# Probing the potential metal binding site in *Escherichia coli* 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (phenylalanine-sensitive)

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**Abstract The active site residues of the proposed metal binding site of DAH 7-P synthase (phe) were probed by site-directed mutagenesis of C61 to glycine and serine, H64 to glycine, and with the double mutant C61H/H64C. While C61S and C61H/H64C were inactive, both C61G and H64G were active. All mutants, regardless of enzymatic activity, bound one equivalent of $Fe^{2+}$ per monomeric unit. Even though C61 and H64 were shown not to be metal ligands for the DAH 7-P synthase (phe), they may provide some of the backbone interactions/secondary structural elements necessary to properly form the metal binding pocket.**



*Key words:* Deoxy-D-*arabino*-heptulosonate 7-phosphate synthase; Metal binding; Phospho*enol*pyruvate; Erythrose 4-phosphate; Site-directed mutagenesis

## 1. Introduction

The enzyme 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAH 7-P) synthase (phe) (EC 4.1.2.15) catalyzes an aldol-type condensation of phospho*enol*pyruvate (PEP) with D-erythrose 4-phosphate (E 4-P) to yield DAH 7-P and inorganic phosphate ($P_i$) in the first committed step in the biosynthesis of chorismate [1] (see Fig. 1). The enzyme 3-deoxy-D-*manno*-octulosonic acid 8-phosphate (KDO 8-P) synthase catalyzes a similar reaction except with D-arabinose 5-phosphate (A 5-P) and PEP as substrates to yield KDO 8-P and $P_i$ (Fig. 1). The facial stereoselectivity of the reactions catalyzed by these two enzymes, with respect to both the PEP and the aldehyde portion of the monosaccharide substrate, is identical [2–5] as is the regioselectivity of the phosphate bond cleavage step [6,7]. However, DAH 7-P synthase (phe) is tetrameric, requires a divalent metal for catalytic activity, and has a feedback inhibitor phenylalanine [8–10]. KDO 8-P synthase is trimeric, does not require a divalent metal for activity nor a feedback inhibitor [11]. There is only 6% amino acid sequence identity [12] between DAH 7-P synthase (phe) and KDO 8-P synthase. This leads to a very interesting question as to how two seemingly very different enzymes can catalyze mechanistically similar reactions.

Sulfhydryl group modification studies with DAH 7-P synthases from different microorganisms have suggested that at least one cysteine residue is essential for catalytic activity [13–15]. Two cysteine residues, cysteine-61 and cysteine-328 (numbers correspond to the *Escherichia coli* phe-sensitive isozyme), are highly conserved among all members of the DAH 7-P synthase super family [12]. Cysteine-61 has been suggested to be both catalytically essential and involved in metal binding based on the results from mutational studies in which four mutants of C61 (C61V, C61S, C61F and C61A) were constructed, expressed, purified and found to be catalytically inactive [16]. There is also an invariant histidine (histidine-64) in close proximity to cysteine-61 which has also been suggested to be involved in metal binding [16].

In the present work, site-directed mutagenesis was used to further explore the potential roles of cysteine-61 and the nearest nucleophilic residue, histidine-64 in both metal binding and catalysis. The following mutants C61G, C61S, H64G, and the double mutant C61H/H64C were constructed and characterized by examining the catalytic activity, gross structural changes, and the metal binding properties of each mutant.

