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Effect of Amino acid Substitutions of *Escherichia coli* γ-Glutamylcysteine Synthetase

Nam, Yong Suk, Joong Soo Kim, Joon Hyeok Kwak, Young In Park* and Se Yong Lee

Department of Agricultural Chemistry
*Department of Genetic Engineering, Korea University, Seoul 136-701, Korea

Escherichia coli γ-Glutamylcysteine Synthetase의 아미노산 치환 효과

남용석 · 김중수 · 곽준혁 · 박영인* · 이세영

고려대학교 농대 농화학과, 유전공학과*

ABSTRACT: Two amino acid residues (Ala[494] and Ser[495]) of *E. coli* γ-glutamylcysteine synthetase have been investigated whether they are the site of feedback inhibition by site specific mutagenesis. Single substitution of serine[495] (S495F), and double substitutions of alanine[494] and serine[495] (A494G-S495F) resulted in the inactivation of the γ-glutamylcysteine synthetase activity. Substitution of alanine[494] with glycine[494] resulted in a higher level of feedback inhibition. These results suggest that serine[495] in γ-glutamylcysteine synthetase is required for its catalytic activity and alanine[494] is presumably related to the feeback inhibition site.

KEY WORDS □ *E. coli* γ-glutamylcysteine synthetase, alanine[494], serine[495], feedback inhibition site, site-specific mutagensis

γ-Glutamylcystein synthetase (GSH-I; EC 6.3.2.2), which catalyzes the formation of γ-glutamylcysteine from L-glutamate and L-cysteine in the presence of ATP, is one of the two enzymes in glutathione biosynthesis. GSH-I is the key enzyme, and its molecular weight has been estimated as about 60,000 daltons in *E. coli* and consists of 517 amino acid residues (Watanabe *et al.*, 1986a). The enzyme synthesis is not repressed by glutathione, but the enzyme activity is inhibited by reduced glutathione (Meister and Anderson, 1983). A mutant strain, however, which was desensitized to feedback inhibition by reduced glutathione, has been reported and its mutant type *gsh*I gene has also been cloned (Murata *et al.*, 1982). And the nucleotide sequence of its varient *gsh*I gene derived from *E. coli* B was determined (Watanabe *et al.*, 1986b). The nucleotide sequence of wild type *gsh*I gene of *E. coli* B, however, has not been reported. Therefore, we have cloned the wild type *gsh*I gene from *E. coli* K-12 and the nucleotide sequence has been determined for comparisons (Nam, Y.S., 1990). Sequence analysis of these two genes have shown that only five bases were found to be different. Therefore, it can be deduced from the nucleotide sequence differences that Ala-Ser at positions, 494 and 495, are substituted to Gly-Phe by changes of these five bases in the mutant type GSH-I. Because no other alteration has been found in the *gsh*I gene sequence, these substitutions might be the principal site of the desensitization of GSH-I to be feedback inhibition by reduced glutatione.

To investigate the sites which are responsible for the feedback inhibition the Ala-Ser residues at positions, 494 and 495, were substituted by other amino acids (gly-ser, ala-phe, gly-phe) using site-specific mutagenesis in this study. The catalytic activities and feedback inhibition of these mutant GSH-I's were investigated.

