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Identification of Amino Acid Residues Involved in Feedback Inhibition of the Anthranilate Synthase in *Escherichia coli*

Joon Hyeok Kwak, Kwang Won Hong†, Sung Haeng Lee, Jin Han Hong and Se Yong Lee*
Graduate School of Biotechnology, Korea University, Seoul, Korea
†Department of Food Science and Technology, Dongguk University, Seoul, Korea

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The first step of the branch pathway in tryptophan biosynthesis is catalyzed by anthranilate synthase, which is subjected to feedback inhibition by the end product of the pathway. The *trpE*^{FBR} gene from a mutant *Escherichia coli* strain coding for anthranilate synthase that was insensitive to feedback inhibition by tryptophan has been cloned. To identify the amino acid changes involved in the feedback regulation of anthranilate synthase, the nucleotide sequence of the mutant *trpE*^{FBR} gene was determined. Sequence analysis of the *trpE*^{FBR} gene revealed that four bases were changed in the structural gene while alteration was not found in the 5′ control region. Among these base changes, only two base substitutions caused the alterations in amino acid sequences. From the results of restriction fragment exchange mapping, the 61st nucleotide, C to A substitution, that changed Pro^{21}→Ser was identified as the cause of the desensitization to feedback inhibition by tryptophan. Additional feedback-resistant enzymes of the *E. coli* anthranilate synthases were constructed by site-directed mutagenesis to examine the effect of the Ser^{40}→Arg^{40} change found in the *trpE*^{FBR} gene of *Brevibacterium lactofermentum*. From the feedback inhibition analysis, the Pro^{21}→Ser and Ser^{40}→Arg mutants maintained about 50% and 90% of their maximal activities, respectively, even at the extreme concentration of 10 mM tryptophan. From these results, we suggest that the Pro^{21} and Ser^{40} residues are involved in the tryptophan binding in the *E. coli* enzyme.

* To whom correspondence should be addressed.
Tel: 82-2-3290-3413; Fax: 82-2-927-9028, 82-2-923-9923
E-mail: sylee@kuccnx.korea.ac.kr

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Introduction

In *Escherichia coli*, the *trp* operon consists of five structural genes, which code for enzymes participating in the biosynthetic pathway from chorismate to tryptophan. They are translated from single polycistronic mRNA of approximately 7.0 kb. The genes encoded from the *trp* operon are called *trpE, trpD, trpC, trpB,* and *trpA*. The first enzyme complex unique to tryptophan biosynthesis is the anthranilate synthase complex [EC 4.1.3.27], a tetrameric molecule consisting of two subunits each of component I and component II, the polypeptide products of *trpE* and *trpD*, respectively. This complex catalyzes the anthranilate synthase reaction as well as the phosphoribosyl transferase reaction. Both of these activities are feedback inhibited by tryptophan. Tryptophan binding to the *trpE* subunit exerts a conformational effect that is transferred to the *trpD* subunit, affecting not only the glutamine binding ability of the latter but also its ability to catalyze the phosphoribosyl transferase reaction (Yanofsky and Crawford, 1987). Either chorismate or glutamine competitively reverses the feedback inhibition by tryptophan (Pabst *et al.*, 1973).

We have isolated a tryptophan-analogue resistant mutant of *E. coli* and cloned the mutant gene (Hong, 1994). The *trpE* gene was resistant to feedback inhibition by tryptophan. In order to identify the amino acid affecting the feedback inhibition level of anthranilate synthase, we determined the nucleotide sequence of the *trpE*^{FBR} DNA isolated from the tryptophan-analogue resistant mutant of *E. coli*. Since the Ser^{40}→Arg substitution in *Brevibacterium lactofermentum* anthranilate synthase resulted in feedback inhibition resistance (Mastui *et al.*, 1987), we constructed additional mutants in which Ser^{40} was substituted with Arg by site-directed mutagenesis and we

then examined the role of this amino acid in the feedback inhibition of *E. coli* anthranilate synthase.

Materials and Methods

Strain and plasmid *E. coli* HB101 (Sambrook *et al.*, 1989) strain was used for the subcloning and MV1193 (Sambrook *et al.*, 1989) and RZ1032 (Kunkel, 1985) were used for the site-directed mutagenesis. For the enzyme assay of anthanilate synthase, *E. coli* LD102 (*4att4trpED*) strain was used. To analyze the sequence of the mutant gene, the 2.0 kb *Pst*I-*Hin*dIII fragment of the $trpE^{FBR}$ gene from pMW10 (pSC101-$trpE^{FBR}DCBA$; Hong, 1994) was cloned into pUC119 phagemid at the same sites. The resulting pUC119-$trpE^{FBR}$ plasmid was used for the DNA sequencing by Sequenase ver. 2.0 (USB, Cleveland, USA) and site-directed mutagenesis. The wild-type *trpED* fragment was cloned into pDR720 vector and the resulting plasmid was called pDT31.