## 2. Materials and methods

### 2.1. Materials

Restriction and DNA modifying enzymes were purchased from Boehringer Mannheim, New England Biolabs and Gibco BRL. The Promega DNA purification kit and the 5 Prime→3 Prime Perfectprep Plasmid DNA Kit were utilized for plasmid isolation and purification. The *E. coli* strain BL21(DE3) was obtained from Novagen. The *E. coli* XL-Blue1 supercompetent cells and the QuikChange Mutagenesis kit were obtained from Stratagene Cloning Systems. *Vent* DNA polymerase was purchased from New England Biolabs and substituted for the recombinant *Pfu* DNA polymerase provided in the QuikChange Mutagenesis kit. The thermal cycling was performed using an MJR Research Thermal cycler. The mutagenic oligonucleotides were synthesized by the University of Michigan Biomedical Research Resources Core Facility using β-cyanoethyl phosphoramidite chemistry on polystyrene support columns. DNA sequencing was performed by the University of Michigan Biomedical Research Resources Core Facility using ABI Model 373A DNA Sequencers. Phospho*enol*pyruvate mono(cyclohexyl)-ammonium salt and manganese(II) chloride were obtained from the Sigma Chemical Company. The 1,3-bis[tris-(hydroxymethyl)-methylamino]propane was purchased from Research Organics. Erythrose 4-phosphate was prepared in our laboratory by lead tetraacetate oxidative degradation of glucose-6-phosphate [17]. Chelex-100 resin (biotechnology grade), 100–200 mesh, sodium form, was purchased from Bio-Rad. Puratronic manganese(II) chloride, iron(II) sulfate, cobalt(II) chloride, nickel(II) chloride, and copper(II) sulfate were obtained from Alfa AESAR. Puratronic zinc(II) sulfate was obtained from Johnson Matthey, Materials Technology.

### 2.2. Wild-type DAH 7-P synthase (phe) expression and purification

Wild-type DAH 7-P synthase (phe) was cloned as described by Sheflyan et al. [18]. *E. coli* BL21(DE3) cells harboring the plasmid pT7-7/*aroG* were grown in 2×TY medium at 37°C. Isopropyl β-D-thioglucopyranoside was added to the culture at the mid logarithmic phase ($A_{600}$ = 0.6) to a final concentration of 400 μM to induce ex-

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*Abbreviations:* BTP, 1,3-bis[tris-(hydroxymethyl)-methylamino]propane; DAH 7-P, 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate; DAH 7-P synthase, 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate synthase; E 4-P, erythrose 4-phosphate; EDTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PEP, phospho*enol*pyruvate


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pression. The cells were harvested 4 h post induction by centrifugation, resuspended in 10 mM BTP buffer (pH 6.8), and sonicated (sonicator W220, Heat Systems Ultrasonics) at 4°C by applying 4×30 s pulses with 1 min delays between pulses. The cell debris was removed by centrifugation at 12 000×*g* for 20 min at 4°C. All further manipulations of the enzyme were performed at 4°C. DAH 7-P synthase was purified by anion exchange chromatography on a High Q Cartridge column (Bio-Rad) as previously reported [18]. Basically, after the protein was loaded onto the column, the column was first washed with 45 ml of 10 mM BTP buffer (pH 6.8) followed by a linear gradient of 0–0.5 M KCl (100 ml total volume) in 10 mM BTP buffer (pH 6.8) at a flow rate of 1.7 ml/min. The fractions (4 ml each) containing protein and exhibiting DAH 7-P synthase activity were pooled, concentrated to 4 mg/ml and stored at −80°C. The protein was determined to be >95% pure by SDS-PAGE analysis (data not shown). Frozen enzyme retained activity for several months.

*2.3. Site-directed mutagenesis*

DAH 7-P synthase (phe) mutants were prepared using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) [19,20]. DNA containing the proper mutagenic sequence, as determine by initial restriction digest (see Table 1), was used to transform chemically competent BL21(DE3) *E. coli* cells. The transformed cells were grown in 2×TY medium, 100 μg/ml ampicillin, at 37°C until the $OD_{600} = 0.4$. The transformed cells (800 μl) were mixed with 800 μl of 50% glycerol and stored at −80°C.

The DAH 7-P synthase (phe) mutants were overexpressed, isolated and purified as described above in Section 2.2 for the wild-type protein.

*2.4. Kinetic procedures*

The continuous UV spectrophotometric method originally reported by Schoner and Herrmann for the measurement of the disappearance of the double bond of PEP, based on the absorbance at $\lambda = 232$ nm ($\varepsilon = 2840\ M^{-1}\ cm^{-1}$), was used to assay DAH 7-P synthase activity [21]. The standard assay mixture contained in a volume of 1 ml: 10 mM BTP buffer (pH 6.8), 150 μM PEP, 500 μM E 4-P, and 1 mM $MnCl_2$. The enzyme reaction mixture was preincubated at 25°C for 5 min and the reaction initiated by addition of 5 μg of DAH 7-P synthase. The initial rates were determined from a least-squares fit of the data recorded for the first 30 s of the reaction (for the initial part of the curve) using Kaleidagraph 3.08d (Synergy Software). The initial rate data were plotted as a function of substrate concentration and fit to the Michaelis-Menten equation with Kaleidagraph.

