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Identification of Essential Cysteine Residues in 3-Deoxy-D-Arabino-Heptulosonate-7-Phosphate Synthase from *Corynebacterium glutamicum*

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Abstract. To ascertain the functional role of cysteine residue in 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (DAHP) synthase from *Corynebacterium glutamicum*, site-directed mutagenesis was performed to change each of the three residues to serine. Plasmids were constructed for high-level overproduction and one-step purification of histidine-tagged DAHP synthase. Analysis of the purified wild-type and mutant enzymes by SDS-polyacrylamide gel electrophoresis showed an apparent protein band with a molecular mass of approximately 45 kDa. Cys^{145}Ser mutant retained about 16% of the enzyme activity, while DAHP synthase activity was abolished in Cys^{67}Ser mutant. Kinetic analysis of Cys^{145}Ser mutant with PEP as a substrate revealed a marked increase in K_m with significant change in k_{cat}, resulting in a 13.6-fold decrease in k_{cat}/K_m^{PEP}. Cys^{334} was found to be nonessential for catalytic activity, although it is highly conserved in DAHP synthases. From these studies, Cys^{67} appears important for synthase activity, while Cys^{145} plays a crucial role in the catalytic efficiency through affecting the mode of substrate binding.

The enzyme 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase (EC 4.1.2.15) catalyzes the condensation of phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P) to produce DAHP and Pi [27]. This reaction is the first step in the biosynthesis of aromatic amino acids in microorganisms and plants. According to the mode of feedback inhibition, the enzyme is classified into phenylalanine-sensitive DAHP synthase (*aroG* product), tryptophan-sensitive DAHP synthase (*aroH* product), and tyrosine-sensitive DAHP synthase (*aroF* product) [26, 35, 37]. The primary sequences of DAHP synthases from the three *Escherichia coli* isoenzymes [5, 21, 24], the two *Saccharomyces cerevisiae* isoenzymes [11, 17], *Candida albicans* [19], *Salmonella typhimurium* [14], and *Corynebacterium glutamicum* [3] show a high degree of similarity throughout the polypeptide, indicating a common ancestry and conserved structural properties.

It has been reported that the phenylalanine-sensitive DAHP synthase of *E. coli* was inactivated reversibly by *p*-hydroxymercuribenzoate and irreversibly by 5,5-dithiobis-2-nitrobenzoate [28]. Some reports also demonstrated similar sensitivity to sulfhydryl modification in DAHP synthases from *S. typhimurium* [15], *Neurospora crassa* [6], and *Brevibacterium* sp. [32]. These observations indicate that the DAHP synthases appear to require at least one cysteine sulfhydryl for enzymatic activity. Although the role of cysteine residues in enzyme function has not yet been established, a reaction mechanism in which an active site cysteine forms a thioester intermediate with PEP was proposed [7]. It has been speculated that Cys^{61} of *E. coli* phenylalanine-sensitive DAHP synthase may play a critical role in metal binding and catalytic activity, and Cys^{328} is nonessential for the enzymatic activity, but it may lie near the active site [30]. Previously, we have reported the molecular cloning and DNA sequence of the DAHP synthase (*dahps*) gene from *C. glutamicum* [3]. The enzyme contains three cysteine residues at positions 67, 145, and 334, respectively. In

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Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype	References
E. coli strains		
DH5α	*supE44Δ lacU169(ϕ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	9
M15	*nal*$^+$ *str*^{s} *rif*^{s} *lac*$^-$ *ara*$^-$ *gal*$^-$ *mtl*$^-$ F$^-$ *recA*$^+$ *uvr*$^+$ *Ion*$^+$	Qiagen Inc.
JM109	*recA supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB)*	36
CJ236	*dut1 ung1 thi-1 relA1/pCJ105(cam*^{r} F′)	10
MV1190	*Δ(lac-proAB) thi* supE *Δ(srl-recA)*306::Tn10 (*tet*^{r}) F′(*traD36 proAB lacI*^{q} *lacZΔM15*)	36
Plasmids:		
pSUDS-18	Km^r Cm^r *dahps* gene	3
pUC19	Ap^r *lacZ*	36
M13mp18	Ap^r *lacZ*	36
pUC-DS	Ap^r *lacZ dahps* gene	This study
pM13-DS	Ap^r *lacZ dahps* gene	This study
pQE-DS	Ap^r *lacZ dahps* gene	This study

this study, we describe the mutational modification of cysteine residues of *C. glutamicum* DAHP synthase to investigate the role of these residues in the enzymatic activity.

