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Essential Cysteines in 3-Deoxy-D-*arabino*-heptulosonate-7-phosphate Synthase from *Escherichia coli*

ANALYSIS BY CHEMICAL MODIFICATION AND SITE-DIRECTED MUTAGENESIS OF THE PHENYLALANINE-SENSITIVE ISOZYME*

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The phenylalanine-sensitive isozyme of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase from *Escherichia coli* was inactivated by the sulfhydryl modifying reagents 5,5-dithiobis-(2-nitrobenzoate), bromopyruvate, and *N*-ethylmaleimide and protected from inactivation by the presence of its metal activator, Mn^{2+}, and substrate, phosphoenolpyruvate. Inactivation by 5,5-dithiobis-(2-nitrobenzoate) was correlated with modification of two of the seven cysteine sulfhydryls of the enzyme monomer. The kinetics of 5,5-dithiobis-(2-nitrobenzoate) modification were altered significantly and distinctively by both substrates (phosphoenolpyruvate and erythrose 4-phosphate), by Mn^{2+}, and by L-phenylalanine, suggesting that ligand binding has significant effects on the conformation of the enzyme. Site-directed mutagenesis was used to create multiple substitutions at the two invariant cysteine residues of the polypeptide, Cys-61 and Cys-328. Analysis of purified mutant enzymes indicated that Cys-61 is essential for catalytic activity and for metal binding. Cys-328 was found to be nonessential for catalytic activity, although mutations at this position had significant negative effects on V_{max}, K_m^{Mn}, and K_m^{PEP}.

The enzyme DAHP synthase (EC 4.1.2.15) catalyzes the condensation of phosphoenolpyruvate (PEP)[1] and erythrose-4-phosphate (E4P) to yield 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (DAHP) and P_i (1). This reaction is the first committed step in the biosynthesis of chorismate, the common precursor of tryptophan, tyrosine, phenylalanine, and other aromatic metabolites in microorganisms and plants. In many organisms DAHP synthase is the primary target of negative feedback regulation of metabolite flow into the aromatic pathway.

The DAHP synthases from *Escherichia coli* (2), *Salmonella typhimurium* (3), *Saccharomyces cerevisiae* (4), and *Solanum tuberosum* (5) have a significant degree of sequence similarity, indicating a common evolutionary origin and common structural properties. Nevertheless, relationships between molecular structure and function in this enzyme are not yet well understood, although a recent mutational study of the tryptophan-sensitive isozyme from *E. coli* has suggested that the active site and the feedback site contain essential residues that are immediately adjacent in the primary sequence and thus are not located in separate structural domains (2).

All DAHP synthases appear to require at least one cysteine sulfhydryl for enzymatic activity. In an early study, the phenylalanine-sensitive isozyme of DAHP synthase (DAHPS(Phe)) from *E. coli* was inactivated reversibly by *p*-hydroxymercuribenzoate and irreversibly by 5,5-dithiobis-(2-nitrobenzoate) (DTNB) and bromopyruvate (BrPy) (6). PEP significantly reduced inactivation, suggesting that the essential cysteine is at the active site of the enzyme. Other work demonstrated similar sensitivity to sulfhydryl modification in DAHP synthase isozymes from *S. typhimurium* (7), *Neurospora crassa* (8), and *Brassica oleraceae* (cauliflower) (9). Later it was found that inactivation of *Brevibacterium* DAHP synthase by DTNB was correlated with modification of one cysteine per enzyme monomer (10).

The identity of the putative essential cysteine of DAHP synthase and its role in enzyme function have not yet been established. A reaction mechanism in which an active site cysteine forms a thioester intermediate with PEP was proposed some years ago (11). The recovery of an inactivating mutation at one of the two conserved cysteines (*i.e.* Cys-326) of the *E. coli* DAHPS(Trp) isozyme after random *in vitro* mutagenesis of the *aroH* gene led to the speculation that this may be the essential residue (2). We describe here studies combining chemical and mutational modification of cysteine residues of *E. coli* DAHPS(Phe) to address these issues in greater detail.

MATERIALS AND METHODS[2]

RESULTS AND DISCUSSION

Inactivation of DAHPS(Phe) by Sulfhydryl Modifying Reagents—The sensitivity of DAHPS(Phe) apoenzyme to var-

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[1] The abbreviations used are: PEP, phosphoenolpyruvate; E4P, erythrose-4-phosphate; Phe, L-phenylalanine; DAHP, 3-deoxy-D-*arabino*-heptulosonate-7-phosphate; DAHPS(Phe), phenylalanine-sensitive isozyme of DAHP synthase; BTP, 1,3-bis[tris(hydroxymethyl)-methylamino]propane; IPTG, isopropyl-D-thiogalactoside; DTNB, 5,5′-dithiobis-(2-nitrobenzoate); NMB, nitromercaptobenzoate; PCMB, *p*-chloromercuribenzoate; NEM, *N*-ethylmaleimide; BrPy, bromopyruvate; SDS, sodium dodecyl sulfate; FPLC, fast protein liquid chromatography; HPLC, high pressure liquid chromatography.

[2] Portions of this paper (including "Materials and Methods," Tables I and II, and Figs. 3 and 5) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

ious sulfhydryl-modifying reagents is summarized in Table I, with an assessment of the protective effects of various ligands, including PEP, Mn^{2+}, and Phe. PCMB completely inactivated the enzyme under all conditions, whereas iodoacetamide was totally ineffective. Treatment with DTNB, BrPy, and NEM resulted in full to partial inactivation that was reversed to different extents by specific ligands. Mn^{2+} afforded complete protection from NEM and BrPy, but was slightly less protective against DTNB inactivation. "High" PEP (increased from 25 μM carried over with the enzyme to 125 μM)[3] partially blocked inactivation by BrPy and NEM but had no effect on DTNB inactivation. Phe enhanced inactivation by BrPy but had little effect on inactivation by DTNB and NEM.