*2.5. Preparation of apo-wild-type and mutant DAH 7-P synthase (phe)*

Two different methods were used to prepare apo-enzyme (metal-free). Both methods resulted in equally metal free enzyme.

Method 1. 5 ml of the protein solution (4 mg/ml) was dialyzed against 1 l of 10 mM BTP buffer pH 6.8 containing 10 mM EDTA for 12 h at 4°C. The EDTA was subsequently removed by dialyzing the above protein solution against three changes of 1 l of metal-free 10 mM BTP buffer pH 6.8 (4 h each) at 4°C.

Method 2. Preparations containing 10 mg of enzyme in 2.5 ml of 10 mM BTP buffer pH 6.8 were treated with EDTA at a final concentration of 10 mM for 30 min at 4°C. The EDTA was removed from the protein sample by gel filtration chromatography utilizing a gravity flow Sephadex G-25 superfine column (1.5×10 cm) with metal-free 10 mM BTP buffer pH 6.8.

Metal-free buffers were prepared by passing the solutions through a Chelex-100 column (1.5×12 cm, total capacity 5 mEq). The apo-enzyme solutions were checked for activity as described in Section 2.4 except in the absence of 1 mM $MnCl_2$ normally present in the assay mixture. The activity of metal-free wild-type and mutants of DAH 7-P synthase (phe) was also assayed by substituting 1 μM of $Cu^{2+}$, $Fe^{2+}$, $Zn^{2+}$, $Co^{2+}$, $Ni^{2+}$ for the 1 mM $MnCl_2$ in the standard assay mixture.

*2.6. Spectroscopic and analytical procedures*

The UV-visible absorption spectra of the metal-free (apo) DAH 7-P synthase wild-type and mutant proteins (40–80 μM per monomer, in 1 ml of 10 mM BTP buffer pH 6.8) were recorded using a Cary 3 Bio spectrophotometer (Varian). The enzyme solution was then titrated with $Cu^{2+}$, up to 10 times the monomer concentration. After each incremental addition of one equivalent (per monomer) of $Cu^{2+}$, the absorption spectrum was recorded.

The CD spectra were recorded on an Aviv Circular Dichroism Spectrophotometer Model 62 DS and deconvoluted by the method of Sreerama and Woody [22] as previously described by Salleh et al. [23]. After the CD spectrum of metal-free enzyme (3 μM per monomer in 300 μl) was obtained, 3 μM of $Cu^{2+}$, in 1 μl of water, was added to the sample still in the cuvet. After mixing by inversion, the sample was incubated at 25°C for 30 min and the CD recorded. A control dilution experiment was performed by adding 1 μl of water to a solution of wild-type enzyme in 300 μl and recording the CD spectrum.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli [24]. The protein concentrations in cell lysates and soluble enzyme preparations were determined with the Bio-Rad protein assay (Bio-Rad, Richmond, CA) using bovine serum albumin as a standard.

*2.7. Metal analysis*

The concentration of $Fe^{2+}$ bound to protein was determined using the method of Fish [25]. Metal-free DAH 7-P synthase (phe) (40–80 μM per monomer) was incubated for 1 h with iron(II) sulfate ($Fe^{2+}$) at 10 times (per monomer) the molar ratio of the DAH 7-P synthase (phe) proteins. The excess Fe(II) was removed from the protein by gel-filtration as described in Section 2.5, method 2. The eluted protein fraction was used for estimating protein-bound iron as well as the protein concentration.

The presence and quantitation of all metals in enzyme preparations were determined on a Finnigan MAT ELEMENT high-resolution inductively coupled plasma-mass spectrometer (HR-ICP-MS) system by Dr. Ted Huston of the Department of Geology at the University of Michigan.