DNA sequencing Ease a Base System (Promega Co., Madison, USA) was used to prepare the sequencing template of the mutant $trpE^{FBR}$ gene. DNA sequence analysis was carried out by Sequenase Ver 2.0 (USB, Cleveland, USA).

Site-directed mutagenesis Site-directed mutagenesis was performed by Kunkel's method with slight modification (Kawk *et al.*, 1998). For the construction of the mutant *trpE* gene, the 1.2 kb *Eco*RI-*Bgl*II fragment of the wild-type *trpE* gene was subcloned to pUC119 phagemid. The 23 mer of phosphorylated mutagenic oligonucleotide (5′-GCTGCTGGAACGCGCAGAT ATCG-3′) was annealed to the uracil-containing single-stranded pUC119-*trpE* DNA. After hybridization, heteroduplex DNA was generated by *in vitro* DNA polymerization. *E. coli* MV1193 was transformed with the heteroduplex DNA and the desired mutant was screened by the DNA sequencing method (data not shown). In order to construct the hybrid plasmid, the 1.0 kb *Eco*RI-*Nde*I fragment of the recombinant plasmid containing wild-type *trpE* gene was replaced with the corresponding fragment of the mutant pUC119-*trpE* phagemid (pUC1193). The resulting plasmid in which Ser^{40} was changed to Arg was called pDT36. In order to substitute the Pro^{21} to Ser, a 17 mer of phosphorylated mutagenic oligonucleotide (5′-GCGACAATTCCACCGCG-3′) was annealed to the uracil-containing pUC1193 single-stranded DNA. The heteroduplex DNA was transformed with MV1193 and the mutant was screened by DNA sequencing (data not shown). The 1.0 kb *Eco*RI-*Nde*I fragment of the pDT36 was replaced with the corresponding fragment of the mutant pUC119-*trpE* phagemid (pUC1194) to construct the hybrid plasmid pDT37, wherein Pro^{21} and Ser^{40} have been substituted with Ser and Arg, respectively.

Preparation of cell free extract Cells transformed with recombinant plasmid grown in VB minimal medium (1 g sodium citrate, 5 g dipotassium phosphate, 1.75 g sodium ammonium phosphate, 0.1 g magnesium sulfate, and 2 g glucose per liter) were harvested by centrifugation at 12000 × *g* for 10 min and washed twice with cold 0.85% saline. Cells were resuspended in an appropriate volume of sonication buffer (50 mM potassium phosphate buffer, pH 7.4) and disrupted by an ultrasonicator. The cell debris was removed by centrifugation at 16000 × *g* for 30 min at 4°C and the supernatant was used as a crude enzyme source for the anthranilate synthase assay.

Assay of anthranilate synthase The enzyme activity was assayed according to the method of Tamir and Srinivasan (1970). The incubation mixture contained 0.05 ml of 10 mM chorismate in 20 mM potassium phosphate buffer (pH 7.0), 0.05 ml of 1 M glutamine, 0.05 ml of 1 M potassium phosphate buffer (pH 7.0), 0.05 ml of 0.2 M of magnesium chloride, and enzyme to a final volume of 1.0 ml. After incubation at 37°C for 10 min, the reaction was stopped by the addition of one-fourth vol of 27% trichloroacetic acid. After centrifugation, the supernatant was treated with 0.03 ml of 0.1% sodium nitrite and allowed to stand at room temperature for 10 min. Then, 0.3 ml of 0.5% ammonium sulfate was added. After 3 min, 0.3 ml of 0.1% N-1-naphtyl-ethylene-diamine HCl in 95% ethanol was added. The color was allowed to develop for an hour at room temperature and its intensity was then measured at 540 nm. Specific activity was expressed as 0.1 μmol of anthranilate formed per mg of protein per 10 min at 37°C.

Results and Discussion

Determination of nucleotide sequence of the mutant $trpE^{FBR}$ gene From the analysis of the nucleotide sequence of the $trpE^{FBR}$ gene, four base substitutions were identified in the open reading frame (data not shown). No alteration was found in the 5′ end control region of the *trp* operon. Among these base substitutions, only two single-base substitutions resulted in Pro^{21}→Ser and Ser^{147}→Gly changes; C→T at the 61st nucleotide and A→G at the 439th nucleotide, respectively.