MATERIALS AND METHODS

Bacterial strains and plasmids

E. coli K-12 BH5262 (*gshI, ara, gal, strA, arg, trxA*

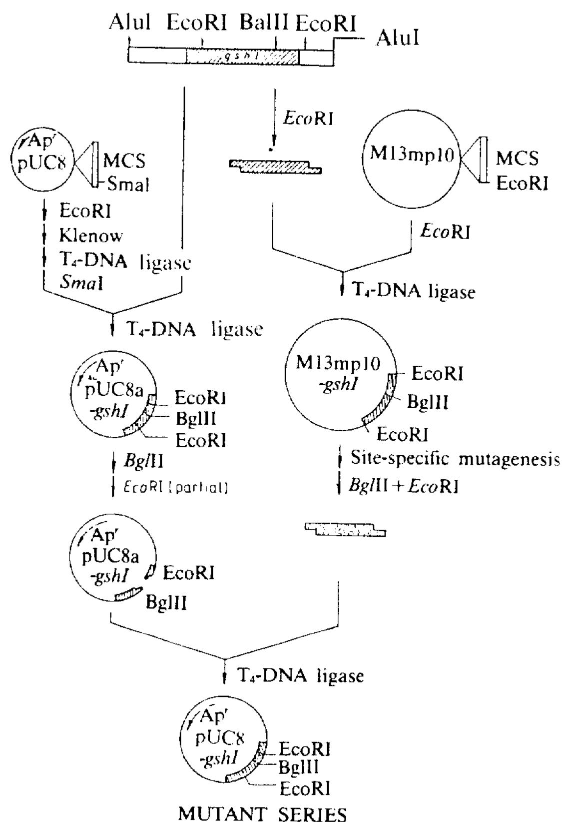

Fig. 1. *Schematic representation for the construction of various recombinant plasmids containing mutated gshI gene.*

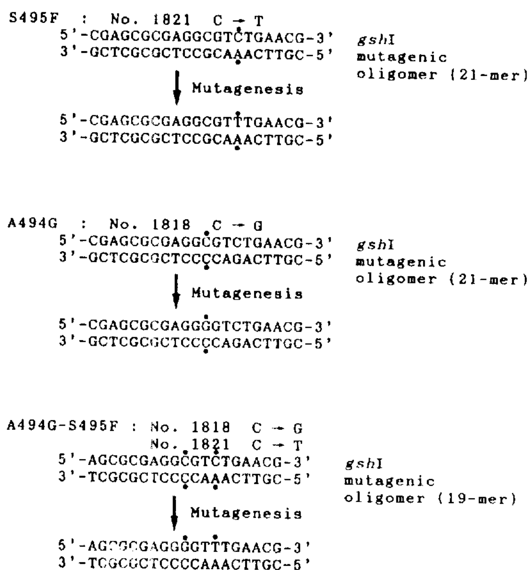

Fig. 2. *Site-specific mutagenesis of gshI gene. The mismatch points are indicated with dots.*

2, Tn::10), which is deficient in the activity of GSH-I, was used for the expression of *gsh*I. For oligonucleotide-directed mutagenesis and nucleotide sequencing, *E. coli* JM103 (Messing *et al.*, 1983), *E. coli* RZ1032 (Kunkel *et al.*, 1985) and M13mp10 phage were used, and for expression of *gsh*I, pUCα plasmid was used. Also pUC8α vector which was filled in by Klenow fragment to disrupt *Eco*RI site of the pUC8 vector in the multiple cloning site was constructed.

Transformation of *E. coli*

$CaCl_2$ treated *E. coli* cells were transformed with plasmid DNA (Maniatis *et al.*, 1982). Transformed cells were selected by plating on the LB-media containing ampicillin (50 μg/m*l*) and incubation overnight.

Preparation of plasmid and M13 phage DNA

Single-stranded DNA of M13 phage was prepared according to the method described by Kunkel (1985). And plasmids and the replicative form (RF) of phage DNA were prepared as described by Bauer *et al.* (1985).

Mutagenic oligonucleotide

Mutagenic oligonucleotide were synthesized with an Applied Biosystem DNA synthesizer Model 391 as described by Fig. 2.

Uracil-containing M13 phage preparation

Cloned M13 templete DNA containing uracil residues was prepared as described by Kunkel (1986). *E. coli* RZ1032 (*ung*⁻, *dut*⁻) cells were grown at 37℃ with a vigorous shaking in YT medium supplemented with deoxyadenosine (100 μg/m*l*) until a cell density of 4×10^8/m*l*. After centrifugation at 2000×g for 15 min, the precipitate was washed with YT medium, resuspended in fresh YT medium containing uridine (0.25 μg/mL), prewarmed at 37℃, and shaken vigorously for 5 min. Then phage (M13mp10-*gsh*I) stock was added at a multiplicity of infection of 5 and incubated at 37℃ for overnight. After centrifugation at 5,000×g, the supernatant was used for the preparation of template DNA.

Site-specific mutagenesis

Site-specific mutagenesis of *gsh*I gene was performed by the uracil-containing templete method (Kunkel, 1985). The 5′-OH of the mutagenic oligonucleotide was phosphorylated in a 20 μ*l* reaction volume containing 50 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, 2 units of T_4 polynucleotide kinase and 9.0 ng of the oligonucleotide. The mixture was incubated at 37℃ for 1 hr, and terminated by the addition of 3 μ*l* of 100 mM EDTA and heating at 65℃ for 100 min.

