Histidine 268 in 3-Deoxy-D-arabino-heptulosonic Acid 7-Phosphate Synthase Plays the Same Role as Histidine 202 in 3-Deoxy-D-manno-octulosonic Acid 8-Phosphate Synthase

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The enzyme 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAH 7-P) synthase (Phe) is inactivated by diethyl pyrocarbonate (DEPC). The inactivation is first order with respect to enzyme and DEPC concentrations with a pseudo-second order rate constant of inactivation by DEPC of 4.9 ± 0.8 m⁻¹ s⁻¹ at pH 6.8 and 4 °C. The dependence on pH and the spectral features of enzyme modified at specific pH values imply that both histidine and cysteine residues are modified, which is confirmed by site-directed mutagenesis. Analysis of the chemical modification data indicates that one histidine is essential for activity. DAH 7-P synthase (Phe) is protected against DEPC inactivation by phosphonoacrylate, whereas D-erythrose 4-phosphate offers only minimal protection. The conserved residues H-172, H-207, H-268, and H-304 were individually mutated to glycine. The H304G and H207G mutants retain some level of activity, whereas the H268G and H172G mutants are virtually inactive. A comparison of the circular dichroism spectra of wild-type enzyme and the various mutants demonstrates that H-172 may play a structural role. Comparison of the UV spectra of the H268G and wild-type enzymes saturated with Cu²⁺ indicates that the metal-binding site of the H268G mutant resembles that of the wild-type enzyme. The residue H-268 may play a catalytic role based on the site-directed mutagenesis and spectroscopic studies. Cysteine 61 appears to influence the pKa of H-268 in the wild-type enzyme. The pKa of H-268 increases from 6.0 to 7.0 following mutation of H-268 (17). The role of cysteine residues in DAH 7-P synthase (Phe) has been investigated using chemical modification, and it was found that at least one cysteine residue is required for DAH 7-P synthase (Phe) activity (18–20). Site-directed mutagenesis studies suggest that C-61 is catalytically essential in DAH 7-P synthase (Phe) and is a metal ligand (21). Recently, Sundaram et al. have reported that C-61 is located near the metal in the active site (22). Park et al. (23, 24) confirmed that C-61 and C-328 reside in the active site of the enzyme based on metal oxidation studies and on the crystal structure of DAH 7-P synthase (Phe).

Chemical modification studies of E. coli KDO 8-P synthase (Phe) reveal that there are two essential cysteine residues (13, 25). Recently, using chemical modification studies and site-directed mutagenesis, it was determined that there is at least one essential histidine, H-202, in KDO 8-P synthase (26). Sequence alignment studies of KDO 8-P synthase and DAH 7-P synthase (Phe) indicate that H-202 in KDO 8-P synthase (Phe) is homologous to H-268 in DAH 7-P synthase (Phe) (17). Both of these enzymes catalyze the same type of reaction and appear to require a cysteine residue for activity, and DAH 7-P contains a histidine residue that aligns with an essential histidine in KDO 8-P synthase. Based on these facts, it would appear that histidine residues might also play a role in DAH 7-P synthase (Phe) activity. In this paper, we investigate the role of histidine residues in DAH 7-P synthase (Phe) using chemical modification and site-directed mutagenesis.

EXPERIMENTAL PROCEDURES

Materials—DNA-modifying enzymes were purchased from Roche Molecular Biochemicals, New England BioLabs, and Life Technologies,
Inc. The Promega Wizard DNA purification kit was utilized for plasmid isolation and purification. The E. coli cells, XL1-Blue, and the QuikChange mutagenesis kit were obtained from Stratagene Cloning Systems. E. coli cells, BL21(DE3), were obtained from Novagen. Thermal cycling was performed using an MJR Research thermal cycler. DNA sequencing and primer synthesis was performed by the University of Michigan Biomedical Resources Core Facility. PEP mono(cyclohexyl)-ammonium salt, E 4-P sodium salt, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), and diethyl pyrocarbonate (DEPC) were obtained from Sigma Chemical Co. The 1,3-bis[tris-(hydroxylmethyl)methylamino]propane (BTP) was purchased from Research Organics, Inc. Biotechnology grade Chelex-100 resin was purchased from Bio-Rad. Manganese(II) chloride, iron(II) sulfate, and copper(II) sulfate, all Puratronic grade, were obtained from Johnson Matthey. EDTA disodium salt was obtained from Mallinckrodt.

