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Site-Specific Mutagenesis of the *gshI* Gene for Increasing the Activity of γ-Glutamylcysteine Synthetase in *Escherichia coli* K-12

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The *gshI* gene from the *Escherichia coli* K-12 strain codes for γ-glutamylcysteine synthetase which mediates the rate-limiting step of glutathione biosynthesis. The isolated *gshI* gene from *E. coli* K-12 has an unusual translation initiation codon, UUG. The 494th amino acid is Ala rather than Gly which was found in a mutant strain *E. coli* B. In order to improve the translational rate of the *gshI* gene of *E. coli* K-12, the initiation codon, UUG, was changed to the usual AUG codon by the site-specific mutagenesis. This change has resulted in a 53% increase of γ-glutamylcysteine synthetase activity. The enzyme activity was also improved by replacing Ala^{494} with Val (A494V) or Leu (A494L). The replacement of Ser^{495} with Thr (S495T) also resulted in a 62% increase of the enzyme activity. Therefore, the specific activity of γ-glutamylcysteine synthetase was increased with the increasing chain length of the aliphathic amino acid at the site of the 494th amino acid (Ala < Val ≤ Leu).

Keywords: γ-Glutamylcysteine synthesis, Glutathione, Site-specific mutagenesis.

Introduction

Glutathione, a γ-glutamylcysteinylglycine, is one of the small thiol compounds found in a living system. It plays many important physiological roles in microorganisms, plants, and animals, such as the maintenance of protein sulfurhydroxyl groups, the formation of the deoxyribonucleotide precursor of DNA, the protection of cells against reactive oxygen compound and free radicals, and detoxification of toxins from the environment (Meister and Anderson, 1983).

γ-Glutamylcysteine synthetase (γ-ECS; E.C. 6.3.2.2.), which catalyzes the formation of γ-glutamylcysteine from L-glutamate and L-cysteine in the presence of ATP, is the rate-limiting step in glutathione biosynthesis (Richman and Meister, 1975). The activity of γ-glutamylcysteine synthetase is controlled through feedback inhibition by the reduced form of glutathione. The gene responsible for this enzyme (*gshI*) is not repressed by gluthathione in *Escherichia coli*. (Watanabe *et al.*, 1986; Nam, 1990).

We have cloned the *gshI* gene from *E. coli* K12 and sequenced it (Nam *et al.*, 1991). The only difference in the base sequence of the *gshI* gene between *E. coli* K12 and a feedback resistant mutant *E. coli* B reported by Watanabe *et al.* (1986) was five bases corresponding to the 494th and 495th amino acids in γ-glutamylcysteine synthetase. The 494th amino acid is Ala rather than Gly, which was found in a feedback mutant strain *E. coli* B (Watanabe *et al.*, 1986). The translational initiation codon of *gshI* of *E. coli* K12 is UUG instead of the usual AUG.

In this work, we tried to improve the activity of γ-glutamylcysteine synthetase in *E. coli* K12 by site-specific mutagenesis. The increased activity of γ-glutamylcysteine synthetase would be useful in the synthesis of glutathione by an *in vitro* bioreactor system. We have also improved the translational rate of the *gshI* gene of *E. coli* K12 by changing the initiation codon UUG to the usual AUG. The enzyme activity was also improved by replacing the 494th and 495th amino acids.

Materials and Methods

Bacterial strains The *gshI* gene cloned from *E. coli* K-12 W3110 strain (supplied by Dr. C. Yanofsky, Stanford University, San Francisco, USA) was used as the DNA template for oligonucleotide-directed mutagenesis. *E. coli* HB101 (F^- *hsd20 recA ara proA2 lacY galK supE44*) was used for cloning of the *gshI*, and *E. coli* BH5262 (F^- *araD139 galU galK hsr+ rpsL argH1 trxA7004 gshI srl::Tn10*; kindly supplied from Kangwon University, Korea; Gleason *et al.*, 1985) was used for the expression of the *gshI* gene. The *E. coli* JM109 (Maniatis, 1989),

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E. coli RZ1032 (Kunkel, 1985), and M13 bacteriophage (Maniatis, 1989) were used for site-specific mutagenesis and nucleotide sequencing.

