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Serine 187 is a crucial residue for allosteric regulation of *Corynebacterium glutamicum* 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase

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Abstract

Corynebacterium glutamicum 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase is sensitive to feedback inhibition by tyrosine. One feedback-insensitive mutant was obtained by in vitro chemical mutagenesis and the mutation was identified as a C→G mutation at nucleotide 560 causing a Ser^{187} to Cys^{187} substitution. Replacing Ser^{187} with cysteine, tyrosine or phenylalanine by site-directed mutagenesis not only reduced the enzymatic activity but also relieved its feedback inhibition by tyrosine, while Ser^{187}Ala exhibited a comparable activity to that of wild-type enzyme and sensitized to allosteric regulation. The His_6-tagged enzymes were expressed in *Escherichia coli* and purified to homogeneity by immobilized nickel-ion affinity chromatography. Kinetic analysis showed that tyrosine is a competitive inhibitor of phosphoenol pyruvate, one of the precursors for DAHP biosynthesis. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: 3-Deoxy-D-arabino-heptulosonate synthase; Allosteric regulation; Site-directed mutagenesis; *Corynebacterium glutamicum*

1. Introduction

The aromatic amino acids tryptophan, phenylalanine, and tyrosine are synthesized by a sequence of reactions starting with the formation of 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) through the condensation of phosphoenol pyruvate (PEP) and erythrose-4-phosphate (E4P). This reaction is achieved by the action of DAHP synthase (EC 4.1.2.15) [1]. This enzyme serves as the primary site for feedback regulation of carbon flow into chorismate synthesis. In *Escherichia coli*, three isoenzymes of DAHP synthase are sensitive to phenylalanine, tyrosine, and tryptophan, respectively [2]. However, the DAHP synthase from *Corynebacterium glutamicum* and its closely related strain are synergistically inhibited by phenylalanine and tyrosine [3].

The high degree of similarity in the primary sequences of microbial DAHP synthases, such as the three *E. coli* isoenzymes [4–6], the two *Saccharomyces cerevisiae* isoenzymes [7,8], *Candida albicans* [9], *Salmonella typhimurium* [10] and *C. glutamicum* [11], is a convincing evidence that the enzymes have a common evolutionary origin and common structural properties. Although a mutational study of tryptophan-sensitive isoenzyme of *E. coli* suggests that the active and feedback sites are immediately adjacent in the primary structure [6], relationships between molecular structure and function in microbial DAHP synthases are not yet well understood. We had cloned DAHP synthase gene *dahps* from *C. glutamicum* [11]. In this study, we found that *C. glutamicum* DAHP synthase is sensitive to tyrosine. An amino acid residue, Ser^{187}, suggested to be involved in the feedback inhibition was identified. In addition, four site-directed mutants (Ser^{187}Ala, Ser^{187}Cys, Ser^{187}Tyr, and Ser^{187}Phe) of *C. glutamicum* DAHP synthase were constructed, purified, and characterized. Our results reveal that this serine residue plays a critical role

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Abbreviations: DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate; E4P, erythrose-4-phosphate; PEP, phosphoenol pyruvate; 3AT, 3-amine-tyrosine

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Table 1
Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties	References
E. coli strains		
DH5α	*supE44*Δ *lacU169*(ϕ*80lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	[14]
M15	nal^+ str^s rif^s lac^- ara^- gal^- mtl^- F^- $recA^+$ uvr^+ lon^+	Qiagen Inc.
C. glutamicum LS1183	Transformable mutant	[13]
Plasmids		
pSUDS-18	Km^r Cm^r *dahps*	[11]
pUC-DS	Ap^r *lacZ dahps*	This study

for the feedback regulation of *C. glutamicum* DAHP synthase.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown at 37°C in Luria–Bertani (LB) medium [14]. Growth was monitored spectrophotometrically at a wavelength of 600 nm. As required, ampicillin and kanamycin were used at concentrations of 100 and 40 μg ml^{-1}, respectively.