Kinetics of Modification and Inactivation by DTNB—Reaction of DAHPS(Phe) with a 10-fold molar excess of DTNB under mildly denaturing conditions (2% SDS) indicated 7.1 ± 0.1 modified cysteines per enzyme monomer (Fig. 1*A*), in agreement with the prediction of 7 cysteine residues per monomer from the nucleotide sequence of *aroG* (13). The enzyme reacted rapidly under these conditions ($T_{½} \approx 1.4$ min). Secondary plots of the second-order rate constant (k), calculated at intervals throughout the reaction, *versus* the mean number of modified cysteine residues indicated that 5 residues reacted with DTNB at equivalent rates (Fig. 1*B*). The remaining 2 residues were less accessible, perhaps due to residual folded structure.

The overall rate of sulfhydryl modification was lower in the native enzyme ($T_{½} \approx 2.8$ min), although no residues were completely protected (Fig. 1*A*). Secondary plots of k as described above (Fig. 1*B*) revealed biphasic reaction kinetics in which the first 1–2 net residues were modified at a rate about 2-fold greater than that of the others. Inactivation of the enzyme by DTNB also followed complex kinetics (Fig. 1*C*). Approximately 90% of enzymatic activity was lost upon modification of the first 2 cysteines (Fig. 2).

In the presence of high PEP (125 μM), the overall rate of modification was significantly reduced ($T_{½} \approx 5.5$ min) (Fig. 1*A*); however, 1 residue remained about 2-fold more reactive than the others (Fig. 1*B*). The rate of inactivation decreased as well (Fig. 1*C*); about half of the activity was lost upon modification of the first residue, and an additional 30% upon reaction of the next 2 residues (Fig. 2). Since at low PEP (25 μM) modification of the first 2 residues resulted in almost 90% inactivation, it is possible that different sets of residues are modified preferentially in the presence of high and low PEP.

The addition of Mn^{2+} (50 μM) also significantly reduced DTNB modification of the enzyme (Fig. 1*A*) and strongly protected it from inactivation (Fig. 1*C*). The simultaneous presence of Mn^{2+} and PEP was most effective, providing almost complete protection from modification and inactivation. These effects were not due to nonspecific masking of free thiol groups by Mn^{2+}, as control experiments showed that excess Mn^{2+} caused no change in DTNB reactivity with free cysteine at equimolar concentrations. Further analysis showed that other metals which activate DAHP synthase, including Fe^{2+}, Co^{2+}, and Zn^{2+} (14), had similar protective effects, whereas nonactivating metals such as Mg^{2+} and Cr^{3+} did not (data not shown).

In contrast to the effects of PEP and Mn^{2+}, saturating E4P

[3] The effects of sulfhydryl modifying reagents could not be assessed in the total absence of PEP, because removal of PEP results in irreversible inactivation of the enzyme *in vitro*. It has been suggested that the unliganded state of DAHP synthases may in fact be nonphysiological since the intracellular concentration of PEP is at least 10 times K_m^{PEP} of the *E. coli* isozymes (12). The amount of PEP carried over with the enzyme is indicated for each experiment.

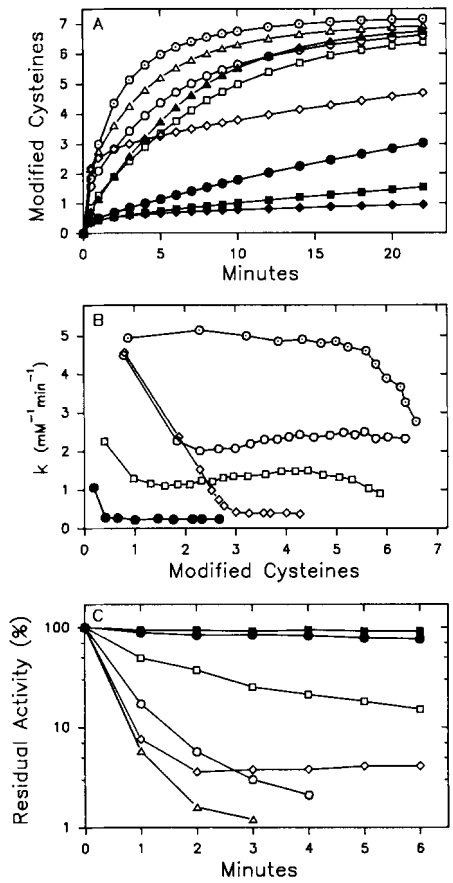

FIG. 1. **Modification and inactivation of DAHPS(Phe) by DTNB in the presence of various ligands.** Initial concentrations were 9 μN DAHPS(Phe) and 100 μM DTNB. All reactions contained 25 μM PEP carried over with the enzyme; additions of PEP and other ligands were as noted. *A* shows the time course of DTNB modification indicating the number of modified cysteines per enzyme monomer. *B* displays secondary plots of second-order rate constants (k) of DTNB modification *versus* the mean number of modified cysteine residues per monomer during the interval for which k was calculated. *C* shows inactivation by DTNB. Additions were as follows: no addition (○); 2% SDS (⊙); 50 μM $MnCl_2$ (●); 100 μM PEP (□); 100 μM PEP, 50 μM $MnCl_2$ (■); 600 μM E4P (△) (DTNB added 1 min after addition of E4P); 600 μM E4P, 50 μM $MnCl_2$ (▲) (DTNB added 1 min after addition of ligands); 100 μM Phe (◇); 100 μM Phe, 100 μM PEP, 50 μM $MnCl_2$ (◆).

