glycine residues should not significantly distort the structure of the rest of the molecule. Because the sequence GGGG could form a β-turn structure and still remain flexible, this replacement ought not to affect the folding of the DELase structure. We described the three-dimensional structure and kinetic properties of DELase.

EXPERIMENTAL PROCEDURES

Materials. Plasmid pKGS00 is a pKK223-3 derivative containing a 1.2-kilobase pair fragment that is coding the GSHase gene (*gsh-II*) from *E. coli* B (Kato et al., 1988).

Bacterial strain *E. coli* JM109 was provided by Takara Shuzo Co., Ltd. (Kyoto, Japan). Restriction enzymes were obtained from Toyobo Co., Ltd. (Osaka, Japan), and New England Biolabs, Inc. (Beverly, MA). Restriction enzymes and DNA-modifying enzymes were purchased from Takara Shuzo Co., Ltd. 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). All other chemicals used in the present work were of the purest grade commercially available.

Construction of a Loopless Mutant of E. coli Glutathione Synthetase. A *Kpn*I–*Bam*HI fragment (453 base pairs) encoding the partial glutathione synthetase gene was isolated from pKGS00 and inserted into bacteriophage M13mp19 to give φKB453 for site-directed mutagenesis to replace the peptide chain from Ile226 to Arg241 with three glycine residues. Site-directed mutagenesis was carried out by the method of Kunkel (1985). Oligonucleotide was synthesized by a solid-phase phosphoramidite method and purified by high-performance liquid chromatography on TSK-Gel DEAE-2SW [Toso Co., Ltd. (Tokyo, Japan)]. Purified oligonucleotide was phosphorylated at the 5′ end with ATP and T4 polynucleotide kinase. The oligonucleotide primer used in the present studies is (the underlined bases encode the three new glycine residues)

5′GCCTGGCGCGTGGTGGTGGTGGTGAACCTCG3′

A short DNA fragment containing the mutation site was excised from the φKB453 mutant with two appropriate restriction enzymes, *Kpn*I and *Eco*RV. After the DNA sequence was verified, this short fragment containing the desired mutations replaced the counterpart in pKGS00 to give the expression vector of the mutant enzyme, pKGSΔ226-241/+226GGG.

Expression and Purification of the Wild-Type and Loopless Mutant GSHases. The wild-type and mutant enzymes were expressed in *E. coli* JM109. Growth and purification were carried out according to Kato et al. (1989). The yield of the expression and purification of the mutant was the same as that of the wild type. The purified protein gave a single band on polyacrylamide gel electrophoresis under denaturing conditions.

Crystallization and Structure Solution. The mutant GSHase was crystallized by microdialysis against 28% saturated ammonium sulfate in 50 mM Tris-HCl buffer, pH 7.5, with 5 mM $MgCl_2$. The crystals are isomorphous with the wild-type enzyme (Kato et al., 1989) with unit cell dimensions $a = 87.8$ Å, $b = 87.8$ Å, and $c = 170.2$ Å and space group $P6_122$. X-ray diffraction data to 2.2-Å resolution were collected on an X-ray imaging plate system, Rigaku R-AXIS IIc [Rigaku (Tokyo, Japan)] (Sato et al., 1992). The data set was 73.2% complete up to 2.2-Å resolution. The initial electron density map was calculated from the diffraction data of DELase and calculated phases of the wild-type enzyme. Crystallographic refinement of the structural model was carried out using the PROLSQ program (Konnert & Hendrickson, 1980). At intervals, the fit of the model to its electron density was analyzed and improved with the molecular graphics program MOLFITG developed at the Institute for Protein Research, Osaka University. The final crystallographic R factor is 0.207 for all data to 2.7-Å resolution.

Enzymatic Activity Measurements. The amount of glutathione synthesized was determined by glutathione reductase (Kato et al., 1987) and the formation of ADP by a coupling system of pyruvate kinase and lactate dehydrogenase (Tanaka et al., 1992). Assays were carried out in the presence of the appropriate concentration of glycine, 10 mM ATP, 10 mM γ-Glu-Cys, 100 mM KCl, 20 mM $MgSO_4$, 100 mM Tris-HCl (pH 7.5), and 3.99 μM DELase at 37 °C. In the case of the measurements of released ADP, the assay mixtures further contained 1 mM phosphoenolpyruvate, 0.24 mM NADH, 25 units of lactate dehydrogenase, and 10 units of pyruvate kinase.