## 3. Results

*3.1. Overexpression and purification of DAH 7-P synthase (phe) wild-type and mutants*

Wild-type DAH 7-P synthase (phe) enzyme was obtained from *E. coli* BL21(DE3) cells harboring the pT7-7/*aroG* plasmid DNA and purified by anion exchange chromatography as previously described [18]. Enzyme activity was monitored by the continuous spectrophotometric method reported by Schoner and Herrmann [21]. The purity of DAH 7-P synthase

Table 1
Oligonucleotides used for the mutagenesis of *E. coli* DAH 7-P synthase

| Target amino acid | Primers 5′→3′ | Restriction site |
|---|---|---|
| C61G | GTGATTGGC*CCAGGC*TCAATTCATGATCCTGTCGCGGC<br>GCCGCGACAGGATCATGAATTGAG*CCTGG*GCCAATCAC | +*Mva*I |
| C61S | GTTGTGATTGGCCCATCCTCAATTCATGATCCTG<br>CAGGATCATGAATTGAGGATGGGCCAATCACAAC | −*Fok*I |
| H64G | GTGATTGGCCCATGCTCAATTGG*GGATCC*TGTCGCGGC<br>GCCGCGACA*GGATCC*CCAATTGAGCATGGGCCAATCAC | +*Bam*HI |
| C61H/H64C | GTGATTGGCCCACACTCAATTTGTGATCCTGTCGCGGC<br>GCCGCGACAGGATCACAAATTGAGTGTGGGCCAATCAC | −*Bsp*HI |

The modified nucleotides are underlined and the newly engineered endonuclease restriction site is shown in italics. The symbol (+) indicates the addition of a new endonuclease restriction site while a (−) signifies the loss of an endonuclease restriction site.

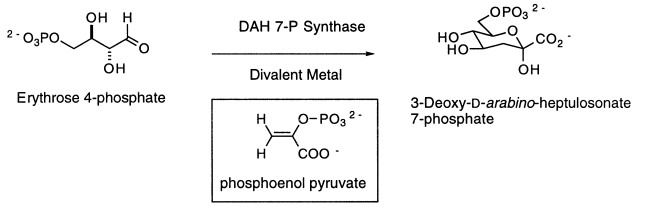


Fig. 1. Reaction catalyzed by *E. coli* DAH 7-P synthase (phenylalanine-sensitive).

(phe) was estimated to be approximately 98% using SDS-PAGE with a monomeric molecular weight of 38 kDa.

Various DAH 7-P synthase (phe) mutants were prepared by the method of Wiener [19,20] using the primers listed in Table 1. The desired mutation in the DNA was first confirmed by restriction endonuclease digestions with the appropriate restriction enzymes (Table 1) and finally by DNA sequencing. The mutants were overexpressed and isolated using the same procedure as for wild-type. The expression levels and purification elution profiles on anion exchange chromatography of all the mutants were *identical* to that of the wild-type protein. The mutants were purified to $>95\%$ purity as determined by SDS-PAGE.

### 3.2. Kinetics for DAH 7-P synthase (phe) mutants

The kinetic parameters obtained for the DAH 7-P synthase mutants are shown in Table 2. While C61G DAH 7-P synthase mutant was active, it demonstrated both a significant decrease in $k_{cat}$ (approximately 50 times lower than the $k_{cat}$ for wild-type) and hindered PEP binding as determined by a $K_m$ for PEP 100 times higher than that of wild-type. The H64G mutant was active and had a $k_{cat}$ almost twice that of wild-type enzyme, however, the $K_m$ for PEP was four-fold higher than wild-type. The C61S and C61H/H64C mutants were basically inactive (Table 2).