2.2. DNA techniques

Plasmid DNA was isolated from *E. coli* by alkali lysis method [14] and further purified by using plasmid preparation kits from Viogene-Biotek Corporation (Taipei, Taiwan). Restriction enzyme digestion, agarose gel electrophoresis, isolation of DNA fragments from agarose gels, transformations and in vitro chemical mutagenesis were carried out by standard methods [14]. PCR amplifications were performed with a Thermal Cycler 9600 (Perkin-Elmer, Norwalk, CT, USA). Conditions used were an initial denaturation for 2 min at 94°C, followed by 30 amplification cycles (denaturation at 94°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 1 min) and a final extension for 10 min at 72°C.

Table 2
Oligonucleotide primers used in PCR and site-directed mutagenesis

Primer for	Sequence
PCR	
DS-1	5′-*GGATCC*CTTCGATATTGAGG-3′ *Bam*HI
DS-2	5′-TGC*GAGCTC*TGTGTTGTTAA-3′ *Sac*I
Site-directed mutagenesis	
Ser^{187}Ala	5′-GGGATG*GCT*ATGCCAATTGG-3′
Ser^{187}Cys	5′-GGGATG*TGT*ATGCCAATTGG-3′
Ser^{187}Tyr	5′-GGGATG*TAT*ATGCCAATTGG-3′
Ser^{187}Phe	5′-ATG*TTT*ATGCCAATTGGTTT-3′

2.3. Plasmid constructions and site-directed mutagenesis

Plasmid pSUDS-18 was used as a template for PCR amplification of the *dahps* gene with primers DS-1 and DS-2 (Table 2). After cleavage of the PCR product with *Bam*HI and *Sac*I, the resulting fragment was ligated to the corresponding sites of pUC18 to make pUC-DS. For site-directed mutagenesis of *dahps* gene, a 1.85-kb *Bam*HI–*Sac*I DNA fragment was excised from pUC-DS and subcloned into replicative form of bacteriophage M13mp18. Mutagenesis was carried out according to Kunkel's method [12] with the Bio-Rad Muta-Gene Version 2 Mutagenesis kit (Bio-Rad Laboratories, Richmond, CA, USA). Oligonucleotides listed in Table 2 were synthesized to alter Ser^{187} to Ala, Cys, Tyr, and Phe. Potential mutants were sequenced by single-stranded DNA sequencing using Sequenase Version 2.0 (United States Biochemical Co., OH, USA) to ensure the successful mutations. The *Bam*HI–*Pst*I DNA fragment of the desired clones was then subcloned into pQE-30 for enzyme characterization.

2.4. Expression and purification of wild-type and mutant DAHP synthases

E. coli M15 (pREP4) cells carrying plasmids described in this work were grown at 37°C in LB containing ampicillin and kanamycin. When the OD_{600} of the culture reached 0.8, IPTG was added at a final concentration of 1 mM, and growth was continued for 3 h. Cells were harvested and washed with 3 ml of cold 20 mM Tris–HCl (pH 7.9) buffer containing 5 mM imidazole and 0.5 M NaCl. Bacterial cells were disrupted by sonication (30-s bursts for 3 min) in the cold, and the resulting suspension was centrifuged at 4°C for 30 min at 15 000 × *g*. The supernatant was then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis and protein purification.

One-step purification procedure for His_6-tagged enzymes was carried out under native conditions according to the protocol of the manufacturer (Qiagen Inc., Valencia, CA, USA).

2.5. Enzyme assays and steady-state kinetics

The catalytic activity was determined by monitoring

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colorimetrically the formation of DAHP from the substrates according to the procedure described by Shiio et al. [15]. Unless otherwise specified, the standard assay mixture consisted of 50 mM Tris–HCl buffer (pH 7.9), 0.5 mM E4P, 0.5 mM PEP, and 10 mM $CoCl_2$. All the assays were initiated with the addition of the enzyme and were performed in triplicate. One unit of activity was defined as the amount of enzyme required to produce 1 μmol of DAHP per min at 30°C, taking the molar extinction coefficient of the DAHP as 4.5×10^4 at 549 nm.

A steady-state kinetics study of wild-type and mutant enzymes was performed with PEP and E4P by systematic variation of the concentrations of both substrates. The K_m and V_{max} values were determined by fitting the initial rates as a function of substrate concentrations to the Michaelis–Menten equation using Grafit software (Sigma Chemical Co., MO, USA).