The phosphorylated oligonucleotide was then mixed with 1 μg (in 0.6 μ*l*) of single-stranded, uracil-containing circular M13mp10 containing

*gsh*I gene and 1.2 μl of 20x SSC(3M NaCl, 300 mM sodium citrate). The reaction mixture was placed in a water bath at 70℃ and allowed to cool to room temperature.

In vitro DNA synthesis was performed in a final volume of 100 μl containing 20 μl of the phosphorylated oligonucleotide mixture, 20 mM HEPES, pH 7.8, 3 mM DTT, 10 mM $MgCl_2$, 500 μM each of dNTP, 1 mM ATP, 2.5 units of Klenow fragment and 2 units of T_4 DNA ligase. The reaction mixture was incubated at 0℃ for 5 min. The tube was placed at room temperature for 5 min, then at 37℃ for 2 hr. The synthesized DNA were then used to transfect *E. coli* JM103 (Messing *et al.*, 1983).

The assay of γ-glutamylcysteine synthetase

Pyruvate kinase-Lactate dehydrogenase (PK-LDH) method was used for feedbak inhibition assay (Gumagai *et al.*, 1982). The standard reaction mixture contained 12.5 μmol of L-glutamate, 12.5 μmol of L-cysteine, 125 μmol of diethanolamine-HCl buffer (pH 9.15), 1.25 μmol of ATP, 12.5 μmol of $MgSO_4 \cdot 7H_2O$, 125 μmol of KCl, 0.75 mg of BSA, 0.25 μmol of NADH, 0.125 μmol of phosphoenolpyruvate, 7.4 units of pyruvate kinase, 12.7 units of lactate dehydrogenase and the enzyme dissolved in 0.05 M Tris-Cl buffer, pH 7.4, in a final volume of 0.8 mL. Incubation was carried out at 37℃ in a cuvette of 1 cm light path. The reaction was started with the addition of enzyme or one of substrate amino acid added and followed by reading the initial change in absorbance at 340 nm.

The activity of γ-glutamylcysteine synthetase was measured by the DTNB method (Jackson *et al.*, 1969). The amount of protein was estimated by the mehtod of Lowry (1951).

DNA sequencing

The nucleotide sequence of mutant *gsh*I gene were determined by the dideoxy chain termination method (Sanger *et al.*, 1977). The dieoxy sequencing reactions were carried out using sequenase version 2.0 from United States Biochemical.

RESULTS

Site-specific mutagenesis

To construct three GSH-I mutants possessing substituted amino acids residues, the oligonucleotide sequences corresponding to the mutagenized amino acid residues were synthesized and used in the site-specific mutagenesis. To mutagenize the *gsh*I gene, a 1.4 Kb *Eco*RI DNA fragment containing the *gsh*I gene was subcloned into *Eco*RI site of M13mp10 vector (Fig. 1.). Single-stranded M13mp10-*gsh*I DNA which contains uracil residues in its genome was prepared from phages grown in *E. coli* RZ1032 (ung^-, dut^-). The

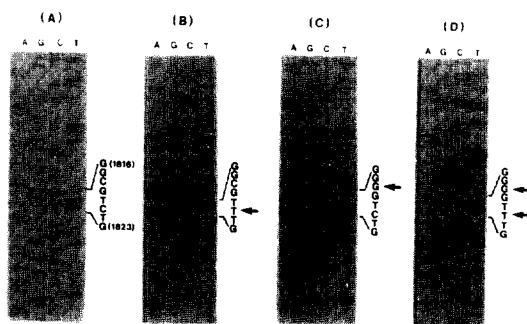

Fig. 3. *Autoradiogram of sequencing polyacrylamide gel showing the mutated gshI genes. The substituted bases are indicated by arrows. The sequencing was performed by M13 dideoxy chain termination method and electrophoresis was carried out on a 6% gel containing 8.3 M urea. Panel (A): wild type gshI gene; panel (B): mutated gshI gene (S495F); panel (C): mutated gshI gene (A494G); panel (D): mutated gshI gene (A494G-S495F).*

double-stranded DNA was synthesized *in vitro* using the mutagenic oligonucleotides and the uracil-containing template with Klenow fragment (Fig. 2), and transfected into *E. coli* JM103 (ung^+, dut^+) host.