To identify which nucleotide substitution caused the desensitization to feedback inhibition of anthranilate synthase by tryptophan, specific DNA fragments were removed from the wild-type *trpED* plasmid (pDT31; Pro^{21} and Ser^{147}) and replaced with the corresponding DNA fragment from the mutant *trpE* gene. The resulting plasmids containing the mutant *trpE* gene were called pDT32 (original mutant, Ser^{21} and Gly^{147}), pDT33 (Ser^{21} and Gly^{147}), pDT34 (Ser^{21} and Ser^{147}), and pDT35 (Pro^{21} and Gly^{147}), (Table 1).

Characterization of anthranilate synthase variants Recombinant plasmids containing the wild-type and mutant *trpE* genes (pDT31, pDT32, pDT33, pDT34, and pDT35) were transformed into *E. coli* strain LD102 ($trpED^-$). Transformants were screened for Trp^+ phenotype on VB minimal agar medium containing 100 μg/ml ampicillin. The anthranilate synthase activities of cell free extracts of LD102 harboring recombinant *trpED* plasmids were assayed for sensitivity to feedback inhibition by tryptophan. As shown in Fig. 1, the mutant enzyme from pDT32 (Ser^{21}, Gly^{147}) was fully active in the presence of 1 mM tryptophan, while the wild-type enzyme from pDT31 (Pro^{21}, Ser^{147}) under the same condition demonstrated less than 10% of the original activity. Furthermore, the activity of the mutant enzyme from pDT32 retained 50% of its original activity in the presence of 10 mM tryptophan while the activity of wild-type

Table 1. Construction of various plasmids containing mutant *trpE* genes.

Plasmid	Nucleotide substitution	Amino acid change
pDT31	—	Wild-type
pDT32*	$C^{61}\rightarrow T$, $A^{439}\rightarrow G$	$Pro^{21}\rightarrow Ser^{21}$, $Ser^{147}\rightarrow Gly^{147}$
pDT33**	$C^{61}\rightarrow T$, $A^{439}\rightarrow G$	$Pro^{21}\rightarrow Ser^{21}$, $Ser^{147}\rightarrow Gly^{147}$
pDT34	$C^{61}\rightarrow T$	$Pro^{21}\rightarrow Ser^{21}$
pDT35	$A^{439}\rightarrow G$	$Ser^{147}\rightarrow Gly^{147}$
pDT36	$T^{118}CC\rightarrow CGC$	$Ser^{40}\rightarrow Arg^{40}$
pDT38	$C^{61}\rightarrow T$, $T^{118}CC\rightarrow CGC$	$Pro^{21}\rightarrow Ser^{21}$, $Ser^{40}\rightarrow Arg^{40}$

*: containing the *trpE* gene cloned from the original mutant.
**: constructed by exchange of the DNA fragment containing the mutated region.

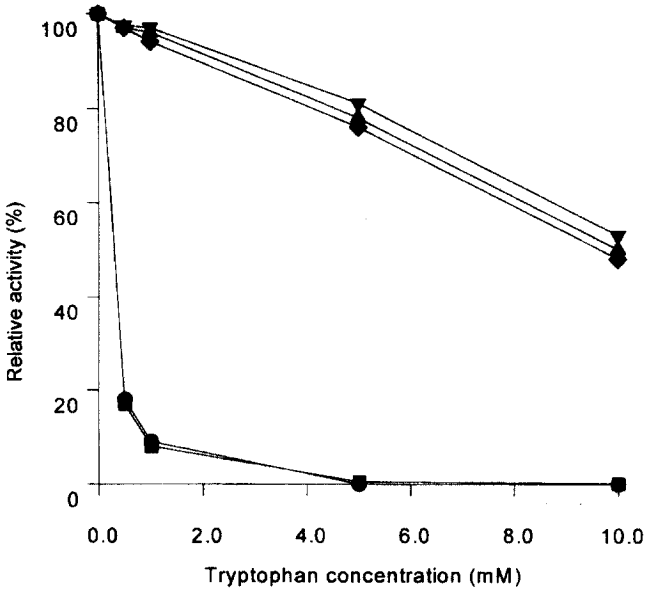

Fig 1. Effects of $Pro^{21}\rightarrow Ser$ and $Ser^{147}\rightarrow Gly$ changes on the tryptophan sensitivity of anthranilate synthase in *E. coli* LD102 (*trpED*$^-$) containing various *trpED* plasmids. The circle indicates wild-type anthranilate synthase (Pro^{21}, Ser^{147}), the square indicates mutant anthranilate synthase (Pro^{21}, Gly^{147}), the triangle indicates mutant anthranilate synthase from the original mutant (Ser^{21}, Gly^{147}), the reverse triangle indicates mutant anthranilate synthase constructed by restriction fragment exchange (Ser^{21}, Gly^{147}), and the diamond indicates mutant anthranilate synthase (Ser^{21}, Ser^{147}). The activity of anthranilate synthase was determined by the method of Tamir and Srinvivasan (1970).