Materials and Methods

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* DH5α was used for plasmid construction and propagation. *E. coli* was cultivated at 37°C on Luria-Bertani medium [22], and, when required for plasmid selection, ampicillin (100 μg/ml) was included.

Enzymes, reagents, and DNA manipulations. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (MA, USA) or Boehringer Mannheim Biochemica (Germany). *Pfu* DNA polymerase was purchased from Promega Co. (Madison, WI, USA). Media and agar were obtained from Difco Ltd. (Detroit, MI, USA). All chemicals for sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS/PAGE) preparation were acquired from Bio-Rad Laboratories Inc. (CA, USA).

DNA manipulations in *E. coli* were conducted as described by Sambrook *et al.* [22]. Restriction endonuclease digestions, ligations, and DNA modifications were performed as recommended by the commercial suppliers. The nucleotide sequence was determined in both directions by the chain termination method [23], by using single-stranded phage DNA and synthetic oligonucleotides as primers.

Plasmid constructions and purification of DAHP synthase. Plasmid pSUDS-18 was used as a template for amplification of the *dahps* gene with primers DS-1 and DS-2 (Table 2). DNA amplification was performed in 100 μl of reaction mixture containing 200 ng of template, 10 μl of a 10× amplification buffer, 2 μM of each primer, 200 μM of each dNTP, 2.5 U of *Pfu* DNA polymerase with a Perkin-Elmer 9600 thermocycler (Perkin-Elmer Cetus, CT, USA). The PCR conditions were as follows: one cycle at 94°C for 3 min followed by 30 cycles of 1 min at 94°C, 2 min at 60°C, and 1 min at 72°C. The amplification reaction was completed with a final extension at 72°C for 10 min. The PCR products were purified from a 1% agarose gel with the Geneclean III kit (Bio101 Inc., CA, USA) as described by the supplier. The recovered products were cloned as a 2.57-kb *Bam*HI-*Xba*I fragment into the corresponding sites of pUC19 to generate pUC-DS.

For overexpression of *C. glutamicum* DAHP synthase, a 1.85-kb *Bam*HI-*Sac*I DNA fragment was excised from pUC-DS and cloned into pQE-30 to yield pQE-DS. The resulting plasmid was introduced into *E. coli* M15, and *dahps* gene expression was induced in exponentially growing cells (optical density at 600 nm, 0.8–1.0) by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After a further 3-h cultivation, cells were harvested by centrifugation at 12,000 *g* for 10 min, resuspended in 3 ml of binding buffer (5 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl; pH 7.9) and disrupted by sonication. Cell debris was removed by centrifugation at 12,000 *g* for 10 min. The resulting supernatants were mixed with nickel nitrilotriacetate (Ni/NTA) resin (Novagen, Madison, WI, USA) pre-equilibrated with binding buffer (5 mM imidazole, 0.5 mM NaCl, and 20 mM Tris-HCl; pH 7.9). The adherent proteins were eluted from the Ni/NTA resin with a buffer containing 1 M imidazole, 0.5 M NaCl, and 20 mM Tris-HCl (pH 7.9).

Table 2. Oligonucleotide primers used for PCR and site-directed mutagenesis

Primer for	Sequence
PCR:	
DS-1	5′-*GGATCC*CTTCGATATTGAGG-3′ *Bam*HI
DS-2	5′-GT*TCTAGA*GCTGTTTGGCGCCGTCTA-GTTC-3′ *Xba*I
Site-directed mutagenesis:	
Cys^{67}Leu	5′-CCT*CTC*TCAGTTCACGATCC-3′
Cys^{67}Ser	5′-CCT*AGC*TCAGTTCACGATCC-3′
Cys^{145}Ser	5′-GGC*AGC*GAATTCCTCGAACC-3′
Cys^{334}Ser	5′-AAG*AGC*ATCGATATTGACAC-3′

