Identification of Essential Histidine Residues in 3-Deoxy-D-*manno*-octulosonic Acid 8-Phosphate Synthase: Analysis by Chemical Modification with Diethyl Pyrocarbonate and Site-Directed Mutagenesis†

Galina Ya. Sheflyan, Henry S. Duewel, Grace Chen, and Ronald W. Woodard*

Interdepartmental Program in Medicinal Chemistry, College of Pharmacy, University of Michigan, Ann Arbor, Michigan 48109-1065

Received December 18, 1998; Revised Manuscript Received August 16, 1999

ABSTRACT: The enzyme 3-deoxy-D-*manno*-octulosonic acid 8-phosphate (KDO 8-P) synthase from *Escherichia coli* that catalyzes the aldol-type condensation of D-arabinose 5-phosphate (A 5-P) and phosphoenolpyruvate (PEP) to give KDO 8-P and inorganic phosphate (P_i) is inactivated by diethyl pyrocarbonate (DEPC). The inactivation is first-order in enzyme and DEPC. A second-order rate constant of 340 M^{-1} min^{-1} is obtained at pH 7.6 and 4 °C. The rate of inactivation is dependent on pH and the pH-inactivation rate data imply the involvement of an amino acid residue with a pK_a value of 7.3. KDO 8-P synthase activity is not restored to the DEPC-inactivated enzyme following treatment with hydroxylamine. Complete loss of KDO 8-P synthase activity correlates with the ethoxyformylation of three histidine residues by DEPC. KDO 8-P synthase is protected against DEPC inactivation by PEP and partially protected against inactivation by A 5-P. To provide further evidence for the involvement or role of the histidine residues in the aldol-type condensation catalyzed by KDO 8-P synthase, all six histidines were individually mutated to either glycine or alanine. The kinetic constants for the three mutants H40A, H67G, and H246G were unaffected as compared to the wild type enzyme. In contrast, H241G demonstrates a >10-fold increase in K_M for both PEP and A 5-P and a 4-fold reduction in k_{cat}, while H97G demonstrates an increase in K_M for only A 5-P and a 2-fold reduction in k_{cat}. The activity of the H202G mutant was too low to be measured accurately but the data obtained indicated an approximate 400-fold reduction in k_{cat}. Circular dichroism measurements of the wild-type and mutant enzymes indicate modest structural changes in only the fully active H67G and H246G mutants. The H241G mutant is protected against DEPC inactivation by PEP and A 5-P to the same extent as the wild-type enzyme, suggesting that the functionally important H241 may not be located in the vicinity of the substrate binding sites. The H97G mutant is protected by PEP against DEPC inactivation to the same degree as the wild-type enzyme but is no longer protected by A 5-P. In the case of the H202G mutant, both A 5-P and PEP protect the mutant against DEPC inactivation but to different extents from those observed for the wild-type enzyme. The catalytic activity of the H97G mutant is partially restored (20% → 60% of wild-type activity) in the presence of imidazole, while a minor amount of activity is restored to the H202G mutant (<1% → 4% of wild-type activity) in the presence of imidazole.

The enzyme 3-deoxy-D-*manno*-octulosonic acid 8-phosphate (KDO 8-P)[1] synthase (EC 4.1.2.16) catalyzes an aldol-type condensation of phospho*enol*pyruvate (PEP) with D-arabinose 5-phosphate (A 5-P) to yield KDO 8-P and inorganic phosphate (P_i) (Scheme 1) (*1*). While KDO was originally described as a site-specific molecule found only in Gram-negative (G−) organisms, its presence in plants has been recently reported (*2*−*5*). In G− bacteria, KDO 8-P is required for the biosynthesis of the lipopolysaccharide (LPS) region of the outer membrane. It has been proposed that KDO 8-P is necessary for maturation and cellular growth since mutants producing incomplete LPS are more susceptible to antibiotics and are less pathogenic (*6*). Therefore, the inhibition of KDO 8-P synthase has become a chemotherapeutic goal in the development of a new generation of antibiotics (*6*).