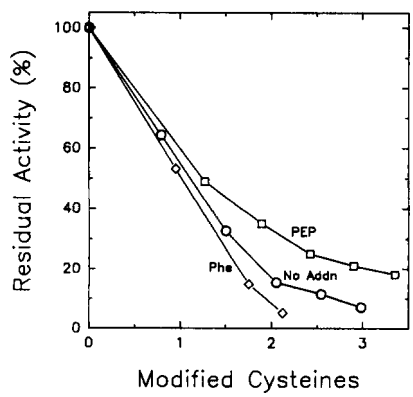

FIG. 2. **Inactivation of DAHPS(Phe) by DTNB as a function of the number of modified cysteines.** Conditions were as described in Fig.1 except that DTNB was at 50 μM in order to slow the rate of modification. Additions were as follows: none (○); 100 μM PEP (□); 100 μM Phe (◇).

(600 μM) enhanced the overall rate of modification by DTNB ($T_{1/2} \approx 2.2$ min), and in particular increased modification of the first 2 residues (Fig. 1*A*). Inactivation was rapid, with 95% loss of activity in 1 min (Fig. 1*C*), concomitant with modification of the first 2 cysteines. These results are consistent with the ordered sequential kinetic mechanism of the DAHP synthase reaction noted earlier (15–17), with binding of PEP preceding that of E4P. Accordingly, the addition of excess E4P to the enzyme would drive the conversion of all available PEP to DAHP, thereby emptying the active site and rendering PEP-protected cysteines more sensitive to DTNB modification. This would not necessarily require the addition of exogenous metal, as the metal-free apoenzyme characteristically contains a low level of catalytic activity (~2% of fully activated enzyme) (14). In fact, it was found that modification in the presence of E4P and Mn^{2+} was slower than in the presence of E4P alone, but faster than in the presence of Mn^{2+} alone (Fig. 1*A*). These results are consistent with the previous observation that the protective effect of Mn^{2+} is enhanced by PEP.

The addition of Phe (100 μM) had distinctive effects on DTNB reactivity of the enzyme (Fig. 1*A*). The first 2–3 cysteine residues were modified more rapidly than the others, whereas modification of the remaining residues proceeded at a rate 5-fold lower than that in the absence of Phe (Fig. 1*B*). Enzyme inactivation was very rapid, attaining 90% in the first minute (Fig. 1*C*), concomitant with modification of the first 2 residues (Fig. 2). Addition of Mn^{2+} and PEP completely reversed the effect of Phe on modification (Fig. 1*A*).

It is likely that the slight increase in the rate of modification of the enzyme by DTNB in the later stages of the reaction at both low and high PEP (Fig. 1*B*) was an artifact due to underestimation of remaining DTNB caused by nonstoichiometric release of nitromercaptobenzoate (NMB). When the thiol groups of the enzyme were titrated by the sequential addition of substoichiometric amounts of DTNB, NMB was released at twice the level of DTNB over the period of the reaction where the first 4 cysteines were modified per monomer (Fig. 3). This most likely reflects the attack of unmodified enzyme thiols on enzyme-NMB disulfides, thereby generating a protein disulfide with the release of a second NMB anion (18). This phenomenon readily occurs when 2 or more cysteines are adjacent in the native structure of the enzyme (19). In support of this possibility, when DAHPS(Phe) was titrated with DTNB in the presence of 2% SDS, the rate of generation and overall yield of NMB was reduced (Fig. 3). Nevertheless, treatment of the enzyme with the fluorescent vicinal sulfhydryl indicator *N*-(1-pyrene)maleimide (20) failed to support the possibility of adjacent cysteine residues. Although the enzyme was rapidly inactivated by this reagent, there was no indication of the formation of the pyrene conjugate in the fluorescence emission spectrum of the reaction mixture (data not shown).

Inactivation of DAHPS(Phe) by Bromopyruvate—The effects of PEP, Mn^{2+}, and Phe on the inactivation of the enzyme by BrPy, an electrophilic reagent which can function as an affinity label for enzymes utilizing pyruvate or PEP (21), were generally similar to those seen with DTNB (Fig. 4), suggesting that the targets of the two reagents may be the same. However, inactivation by BrPy followed pseudo-first order kinetics, indicating modification of a single essential cysteine. The derived T constant was 0.4 min (where T is the extrapolated time for half-maximal inactivation at infinite concentrations of modifying agent (21)). Inactivation was competitive with respect to PEP; K_i^{BrPy} was 0.7 mM. The sensitivity of DAHPS(Phe) to BrPy inactivation was greater than that

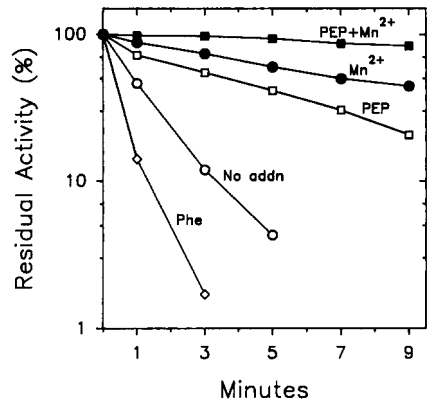

FIG. 4. **Inactivation of DAHPS(Phe) by bromopyruvate.** Concentrations were as follows: DAHPS(Phe), 0.6 μN; bromopyruvate, 0.4 mM. All reactions contained 25 μM PEP carried over with the enzyme. Additions were as follows: no addition (○); 50 μM $MnCl_2$ (●); 100 μM PEP (□); 100 μM PEP, 50 μM $MnCl_2$ (■); 100 μM Phe (◇).

found in an earlier study (T of 1.5 min and K_i^{BrPy} of 20 mM) (22); it is likely that this was due to the absence of protective metal ions in the enzyme preparation used here.