Site-specific mutagenesis Site-specific mutagenesis of the *gshI* gene was performed by the uracil-containing template method (Kunkel, 1985). For the construction of a mutant *gshI* having the usual translational initiation codon, an oligonucleotide corresponding to the desired nucleotide sequence was synthesized and used in the site-specific mutagenesis (Table 1).

A 1.95 kb *AluI* DNA fragment containing the *gshI* gene was subcloned into the *SmaI* site of the plasmid pUC8α vector, in which the *Eco*RI site of pUC8 has been deleted (Nam *et al.*, 1991). For site-specific mutagenesis, a 0.27 kb *StuI–Eco*RI DNA fragment containing the site to be mutagenized of the *gshI* gene was subcloned into the *SmaI–Eco*RI site of M13mp19 vector. Single-stranded M13mp19–*gshI* DNA containing a uracil residue in its genome was prepared from phages grown in *E. coli* RZ1032 (dut^-, ung^-). The double-stranded DNA was prepared from DNA synthesis using mutagenic oligonucleotides and uracil containing template *in vitro*. *E. coli* JM109 (ung^+, dut^+) host was transfected with the double-stranded DNA synthesized by sequenase *in vitro*. In the JM109 host, the uracil-containing template is lethal because of the presence of uracil-N-glycosylase and only ssDNA synthesised *in vitro* can replicate in JM109 and form plaques. Therefore, plaques formed in this host should be variant plaques with the desired nucleotide change. The plaques were randomly selected and phage single-stranded DNAs were isolated. In order to confirm the desired mutation, the phage DNA identified by selective track sequencing was subjected to whole sequencing. The pGH2010 DNA containing a single *Bst*EII site and two *Eco*RI sites in the *gshI* gene was digested completely with *Bst*EII and partially with *Eco*RI restriction endonuclease. A 4.5kb DNA fragment from pGH2010α containing the *gshI* gene, in which 0.2 kb N-terminal region of the *gshI* gene has been removed, was recovered from agarose gel. The 0.2 kb *Bst*EII–*Eco*RI fragment containing the mutagenized site isolated from a double-stranded mutant, M13mp19–*gshI* DNA, was recombined to this 4.5 kb DNA fragment of pGH2010α. To substitute the 494–495 amino acids codons of *gshI* with codons for other amino acids, three oligonucleotides were synthesized (Table 1). The mutagenesis was carried out by the previous method (Nam *et al.*, 1991).

The assay of γ-glutamylcysteine synthetase The activity of γ-glutamylcysteine synthetase was measured by the DTNB [5,5′-dithionitro-bis(2-benzoic acid)] method of Jackson (1969) with a slight modification (Nam *et al.*, 1991), unless otherwise specified.

Table 1. Mutagenic oligonucleotides used for making a mutant of γ-glutamylcysteine synthetase (*gshI*).

Sequence of oligonucleotides[a]	Codon substitution
5′-GaggTCAATATGATCCCGGACGT-3′[b]	TTG ATG
5′-CGTTCAGACACCTCGCGCTCGGC-3′	Ala^{494} Val(A494V)
5′-CGTTCAGACTGCTCGCGCTCGGCT-3′	Ala^{494} Leu(A494L)
5′-CGTTCAGTCGCCTCGCGCTCGGC-3′	Ser^{495} Thr(S495T)

[a] Underlined nucleotides are difference from wild-type.
[b] Lower case lettered nucleotides indicate the ribosome binding site (*rbs*).

One unit of enzyme activity of γ-glutamylcysteine synthetase is defined as the amount of enzyme producing 1 mol of γ-glutamylcysteine per hour under the assay condition. Its specific activity was expressed in terms of units per mg of protein.