KDO 8-P synthase, like the key enzyme in aromatic amino acid biosynthesis 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAH 7-P) synthase (EC 4.1.2.15), catalyzes the attack of the *si* face of the C3 of PEP on the *re* face of the carbonyl carbon of the phosphorylated monosaccharide substrate (*7*−*10*). Concurrent with the condensation is the breaking of the C−O−P bond of PEP between the C and O to form inorganic phosphate (*11*, *12*) and to produce a new phosphorylated 3-deoxy-α-keto sugar acid three carbons longer. The mechanistic details of two other biologically important PEP-utilizing enzymes, EPSP (5-*enol*pyruvylshikimate 3-phos-

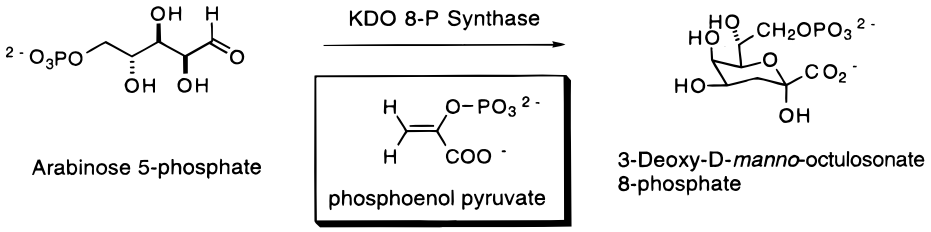
† This investigation was supported by Public Health Service Grant GM 53069 awarded by the Department of Health and Human Services. A preliminary account of this work was presented at the XVIIth Midwest Enzyme Chemistry Conference at Loyola University of Chicago, Chicago, IL, October 18, 1997.

* To whom correspondence should be addressed.

[1] Abbreviations: A 5-P, D-arabinose 5-phosphate; BTP, 1,3-bis[tris(hydroxymethyl)methylamino]propane; DEPC, diethyl pyrocarbonate; KDO 8-P, 3-deoxy-D-*manno*-octulosonic acid 8-phosphate; PEP, phospho*enol*pyruvate; Tris, tris(hydroxymethyl)aminomethane.

10.1021/bi9829884 CCC: $18.00
Published on Web 10/09/1999

Scheme 1: Reaction Catalyzed by KDO 8-P Synthase



phate) synthase and UDP-*N*-acetylglucosamine *enol*pyruvate transferase (EPTase), which also promote C−O bond cleavage, have been recently reported (*13*). Both these latter enzymes catalyze the addition of the intact carboxyvinyl portion of PEP to the substrate alcohol through an addition−elimination-type mechanism concurrent with phosphate cleavage.

Although the DNA coding sequence for KDO 8-P synthase (*14*−*16*), and thus the complete amino acid sequence of KDO 8-P, has been known for over a decade and a number of mechanistic studies have been reported, little information concerning the identity of the active-site amino acids involved in substrate binding and/or catalysis is available. Studies on the role of C38 and C166 in KDO 8-P synthase and the effect of several sulfhydryl modifying reagents have been reported (*12*, *17*). Beyond these recent observations on the potential role of cysteine residues, studies to determine the role of other active-site amino acids are lacking. To obtain information regarding the possible role of other amino acids at the active site, a systematic study has been initiated to identify these residues and ascertain their role in either substrate binding or catalysis.

In the present paper, we provide chemical and kinetic evidence for the involvement of histidine residues in KDO 8-P synthase catalysis as evidenced by the pH dependence of inactivation, substrate protection studies, and detection of *N*-carbethoxyhistidine by UV difference spectra. Results from mutagenic studies further verify these findings and demonstrate the importance of the three conserved histidine residues, H97, H202, and H241.

MATERIALS AND METHODS

Materials. Restriction and DNA modifying enzymes were from Boehringer Mannheim (Indianapolis, IN), New England Biolabs (Beverly, MA), and Gibco BRL (Grand Island, NY). The Promega (Madison, WI) DNA purification kit and the Perfectprep plasmid DNA kit (5Prime→3Prime, Inc., Boulder, CO) were utilized for plasmid isolation and purification. The *Escherichia coli* strain BL21(DE3) was obtained from Novagen (Madison, WI). The XL1-Blue *E. coli* supercompetent cells and the QuikChange mutagenesis kit were obtained from Stratagene Cloning Systems (La Jolla, CA). *Vent* DNA polymerase was purchased from New England Biolabs and substituted for the recombinant *Pfu* DNA polymerase called for in the QuikChange mutagenesis kit. Purine-nucleoside phosphorylase was overexpressed in *E. coli* strain BL21(DE3) harboring plasmid PSE380 DeoD and purified by anion-exchange chromatography (*18*). The PSE380 DeoD plasmid was obtained from Joanne Turnbull (Concordia University, Montreal, Quebec, Canada). Thermal cycling was performed on an MJR Research Thermal cycler. The construction of plasmid pT7-7/*kdsA* harboring the gene for *E. coli* KDO 8-P synthase has been previously described (*11*). Mutagenic oligonucleotide synthesis and DNA sequencing were services provided by the University of Michigan Biomedical Research Resources Core Facility. Phospho*enol*pyruvate mono(cyclohexyl)ammonium salt, D-arabinose 5-phosphate disodium salt, imidazole, sodium arsenite, sodium periodate, tris(hydroxymethyl)aminomethane (Tris), 2-thiobarbituric acid, 7-methylinosine, and diethyl pyrocarbonate were obtained from the Sigma Chemical Co. (St. Louis, MO). Hydroxylamine hydrochloride was purchased from Aldrich Chemical Co. (Milwaukee, WI), and 1,3-bis[tris(hydroxymethyl) methylamino]propane (BTP) was purchased from Research Organics (Cleveland, OH).