Site-directed Mutagenesis—The construction of a plasmid containing the aroG gene coding for wild-type DAH 7-P synthase (Phe), pT7-7/aroG, has been previously reported (27). This plasmid served as the template for polymerase chain reaction mutagenesis utilizing the QuikChange site-directed mutagenesis kit (28, 29) and the primers given in Table I. The potential mutagenic plasmids were used to transform competent XL1-Blue cells. The purified mutant plasmid DNA from selected clones was characterized by restriction digestion followed by DNA sequencing. The plasmid containing the proper mutant sequence was used to transform competent E. coli BL21(DE3) cells for the expression of the desired mutant protein.

Wild-type and Mutant DAH 7-P Synthase (Phe) Expression and Purification—E. coli BL21(DE3) cells containing the pT7-7/aroG plasmid, with either the histidine to glycine mutant gene or the wild-type gene, were cultured, and the proteins were isolated and purified as previously reported (27). All proteins were >95% pure as determined by SDS-polyacrylamide gel electrophoresis analysis (data not shown).

Analytical Procedures—Protein concentrations were determined by the Bradford assay (30) using the Bio-Rad protein assay reagent with bovine serum albumin as a standard. Protein concentrations were determined using the calculated monomer molecular weight (38 kDa) based on the amino acid sequence of DAH 7-P synthase (Phe). Optical spectroscopy was performed using a Cary 3 Bio UV-visible spectrophotometer (Varian). The circular dichroism spectra of the wild-type and the mutant enzymes were obtained using an Aviv circular dichroism spectrophotometer Model 62 DS and deconvoluted using the method of Sreerama and Woody (31) as described by Saleeh et al. (25).

DAH 7-P Synthase (Phe) Assay—The continuous UV spectrophotometric method reported by Schoner and Hermann (32), based on the absorbance of the double bond of PEP (A = 232 nm; ε = 2840 cm⁻¹ mol⁻¹), was used to assay DAH 7-P synthase (Phe) activity for both wild-type enzyme and the histidine mutants. The standard assay mixture (1 ml) contained 10 mM BTP buffer (pH 6.8), 150 μM PEP, 500 μM E 4-P, and 1 mM MnCl₂. The reaction mixture was preincubated at 25 °C for 5 min and initiated by the addition of DAH 7-P synthase (Phe) (125 nm). To determine the kinetic parameters, the concentration of one substrate was held constant (10 × Kₘ) while the other was varied over the range of 0.1 to 10 × Kₘ. The initial rates were determined from a least-squares fit of the data recorded for the first 30 s of the reaction using Kaleidagraph version 3.08d (Synergy Software). Best fits of Kₘ and Vₘₐₓ were determined by fitting the initial velocity versus substrate concentration using a non-linear least squares fit to the Michaelis-Menten equation.

DAH 7-P Synthase (Phe) Assay for the C61G Mutant—An alternate method for determining the activity of this mutant was utilized due to its diminished activity. The assay mixture contained 10 mM BTP (pH 6.8), 3 mM E 4-P, 3 mM PEP, 1 mM MnCl₂, and the C61G mutant (125 nm) in a total volume of 150 μl. The mixture was incubated at 37 °C for 10 min and then quenched with 150 μl of 10% trichloroacetic acid. The DAH 7-P synthase (Phe) activity was measured by determining the amount of DAH 7-P produced using a slight modification of the periodate-thiobarbituric acid assay (3, 33). The oxidation step was performed at 60 °C instead of at room temperature.

Preparation of Metal-free Wild-type and Mutant DAH 7-P Synthases (Phe)—Two ml of the protein solution (4 mg/ml) was dialyzed against 1 L of 10 mM BTP (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 1 L of metal-free 10 mM BTP (pH 6.8) (3 × 4 h each) at 4 °C. The latter buffer was rendered metal free by passage over a Chelex-100 column. The activity of metal-free wild-type DAH 7-P synthase (Phe) was reduced to 5% of the Mn²⁺-DAH 7-P synthase (Phe).

Iron Determination—The concentration of iron bound to protein was determined using the colorimetric method developed by Fish (34). Metal-free DAH 7-P synthase (Phe) (wild-type and mutants) (12.5 μM) was incubated for 1 h with 10 equivalents of iron (II) sulfate in 10 mM BTP (pH 6.8). The excess iron was removed by dialyzing against 1 L of metal-free 10 mM BTP (pH 6.8) (3 × 4 h each) at 4 °C. The dialyzed protein fractions were analyzed for iron and protein concentrations.