The enzyme activity of γ-glutamylcysteine synthetase during the feedback inhibition analysis was determined by measuring the amount of released inorganic phosphate (Richman and Meister, 1975). The released inorganic phosphate was determined by Fiske's method (Cooper, 1975). The intensity of the blue color obtained was determined at 720 nm.

Results and Discussion

Initiation codon substitution of γ-glutamylcysteine synthetase gene The lysate of *E. coli* BH5262 cell transformed with the mutant pUC8α–*gshI* DNA was analyzed for γ-glutamylcysteine synthetase activity. The γ-glutamylcysteine synthetase activity of mutant and wild-type is shown in Table 2. The activity of the mutant, in which UUG was substituted with the usual translational initiation codon, AUG, was increased up to 50% compared with that of wild-type.

A study on the relationship between the initiation codon and the translational efficiency has been studied for the genes of β-galactosidase (Munson *et al.*, 1984) and adenylate cyclase (Reddy *et al.*, 1985). As a site-specific mutation occurred at the initial codon from AUG to GUG in the β-galactosidase gene, its activity was decreased to 90% compared with wild-type and the frequency of the translational initiation at this codon was altered. When the initiation codon of the adenylate cyclase gene was changed from UUG to AUG, the translational efficiency was improved more than three fold (Reddy *et al.*, 1985). It implies that the activity increment of γ-glutamylcysteine synthetase is closely related to the translation efficiency. Although the increase of enzyme expression in *gshI* was smaller than those of the β-galactosidase gene and adenylate cyclase gene, the 50% increment was substantial for the *in vitro* production of glutathione. The *gshI* contains the 5′-AGG-3′ sequence known as the typical ribosome binding site (*rbs*). It was thought that the *rbs* sequence of the γ-glutamylcysteine synthetase gene binds strongly to 16S ribosomal RNA while the β-galactosidase gene does not possess such a well-matching consensus *rbs* sequence.

Table 2. Specific activities of γ-glutamylcysteine synthetase mutants by changing the unusual codon (TTG) with the usual codon (ATG).

Strain	Specific activity[a]	Relative activity (%)	Characteristics
BH5262	N.D.[b]	—	
BH5262/pGH2010	1.84	100	TTG
BH5262/pGH201A	2.94	154	ATG

[a] Enzyme assayed by the DTNB method.
[b] N.D. = not detected.

Table 3. Feedback assay of γ-glutamylcysteine synthetase mutants at the various concentrations of reduced glutathione which were determined by the inorganic phosphate assay.

Strain	Characteristics	Relative Activity of *gshI* (%)				
		Glutathione Concentration (mM)				
		0	2.5	5	7.5	10
BH5262/pGH2010 (wild-type)	494^{Ala}–495^{Ser}	100	100	83	82	73
BH5262/pGH2014 (A494V)	494^{Val}–495^{Ser}	100	100	92	80	72
BH5262/pGH2016 (A494L)	494^{Leu}–495^{Ser}	100	99	98	72	60
BH5262/pGH2015 (S495T)	494^{Ala}–495^{The}	100	95	86	76	76

In addition, *gshI* has complementary sequences which fit into the base sequences of the T-loop and D-loop of the clover leaf structure of an initiator tRNA on both sides of the initiation codon UUG (Ganoza *et al.*, 1985). It suggests that the region around the initiation codon, UUG of *gshI*, was adapted to favor initiation of translation (Watanabe *et al.*, 1987).

Feedback inhibition of γ-glutamylcysteine synthetase mutants The enzyme activities of one wild-type and three mutants for the γ-glutamylcysteine synthetase were also determined by the inorganic phosphate assay method (Richman and Meister, 1975) in the presence of reduced glutathione, at concentrations ranging from 0 mM to 10 mM, as shown in Table 3. The activity of wild-type γ-glutamylcysteine synthetase at 10 mM reduced glutathione was decreased up to about 70% of original activity, and the activities of γ-glutamylcysteine synthetase mutants was also decreased to 60~75% of original activity. There was no difference in feedback inhibition levels with reduced glutathione between wild-type and mutants of γ-glutamylcysteine synthetase.