### 3.3. Metal analysis

Metal-free enzymes were prepared and assayed for DAH 7-P synthase activity. In the absence of exogenously added metal, apo-enzymes retained less than 5% of the activity of holo-enzymes. This activity is most likely due to the presence of residual amounts of $Zn^{2+}$ still present in the apo-enzyme sample as determined from HR-ICP-MS. Enzymatic activity was restored to wild-type DAH 7-P synthase as well as the C61G and H64G mutants upon addition of $Mn^{2+}$ into the assay mixture to a final concentration of 1 mM. The ability of other divalent metal ions to stimulate the DAH 7-P synthase activity in the mutants was tested by measuring DAH 7-P synthase activity in the presence of various divalent metal ions (see Table 3). Previous workers have demonstrated the ability of a wide variety of divalent metals to support catalysis albeit at various rates [8]. While the $K_m$s of all the various metals for DAH 7-P synthase were not reported, a displacement study did establish a rank order of metal binding. Since DAH 7-P synthase displayed the highest activity with $Mn^{2+}$, its $K_m$ for wild-type DAH 7-P synthase was chosen to establish the metal binding properties of the various mutants. The relative activities of mutants were measured in the presence of 1 μM divalent metal. The results are presented in Table 3. Interestingly, at the metal concentrations tested, the mutants C61G and H64G had altered metal preferences as compared to wild-type and were most active in the presence of $Zn^{2+}$. These results could be indicative of changes in structure and/or electronic environment of the metal binding site in these mutants. The C61S and C61H/H64C DAH 7-P synthase mutants were found to be inactive irrespective of the divalent metal ion included in the assay mixture.

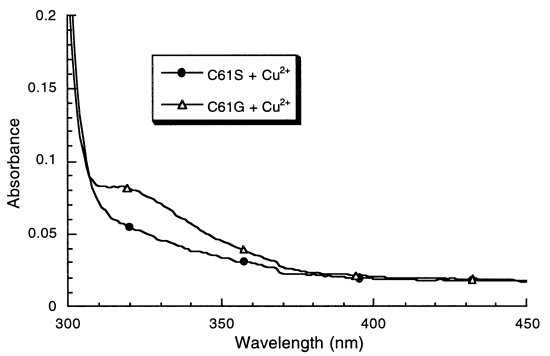


Fig. 2. The UV absorption spectra of the DAH 7-P synthase mutants C61S (●) and C61G (△) in the presence of 5 molar equivalents of $Cu^{2+}$ per monomer of protein.

Table 2
Kinetic constants of DAH 7-P synthase (phe)

| Enzyme[a] | $k_{cat}$ ($s^{-1}$) | $K_M^{PEP}$ (μM) | $K_M^{E4-P}$ (μM) |
|---|---|---|---|
| Wild-type[b] | 32 | 9 | 86 |
| C61G | 0.6 | >1000 | 45 |
| C61S | na[c] | na | na |
| H64G | 53 | 40 | 140 |
| C61H/H64C | na | na | na |

[a]The mutant enzymes are designated by the identity of the wild-type residue mutated, followed by the numerical position of that residue, then the single amino acid code of the replacement amino acid.
[b]Kinetic values obtained from the continuous assay described in Section 2.
[c]na = not active.

The metal binding capability of the DAH 7-P synthase mutants was analyzed for their ability to bind $Fe^{2+}$ using the Fish assay [25]. Metal-free enzyme was incubated with a 10-fold excess of iron ($Fe^{2+}$) for 1 h, after which the unbound $Fe^{2+}$ was removed by gel filtration chromatography. The DAH 7-P synthase-containing fractions were assayed for iron content and protein concentration. Wild-type DAH 7-P synthase (phe) and *all four mutants* were found to bind 1 mol of iron per mol of enzyme monomer, irrespective of their ability to catalyze the condensation reaction. The stoichiometry was verified utilizing HR-ICP-MS.

It has been reported that DAH 7-P synthase is a natural $Cu^{2+}$-containing enzyme [26]. Other reports verify that $Cu^{2+}$-containing DAH 7-P synthase is catalytically active [8,27]. Based on these reports, we decided to determine if our mutant enzymes could bind $Cu^{2+}$ and if they did, to determine the stoichiometry of their metal binding. We incubated wild-type and all four mutant DAH 7-P synthases with a 10-fold excess of copper(II) sulfate for various lengths of times, at various temperatures, and then removed the non-bound copper by either of the two methods reported in the experimental section. While the wild-type enzyme as well as *all* four mutants (C61G, C61S, H64G and C61H/H64C) were able to bind $Cu^{2+}$, we were unable to obtain a reproducible ratio for