Table 1: Oligonucleotides Used for the Mutagenesis of *E. Coli* KDO 8-P Synthase[a]

Target Amino Acid	Primers 5′—>3′	Resulting Amino Acid	Restriction site
H40	GATGCGCATTTGCGA*GGCCT*ACGTAACTGTCAC GTGACAGTTACGTA*GGCCT*CGCAAATGCGCATC	A	+StuI
H67	CCAACCGCTCCTCCATC*GGATCT*TATCGTGGACCG CGGTCCACGATAA*GATCC*GATGGAGGAGCGGTTGG	G	+BstYI
H97	GAAAATTATCACCGACGTT*GGGGAACC*AAGTCAGGCAC GTGCCTGACTT*GGTTCCCC*AACGTCGGTGATAATTTTC	G	+NlaIV
H202	GTGATTTTCGACGTGACC*GGTGCAC*TGCAATGCCGCGATC GATCGCGGCATTGCA*GTGCACC*GGTCACGTCGAAAATCAC	G	+ApaLI
H241	GTTTATTGAAGCG*GGCC*CGGATCCGGAACATG CATGTTCCGGATCC*GGGCCC*GCTTCAATAAAC	G	+ApaI
H246	GCATCCGGATCCGGAA*GGT*GCGAAATGTGATGGTCC GGACCATCACATTTCGC*ACC*TTCCGGATCCGGATGC	G	-NspI

[a] The mutated nucleotides are underlined and the newly engineered restriction site is shown in italic type. The symbol (+) indicates the addition of a new endonuclease restriction site, while a (−) indicates the loss of a endonuclease restriction site.

Preparation and Isolation of Wild-Type and Mutant KDO 8-P Synthases. The histidine to glycine/alanine mutants of *kdsA* were prepared by the parental-strand-only replication methodology (*19*, *20*). Two oligonucleotide primers, each containing the desired mutagenic replacement codon (see Table 1), and miniprep wild-type plasmid DNA (pT7-7/*kds*A) were temperature-cycled 15 times with high-fidelity *Vent* DNA polymerase in the presence of buffer and dNTPs through the following cycle program: 94 °C for 1 min, 45 °C for 1 min, and 72 °C for 3.5 min. The thermal-cycled reaction mixture containing the mutated plasmid with staggered nicks was treated with *Dpn*I to digest the parental pT7-7/*kdsA* DNA template. The *Dpn*I digestion reaction mixture, containing the nicked mutagenic DNA, was used to transform supercompetent *E. coli* XL1-Blue cells. Plasmid DNAs were isolated and purified from each of the clones and initially characterized by restriction digestion and then DNA se-

quencing. DNA containing the proper mutagenic sequence was used to transform chemically competent *E. coli* BL21-(DE3).

The *E. coli* BL21(DE3) cells harboring either the pT7−7/*kdsA* plasmids containing the wild-type or mutant sequences were grown and the proteins were isolated and purified by the methods previously described from this laboratory (*11*).

KDO 8-P Synthase Assay. Method 1: Formation of KDO 8-P. Unless otherwise stated, the assay mixture contained 100 mM Tris−acetate (pH 7.5), 3 mM A 5-P, 3 mM PEP, and KDO 8-P synthase (approximately 0.1−3.5 μM as specified) in a total volume of 150 μL. This mixture was incubated at 37 °C for 10 min and then quenched with 10% trichloroacetic acid (150 μL). KDO 8-P synthase activity was measured by determining the amount of KDO 8-P produced by the periodate−thiobarbituric acid assay as previously reported (*1*, *21*).