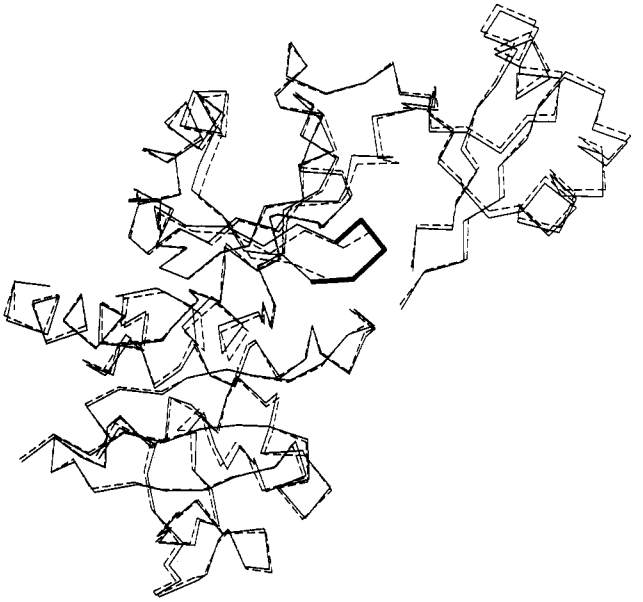


FIGURE 2: Comparison of a Cα backbone tracing of wild-type (dashed line) and mutant glutathione synthetase (solid line). The residues replaced in the mutant structure are shown in thick lines. There was no tracing of the flexible loop in the wild-type enzyme. The structure of the wild-type GSHase is from Yamaguchi *et al.* (1993).

Protein concentration was calculated from the absorption coefficient $A^{1\%}_{1cm} = 9.02 \pm 0.44$ at 280 nm in 0.05 M potassium phosphate buffer (pH 7.0) (Kato et al., 1987).

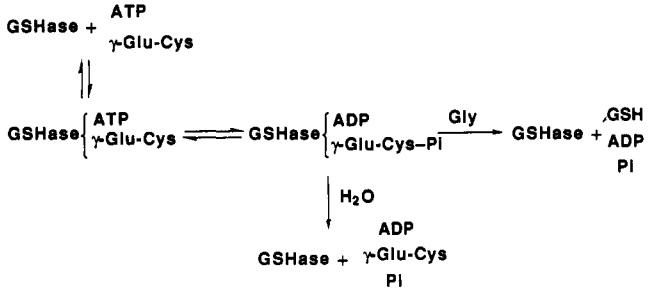
K_d Determination for Ant-ATP. Binding of 3′-*O*-anthraniloyl-ATP (Ant-ATP; Hiratsuka, 1983) to the wild-type and mutant GSHases was analyzed with a Hitachi A-3000 spectrofluorometer at 25 °C using 0.5 × 0.5 × 3 cm UV-grade quartz cuvettes. Appropriate concentrations of Ant-ATP were added to the solution containing 7.5 μM GSHase, 0.1 M Tris-HCl (pH 7.5), and 5 mM $MgCl_2$. Fluorescence was monitored with excitation at 390 nm and emission at 420 nm. After data from a titration in the absence of the enzymes were subtracted, the data were analyzed by a nonlinear least squares fit as described by Hiromi (Kakitani et al., 1989).

RESULTS AND DISCUSSION

Crystal Structure of DELase. DELase crystals, isomorphous with those of the wild-type enzyme, were obtained from Tris-HCl buffer (pH 7.5) with ammonium sulfate as a precipitant. The crystals diffracted X-rays to 2.1-Å resolution. The crystal structure of DELase at 2.7-Å resolution is shown in Figure 2 together with that of the wild-type enzyme. The root-mean-square (rms) deviation between the wild-type and the DELase structures was 0.58 Å for their corresponding Cα atoms excluding the loop region (residues Ile226 to Gly242, referred to the sequence number for the wild-type enzyme). Thus, the structure of DELase is essentially identical with the wild-type GSHase structure. The flexible loop seems to be independent of the rest of the GSHase structure.