Table 3
Activity[a] of wild-type DAH 7-P synthase and mutants in the presence of 1 μmol of the indicated metal

| Enzyme | $Mn^{2+}$ | $Cu^{2+}$ | $Fe^{2+}$ | $Zn^{2+}$ | $Co^{2+}$ | $Ni^{2+}$ |
|---|---|---|---|---|---|---|
| Wild-type | 1.0 | 1.1 | 1.0 | 0.8 | 1.2 | 1.8 |
| C61G | 0.3 | 0.1 | 0.1 | 0.4 | 0.1 | 0.3 |
| C61S | na[b] | na | na | na | na | na |
| H64G | 0.7 | 1.2 | 0.5 | 1.2 | 1.1 | 1.3 |
| C61H/H64C | na | na | na | na | na | na |

[a]Activity is reported as μmol of product formed/(min × mg).
[b]na = not active.

DAH 7-P synthase:$Cu^{2+}$ via HR-ICP-MS analysis. The values ranged from 0.1 to 4. These results are consistent with previous reports that $Cu^{2+}$ is a very weak metal ligand [8].

At least three groups studying $Cu^{2+}$-containing DAH 7-P synthase (one equivalent of $Cu^{2+}$ per monomer in the presence of PEP) reported that the UV spectrum showed an absorbance near 350 nm [8,26,27]. This absorbance has been attributed to a ligand-to-metal charge transfer process between $Cu^{2+}$ and DAH 7-P synthase [8]. It has been reported that four 'inactive' C61 DAH 7-P synthase mutants (C61A, C61V, C61F and C61S) do *not* display this absorbance at $\lambda = 350$ nm where as the four 'active' C328A/V/F/S mutant DAH 7-P synthases do display the peak [16]. These observations lead the authors to conclude that $Cu^{2+}$ must ligate to C61 for catalytic activity as well as causing the $\lambda = 350$ nm absorbance. A similar band at $\lambda = 320$–325 nm has been observed in the present studies for our recombinant *E. coli* (phe) DAH 7-P synthase when incubated with $Cu^{2+}$ either in the presence or in the absence of PEP. We have also shown that in the presence of $Cu^{2+}$ the DAH 7-P synthase C61G mutant displays the $\lambda = 325$ nm peak. We interpret this to mean that the thiolate of cysteine 61 is *not* participating in $Cu^{2+}$ (metal) ligation. Our enzymatically 'inactive' DAH 7-P synthase mutants C61S and C61H/H64C did *not* exhibit the peak at $\lambda = 325$ nm whereas our enzymatically 'active' H64G mutant did exhibit the peak. The UV absorption spectra of the DAH 7-P synthase mutant C61G (active) and C61S (inactive) in the presence of an excess (5 molar equivalents per monomer) of $Cu^{2+}$ is shown in Fig. 2. Re-examination of the finding by the previous workers in the light of our new results strongly suggest that in the C61A, C61V, C61F and C61S mutants (and our C61H/H64C) the replacement of the sulfur atom of cysteine with a different side chain from the replacement amino acid must drastically alter the environment of the $Cu^{2+}$ (metal) binding site.

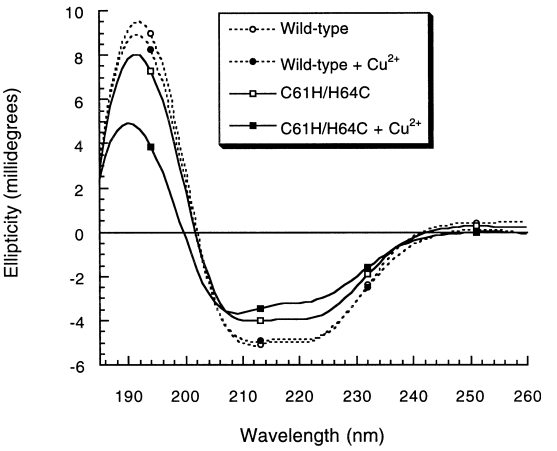


Fig. 3. The CD spectra of apo-wild-type (○) and mutant C61H/H64C (□) DAH 7-P synthase in the absence of metal and in the presence of 1 molar equivalent of $Cu^{2+}$ [holo-wild-type (●) and holo-C61H/H64C (■)].