To compare the net number of cysteine residues modified by BrPy under various conditions, enzyme was treated for 15 min with a 10-fold molar excess of BrPy to enzyme in the presence of low (25 μM) and high (125 μM) PEP, Mn^{2+} (50 μM), or Phe (100 μM). The modified protein was separated from unreacted BrPy and ligands by gel filtration, and the number of unreacted cysteines per enzyme monomer was then determined by titration with DTNB. Three residual cysteines were detected following BrPy treatment in the presence of low PEP, six after treatment in the presence of high PEP and Mn^{2+}, and four after treatment in the presence of Phe. These results are very similar to those obtained in the analogous experiments with DTNB (Fig. 1*A*).

These DTNB and BrPy modification results indicate that DAHPS(Phe) most likely has a single cysteine at its active site that is essential for catalytic activity and that is protected from chemical attack by the binding of PEP and Mn^{2+}. Furthermore, the dramatic effects on net sulfhydryl reactivity exerted by PEP, Mn^{2+}, and Phe suggest that significant and distinct conformational changes occur in the enzyme upon binding of these ligands. Ligand-associated conformational effects have also been indicated by the strong cooperativity in the reaction kinetics of the enzyme with respect to PEP, E4P, and Phe (14), as well as by the increased thermal stability of the enzyme in the presence of PEP and Phe (6, 22). Thus it is possible that in the presence of different ligands not only do alterations in the kinetics of cysteine modification occur, but also changes in the identity of the residues modified could arise due to variations in sulfhydryl accessibility in different enzyme conformers.

Site-directed Mutagenesis of Invariant Cysteines in DAHPS(Phe)—In view of the universal sensitivity of DAHP synthases to sulfhydryl modifying agents, it is reasonable to speculate that the putative essential cysteine of DAHPS(Phe) would be highly conserved between members of the DAHP synthase family. In fact, there are two invariant cysteines in the six known bacterial and fungal DAHP synthase sequences. In DAHPS(Phe) these are Cys-61 and Cys-328. Interestingly, an inactivating change at the latter position in DAHPS(Trp) (namely, Cys-326 → Tyr) was previously recovered upon random mutagenesis of the *E. coli aroH* gene (2). In order to test more systematically whether one or both of these invar-

iant cysteines might be essential for activity, multiple replacements were made at each by oligonucleotide-directed mutagenesis. Cys-61 was replaced with serine, alanine, valine, and Phe, and Cys-328 was changed to serine, alanine, valine, and leucine.

The eight mutant enzymes were initially assessed for catalytic activity *in vivo* by the ability of the plasmid-borne mutant genes to restore aromatic prototrophy to *E. coli* strain CB717, which has all three chromosomal DAHP synthase genes inactivated. Considering the degree of overexpression obtained in the plasmid constructions, it was estimated that as little as 0.1% of wild-type catalytic activity would give a prototrophic phenotype. Significantly, each of the four Cys-328 mutant plasmids rendered CB717 prototrophic, while all four strains carrying the Cys-61 mutant plasmids remained auxotrophic.

The eight mutant enzymes were then overproduced in these host-vector constructions and purified to >90% homogeneity, as assessed by SDS-polyacrylamide gel electrophoresis. All of the enzymes were found in the soluble fraction of the cellular extract and all fractionated like the wild-type enzyme throughout the purification, including anion exchange FPLC. Treatment of the mutant enzymes with DTNB under denaturing conditions (2% SDS) confirmed the loss of a single cysteine residue in each.[4] Size exclusion HPLC of the enzymes revealed that the native molecular weight of each was essentially identical (±10%) to that of the wild-type enzyme (M_r = 140,000), indicating that all retained the tetrameric structure. These results establish that none of the amino acid substitutions led to defective folding of the polypeptide or caused major distortions in the structure of the native subunit.

Effects of Mutations at Cys-328—As expected from the *in vivo* results, the four Cys-328 mutant enzymes were catalytically active; however, all had altered kinetic properties. The specific activities of the purified enzymes ranged from 20 to 80% of the wild type, and their apparent K_m constants for both PEP and Mn^{2+} were increased significantly (Table II). However, their apparent affinity for E4P[5] and their sensitivity to feedback inhibition by Phe was unaffected (not shown). The enzymes were somewhat less stable than the wild type; their activities decreased 20–40% during storage on ice for 1 week, conditions under which the activity of the wild-type enzyme was essentially constant. It is interesting that the effects of the conservative Cys-328 → Ser substitution on the kinetic properties of the enzyme were more pronounced than those of the Cys-328 → Val and Cys-328 → Ala substitutions. On the other hand, the fact that the Cys → Leu substitution, having the bulkiest side chain, had the most severe effects is consistent with the previous finding that the Cys-326 → Tyr change in the DAHPS(Trp) isozyme was completely inactivating *in vivo* (2).

The Cys-328 mutant enzymes all displayed increased sensitivity to modification and inactivation by DTNB (Fig. 5). The rate constants calculated for modification of the first 2–3 cysteines of the native enzymes (with carryover of 40 μM PEP) were 2–5-fold that of the wild-type enzyme in the presence of 25 μM PEP. Under these conditions, 99% of activity was lost within 1 min. Higher concentrations of PEP (140 μM), even in the presence of Mn^{2+} (50 μM), were much less effective in protecting the mutant enzymes relative to the wild-type enzyme. Lack of protection was most evident with the C328L enzyme, consistent with its more severely altered kinetic properties. In contrast, modification of the mutant enzymes in the presence of Phe was similar to that of the wild-type enzyme.