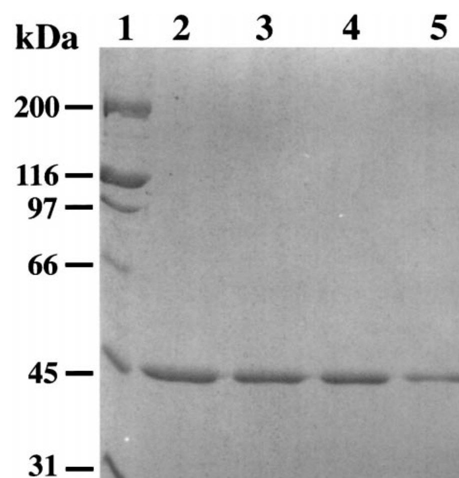

Fig. 1. SDS–PAGE analysis of the wild-type and mutant DAHP synthases. Lanes: 1, protein size markers; 2, purified wild-type DAHP synthase; 3, purified Ser^{187}Ala; 4, purified Ser^{187}Cys; 5, purified Ser^{187}Tyr.

3. Results and discussion

3.1. Mutational analysis and expression of *C. glutamicum* DAHP synthases

In vitro mutagenesis of the *dahps* gene was used to probe the key amino acid residue important for regulatory function. The 1.85-kb *Bam*HI–*Sac*I fragment containing *dahps* gene was prepared from plasmid pUC-DS and then mutagenized with nitrous acid. The mutated products were cloned into *Bam*HI–*Sac*I restricted pSUMN18, an *E. coli* coryneform bacteria shuttle vector [11]. The recombinant plasmids were transformed into *C. glutamicum* LS1183 [13]. Transformants were selected by their ability to grow on the CM plates [16] containing chloramphenicol (50 μg ml^{-1}). Cm^r transformants were then replicated on MM plates [16] containing 3-aminotyrosine (3AT, 3 mM) and/or *m*-fluorophenylalanine (7 mM). One $3AT^r$ Cm^r transformant was obtained from about 10 000 Cm^r transformants from which a recombinant plasmid, designated pSUDSM, was isolated. The wild-type and mutant plasmids were transformed into *E. coli* DH5α to compare their DAHP synthase activities. The crude enzyme preparations were assayed for DAHP synthase activities in the presence of 300 μM Tyr. *E. coli* DH5α (pSUDS-18) exhibited a significant inhibition with an inhibition efficiency of about 87%, whereas less than 5% inhibition was found in bacterial cells containing pSUDSM. To elucidate the genotypic change in mutant *dahps* gene, plasmid pSUDSM was used as a template for DNA sequencing. When compared with the *C. glutamicum dahps* gene [11], a C→G mutation was observed at nucleotide 560, resulting in Ser^{187} to Cys substitution.

To confirm the role of Ser^{187} in *C. glutamicum* DAHP synthase, we generated four mutations at this position by site-directed mutagenesis. The wild-type and mutant *dahps* genes were cloned into pQE-30 and expressed in *E. coli* M15 (pREP4). As shown in Fig. 1, the wild-type and mutant enzymes exhibited a protein band with a molecular mass of approximately 45 kDa.

3.2. Characterization of wild-type and mutant enzymes

The specific activity was reduced by the mutation introduced at position 187 of DAHP synthase. Losses of 6, 40, 89, and 92% were observed for Ser^{187}Ala/Cys/Phe/Tyr, respectively. The decrease in enzymatic activity could be due to steric hindrance caused by the branch chain from substituted amino acid residue since the activity has a reverse proportion to the size of side chain.

Table 3
Effect of phenylalanine and tyrosine on the specific activity of *C. glutamicum* DAHP synthase

Enzyme	Specific activity (U mg^{-1} of protein)[a]			
	None	Tyr[b]	Phe[b]	Phe+Tyr[b]
Wild-type	159.6 ± 13.9	10.7 ± 0.9	147.0 ± 10.9	11.0 ± 1.0
Ser^{187}Ala	150.7 ± 10.3	12.5 ± 1.1	150.7 ± 13.4	13.3 ± 0.9
Ser^{187}Cys	95.9 ± 8.7	140.5 ± 12.9	112.9 ± 10.1	131.2 ± 11.9
Ser^{187}Tyr	16.9 ± 1.3	40.7 ± 2.1	21.6 ± 3.9	36.8 ± 3.3
Ser^{187}Phe	18.3 ± 1.9	37.9 ± 1.8	20.4 ± 2.1	33.7 ± 2.7

[a]The data represent the average values of three measurements.
[b]Tyrosine and phenylalanine concentrations in the reaction mixture were 300 μM and 700 μM, respectively.