Screening of mutant *gsh*I gene

The single-stranded phage DNAs were isolated from the phages produced by transfected *E. coli* JM103 and then subjected to selective track sequencing for the identification of varient phage containing the mutated *gsh*I gene. In this study, two serial codons 5′-GCGTCT-3′ coding for Ala-Ser at positions of 494 and 495 were substituted by either 5′-GCGTTT-3′, 5′-GGGTCT-3′, or 5′-GGGTTT-3′, which code for Ala-Phe (S495F), Gly-Ser (A494G), or Gly-Phe (A494G-S495F), respectively. For mutant S495F, three of ten phages isolated were detected in a "A" track, for mutant A494G, one of ten phages isolated in a "C" track, and the double mutant A494G-S495F, two of five phages isolated in a "A" track. The phage DNAs identified by the selective track sequencing were subjected to the entire sequence analysis in order to confirm the desired mutation at the specific sites (Fig. 3).

Construction of mutant *gsh*I gene

The 0.4 Kb *Bgl*II-*Eco*RI fragment of pGH2010 plasmid (pUCα-gshI), which contains 1.95 Kb *gsh*I DNA fragment on pUC8*a* vector, was replaced by the corresponding fragment isolated from the mutated M13mp10-*gsh*I RF DNAs (Fig. 1). This process was thought to be necessary to eliminate the possibility that other sites in the structural region might be mutated by errors during the site-specific mutagenesis. By this way, various recombinant plasmids containing the mutated

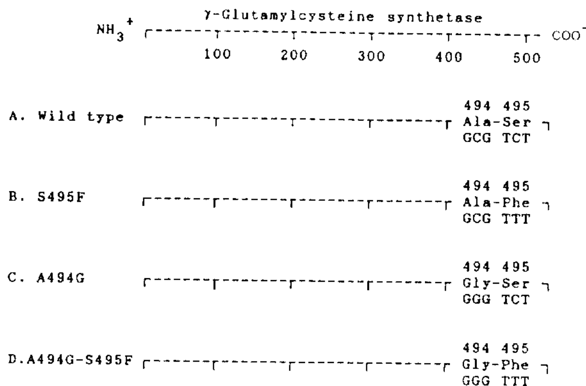

Fig. 4. *Comparison of amino acid residue differences between E. coli wild type and mutant enzymes. Scale of the residue positions in the γ-glutamylcysteine synthetase and the position of nucleotide changes are also indicated. (A) shows amino acid residues of wild γ-glutamylcysteine synthetase. (B), (C) and (D) shows amino acid residues of mutant γ-glutamylcysteine synthetases in the specific region of the polypeptide chain.*

*gsh*I gene such as pGH2011 (for S495F), pGH2012 (for A404G), and pGH2013 (for A494G-S495F) plasmids, were obtained (Fig. 4).

Expression of mutant *gsh*I gene

GSH-I activities of *E. coli* BH5262 cells tranformed with the mutated pUC8*a*-*gsh*I DNAs were determined. No enzyme activities were detected in S495F strain and A494G-S495F. But A494G strain harboring pGH2012 exhibited 96% of wild type activity (Table 1).

Properties of mutant GSH-I

The GSH-I activity of A494G strain was measured by the PK-LDH method in the presence of reduced glutatione in the range of 0 to 10 mM (Table 2). The enzyme activity of A494G was observed as 21% of its original activity in the presence of 5 mM of reudced glutatione. In the presence of 10 mM of reduced glutathione, 93% of its original activity was inhibited. However, the wild type (pGH2010) exhibited the 0.05% and 66.8 % inhibition of its original activity, respectively. These results suggest that GSH-I of A494G enzyme is more sensitive to the feedback inhibition by reduced glutathione than those of both *E. coli* strain B (Murata *et al.*, 1982) and K-12 (Nam, 1990) wild types.