The fact that the mutant enzymes retain catalytic activity excludes the possibility that Cys-328 is an essential catalytic residue. However, the effects of the mutational changes on the kinetic properties of the enzyme suggest that Cys-328 is positioned in the native structure such that its replacement by other residues can transmit structural defects which impact on k_{cat} as well as on PEP and metal binding. It is noteworthy that Cys-328 resides in a stretch of residues predicted to form a β-strand motif, and all four substitutions are predicted to cause perturbation of this structure.

Effects of Mutations at Cys-61—Consistent with the *in vivo* results, crude extracts of strains producing the four Cys-61 mutant enzymes had no detectable DAHP synthase activity. Likewise, purified enzyme preparations were inactive, even with elevated substrate and metal concentrations in the reaction mixture (250 μM PEP, 1 mM E4P, and either 250 μM Mn^{2+} or 100 μM Zn^{2+}). The detection limit of the assay was estimated to be about 0.01% of the wild-type activity.

DTNB modification patterns of the purified Cys-61 mutant enzymes are shown in Fig. 6. Unlike with the wild-type enzyme (Fig. 1), PEP and Mn^{2+} gave virtually no protection of the C61V and C61F enzymes, suggesting that the bulky substitutions of the enzymes eliminated binding of both ligands at the active site. The modification patterns in the presence of Phe were essentially unaffected. In contrast, the C61S and C61A enzymes were moderately resistant to modification at 40 μM PEP, and were strongly protected at 140 μM PEP, indicating that these two conservative substitutions did not eliminate PEP binding. However, protection by Mn^{2+} was totally absent in these two enzymes, as was the characteristic rapid modification of the first two residues in the presence of Phe.

The loss of enzymatic activity and the failure of Mn^{2+} to protect the four Cys-61 mutant enzymes from DTNB modification strongly suggests that Cys-61 is an essential residue and that it plays a critical role in metal binding. Additional support for this conclusion was gained from the results of

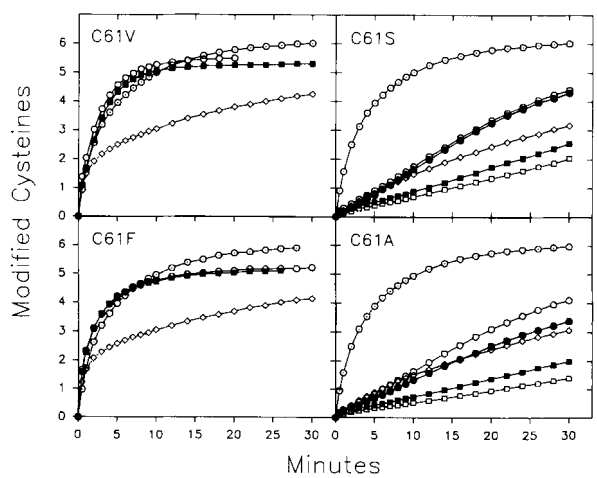

FIG. 6. **DTNB modification of Cys-61 mutant enzymes.** Reaction conditions were identical to those described in the legend of Fig. 5. Additions were as follows: no addition (○) ; 2% SDS (⊙); 100 μM $MnCl_2$ (●); 100 μM PEP (□); 100 μM PEP, 100 μM $MnCl_2$ (■); 100 μM Phe (◇). The data indicate the number of modified cysteines per enzyme monomer.

[4] The number of detected sulfhydryls in the eight enzymes ranged from 5.3 to 6.0 per monomer. This finding of non-integer numbers of cysteines in some of the mutant enzymes was possibly due to slight increases in ϵ^{M}_{280} nm, as protein concentrations were determined using the wild-type value.

[5] The kinetic properties of C328L with respect to E4P was not examined in steady state experiments; however, it was established that 600 μM E4P was saturating under standard assay conditions.

spectrophotometric studies of the mutant enzymes. We had shown previously that the wild type apoenzyme develops a strong ligand-to-metal charge-transfer absorption band at 350 nm upon saturation with Cu^{2+} (14). Significantly, no absorption band arose in any of the four Cys-61 mutant enzymes when treated with Cu^{2+}, even after extended incubation at elevated concentrations. In contrast, and consistent with their catalytic properties and DTNB modification patterns, the four Cys-328 mutant enzymes displayed wild-type absorption properties after incubation with Cu^{2+} (not shown).

Further evidence for the postulated role of Cys-61 was obtained from direct metal binding experiments. Samples of purified wild-type and C61V apoenzymes were treated with an 8-fold molar excess of Mn^{2+} or Cu^{2+}. We had previously demonstrated a functional stoichiometry of one metal atom per wild-type monomer in the fully activated holoenzyme (14). After separation of unbound metal by gel filtration, atomic absorption analysis of the samples indicated binding of 0.8 g-atoms manganese per mol of wild-type monomer, but only $\leq$0.1 g-atoms/mol of C61V monomer. The values for bound copper were very similar.

It is noteworthy that the topography of Cys-61 in the primary structure of DAHPS(Phe) is appropriate for a metal coordination site, as it lies in a predicted turn motif and is separated by 2 residues from an invariant histidine (His-64). We are now probing structure-function relationships throughout this region of the polypeptide by saturation mutagenesis. If the hypothesized role for Cys-61 in metal binding is correct, the catalytic mechanism for DAHP synthase proposed by Ganem (11), which involves a cysteine sulfhydryl functioning as an essential nucleophile at the active site, is unlikely as there is no other invariant cysteine to fulfill this role. Nevertheless, a similar mechanism invoking an alternative nucleophile, as previously suggested (15), may be applicable.