### 3.4. CD spectra

The influence of each mutation on the gross structure of apo-DAH 7-P synthase (phe) was assessed using circular dichroism spectroscopy. In the case of apo-enzymes, all mutations, except H64G, evoked only minor decreases in the content of α-helix with a concomitant increase in β-sheet content (Table 4) as compared to wild-type. A large increase in the α-helix content was observed for the H64G. Addition of 1 equivalent of $Cu^{2+}$ to apo-wild-type DAH 7-P synthase and the apo-mutant C61G, caused only a small increase in the content of α-helix (1–4%). A small decrease in α-helix content was observed with the C61S mutant upon addition of $Cu^{2+}$ while a large decrease was observed with holo-H64G. While the α-helix, β-sheet and β-turn content of apo-C61H/H64C DAH 7-P synthase was similar to that of the other mutants except H64G (Table 4), the presence of $Cu^{2+}$ caused such a significant structural change that deconvolution was not possible utilizing the self consistent method (SELCON program) described by Sreerama and Woody [22] (Fig. 3).

## 4. Discussion

The amino acid sequences of DAH 7-P synthase from *E. coli*, *Salmonella typhimurium*, *Saccharomyces cerevisiae* and other diverse microorganisms show a high degree of similarity, suggesting a conserved active site structural motif [12,16,28]. Cysteine-61 has been suggested to be both catalytically essential as well as involved in metal binding [16]. The

Table 4
Deconvoluted CD data of apo-DAH 7-P synthase and apo-mutants in the presence of 1 molar equivalent of $Cu^{2+}$

| DAH 7-P synthase | α-Helix (%) | β-Sheet (%) | β-Turn (%) |
|---|---|---|---|
| Wild-type | 19.7 | 17.7 | 18.0 |
| Wild-type+1 eq. $Cu^{2+}$ | 19.9 | 15.6 | 17.5 |
| C61G | 14.4 | 21.5 | 16.5 |
| C61G+1 eq. $Cu^{2+}$ | 15.0 | 19.9 | 16.0 |
| H64G | 22.3 | 16.3 | 19.6 |
| H64G+1 eq. $Cu^{2+}$ | 17.9 | 20.5 | 18.4 |
| C61S | 15.3 | 20.3 | 17.3 |
| C61S+1 eq. $Cu^{2+}$ | 14.9 | 20.9 | 16.3 |
| C61H/H64C | 14.2 | 22.3 | 16.8 |
| C61S/H64C+1 eq. $Cu^{2+}$ | nd[a] | nd | nd |

[a]nd = not determinable by the method of Sreerama and Woody [22].

roles of cysteine-61 and the invariant histidine-64 in close proximity have been further examined in the present study in order to better understand their role in DAH 7-P synthase.

We have confirmed that C61S is inactive as previously reported [16]. We were able to demonstrate for the first time that elimination of the sulfur atom of cysteine-61 even in the inactive mutants (C61S and C61H/H64C) does not result in loss of metal binding. Wild-type DAH 7-P synthase as well as the mutants C61G, C61S, H64G and C61H/H64C bind $Fe^{2+}$ in the ratio of 1 iron ion per monomer of the protein whereas wild-type DAH 7-P and the mutants C61G, C61S, H64G and C61H/H64C bind $Cu^{2+}$ but not stoichiometrically. Spectroscopic studies suggest that wild-type enzyme and active mutants undergo a significant change in structure upon binding of $Cu^{2+}$. Wild-type DAH 7-P as well as the active mutants C61G and H64G, in the presence of $Cu^{2+}$, display an absorption band at $\lambda = 325$ nm whereas the inactive mutants C61S and C61H/H64C do not.

The histidine-64, though invariant in DAH 7-P synthases [16], seems not to be involved in the binding of metal as demonstrated by the kinetic and spectroscopic studies of the H64G mutant. The C61H/H64C mutant exhibits complete loss of activity probably due to the bulky substitution of cysteine-61 by histidine as was previously observed [16].

Thus, the mutagenic and spectroscopic studies presented here imply that cysteine-61 and histidine-64 are not directly involved in metal binding in DAH 7-P synthase (phe), but that these residues may be near the metal binding site or in some way influence the geometry of the metal binding site. The kinetic data suggest that these residues may be near the binding site of the substrates and/or involved directly in their binding at the active site.

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