Table 1. *Specific activity of mutant γ-glutamylcysteine synthetase*

Strain	Specific activity[a]*	Changed amino acids
BH5262/pGH2010	3.48	Ala494.Ser 495
BH5262/pGH2011	0.00	Ala494.Phe495
BH5262/pGH2012	3.34	Gly494.Ser 495
BH5262/pGH2013	0.00	Gly494.Phe495

All strains were grown on LB medium containing Ap (50 μg/ml). The enzyme activity was determined by DTNB method.
[a]*: μmol/h/mg protein

Table 2. *Feedback inhibition of mutant γ-glutamylcysteine synthetase by reduced glutathione*

Glutathione (mM)	Relative activity (%)	
	A494G	Wild type
None	100.0	100.0
2.5	41.3	100.0
5.0	21.3	99.5
7.5	10.0	64.6
10.0	7.0	43.2

Enzyme activity was determined by the PK-LDH method and enzyme activity is expressed relative to that determined in the absence of reduced glutathione.

DISCUSSION

Without any understanding of three-dimensional structure, we had introduced base substitution mutations into the segment of the *gsh*I gene corresponding to the polypeptide coding region which is considered to be the site of feedback inhibition.

A494G-S495F and S495F mutants which have substituted Ser-495 residue to Phe-495, showed no detectable enzyme activity. The hydroxyl group of seryl residue has generally been known to play an important role in the maintenance of tertiary structure through hydrogen bonding and participate in catalytic mechanism of enzymes such as serine protease (Carter er al., 1988). In this regard, Ser-495 residue presumably plays the key role in the GSH-I. Alternatively, it may infer that Ser-495 residue is located near the active site and the replacement of Ser with Phe induces remote conformational change in the overall structure of enzyme (Louri and Pielak, 1988; Dembowski *et al.*, 1990). From these reports, they still could have some effect to mintain the catalytic activity, cooperatively, or three dimentional structure of enzyme, although Ser or Phe may not be located at the active site. Replacement of the Ser-495 with a bulky, nonpolar amino acid such as Phe might abolish enzyme activity due to a great conformation change.

A mutant type GSH-I of A494G, which replaced Ala-494 residue with glycine, lost only 4% of its wild type activity (Table 1), but its activity has

been inhibited more by reduced glutatione compared to wild type activity (Table 2).

Because glycyl residue favors a turn-like proryl residue and its side chain is small, it is mainly located at bent region and offer a space for binding of ligand (Chou *et al.*, 1974; Levitt *et al.*, 1978; Chen *et al.*, 1990; Craik *et al.*, 1985). Moreover, in spite of small size difference of R group between alanine and glycine, a change in conformation has also been observed (Villafranca *et al.*, 1990; Estell *et al.*, 1986). Therefore, our results suggest the possibility that a change of Ala-494 to Gly can cause a release from the feedback inhibition by reduced glutathione.

In order to investigate a role of Ser-495, it is necessary to substitute Ser residue to such as Ala, Gly, Thr, Cys or Tyr. This substitution will elucidate whether the loss of activity is due to the removal of a hydroxyl group of Ser or the conformational change by the substitution of hydroxyl group with a bulky phenolic group.

Moreover, to investigate the exact role of Ala-494, it might be necessary to substitute Ala to Val or Leu. Because the only difference between Ala and Gly lies in different R groups, the different space for binding of reduced glutatione by increasing of the size of nonpolar R group would release the feedback inhibition.

적 요

γ-Glutamylcystein synthetase는 글루타치온의 생합성에 관여하는 첫번째 효소로서 환원형 글루타치온에 의해서 feedback inhibition을 받는다. *gsh*I 유전자의 염기서열로 부터, wild type (*E. coli* K-12)과 feedback resistance mutant strain (*E. coli* B) 사이에 두개의 연속된 아미노산 잔기가 다르다는 것을 확인 하였다. 따라서 야생형에 있는 아미노산 잔기 (alanine-494, serine-495)가 feedback inhibition에 관계되는 지를 확인하기 위해서 feedback resistance mutant strains에 있는 아미노산 잔기 (glycine-494, phenylalanine-495)로 차례로 치환하였다. Alanine-494와 serine-495가 glycine-494와 phenylalanine-495로 함께 치환된 효소 (A494G-S495F)와 serine-495가 phenylalanine-495 (S495F)로 치환된 효소는 활성이 없었고, alanine-494가 glycine-494로 치환된 효소는 활성이 4% 감소 하였으며, 또한 alanine-494가 glycine으로 치환된 효소는 wild type 보다 glutathione에 의해서 inhibition을 더 받았다. 따라서 γ-glutamylcysteine synthetase에 있는 serine-495 잔기가 효소활성을 관여하며 alanine-494잔기가 feedback inhibition에 관여하는 위치라고 생각된다.

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