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Table 4
A comparison of the kinetic data of the wild-type and the mutant forms of DAHP synthase[a]

Enzyme	Tyrosine (μM)	V_{max} (μmol^{-1} min^{-1} mg^{-1})	K_m (μM)	
			PEP	E4P
Wild-type	0	5.85 ± 0.41	3.24 ± 0.29	17.87 ± 1.05
	50	4.90 ± 0.23	15.82 ± 1.37	16.03 ± 1.23
	100	4.31 ± 0.15	37.05 ± 2.89	17.42 ± 1.75
	200	3.97 ± 0.19	69.94 ± 5.91	16.53 ± 0.98
	300	3.51 ± 0.17	83.24 ± 7.01	17.15 ± 1.91
Ser^{187}Ala	0	5.36 ± 0.48	0.91 ± 0.05	6.30 ± 0.53
	50	5.33 ± 0.39	3.01 ± 0.32	6.20 ± 0.39
	100	5.75 ± 0.27	10.24 ± 1.05	6.12 ± 0.51
	200	4.94 ± 0.24	30.95 ± 2.58	6.37 ± 0.60
	300	4.24 ± 0.39	41.01 ± 3.97	6.44 ± 0.63
Ser^{187}Cys	0	2.27 ± 0.19	1.76 ± 0.12	8.21 ± 0.59
	50	2.29 ± 0.11	1.95 ± 0.18	8.97 ± 0.78
	100	2.50 ± 0.17	1.68 ± 0.10	9.21 ± 0.91
	200	2.74 ± 0.20	1.90 ± 0.19	9.05 ± 0.69
	300	2.89 ± 0.23	2.01 ± 0.16	8.98 ± 0.58

[a]The kinetic parameters were determined as described in Section 2 at different concentrations of tyrosine. The data represent the average values of three measurements.

The purified enzymes were assayed for feedback sensitivity to Tyr and Phe, respectively. As shown in Table 3, the wild-type enzyme and Ser^{187}Ala were sensitive to Tyr with an approximately 93 and 92% inhibition efficiency, respectively, whereas the other mutant enzymes were completely resistant to Tyr inhibition. Like the wild-type DAHP synthase, the mutant enzymes were active in the presence of 700 μM Phe. These results indicate that *C. glutamicum* DAHP synthase is a Tyr-sensitive enzyme. It is worth to note that Ser^{187}Cys/Tyr/Phe was not only resistant to Tyr inhibition but also activated by the end products, Tyr and Phe (Table 3). Interestingly, the enhancement of enzymatic activity by the end product was also reported in Phe-insensitive AroG [17].

A steady-state kinetics study of wild-type and mutant enzymes was performed with PEP and E4P by systematic variation of the concentrations of both substrates. As shown in Table 4, the apparent K_m value of the wild-type enzyme for PEP was 3.24 mM. The value was remarkably increased to 83.24 mM in the presence of 300 μM Tyr. Similarly, Ser^{187}Ala exhibited an increased K_m value even at concentration as low as 50 μM; however, no significant alteration was found in the apparent K_m value of Ser^{187}Cys. With E4P as the substrate, Tyr did not cause a significant change in V_{max} and K_m values of wild-type and mutant enzymes. These results clearly indicated that Tyr is a competitive inhibitor of *C. glutamicum* DAHP synthase for PEP. Similarly, feedback inhibition of tyrosine-sensitive DAHP synthase by Tyr has been shown to be competitive with the substrate PEP [18].