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Supplementary Material To:

ESSENTIAL CYSTEINES IN 3-DEOXY-D-*ARABINO*-HEPTULOSONATE-7-PHOSPHATE SYNTHASE FROM *ESCHERICHIA COLI*

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MATERIALS AND METHODS

Chemicals and enzymes. PEP, E4P, DTNB, BrPy, N-ethylmaleimide (NEM), iodoacetamide, isopropyl β-D-thiogalactoside (IPTG), tris(hydroxymethyl)-aminomethane (Tris), 1,3-bis[tris(hydroxymethyl)methylamino]propane (BTP), amino acids, vitamins and antibiotics were from Sigma. p-chloromercuribenzoate (PCMB) from United States Biochemical Corp., N-(1-pyrene)maleimide from Molecular Probes, nucleotides from Pharmacia LKB, restriction endonucleases and DNA modifying enzymes from Bethesda Research Labs or Boehringer Mannheim.

Bacterial strains and plasmids. *E. coli* strains were as follows: CB198 (W3110 *trpR tnaA lacI*^{q}) (23); CB717 (C600 Δ(*gal-aroG-nadA*) *aroF*::cat Δ*aroH*::neo/ F'*lacI*^{q}ZΔM15 *proA*'B' Tn10(Tet^R); BMH 71-18 (*mutS*::Tn10 Δ(*lac-proAB*) *thi supE/F' proA*'B' *lacI*^{q}ZΔM15) (purchased from Promega) and JM107 (24). Phagemid pttG1 is a derivative of expression vector pttS7 (14) and carries the wild type *aroG* gene under the control of the *tac* promoter. Phagemids pttS9 and pttS10 are slightly modified versions of pttS7, in which the M13 origin of replication is of the opposite orientation and, in the case of pttS10, a vector ClaI site has been destroyed.

Enzyme preparation and assay. Wild-type DAHPS(Phe) was overproduced by IPTG induction of the *aroG* gene of plasmid pttG1 in host strain CB198 grown in minimal salts medium supplemented with 5 g/l glucose, 1 g/l casein hydrolysate and 25 mg/l ampicillin (14). Mutant DAHP synthases were overproduced from their respective plasmids in host strain CB717 grown in the same medium but supplemented additionally with 25 mg/l leucine, 6 mg/l thiamine and 6 mg/l nicotinic acid. IPTG induction was as with CB198/pttG1. The wild type enzyme was purified as previously described (14). The purification protocol for the mutant enzymes was the same except that the acetone precipitation was done in two steps. Acetone was first added to 20% and precipitated material removed by centrifugation (12000 x g for 10 min). The acetone concentration of the supernatant was then raised to 30% and precipitated material, which included DAHP synthase, was collected by centrifugation (12000 x g for 20 min).

Metal-free enzyme was prepared by adding one-tenth volume of a slurry of neutralized Chelex 100 resin (BioRad Laboratories) to the enzyme solutions and stirring for 12 h at 0°C. Samples were then dialyzed against two changes of 200 volumes of Chelex-treated BPP buffer (10 mM BTP, pH 6.8, 200 μM PEP). Catalytic activity assayed in the absence of metal ions was routinely 1 to 2% of that in the presence of 50 μM Mn^{2+}, the most efficient metal activator (14).

DAHP synthase activity was assayed by measuring the disappearance of PEP at 232 nm in a Varian DMS 200 double-beam recording spectrophotometer with a thermostated cuvette holder held at 25°C. The standard reaction mixture contained 150 μM PEP, 600 μM E4P, 50 μM $MnCl_2$, 10 mM BTP, pH 6.8, in a final volume of 1.0 ml. Reactions were initiated by addition of enzyme. All solutions for enzymatic analysis were depleted of trace metals immediately before use by passage over Chelex 100 resin.

The concentration of DAHPS(Phe) preparations, expressed in terms of enzyme monomer (i.e. μN), was determined spectrophotometrically using ϵ^M_{280nm}= 40,500, a constant derived empirically with the wild type enzyme (14). For the Cys-61 and Cys-328 mutant enzymes, use of this constant in the calculation of total cysteine residues by DTNB modification yielded 5.3 to 6.0 cysteines per monomer (instead of the expected 6.0). We assumed that these non-integer values resulted from slight increases in ϵ^M_{280nm} for the mutant enzymes. For sake of comparison in Fig. 5 and 6, concentrations for the mutant enzymes were calculated with the following extrapolated values for ϵ^M_{280nm} (calculated by normalizing all of the mutant enzymes to 6.0 cysteines per monomer): C61F, 46,000; C61V, 43,000; C61S, 42,600; C61A, 41,600; C328V, 42,300; C328A, 43,200; C328S, 40,500; C328L, 45,300.

Molecular weight determinations. Size exclusion chromatography for the estimation of the native molecular weight of the wild-type and mutant DAHPS(Phe) enzymes employed a Superose 12 HR10/30 FPLC column (Pharmacia LKB) developed at a flow rate of 0.5 ml/min with 10 mM BTP, pH 6.8, 100 mM KCl. Molecular weight standards were as follows: *S. typhimurium* anthranilate synthase complex, 232,000; *S. typhimurium* anthranilate synthase partial complex, 165,000; yeast alcohol dehydrogenase, 150,000; bovine serum albumin, 67,000; ovalbumin, 45,000; chymotrypsinogen, 25,000; and cytochrome C, 12,500. Blue dextran was used to determine the void volume (V_o) of the column. The molecular weight of DAHPS(Phe) samples was estimated from a standard curve prepared by plotting V_e/V_o versus the log of the molecular weight of the standard proteins.