Our present and previous data suggest that the 494th amino acid is not related with the feedback inhibition by reduced glutathione (Nam *et al.*, 1991).

Amino acid substitution of the γ-glutamylcysteine synthetase gene Lysates of *E. coli* BH5262 cells transformed with the mutated pUC8α–*gshI* DNA were analyzed for γ-glutamylcysteine synthetase activity. Three mutants of γ-glutamylcysteine synthetase genes (A494V, A494L, and S494T) by amino acid substitution showed considerable increase in enzyme activity, as shown in Table 4. Substitution of Val or Leu for Ala^{494} in γ-glutamylcysteine synthetase resulted in a 60% increase in the specific activities of γ-glutamylcysteine synthetase.

The activities of all mutant γ-glutamylcysteine synthetases of which the Ala^{494} amino acid was substituted with other amino acids were increased, as shown in Table 3. The size of the R-residue in each substituted amino acid is larger than that of the original amino acid, whether Ala or Ser. All substituted amino acids such as Val and Leu were more hydrophobic than the original amino acids.

The previous data that enzyme activity of the mutant in which Ala^{494} was replaced with Gly was reduced more than that of the wild-type (Nam *et al.*, 1991) was consistent with the present result, since the side chain of Ala is bigger than that of Gly. The role of the individual amino acid residue for the protein stability was investigated by Yutani *et al.* (1984). They suggested that the conformation of the protein stability tended to increase with the hydrophobicity

Table 4. Specific activities of γ-glutamylcysteine synthetase mutants which were produced by site-directed mutagenesis.

Strain (mutant *gshI*)	Specific activity[a] (unit/mg)	Relative activity (%)	Characteristics
BH5262	N.D.[b]	—	
BH5262/pGH2010(wild-type)	1.84	100	494^{Ala}–495^{Ser}
BH5262/pGH2014(A494V)	3.06	166	494^{Val}–495^{Ser}
BH5262/pGH2016(A494L)	3.05	165	494^{Leu}–495^{Ser}
BH5262/pGH2014(S495T)	2.98	162	494^{Ala}–495^{Thr}

[a] Enzyme assayed by the DTNB method.
[b] N.D. = not detected.

of the substituted residue (Gln, Met, Val, Tyr, Leu, Ser, Lys). The conformation of the globular protein was stabilized by the hydrophobic residue in its interior (Yutani *et al.,* 1984).

In fact, a Gly^{77} to Ala substitution mutant of DNA ligase increased the protein stability by the enhancement of its α-helical propensity (Mattews *et al.,* 1987), and Pro to Leu substitution in *lac* permease did affect enzyme activity (Lolkema *et al.,* 1988). It implies that a change of conformation by substitution of Ala with either Val or Leu might increase the specific activity of the mutant γ-glutamylcysteine synthetase by the change of the tertiary structure of the protein conformation through the decrease of unfolding backbone entropy (Matthews *et al.*, 1987; Goodenough, 1995).

Substitution of Ser^{495} to Thr in γ-glutamylcysteine synthetase also increased its specific activity up to 60% (Table 3). This replacement should also have resulted in the increase of the protein stability through the decrease of entropy of protein structure. However, in the case of F_1-ATPase of yeast *Saccharomyces cerevisiae*, the Thr^{197} to Ser substitution increased the activities of these mutant enzymes through the increase of protein flexibility (Mueller, 1989).

From these results, it can be concluded that a substitution to a larger or more hydrophobic molecule in γ-glutamylcysteine synthetase at 494–495 stabilizes the protein tertiary structure by decreasing the entropy of the protein or by altering the protein conformation for a higher specific activity. However, further structural studies are necessary to confirm this conclusion.

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