Modification of DAH 7-P Synthase (Phe) with DEPC—Stock solutions of DEPC (10 mM) were prepared in absolute ethanol immediately before use. The inactivation of DAH 7-P synthase (Phe) was performed in a reaction mixture containing 2 μM metal-free DAH 7-P synthase (Phe), 10 mM BTP (pH 6.8), and DEPC (0.2–1.4 mM) at 4 °C. At various times over 15 min (12 min effective time), aliquots (5 μl) were removed and assayed for residual enzyme activity. The initial concentration of DEPC in the reaction solutions was determined spectrophotometrically by reacting an aliquot with imidazole (10 mM) and measuring the increase in absorbance at 240 nm (ε = 3200 M⁻¹ cm⁻¹) (35). The rate of hydrolysis of DEPC was determined by incubating DEPC (1 mM) in 10 mM BTP (pH 6.8) at 4 °C. Aliquots were assayed for the amount of DEPC remaining at various time intervals using the imidazole assay described above. The enzyme-DEPC inactivation data were fit to the following equation:

$$\ln(A/A_0) = -\frac{k_p}{k_e} \log(t - t_0)$$

(Eq. 1)

where A/A₀ is the fractional activity remaining at time t, t₀ is the initial concentration of DEPC, kₚ is the pseudo-first order rate constant for the inactivation of DAH 7-P synthase (Phe) by DEPC, and kₑ is the pseudo-first order rate constant for the hydrolysis of DEPC (36). The value of kₑ was determined to be 0.026 min⁻¹ at pH 6.8.

Spectroscopic Characterization of DEPC-modified DAH 7-P Synthase (Phe)—DAH 7-P synthase (Phe) (25 μM) was incubated in the presence of DEPC (1 mM) in 10 mM BTP of various pH values (6.8–7.2) at 23 °C. After 15 min, the difference UV spectrum was acquired using an identical enzyme solution lacking DEPC as a control.

Correlation between Enzyme Activity and Histidine Residues Modified by DEPC—DAH 7-P synthase (Phe) (25 μM) and DEPC (1 mM) were incubated in the absence or presence of Mn²⁺ (80 μM) in 10 mM BTP (pH 6.8) at 4 °C. At 3-min intervals over 15 min, aliquots were removed and the activity was determined. In a parallel experiment, the number of histidine residues modified at each time interval as above was determined by measuring the increase in the difference spectra at 240 nm. The data were evaluated according to the following equation as described by Tsou (37, 38):

$$\log \left[ \frac{n_k}{A/A_0} - p \right] = \log(n - p) + \left( \frac{a - 1}{l} \right) \log \left( \frac{A}{A_0} \right)$$

(Eq. 2)

where A/A₀ is the fraction of enzyme activity remaining, n is the number of modifiable residues of which p residues, including i essential residues, react with DEPC at a pseudo-first order rate constant kᵢ and (n − p) nonessential residues react at a rate constant kᵢ (kᵢ = a/kₑ), and x is the number of residues remaining after the DEPC inactivation.

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reaction. The number of essential residues is determined by plotting log \((\text{ln}(\text{A}/\text{A}_0))\) versus log \((\text{DEPC})\). The variables, \(p\) and \(i\), are varied to give the best linear fit, and the value of \(\alpha\) is determined from the slope.

**pH Dependence of Inactivation—DEPC** was added (1 mM final concentration) to various DAH 7-P synthase (Phe) samples (2 \(\mu\)M) in 10 mM BTP buffers with pH values ranging from 5.8 to 8.5. Aliquots were removed at various times and assayed for residual enzyme activity. The pseudo-first order rate constant of DEPC hydrolysis \(k'\) was determined independently at each pH. The \(k'\) value increased approximately 5-fold at pH 8.5 as compared with pH 6.8 (0.101 versus 0.026 \(\text{min}^{-1}\)). The pseudo-first order rate constant of DEPC inactivation of DAH 7-P synthase (Phe) was determined at each pH value as described previously. The data were fit to the following equation:

\[
\ln(A/A_0) = K_{a}a[H^+] - K_{a}a[H^+] \text{obs(max)} - K_a a[H^+] \text{obs(min)}
\]

where \(K_a\) is the acid dissociation constant of the reacting group, and \(K_a a[H^+] \text{obs(max)}\) is the pseudo-first order rate constant of the unprotonated reacting group (39).