Method 2: Liberation of Inorganic Phosphate. A continuous spectrophotometric method for the measurement of phosphate release by KDO 8-P synthase, based on a purine-nucleoside phosphorylase-coupled phosphate assay reported by Webb (*22*) and recently modified by Turnbull and coworkers (*18*), was used to determine kinetic parameters. The standard assay mixture contained 100 mM Tris−acetate (pH 7.5), 200 μM 7-methylinosine, 0.2 μM purine-nucleoside phosphorylase, and various concentrations of PEP and A 5-P as follows. Initial velocities were determined separately for at least six concentrations of one substrate (varied between 0.1 and 10 times K_m) while the concentration of the other substrate was held constant at saturating levels (>15 K_m). The first five reagents were preincubated at 37 °C for 5 min and the reactions were initiated by the addition of enzyme (0.2 μM for wild type, H40A, H67G, H97G, and H246G and 4.0 μM for H202G). The decrease in absorbance of the reactions was monitored at 280 nm ($\epsilon_{280} = 5091$ M^{-1} cm^{-1}). Initial rates were determined by least-squares fitting of the linear portion of the progress curve to a straight line. Values for K_M and k_{cat} were determined by fitting the reaction rate versus the substrate concentration to the Michaelis−Menten equation utilizing the KaleidaGraph software (v3.08d, Synergy Software).

Chemical Modification of KDO 8-P Synthase with Diethyl Pyrocarbonate. Stock solutions of diethyl pyrocarbonate (DEPC) were freshly prepared in absolute ethanol immediately before use. The concentration of DEPC was determined spectrophotometrically by reacting an aliquot with 10 mM imidazole in 20 mM Tris-HCl (pH 7.6) and measuring the increase in absorbance at 240 nm due to the formation of the *N*-carbethoxyimidazole derivative ($\epsilon_{240} = 3200$ M^{-1} cm^{-1}) (*23*). Modification of KDO 8-P synthase was performed in a reaction mixture containing 3.4 μM KDO 8-P synthase, 20 mM Tris-HCl (pH 7.6), and various concentrations of DEPC (0.03−0.72 mM) at 4 °C. At various times, aliquots (5 μL) were removed and immediately assayed for residual enzyme activity by method 1.

Substrate Protection. Wild-type KDO 8-P synthase, the H97G and H241G mutants (3.4 μM), or the H202G mutant (35 μM) were individually mixed with 3 mM of either PEP or A 5-P in 20 mM Tris-HCl (pH 7.6) prior to the addition of 3.5 mM DEPC. After the addition of the modification reagent, aliquots (15 μL) were removed at various times and the enzyme was assayed for residual activity by method 1.

Reaction with Hydroxylamine. KDO 8-P synthase (3.4 μM) was incubated in the absence or presence of 0.26 or 0.68 mM DEPC in 20 mM Tris-HCl (pH 7.6) at 4 °C. After 20 min, the reaction mixtures were quenched with 2 mM imidazole. A solution of hydroxylamine hydrochloride (adjusted to pH 7.6) was added to each of the quenched mixtures to achieve a final concentration of 20 mM hydroxylamine and the mixtures were incubated for 30 min at 4 °C. An aliquot was removed prior to and following hydroxylamine treatment and the residual activity was determined by method 1. In a separate experiment, KDO 8-P synthase was directly incubated with different concentrations of hydroxylamine as described above and the enzymatic activities were determined by method 1.

Correlation between Enzyme Activity and Histidine Residues Modified by DEPC. KDO 8-P synthase (12.5 μM) in 20 mM Tris-HCl (pH 7.6) was incubated with increasing incremental concentrations of DEPC (0.03−3 mM final concentration) in a 1 mL quartz cuvette at ambient temperature. An identical solution containing an equivalent amount of ethanol without DEPC served as a blank. The progress of the inactivation reaction was followed by monitoring the formation of the *N*-carbethoxyimidazole derivative of the histidine at 240 nm. Prior to the addition of each increment of DEPC, an aliquot was withdrawn to determine the remaining activity of the modified enzyme by method 1.

pH Dependence of Inactivation. KDO 8-P synthase (3.4 μM) samples were prepared in 100 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane (BTP) buffers over the pH range of 6−9 or in 100 mM potassium phosphate buffers over the pH range of 6.4−9.6. DEPC was added to each sample to a final concentration of 1 mM. Aliquots (15 μL) were removed at various times and assayed for residual enzyme activity by method 1.