Kinetic Behavior of DELase. The glutathione synthetic activity of DELase was reduced to 0.1% that of the wild-type

Scheme 1



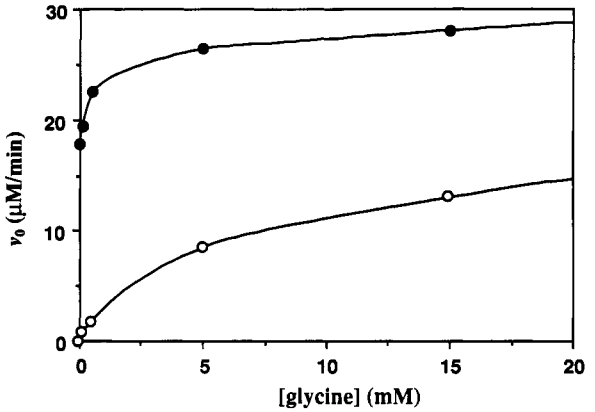


FIGURE 3: Kinetics of the DELase reaction. Glycine saturation curves of the DELase reaction. Initial rates (v_0) of the reaction were measured by the synthesis of glutathione (○) and the formation of ADP (●). The amount of glutathione synthesized was determined by glutathione reductase and the formation of ADP by a coupling system of pyruvate kinase and lactate dehydrogenase. Assays were carried out in the presence of the appropriate concentration of glycine, 10 mM ATP, 10 mM γ-Glu-Cys, 100 mM KCl, 20 mM $MgSO_4$, 100 mM Tris-HCl (pH 7.5), and 3.99 μM DELase at 37 °C. In the case of the measurements of released ADP, the assay mixtures further contained 1 mM phosphoenolpyruvate, 0.24 mM NADH, 25 units of lactate dehydrogenase, and 10 units of pyruvate kinase.

enzyme. The catalytic mechanism of GSHase has been proposed to proceed via phosphorylation of the carboxylate of the cysteine moiety of γ-Glu-Cys to yield γ-L-glutamyl-L-cysteinyl phosphate (Meister, 1974). This intermediate is subsequently attacked by glycine, followed by loss of inorganic phosphate, leading to product formation (Nishimura et al., 1964). A similar intermediate, acyl phosphate, is also involved in the reaction of the other C–N bond ligases (Mullins et al., 1990; Meek & Villafranca, 1980; Bass et al., 1984). On the basis of this putative mechanism, we proposed that the loop would stabilize the acyl phosphate intermediate by preventing its decomposition by hydrolysis with water. According to this presumption, it was expected that the loopless mutant would have γ-Glu-Cys-dependent ATP hydrolysis activity, despite its reduced glutathione synthetic activity (Scheme 1).

Kinetics of the enzymatic reaction of DELase were determined with two different assays: determination of the amount of ADP released (Tanaka et al., 1992) and determination of the amount of glutathione synthesized (Kato et al., 1987) (Figure 3). The rate of glutathione synthesis follows the Michaelis–Menten model when measured against the varied concentration of glycine with excess and fixed concentrations of ATP and γ-Glu-Cys, whereas the rate of formation of ADP does not. The rate of release of ADP was significant at [glycine] = 0, and the rate was saturated at lower concentrations of glycine. The rate of ADP released at saturation was about twice that of the glutathione synthesis. Thus, DELase catalyzes ATP hydrolysis in a manner that the significant amount of ADP release is essentially uncoupled

Table 1: Apparent Kinetic Properties for Wild-Type and Loopless Mutant GSHases

kinetic parameter		wild-type enzyme	DELase	ratio (wild type/DELase)
		GSH Synthesis		
K_m (mM)	ATP	0.24 ± 0.1	1.54 ± 0.21	0.16
	γ-Glu-Cys	0.24 ± 0.1	0.70 ± 0.49	0.34
	Gly	0.91 ± 0.4	29.8 ± 4.7	0.031
k_0 (s^{-1})		151 ± 9	0.163 ± 0.010	930
		ATP Hydrolysis[a]		
K_m (mM)	ATP	0.085 ± 0.014	1.35 ± 0.16	0.063
	γ-Glu-Cys	0.14 ± 0.04	0.72 ± 0.02	0.20
k_0 (s^{-1})		3.04 ± 0.09	0.100 ± 0.004	30
Selectivity (k_0 of GSH Synthesis/k_0 of ATP Hydrolysis)		50	1.6	31
		Binding of Ant-ATP		
K_d (mM)		0.045 ± 0.006	1.74 ± 0.74	0.026