**Determination of Cysteine Residues Modified by DEPC—Metal-free DAH 7-P synthase (Phe) (2 \(\mu\)M) in 10 mM BTP (pH 7.0 or 7.2) was incubated in the presence or absence of 1 mM DEPC for 15 min. An aliquot (100 \(\mu\)l) was diluted with 0.5 mM DTNB, 4 M guanidinium hydrochloride, 150 mM Tris (pH 7.6) (700 \(\mu\)l). Total free thiold concentration was determined from the absorbance at 412 nm \((e = 13,600 \text{ M}^{-1} \text{ cm}^{-1})\). Appropriate control solutions were prepared to correct for background absorption.

**Substrate Protection—DAH 7-P synthase (Phe) or its mutants (2 \(\mu\)M) were mixed with an 80 \(\mu\)M concentration of either PEP, E 4-P, or MnCl\(_2\) prior to addition of 1 mM DEPC. After the addition of DEPC, aliquots (5 \(\mu\)l) were removed at various times and the enzyme was assayed for residual activity.

**Spectroscopy of H268G Mutant DAH 7-P Synthase (Phe)—**The UV-visible absorption spectra of the metal-free H268G mutant enzyme (80 \(\mu\)M) in the absence or presence of CuSO\(_4\) (0.8 mM) in 10 mM BTP buffer (pH 6.8) were recorded.

**RESULTS**

**Inactivation of wild-type DAH 7-P Synthase (Phe) by DEPC—**Metal-free DAH 7-P synthase (Phe) is inactivated in a time-dependent manner by DEPC as shown in Fig. 1. The pseudo-first order rate constants vary linearly with DEPC concentration (Fig. 1, inset). The second order rate constant for the inactivation of DAH 7-P synthase (Phe) by DEPC obtained from the slope of the plot is 4.9 \(\pm\) 0.8 \(\text{M}^{-1} \text{ s}^{-1}\) (Fig. 1, inset).

**Correlation between Enzyme Activity and Histidine Residues Modified by DEPC—**Plots of residual enzyme activity as a function of the number of histidine residues modified by DEPC at pH 6.8 with and without Mn\(^{2+}\) (Fig. 2) indicate that activity decreases with an increase in the number of modified histidine residues. After a 15-min incubation of DAH 7-P synthase (Phe) with 1 mM DEPC, five out of the eleven histidine residues were modified with 80% loss of activity. However, over the same time period, seven histidine residues are modified in the presence of Mn\(^{2+}\) with 100% loss of activity (Fig. 2). The number of essential histidine residues as determined using Eq. 2 is one in the presence or absence of Mn\(^{2+}\) (Fig. 3). The values of \(\alpha\), the constant relating the reaction rates of the essential and nonessential residues with and without metal present, are 0.31 and 0.33, respectively. These results indicate that one essential histidine is present and reacts approximately three times faster than the nonessential residues.

**Characterization of DEPC-modified Enzyme—**When metal-free DAH 7-P synthase (Phe) is modified with DEPC, the difference spectrum between the modified and untreated enzymes at pH 6.8 (not shown) or pH 7.0 (Fig. 4A) shows an absorption maximum at 240 nm characteristic of the carbethoxylolation of histidine (40). The lack of a trough at 278 nm (42) as discussed below, cysteine residues are not carbethoxylated (41). Cysteine residues are not modified under these conditions as evidenced by the lack of a peak at 230 nm (42). However, as discussed below, cysteine residues appear to be modified at higher pH values or in the presence of Mn\(^{2+}\) (Fig. 4, B and C).