3.3. The role of Ser^{187} in allosteric mechanism

The fusion of a common catalytic domain with Trp-, Phe- or Tyr-binding domains has led to the attainment of specific feedback regulation in each isoenzyme [19]. However, the simultaneous loss of catalytic activity and feedback inhibition of Trp-sensitive DAHPS implies that functionally important regions probably exist in the overlapping sequences [7]. The mutational study on Phe-sensi-

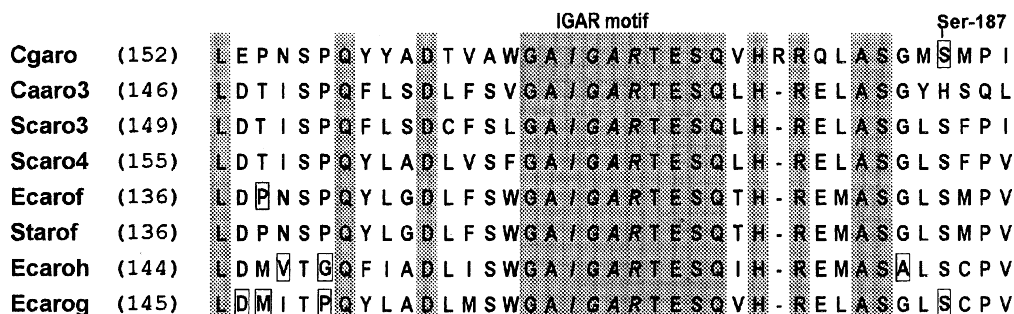

Fig. 2. Sequence comparison of DAHP synthases surrounding the IGAR motif. Amino acid residues are expressed in one-letter codes and numbered from the translational methionine of each enzyme. Conserved amino acid residues are in gray boxes. The amino acid residues involved in allosteric regulation are in open boxes. Cgaro, *C. glutamicum* DAHP synthase; Caaro3, *C. albicans* DAHP synthase; Scaro3 and Scaro4, *S. cerevisiae* DAHP synthase isoenzymes; Starof, *S. typhimurium* DAHP synthase; Ecarof, Ecaroh and Ecarog, *E. coli* DAHP synthase isoenzymes.

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tive DAHP synthase also reveals that the catalytic and feedback inhibition site is located in the same domain [20]. In our case, the mutant enzymes were insensitive to Tyr and had a reduced specific activity. Therefore, it seems likely that the catalytic and regulatory sites are overlapped in the enzyme. As defined by Walker et al. [21], type I DAHP synthase as *E. coli*-like homologs having a subunit M_r of approximately 39 kDa. The conspicuous motifs GPCS, KPRTS/T and IGAR in these enzymes are proposed to be important for catalytic activity [22]. Using in vitro chemical mutagenesis, the residues responsible for feedback regulation of *E. coli* DAHP synthases are identified [6,23]. As shown in Fig. 2, some of the key amino acid residues are around the IGAR motif. It is evident that tyrosine acts as a competitor for the binding of PEP to the tyrosine-sensitive DAHP synthase and phenylalanine plays as a competitor for the binding of E4P to the phenylalanine-sensitive enzyme [18,24]. Based on the above facts, it is reasonable to speculate that the residues shown to be important for feedback inhibition are uniquely correlated with the specificity of feedback inhibitor. As shown in Fig. 2, except the ARO3 from *C. albicans*, the encompassing residue of Ser^{187} of *C. glutamicum* DAHP synthase is well conserved among different enzymes. Thus, this residue does not seem to participate directly in the feedback specificity of DAHPS isoenzyme. In *C. glutamicum* DAHP synthase, the replacement of Ser^{187} with Cys, Phe or Tyr resulted in a marked resistance to feedback inhibition. Since the insensibility of the enzyme to tyrosine is related to the bulk of substituted amino acids, the steric hindrance seems to be important in the feedback inhibition process. To function as part of a direct binding site, amino acid residues of the enzyme should be in the substrate-accessible hydrophilic domain. In fact, Ser^{187} of *C. glutamicum* DAHP synthase is located in a hydrophobic domain (data not shown), indicating it would not have a direct interaction with Tyr during allosteric regulation.

In conclusion, we have demonstrated that Ser^{187} of *C. glutamicum* DAHP synthase is an essential residue for the feedback inhibition. Rather than being directly involved in the binding of competitive inhibitor, it may play a role in intramolecular interactions linked to feedback inhibition.

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