[a] ATP hydrolase activity was measured in the absence of the substrate glycine.

with glutathione synthesis. This ATP hydrolysis requires the presence of γ-Glu-Cys because ADP release was observed only when both ATP and γ-Glu-Cys were added in the reaction mixtures. In contrast, the wild-type enzyme showed little γ-Glu-Cys-dependent ATP hydrolysis activity without glycine, less than 2% of its glutathione synthetic activity. These data strongly support the mechanism in Scheme 1. In the case of the DELase reaction, glycine and water molecules compete with each other in the reaction with the acyl phosphate intermediate. The loop is therefore responsible for stabilization of the acyl phosphate intermediate.

Apparent steady-state kinetic constants of DELase were determined for both the glutathione synthesis and ATP hydrolysis activities (Table 1). To allow comparison, the values of the wild-type GSHase are also included. In the absence of glycine, the kinetics of the ATP hydrolysis obeyed the Michaelis–Menten equation with respect to the concentrations of ATP and γ-Glu-Cys. As a reference experiment, we measured the dissociation constant K_d of 3′-anthraniloyl-ATP, a fluorescent ATP analog, with both DELase and the wild-type enzyme (Table 1). These K_d values corresponded well to the K_m values in the ATP hydrolysis of the wild-type enzyme and of DELase and so are good indication of the affinity of the substrate with the enzymes. In the case of the glutathione synthesis activities, the replacement of the loop structure led to increases in the K_m values, especially for the glycine, and a 930-fold decrease in the k_0 value. Hence, DELase was 3 × 10^4 less active in terms of its specificity constant (k_0/K_m) for glycine than the wild-type enzyme. This result suggests that the loop enhances the recognition of glycine as well as stabilizes the acyl phosphate intermediate so that the intermediate rapidly reacts with glycine.

The ratio of catalytic constant k_0 values of glutathione synthesis to that of ATP hydrolysis in the wild-type enzyme, 50, is an indication of the selectivity in the reaction of the acyl phosphate intermediate with glycine compared with H_2O. This ratio is the sum of the selectivity due to both the loop and the rest of the structure of the wild-type enzyme. The contribution of the latter to selectivity is estimated as 1.6 from the selectivity of the DELase mutant. Consequently, the net acceleration with the loop structure to the product formation from the acyl phosphate intermediate and glycine is 31-fold.

In the DELase reaction, the k_0 for ATP hydrolysis was not equal to that of the wild type but decreased about 30-fold. It is thus interpreted that the loop structure accelerates not only

Table 2: Substrate Specificity for Glycine Analogs in DELase and the Wild-Type GSHases[a]

glycine analogs	relative activity (%)	
	wild type	DELase
glycine	100	100
β-alanine	42	42
glycine methyl ester	55	109
glycine ethyl ester	44	144
glycylglycine	ND[b]	8.5
glycinamide	ND[b]	12.6

[a] Assays on GSH synthetic activity were carried out by the measurement of the formation of ADP with pyruvate kinase and lactate dehydrogenase as a coupling system in the presence of 15 mM glycine analogs. Data from a measurement in the absence of the glucine analogs were subtracted. [b] ND, not detectable.

the GSH formation from the acyl phosphate intermediate but also the formation of the intermediate from ATP and γ-Glu-Cys. A total acceleration of 930-fold is achieved by the loop structure.

We also examined the glycine specificity of DELase. Table 2 shows relative specific activities with glycine analogs for DELase and the wild-type enzyme reactions. These results indicated that DELase is less specific for glycine than the wild-type enzyme.

The structural and kinetic studies reported here indicate a clear functional difference between GSHases with and without the flexible loop. The loop wraps around each active species that exists along the GSHase reaction. It is conceivable that the loop has a hyperfunction that is not only stabilization of the transition states and/or the intermediate of the reaction but also the coupling of ATP hydrolysis with glutathione synthesis through the recognition of the substrate glycine. In this way, the loop may regulate the ordering of the reaction so that each partial reaction proceeds sequentially and both of the partial reactions are concerted with each other.

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