**pH Dependence of Inactivation—**DEPC inactivation of DAH 7-P synthase (Phe) was studied in the pH range of 5.8–8.5. A representative plot of DEPC inactivation of metal-free DAH 7-P synthase (Phe) as a function of pH is depicted in Fig. 5A, which reveals that the data are discontinuous. Although the observed discontinuity is not expected for this type of experiment, several repeated attempts produced analogous results. The data are discontinuous between pH 7.0 and 7.2, and two \(pK_a\) values can be calculated using Eq. 3. The \(pK_a\) values are approximately 6.0 and 7.5, as determined from the available data. Metal-free DAH 7-P synthase (Phe) modified by DEPC at pH values immediately before and after the discontinuity (pH 7.0 and 7.2, respectively) were characterized using UV-difference spectroscopy (Fig. 4, A and B). Cysteine residues appear to be carbethoxylated at pH 7.2, based on the appearance of a
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Various mutants is given in Table II. Substantial changes in structural changes for the mutants H172G, H207G, and H304G. The CD spectra of the H268G and C61G mutants are circular dichroism spectroscopy. There appear to be significant discontinuity in the pH-DEPC inactivation plot (Fig. 5A) (42). Of the seven cysteine residues in DAH 7-P synthase (Phe), the number remaining after DEPC modification at pH values 7.0 and 7.2 are 6.7 ± 0.4 and 5.8 ± 0.3, respectively, implying that one cysteine residue is modified by DEPC at the higher pH. In the presence of Mn²⁺, DEPC reacts with this cysteine residue at pH 7.0 (Fig. 4C), and the discontinuity in the pH-DEPC inactivation plot (Fig. 5B) now occurs between pH 6.8 and 7.0. The UV-difference spectra of the metal-free C61G mutant (22) modified at pH 7.2 is shown in Fig. 4D. The shoulder at 230 nm is no longer present, indicating that for the C61G mutant, in contrast to wild-type enzyme at pH 7.2, no cysteine residues are modified by DEPC. From these observations, it appears that C-61 is the cysteine residue essential for the catalytic activity of DAH 7-P synthase (Phe) mutants. The effect of pH on the DEPC inactivation of the C61G mutant is shown in Fig. 5C. The rate of DEPC inactivation increases with increasing pH, and the discontinuity in the DEPC versus pH plot observed with the wild-type enzyme is no longer observed with the C61G mutant. The rate of inactivation by DEPC for the C61G mutant enzyme is approximately the same obtained for wild-type DAH 7-P synthase (Phe) below pH 7 (Fig. 5, A and C). The pHₕ of the essential histidine residue in the C61G mutant is 7.0, as compared with 6.0 in the wild-type enzyme. The activity of the C61G mutant as a function of pH indicates that the C61G mutant appears to have a pH optimum greater than 8 (Fig. 5D). In contrast, the pH optimum of wild-type DAH 7-P synthase (Phe) is 6.8 (43).

Circular Dichroism Studies—The conformational integrity of each mutant DAH 7-P synthase (Phe) was analyzed using circular dichroism spectroscopy. There appear to be significant structural changes for the mutants H172G, H207G, and H304G. The CD spectra of the H268G and C61G mutants are similar to wild-type DAH 7-P synthase (Phe) (22) (data not shown).

Kinetic Studies—A summary of the kinetic constants for the various mutants is given in Table II. Substantial changes in \( K_{m} \) values for E 4-P are seen for the H172G, H207G, and H304G mutants, whereas only the H172G enzyme, and to a lesser extent the H304G mutant, demonstrate any significant increase in \( K_{m} \) for PEP. Mutation of H-268 to glycine renders DAH 7-P synthase (Phe) inactive. With the exception of the C61G and H268G mutants, only modest effects on \( k_{cat} \) are observed (Table II) for the other histidine to glycine DAH 7-P synthase (Phe) mutants.

Metal Analysis—Metal-free enzymes were prepared and assayed for DAH 7-P synthase (Phe) activity. DAH 7-P synthase (Phe) is rendered inactive following treatment with 10 mM EDTA. The enzyme regains partial activity upon further treatment to remove the EDTA from the system most likely due to residual contaminating metals present in the buffers (22). The activity of the apoenzyme is reduced to less than 5% of the Mn²⁺ form of the enzyme after removal of the EDTA. Enzymatic activity is completely restored upon addition of Mn²⁺ to the assay mixture. In comparison to several divalent cations, Mn²⁺ gives the highest activity with all three DAH 7-P synthase isozymes (14) and was chosen as the activating metal for enzyme assays. However, there have been conflicting reports as to which divalent metal is utilized by DAH 7-P synthase (Phe) in vivo. Two divalent metals, Fe²⁺ and Cu²⁺, have been proposed to serve as the metal cofactor for the native enzyme (14, 44).