anthranilate synthase was completely inhibited by tryptophan at this concentration. The feedback inhibition pattern of mutant anthranilate synthase encoded by pDT33 was similar to the original mutant, pDT32. Therefore, it may be suggested that the changes of two amino acids conferred the feedback inhibition resistance by tryptophan to anthranilate synthase. The activity of the hybrid enzymes from pDT34 (Ser^{21}, Ser^{147}) was nearly as insensitive to tryptophan as the mutant enzyme from pDT32. However, the other hybrid mutant from pDT35 (Pro^{21}, Gly^{147}) showed a similar feedback-inhibition pattern to the wild-type enzyme from pDT31. From these results, a single nucleotide change ($C^{61}\rightarrow T$), which resulted in $Pro^{21}\rightarrow Ser$ changes, was identified as the cause of the desensitization of *E. coli* anthranilate synthase to feedback inhibition. The Pro residue in protein decreases protein flexibility and changes the α-helical structure (Matthews *et al.*, 1987). The $Pro^{21}\rightarrow Ser$ substitution might have affected the protein folding structure and decreased the binding affinity of anthranilate synthase for tryptophan. Since Pro^{21} is near the anthranilate synthase conserved region which was known as a binding site for tryptophan (Graf, *et al.*, 1993), the substitution of Ser^{21} for Pro might affect on the structure of the binding site.

Construction of *trpE*^{FBR} mutants by site-directed mutagenesis In order to know the amino acids related to the feedback inhibition in detail, Pro^{21} and Ser^{40} were changed to other amino acids. Since the amino acid Ser^{40} was related to feedback inhibition resistance of anthranilate synthase in *B. lactofermentum* (Matsui *et al.*, 1987) and the amino acid was conserved in *E. coli* anthranilate synthase, Ser^{40} was substituted with Arg by site-directed mutagenesis (pDT36; $Ser^{40}\rightarrow Arg$). Since, in the above experiment, the $Pro^{21}\rightarrow Ser$ mutation increased feedback inhibition resistance, it was also substituted with Ser by site-directed mutagenesis (pDT37; $Pro^{21}\rightarrow Ser$, $Ser^{40}\rightarrow Arg$). The mutant and wild-type *trpED* plasmids were transformed into *E. coli* LD102 (*trpED*$^-$) strain.

Effect of Ser^{40} to Arg^{40} change on the tryptophan sensitivity of anthranilate synthase The activities of the wild-type and the mutant anthranilate synthases were analyzed in the presence of tryptophan as shown in Fig. 2. The activity of the mutant enzyme from pDT32 (Ser^{21}, Gly^{147}) was maintained to a maximum of about 50% in the presence of 10 mM tryptophan, while that of the wild-type enzyme from pDT31 (Pro^{21}, Ser^{40}) under the same condition was almost completely inhibited. However, the activities of pDT36 (Pro^{21}, Arg^{40}) enzyme and pDT37 (Ser^{21}, Arg^{40}) enzyme were maintained at about 90% of the