Site-directed mutagenesis of *C. glutamicum* DAHP synthase. A 1.85-kb *Bam*HI-*Sac*I fragment containing *C. glutamicum dahps* gene in plasmid pUC-DS was subcloned into phage M13mp19 to yield pM13-DS. The uridinylated DNA templates of pM13-DS were prepared in *E. coli* CJ236. Site-specific mutagenesis was performed according to the method of Kunkel *et al.* [10], using the Muta-Gene M13 in vitro Mutagenesis kit from Bio-Rad. Synthetic oligonucleotides used as primers to carry out the reactions are listed in Table 2. The phages containing mutant *dahps* genes were screened and sequenced to confirm the successful mutations. The *Bam*HI-*Pst*I fragments of the desired

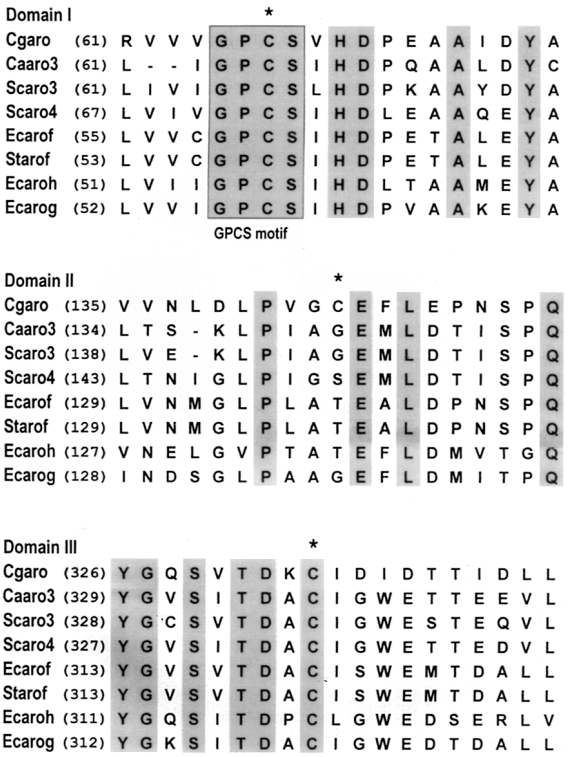

Fig 1. Amino acid sequence alignment of *C. glutamicum* DAHP synthase with other microbial enzymes. Invariant residues are in gray boxes. Cys^{67}, Cys^{145}, and Cys^{334} are noted by stars. Cgaro, *C. glutamicum* DAHP synthase [12]; Caaro3, *C. albicans* DAHP synthase [10]; Scaro3 and Scaro4, *S. cerevisiae* DAHP synthase isoenzymes [8, 9]; Starof, *S. typhimurium* DAHP synthase [11]; Ecarof, Ecaroh, and Ecarog, *E. coli* DAHP synthase isoenzymes [5–7].

clones were then subcloned into pQE-30 for enzyme purification and characterization.

Electrophoretic analysis and spectroscopic procedure. SDS/PAGE was performed by using a Mini-Protean gel system (Bio-Rad Laboratories, MA, USA) essentially according to Laemmli [12]. Protein bands were visualized by staining with Coomassie brilliant blue G-250. A molecular mass standard mixture (21.5–200 kDa) from Novex Experimental Technology (CA, USA) was used to determine the apparent molecular masses of the samples.

The UV-visible absorption spectra of wild-type and mutant DAHP synthases were performed according to the procedure of Sundaram *et al.* [33] with a Beckman spectrophotometer.

Enzyme assay and determination of kinetic parameters. The DAHP synthase activity was determined with a stop assay based on the absorbance at 549 nm as described by Shiio *et al.* [25]. One activity unit is defined as the disappearance of 1 μmole of PEP per min at 30°C. Protein concentration was estimated by the method of Bradford [2] with bovine serum albumin as a standard.

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 V_{max} and K_m values were determined by fitting the initial rates as a function of substrate concentrations to Michaelis-Menten equation by using Grafit software (Sigma Co., St. Louis, MO, USA).