For the present study, the metal binding site of the mutant enzymes was investigated using methodology described previously (22). The procedure involves evaluating the ability of the mutant protein to bind a divalent metal (Fe²⁺) and to further probe the environment of the metal-binding site utilizing spectroscopic properties of the Cu²⁺ form of the enzyme. When treated with excess Fe²⁺ followed by dialysis, each of the mutants studied were found to bind 1 mol equivalent of iron per monomer, as is the case for the wild-type protein. The absorption spectra of the Cu²⁺ form of each DAH 7-P synthase (Phe) mutant displayed the characteristic absorption observed at \( \lambda = 350 \) nm for wild-type DAH 7-P synthase (Phe) (Cu²⁺) (14, 44). The mutant proteins studied appear to display similar metal binding properties of the wild-type DAH 7-P synthase (Phe),
indicating that the metal-binding site is not significantly altered in each mutant enzyme.

**Substrate Protection of Wild-type and Histidine Mutants of DAH 7-P Synthase (Phe) against DEPC inactivation—**

**PEP, E 4-P, and various divalent metals were evaluated for their ability to protect wild-type and mutant DAH 7-P synthases from inactivation by DEPC (Table III).** When wild-type DAH 7-P synthase (Phe) is modified by DEPC in the presence of PEP (80 μM), $k_{\text{obs}}$ decreases by approximately 80%. PEP provides approximately the same protection to the other histidine mutants (Table III). Thus, PEP appears to prevent modification of the histidine residue(s) by interfering with the inactivation either directly or indirectly by inducing conformational changes that make the histidine residue(s) inaccessible. In contrast to PEP under similar conditions, E 4-P offers only minimal protection to the wild-type and H64G enzymes reducing $k_{\text{obs}}$ by approximately 20%, but does not appear to protect the H207G and H304G mutant enzymes (Table III).

Various divalent metals have been reported to activate DAH 7-P synthase (Phe) to differing degrees of activity (14). The divalent metals $\text{Co}^{2+}$ and $\text{Cu}^{2+}$ also activate DAH 7-P synthase (Phe), but to a lesser extent as compared with $\text{Mn}^{2+}$ ($\text{Mn}^{2+} > \text{Co}^{2+} > \text{Cu}^{2+}$) (14). Initial experiments indicated that $\text{Mn}^{2+}$ increases the rate of DEPC inactivation for the wild-type enzyme (Table III). For this reason, the effect of other divalent metals on DEPC inactivation was investigated (Table III). The effect of $\text{Co}^{2+}$ is similar to that of $\text{Mn}^{2+}$, whereas $\text{Cu}^{2+}$ does not significantly affect the rate of inactivation (Table III). Nonetheless, the rate of increased DEPC inactivation follows the same trend as the ability of the metals to activate DAH 7-P synthase (Phe).

**DISCUSSION**

In this study, chemical modification and site-directed mutagenesis was used to investigate the potential role of histidine residues in DAH 7-P synthase (Phe). Classic DEPC inactivation experiments have demonstrated that histidine residues are important for DAH 7-P synthase (Phe) activity. Detailed examination of the data indicates that the observed inactivation results primarily from the modification of a single histidine residue with a $pK_a$ of approximately 6.0. Mutation of the conserved histidine residues in DAH 7-P synthase (Phe) suggests that H-268 is the essential residue. Additional observations recorded in this study appear to substantiate the involvement of H-268.

Sequence alignments between DAH 7-P synthases and KDO 8-P synthases indicate that H-268 is conserved across both enzymes, whereas H-64, H-172, and H-207 are highly conserved among the DAH 7-P synthases (17). The residue H-304 was selected for mutation, because the homologous residue in *E. coli* KDO 8-P synthase (H241) is highly conserved among KDO 8-P synthases but not among DAH 7-P synthases. Recently, it has been demonstrated that histidine residues are important for the reaction catalyzed by KDO 8-P synthase (26). One of the two essential histidines in KDO 8-P synthase, H-202, is homologous to H-268 in DAH 7-P synthase (Phe). Similarly, H-202 is conserved across all KDO 8-P synthases (17) and is essential for activity (26).

During the preparation of this manuscript, the crystal structure of DAH 7-P synthase (Phe) was reported by Shumilin et al. (24). Phosphoenolpyruvate and a divalent metal ($\text{Pb}^{2+}$) were bound to the crystallized enzyme. The activation conferred to DAH 7-P synthase (Phe) by $\text{Pb}^{2+}$ is only 3% of the level of $\text{Mn}^{2+}$.