Treatment of DAHPS(Phe) with sulfhydryl-modifying reagents. All modification experiments were done at 25°C in 10 mM BTP, pH 6.8, in a final volume of 1 ml. Purified, metal-free enzyme was diluted with buffer and the concentration was determined spectrophotometrically. Ligands were added to the diluted enzyme, samples were assayed to determine initial activity, and the reaction was started by the addition of modifying reagent (10 µl of 10 mM solutions in dimethylformamide, except for PCMB which was prepared in 0.1 N NaOH). Samples (2-20 µl) were removed and assayed at 1 to 5 minute intervals thereafter.

DTNB modification reactions were monitored continuously by measuring the increase in absorbance at 412 nm (25). Absorbance values were recorded automatically at 30 s intervals and converted to the number of modified cysteine residues using ϵ^{M}_{412nm}= 14,500 for the released nitromercaptobenzoate (NMB) anion. This value was determined empirically using known concentrations of DTNB and L-cysteine reacted under conditions identical to those used for enzyme modification. The second-order rate constant, *k*, was calculated for intervals of the reaction using the equation: $k = \Delta[Cys^-]/\Delta t[Cys][DTNB]$, where $[Cys^-]$ = concentration of modified cysteine residues at the end of each interval, and Δt = duration of interval (min). [Cys] = $[Cys]_{initial}$-$[Cys^-]$, and [DTNB] = $[DTNB]_{initial}$-$[Cys^-]$. $[Cys]_{mean}$ and $[DTNB]_{mean}$ are the mean concentrations of these two reactants during the time interval, determined by averaging the initial and final [Cys] and [DTNB] values for the interval. Enzymatic activity during DTNB modification was monitored in separate but identical reaction mixtures incubated at 25°C. Samples were withdrawn and assayed for DAHP synthase activity at intervals after addition of DTNB.

Mutagenesis of Cys-61 and Cys-328 of DAHPS(Phe). Oligonucleotide-directed mutagenesis of *aroG* was carried out using the "Altered Sites" system (Promega). Standard molecular cloning techniques (26) were used throughout. The *E. coli aroG* gene (13) was cloned as a 1.5 kb *Eco*RI/*Hin*dIII restriction fragment into plasmid pSelect1 (Promega) digested with *Eco*RI and *Hin*dIII. This construction was designated pSelG1. Single-stranded DNA was recovered from JM107/pSelG1 by infection with M13KO7 helper phage (27).

The sequence of the 22-mer mutagenic oligonucleotide used to change the Cys-61 codon to Ser, Ala, Val and Phe was the following: ^{5}GATTGGCCCA T/G T/C CTCAATTCAT^3. Nucleotides 11 and 12 are the first two positions of the Cys-61 codon, which are T and G, respectively, in the wild-type gene; the oligonucleotide was synthesized with a 1:1 mixture of the bases indicated at these positions. The sequence of the 30-mer used to change the Cys-328 codon to Ser, Ala, Val and Leu was the following: ^{5}GCATCACCGATGCC T/G T/C GATAGGCTGGGAAG^3. Residues 15 and 16 are the first two bases of the Cys-328 codon, which in the wild-type gene are T and G, respectively (13); the oligonucleotide was synthesized with a 1:1 mixture of the bases indicated at these positions. Positions 17 and 20 were synthesized with non-wild-type bases to reduce the stability of a stem-loop structure predicted for the wild-type sequence in this region. This was necessary as we were unable to recover mutants at Cys-328 with an oligonucleotide containing only the changes at positions 15 and 16, which lie in the "loop" region of the structure. Position 17 is the third base of the mutagenized Cys codon, while the change of C (wild-type) to A at position 20 is a silent change in the Ala-329 codon.

Single-stranded pSelG1 template DNA (0.6 pmoles) was mixed with the desired mutagenic oligonucleotide (1.2 pmoles) and the Amp^R-repair oligonucleotide (0.5 pmoles, purchased from Promega) in 50 mM Tris-HCl (pH 7.5), 50 mM $MgCl_2$, 50 mM NaCl, 2.5 mM DTT in a final volume of 20 µl. The mixture was incubated at 80°C for 10 min, cooled to room temperature for 15 min and then diluted into a final volume of 100 µl for the polymerization/ligation reaction, which contained 30 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM NaCl, 0.5 mM DTT, 1 mM ATP, 0.5 mM dATP, 0.5 mM dCTP, 0.5 Mm dGTP, 0.5 mM dTTP, 2 units T4 DNA polymerase (Boehringer Mannheim) and 5 units T4 DNA ligase (Bethesda Research Laboratories). Reactions were incubated at 37°C for 2 h. The preparation (10 µl) was used to transform *E. coli* BMH 71-18 with selection of transformants on L agar containing 50 µg/ml ampicillin and 10 µg/ml tetracycline (L/Amp/Tet). Plasmid DNA was prepared from several isolates by alkaline lysis and transformed into *E. coli* JM107. Transformants were selected and purified on L/Amp/Tet plates. Single-stranded DNA was isolated after M13K07 infection of the transformants, and mutations were identified by DNA sequencing (28) of the relevant regions of *aroG* using a 17-mer oligonucleotide primer (^{5}GGAAGTATTGCATTCAC^3) equivalent to bases -47 to -31 relative to the start of the *aroG* coding sequence for mutations at Cys-61, or a 16-mer oligonucleotide primer (^{5}CAGCCATGCTAACTCG^3) equivalent to bases 797-812 of the coding sequence for mutations at Cys-328.

The Cys-61 mutations were sub-cloned from pSelG1 on a 0.7 kb *Eco*RI/*Cla*I restriction fragment containing the 5' half of *aroG* into expression vector pttS10 by replacing the *Eco*RI/*Cla*I fragment of the wild-type *aroG* gene. Cys-328 mutations were sub-cloned on a 1.5 kb *Eco*RI/*Hin*dIII fragment containing the entire *aroG* coding sequence into pttS9. The relevant portions of the *aroG* inserts of all constructions were resequenced to confirm the presence of the mutations.