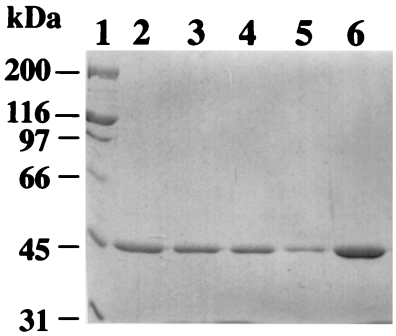

Fig 2. SDS/PAGE analysis of DAHP synthases purified by Ni-NTA resin. Lanes:1, protein size marker; 2, wild-type DAHP synthase; 3, Cys^{67}Leu mutant; 4, Cys^{67}Ser mutant; 5, Cys^{145}Ser mutant; 6, Cys^{334}Ser mutant.

Table 3. Specific activity and kinetic parameters of wild-type and mutant DAHP synthases[a]

Enzyme	Specific activity (U/mg)	K_m^{PEP} (μM)	k_{cat} (min^{-})	k_{cat}/K_m^{PEP} (min^{-1} mM^{-1})
Wild-type	139.3	4.29	0.21	48.95
Cys^{67}Leu	8.1	40.44	0.13	3.21
Cys^{67}Ser	0.0	ND[b]	ND	ND
Cys^{145}Ser	22.8	23.85	0.08	3.35
Cys^{334}Ser	131.5	3.77	0.18	47.74

[a] The data represent the average values of three measurements, and the standard deviations are estimated to be ±15%.
[b] ND, not determined.

Results and Discussion

Sequence comparison and expression of recombinant DAHP synthases. There are two distinct types of DAHP synthase in the current database. The *E. coli*-like DAHP synthases have a subunit M_r of about 39 kDa, while the plant-like enzymes have a subunit M_r of about 54 kDa [34]. The type I DAHP synthases (*E. coli*-like enzymes) were further subdivided into two subfamilies, and the levels of overall identities between individual members of subfamilies are sufficient low [31]. It has been proposed that GPCS, KPRTS/T, and IGAR motifs may correspond to the active-site residues of DAHP synthases [21]. In fact, all these motifs are conserved in DAHP synthase from *C. glutamicum*. On the basis of the universal sensitivity of DAHP synthases to sulfhydryl modifying agents, it is reasonable to speculate that the putative essential cysteine would be well conserved among the enzymes. The enzyme of *C. glutamicum* contains two invariant cysteine residues at positions 67 and 334, respectively (Fig. 1). Cys^{67} (corresponds to Cys^{61} of *E. coli* phenylalanine-sensitive DAHP synthase) is located within the GPCS motif, and Cys^{334} (corresponds to

Table 4. Activity of wild-type and mutant DAHP synthases in the presence of the indicated metal[a]

Enzyme	Specific activity (U/mg protein)					
	None	Co^{2+}	Ni^{2+}	Mg^{2+}	Zn^{2+}	Mn^{2+}
Wild-type	103.40	965.48	447.99	92.28	98.53	76.26
$Cys^{67}Leu$	7.35	24.90	16.78	8.28	12.70	10.13
$Cys^{67}Ser$	0.00	0.00	0.00	0.00	0.00	0.00
$Cys^{145}Ser$	17.62	149.33	57.50	8.28	5.75	16.78
$Cys^{334}Ser$	98.26	894.90	388.26	71.32	75.97	72.63

[a] The data represent the average values of three measurements and the standard deviations are estimated to be ±10%.

Cys^{328} of *E. coli* phenylalanine-sensitive DAHP synthase) exists in the carboxyl-terminal of the enzyme. A third variant cysteine is present at position 145 of *C. glutamicum* DAHP synthase, while other DAHP synthases contain mostly glycine or tryptophan residue at the equivalent position (Fig. 1). In order to investigate which cysteine residue might be essential for activity, replacement of each residue by serine was performed by oligonucleotide-directed mutagenesis. The altered sequences of the *dahps* gene in the plasmids were confirmed by DNA sequencing. *E. coli* M15 containing the recombinant plasmids was evaluated for the synthesis of DAHP synthases by IPTG induction of a shake flask culture. Synthesis of a protein of about 45 kDa was detected on SDS/PAGE gel following IPTG induction, whereas no protein band was observed in the uninduced samples (data not shown). The expressed enzymes in which a six-histidine tag is present at their C-terminal were purified by Ni-NTA resins and showed identical mobility on SDS/PAGE (Fig 2). Protein secondary structures of wild-type and mutant DAHP synthases were compared by using the PEPTIDE STRUCTURE and PLOT STRUCTURE programs based on the Chou-Fasman algorithm [4]. The results showed that no obvious change was found in the overall secondary structure of the wild-type and mutant enzymes. Additionally, comparison of the mutant proteins with wild-type DAHP synthase by circular dichroism in the far-UV region did not reveal any significant difference (data not shown). These observations indicated that the alteration of cysteine residues is unlikely to cause any disruption to the global conformation of *C. glutamicum* DAHP synthase.