![Difference spectra of DEPC modified DAH 7-P synthase (Phe).](image-url)
activation, implying that the Pb^{2+} coordination observed in the published crystal structure may not represent the metal coordination of the other active divalent metals in DAH 7-P synthase (Phe) (24). The results presented in the present study corroborate information provided by the crystal structure and provide additional information about the active site. The locations of the four conserved histidine residues in DAH 7-P synthase (Phe) are shown in Fig. 6. As illustrated by this figure, only H-268 is located at the active site and is in close proximity to PEP and the divalent metal. The \( Ne^2 \) of H-268 is located 2.8 Å from the divalent metal. Histidine 268 also appears to interact with the \( p \) orbitals of PEP. The distance between \( Ne^2 \) of H-268 and C1, C2, C3, and the bridging oxygen atom of PEP are 3.9, 3.2, 3.4, and 3.4 Å, respectively (24). We have shown that PEP offers protection against DEPC inactivation for the wild-type and the active histidine mutants (H64G, H207G, and H304G) providing additional evidence that H-268 is indeed in proximity to the PEP binding site (Table III). This finding offers validation that the crystal structure of DAH 7-P synthase (Phe) depicts the structure of DAH 7-P synthase (Phe) in solution. Mutation of H-268 to glycine renders the enzyme inactive, highlighting the importance of the active site location of H-268. Additional observations indicate that the loss of activity is not due to perturbations in the overall structure or the metal-binding environment. First, the CD spectrum of the H268G mutant is indistinguishable from the wild-type enzyme. Second, several studies have shown a unique absorption for DAH 7-P synthase (Phe) reconstituted with Cu^{2+} (14, 44). The spectra of Cu^{2+}-H268G shows the characteristic absorption associated with the wild-type enzyme, suggesting that the ligand environment around Cu^{2+} for the mutant has not been altered. The H268G mutant binds one equivalent of iron per
monomer, as is the case for wild-type DAH 7-P synthase (Phe), providing additional evidence that the metal binding environment has not been altered in the H268G mutant. These data imply that H-268 plays a catalytic role in DAH 7-P synthase (Phe).

Additional correlation between this study and the crystal structure is provided by the protection to DEPC inactivation afforded by E 4-P and the kinetic analysis of the histidine to glycine mutants. Although E 4-P is not present in the crystal structure, an inorganic sulfate ion is present and is thought to represent the phosphate binding location in the E 4-P binding site (24). In this regard, E 4-P would appear to bind at the opening of the active site and extend into the active site bringing the carbonyl group in close proximity to PEP. As such, some protection by E 4-P is expected but not to the same extent as PEP, because E 4-P is located in the active site but does not bind near H-268 as PEP does. Our observation that E 4-P offers only minimal protection against inactivation (Table III) supports this argument. The histidine mutants H172G and H207G have increased values for E 4-P (Table II). These data are again in agreement with the crystal structure, because H-172 and H-207 are located near the proposed E 4-P phosphate binding region in the crystal structure (Fig. 6).

Recently, our laboratory in collaboration with Dr. Dominico Gatti (Wayne State University) have reported the crystal structure for E. coli KDO 8-P synthase (45). Both DAH 7-P synthase (Phe) and KDO 8-P synthase adopt a β/α barrel topology and share several other similarities (45). The essential histidine in KDO 8-P synthase, H-202, is located at the active site. The H202G mutant of KDO 8-P synthase is essentially inactive, and although the exact role of H-202 in KDO 8-P synthase has yet to be fully elucidated, H-202 is thought to be involved in the catalytic mechanism as a general acid or base (26). As discussed earlier, H-202 aligns with H-268 in DAH 7-P synthase (Phe) in the primary sequence. When the active sites of the two enzymes are superimposed, H-202 and H-268 occupy essentially analogous positions (Fig. 7). These observations indicate that both enzymes require a histidine residue and the histidine residue may perform the same function in both enzymes.