Analysis of metal binding by wild-type and mutant DAHPS(Phe). Samples of the purified wild type and C61V mutant enzymes (estimated by SDS-PAGE (29)) to be about 90% homogeneous) were depleted of endogenous metals by Chelex treatment and then incubated with an 8-fold molar excess of $MnCl_2$ or $CuSO_4$ (1 ml final volume) for 30 min at 22°C. Unbound metal was removed by passage of the preparations over 10 ml Sephadex G-50 columns (Pharmacia LKB) which had been pre-washed with 1 mM EDTA and equilibrated with metal-free buffer. Enzyme and buffer control samples were analyzed for metal content using a Perkin-Elmer model 5100 atomic absorption spectrometer equipped with a graphite furnace. The concentrations of the analyzed preparations were 47 µN for the wild-type enzyme and 15 µN for the C61V enzyme. The limit of detection for manganese was 18 nM and for copper was 8 nM.

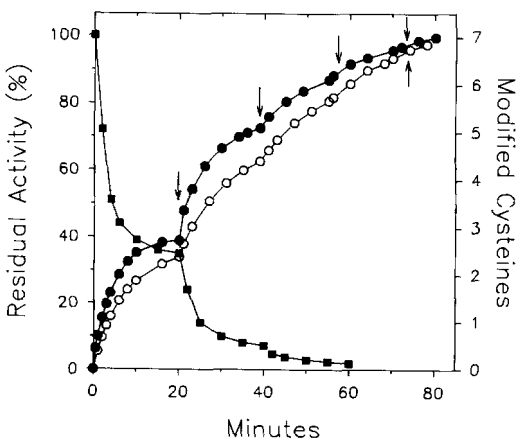

Fig. 3. Titration of DAHPS(Phe) cysteines with DTNB. DAHPS(Phe) was at 9 µN. At zero time, DTNB was added to 12 µM. At the times indicated by the arrow, aliquots of DTNB were added to increase the concentration of DTNB by increments of 12 µM. All reactions contained 25 µM PEP carried over with the enzyme. Modified cysteines per monomer in the native enzyme (●) and in enzyme in the presence of 2% SDS (○); DAHP synthase activity of native enzyme (■).

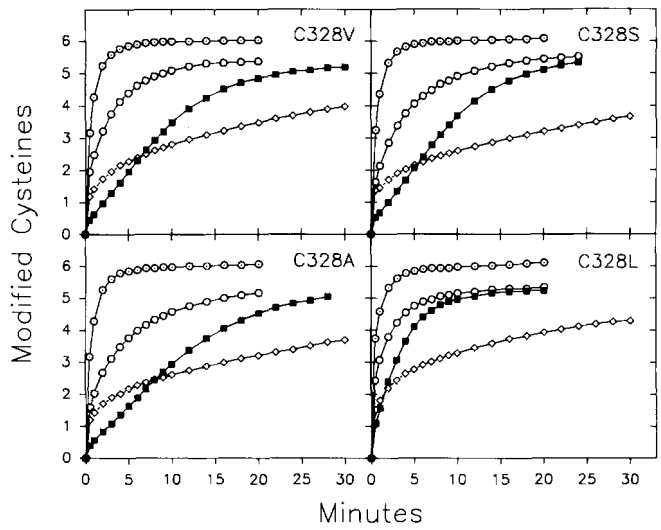

Fig. 5. DTNB modification of Cys-328 mutant enzymes. Initial concentrations: DAHPS(Phe), 2 to 3.5 µN; DTNB, 50 µM. Mutant enzyme concentrations were estimated as outlined under "Materials and Methods". All reactions contained 40 µM PEP carried over with the enzyme. Additions were as follows: no addition (○); 2% SDS (⊙); 100 µM PEP, 50 µM $MnCl_2$ (■); 100 µM Phe (◇). The data indicate the number of modified cysteines per enzyme monomer.

TABLE I

Effect of sulfhydryl-modifying reagents on DAHPS(Phe) activity

DAHPS(Phe) (10 µN) was incubated for 15 minutes with modifying reagent after which samples were removed and assayed. Activities shown are relative to that measured prior to addition of the modifying reagent. Assays were done at least in duplicate, with variations no greater than ± 5%. All reactions contained 25 µM PEP carried over with the enzyme.

Reagent	Added ligand			
	None	PEP[a]	Mn^{2+}[b]	Phe[a]
	Fraction activity remaining			
None	0.9	1.0	1.0	0.9
p-Chloromercuribenzoate[a]	<0.05	<0.05	<0.05	<0.05
5,5'-Dithio-bis-(2-nitrobenzoate)[a]	<0.05	<0.05	0.8	0.1
Bromopyruvate[a]	0.3	0.4	1.0	<0.05
N-Ethylmaleimide[a]	0.6	0.9	1.1	0.5
Iodoacetamide[c]	0.9	0.9	1.0	1.1

[a]100 µM
[b]50 µM
[c]1 mM

TABLE II

Kinetic constants of mutant DAHPS(Phe) synthases with changes at Cys-328

Enzyme[a]	Specific activity	K_m^{PEP}	K_m^{Mn}
	units/mg	µM	µM
Wild type	93.3	1.5	0.5
C328V	73.5	3.0	1.9
C328A	70.7	6.0	2.9
C328S	55.1	9.7	6.0
C328L	18.8	23	12.7

[a]Mutant enzymes are designated by the identity of the wild type residue followed by the position of the residue and then the identity of the mutant residue, using the single amino acid code.