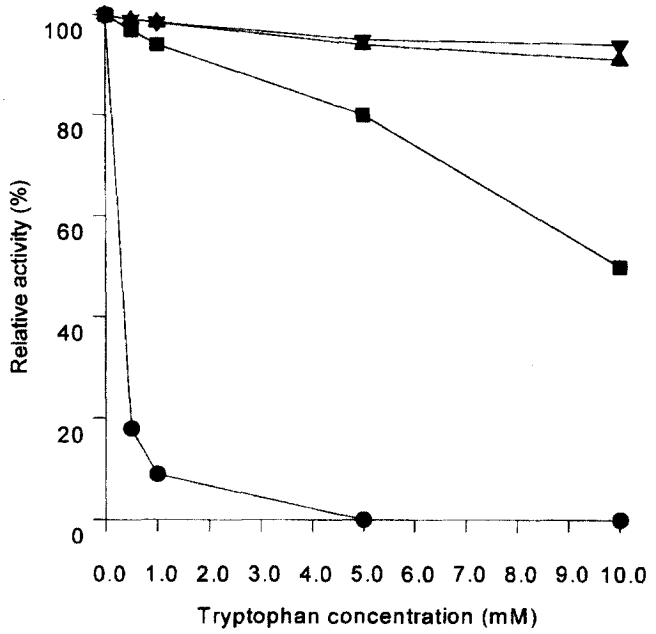

Fig. 2. Effects of Ser^{40} to Arg change on the tryptophan sensitivity of anthranilate synthase in *E. coli* LD102 (*trpED*$^-$) containing various *trpED* plasmids. The circle indicates wild-type anthranilate synthase (Pro^{21}, Ser^{40}), the square indicates mutant anthranilate synthase (Pro^{21}, Ser^{40}), the triangle indicates mutant anthranilate synthase (Ser^{21}, Arg^{40}), and the reverse triangle indicates mutant anthranilate synthase (Pro^{21}, Arg^{40}). The activity of anthranilate synthase was determined in the presence of tryptophan by the method of Tamir and Srinvivasan (1970).

maximum activities in the presence of 10 mM tryptophan. These results indicated that the alterations of Ser^{40}→Arg and Pro^{2}→Ser were related do the desensitization of feedback inhibition by tryptophan. Furthermore, the alteration of Ser^{40}→Arg was more effective than that of Pro^{21}→Ser for desensitization of feedback inhibition by tryptophan. Since the amino acid sequences around Ser^{40} (LLES) were conserved in many organisms, it would play an important role in the enzyme reaction (Fig. 3). Mutations of anthranilate synthase causing feedback resistance have been characterized in *B. lactofermentum*, *Saccharomyces cerevisiae*, and *Salmonella typhimurium* (Matsui *et al.*, 1987; Graf *et al.*, 1991; Caligiuri and Bauerle, 1993). Mutation of Ser^{40}→Phe in *S. typhimurium* anthranilate synthase caused an alteration in the tryptophan-induced conformational change and decreased kinetic response to tryptophan binding (Caligiuri and Bauerle, 1993). In this case, tryptophan binding to the enzyme was completely inhibited while the binding affinity for substrate was not changed. Graf *et al.* (1991) showed that a single amino acid change (Ser^{76}→Leu) among (LLES) amino acids, which were conserved in *E. coli* anthranilate synthase, caused desensitized feedback inhibition in *S. cerevisiae*. Therefore, it was concluded that Ser^{40}→Arg substitution in *E. coli* anthranilate synthase

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EC   1 -MQTQKPTLELLTCEGAYRDNPTALFHQLCGDRPA-TLLLESADIDSKDDLKSLLLVDSA 58
BL   1 ----MSTNPHVFSLDVRYHEDASALFAHLGGTTADDAALLESADITTKNGISSLAVLKSS 55
ST   1 -MQTPKPTLELLTCDAAYRENPTALFHQVCGDRPA-TLLLESADIDSKDDLKSLLLVDSA 58
HP   1 ---------MISLIEKAPYIPYPLALYEKLEQPHTL---LFESAEIESKAHTKSLLMAKAC 49
HI   1 MNIQTQAFIAVTAQPIPYYADTTAIFNTLCQSNSN-SLLLDSAEIGSKNSLQSLILVNAA 59
BA   1 -MKKSAYPIEIIQKQAPYHPDPTMIFNHLCESRSE-TLLLETADNK-KKRSRKIMIIDSA 57
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Fig. 3 Alignment of anthranilate synthase domains from various organisms. The asterisk indicates Ser^{40} in the conserved region. EC, *E. coli;* BL, *B. lactofermentum;* ST, *S. thyphimurium;* HP, *Helicobacter pylori;* HI, *Haemophilus influenza;* BA, *Buchinai aphidicola.* Numbers indicate amino acids.

decreased the binding affinity for tryptophan by changing the conformation of the anthranilate synthase-tryptophan complex (Caligiuri and Bauerle, 1991).

The specific activities of the mutant anthranilate synthetases were much higher than that of the wild-type enzyme at the 10 mM tryptophan level although the substitutions of Pro^{21}→Ser and Ser^{40}→Arg decreased the activities of the mutant by about 65% at the 0 mM tryptophan level. In plant, $trpE^{FBR}$ mutation increased the tryptophan contents in arabidopsis (Li and Last, 1995). Therefore, the feedback-resistant mutant of anthranilate synthase would be useful for tryptophan production.

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