TABLE III

| Enzyme     | Substrate added | Relative $k_{cat}$ |
|------------|-----------------|--------------------|
| Wild-type  | None            | 1.0                |
|            | E4P             | 0.8                |
|            | PEP             | 0.2                |
|            | Mn$^{2+}$       | 1.2                |
|            | Co$^{2+}$       | 1.1                |
|            | Cu$^{2+}$       | 0.9                |
| H64G       | None            | 1.0                |
|            | E4P             | 0.8                |
|            | PEP             | 0.4                |
|            | Mn$^{2+}$       | 1.1                |
| H207G      | None            | 1.0                |
|            | E4P             | 1.1                |
|            | PEP             | 0.5                |
|            | Mn$^{2+}$       | 0.9                |
| H304G      | None            | 1.0                |
|            | E4P             | 0.9                |
|            | PEP             | 0.2                |
|            | Mn$^{2+}$       | 1.0                |

FIG. 6. Crystal structure of PEP and E 4-P binding sites of DAH 7-P synthase (Phe)-PEP-Pb$^{2+}$ monomer with the various histidine residues highlighted.

FIG. 7. The three-dimensional superposition of the essential histidines of DAH 7-P synthase (Phe) (H-268, blue) and KDO 8-P synthase (H-202, green) is depicted.

Examination of the DAH 7-P synthase (Phe) structure indicates that, together with Glu-302 and Asp-326, H-268 and C-61 serve as ligands for the Pb$^{2+}$ ion (24). Results from the current study suggest that C-61 serves an additional function to simply being a metal ligand. The activity of the C61G mutant is low ($k_{cat} = 0.6 \text{ s}^{-1}$) at pH 6.8 as compared with the wild-type enzyme (22). In addition, the pH optimum of the C61G mutant increases to greater than 8 (Fig. 5D), as compared with a pH optimum of 6.8 for the wild-type enzyme (43). Furthermore, in the absence of C-61, the $pK_a$ of H-268 increases from 6.0 to 7.0 (Fig. 5D). These findings suggest that C-61 is altering the $pK_a$ of H-268 in wild-type DAH 7-P synthase (Phe) affecting the pH optimum of the enzyme. In E. coli KDO 8-P synthase, the $pK_a$ of the catalytically essential histidine, H-202, is 7.3 (26). In the C61G DAH 7-P synthase (Phe) mutant, the $pK_a$ of H-268 is altered to resemble that of H-202 in KDO 8-P synthase (7.0 versus 7.3) (3), again providing additional evidence that H-268 and H-202 perform similar roles in their respective enzymes. The neutral form of the essential histidine appears important for the activity of both enzymes, based on the pH optimum of each respective enzyme and the $pK_a$ of the corresponding essential histidine residue, implying that the essential histidines are potentially acting as general bases.

DAH 7-P synthase (Phe) requires a divalent metal for activity. The divalent metal is located 2.8 Å from Nε2 of H-268 and
is 3.2 Å from Sγ of C-61 (24). The effect of the divalent metal on the rate of DEPC inactivation as a function of pH (Fig. 5B), and the increase of DEPC inactivation in the presence of the divalent metal (Table III) confirms that the divalent metal is located near H-268 and C-61. Again, additional data substantiate that the crystal structure of DAH 7-P synthase (Phe) is representative of the enzyme in solution. The divalent metal also causes DEPC to react with C-61 at lower pH values (Fig. 4C) and may explain why DEPC inactivation is increased in the presence of Mn2+. The increase in the rate of DEPC inactivation caused by divalent metals follows the same trend as the metal’s ability to activate DAH 7-P synthase (Phe) activity (Mn2+ > Co2+ > Cu2+) (14). These data imply that the metal plays a catalytic role in DAH 7-P synthase (Phe) and not merely a structural role. The exact role of the metal in the mechanism is currently being studied.

Chemical modification and site-directed mutagenesis studies on DAH 7-P synthase (Phe) have shown that there is one essential histidine residue (H-268) and suggest that cysteine 61 modulates the $pK_a$ of H-268. The residues H-268 and C-61 appear to interact with one another, possibly through the metal ion. This interaction between H-268 and C-61 is important for the catalytic activity of DAH 7-P synthase (Phe). The roles of these two essential residues in DAH 7-P synthase (Phe) are currently under investigation to further define their function in the catalytic mechanism.

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Histidine 268 in 3-Deoxy-d-arabino-heptulosonic Acid 7-Phosphate Synthase Plays the Same Role as Histidine 202 in 3-Deoxy-d-manno-octulosonic Acid 8-Phosphate Synthase

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