Enzymatic properties. The purified wild-type enzyme had a specific activity of 139.3 U per mg of protein (Table 3). The activity was completely abolished in $Cys^{67}Ser$ mutant, while the $Cys^{145}Ser$ mutant retained about 83.6% of the wild-type enzyme. It is worth noting that $Cys^{334}Ser$ mutant showed no apparent change in enzymatic activity, indicating Cys^{334} residue is not essential for DAHP synthase activity. The optimum pH for wild-type and mutant DAHP synthases was the same (pH 6.5), and the enzymes exhibited similar patterns in activity over a pH range of 1.5 to 8.5. The wild-type and mutant enzymes were active over a temperature range of 25–50°C and had optimum activity at 30°C. These results revealed that the wild-type and mutant enzymes had a similar biochemical characteristic.

The kinetic parameters of wild-type and mutant enzymes are listed in Table 3. Compared with the wild-type enzyme, the $Cys^{145}Ser$ mutant showed a 1.6-fold decrease in k_{cat}, and a 4.6-fold increase in K_m^{PEP}. It should be noted that the mutation of Cys^{334} to serine did not significantly change kinetic properties of the enzyme.

Effect of metal ions on wild-type and mutant DAHP synthases. The effect of metal ions on the activity of *C. glutamicum* DAHP synthase was examined by the addition of 1 mM metal ions to the enzyme reaction. As shown in Table 4, the wild-type and mutant enzymes had a similar metal preference and were most active in the presence of Co^{2+}. It is worth noting that $Cys^{67}Ser$ was found to be inactive irrespective of the divalent metal ion included in the assay mixture. Although the role of the metal ion remains unclear, it is generally known that DAHP synthases require a divalent metal cofactor for activity. Previous reports indicated that the three isozymes from *E. coli* are ion dependent [13, 20]. DAHP synthases from other bacteria [8, 15] and from some eukaryotes [16, 18] have also been shown to be EDTA sensitive and stimulatable by various divalent cations.

Many researchers speculated that DAHP synthase from *E. coli* is a natural Cu^{2+}-containing enzyme and shows an absorbance near 350 nm in the UV spectrum [1, 20, 29]. The absorbance has been attributed to a ligand-to-metal charge transfer process between DAHP synthase and Cu^{2+} [29]. Previously, it has been shown that four inactive Cys^{61} mutants do not exhibit the typical absorbance at $\lambda = 350$ nm, whereas the Cys^{328} mutants do display the peak [30]. In the present study, a similar peak at $\lambda = 350$ nm was observed in wild-type *C. glutamicum* DAHP synthase when it was incubated with

Cu^{2+} either in the presence or in the absence of PEP (data not shown). Interestingly, our inactive mutant, Cys^{67}Ser, did not have the typical peak for Cu^{2+}-containing DAHP synthase, whereas Cys^{67}Leu displayed the $\lambda = 350$ nm peak, indicating that the thiolate of Cys^{67} is not participating in Cu^{2+} binding. Although Cys^{61} of *E. coli* phenylalanine-sensitive DAHP synthase has been proposed to play a critical role in metal binding and catalytic activity [30], the work of Sundaram *et al.* [33] suggested that this residue is not directly involved in metal binding, but it may influence geometry of the metal binding site.

Conclusion

From the above fact, it is clear that Cys^{67} of *C. glutamicum* DAHP synthase is required for the catalytic mechanism of the enzyme, since its mutation resulted in a complete loss of activity. Because no dramatic change in enzymatic activity was found in Cys^{334}Ser, we proposed that residue Cys^{334} is not essential in catalytic activity. It is likely that Cys^{145} may be involved in substrate binding, as suggested by the increased K_m value for PEP. Determination of the three-dimensional structure of *C. glutamicum* DAHP synthase is in progress. The resultant information will help to clarify their roles in the enzyme.

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