Imidazole Rescue Studies. The H97G, H202G, and H241G mutants (3.4 μM) were preincubated in the absence or presence of 50 mM imidazole in 20 mM Tris−acetate (pH 7.5) at 4 °C for 30 min. Following this preincubation, aliquots (15 μL) were removed and assayed by method 1 (resulting in a 10-fold dilution of the preincubation mixture) in which the assay solution contained either 0 or 50 mM imidazole. For these experiments, the assay mixture contained 0.3 mM PEP and 0.6 mM A 5-P instead of the standard 3 mM concentration of the substrates. In a separate experiment, the H97G mutant was preincubated with 50 mM imidazole in the presence of either 3 mM A 5-P or 3 mM PEP and assayed as described above.

Analytical Methods. Protein concentrations were determined with the Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin as the standard. Optical spectroscopy was performed on a Cary 3 Bio UV−visible spectrophotometer (Varian Associates). The circular dichroism (CD) spectra of the wild-type and mutant enzymes were measured as described previously (*17*). The estimated percentages of secondary structure were calculated from the CD spectra by the self-consistent method (SELCON program) described by Sreerama and Woody (*24*).

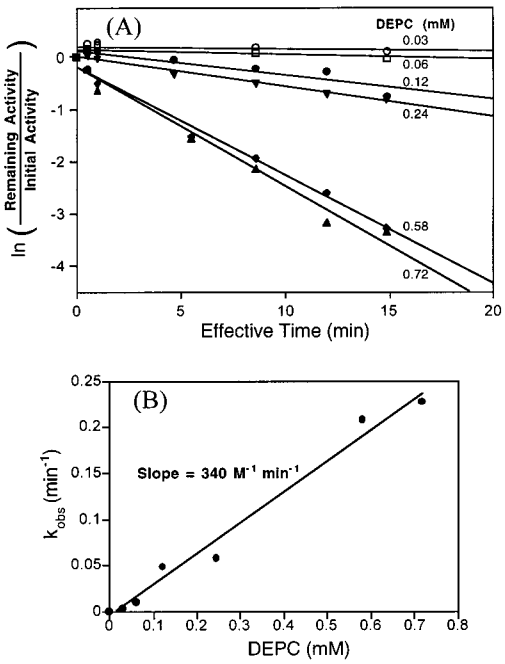


FIGURE 1: Inactivation of KDO 8-P synthase by diethyl pyrocarbonate. (**A**) KDO 8-P synthase (3.4 μM) in 20 mM Tris-HCl (pH 7.6) was incubated with different concentrations of DEPC as indicated at 4 °C. At various times, aliquots were removed and assayed for residual enzyme activity by method 1 and the data were plotted according to eq 1. (**B**) Determination of the second-order rate constant. The pseudo first-order rate constants (k_{obs}) obtained from the linear plots in panel A were plotted against DEPC concentration.

RESULTS

Inactivation of KDO 8-P Synthase by DEPC. KDO 8-P synthase was treated with increasing concentrations of DEPC and the time course of the loss of enzymatic activity of the DEPC-treated enzyme was followed. Since DEPC undergoes hydrolysis in aqueous solutions, the inactivation process is corrected for the spontaneous hydrolysis of DEPC as described by

$$\ln (A/A_0) = -(k/k')I_0(1 - e^{-k't}) \qquad (1)$$

where A/A_0 is the fractional activity remaining at time t, I_0 is the initial concentration of DEPC, k is the second-order rate constant for the inactivation of KDO 8-P synthase by DEPC, and k' is the pseudo-first-order rate constant for the spontaneous hydrolysis of DEPC (*25*). To estimate the value of k', DEPC was incubated in 20 mM Tris-HCl (pH = 7.6) at 4 °C. At various time intervals, aliquots were removed and the amount of DEPC remaining was determined by reaction with excess imidazole and measuring the absorbance increase at 240 nm as described under Materials and Methods. The value of k' was estimated to be 0.031 min^{-1}. Plots of ln A/A_0 versus $(1 - e^{-k't})/k'$ at various DEPC concentrations were linear and the inactivation process followed pseudo-first-order rate kinetics (Figure 1A). A second-order rate constant (k) of 340 min^{-1} M^{-1} was obtained from the slope of the plot of k_{obs} versus DEPC concentration (Figure 1B). The values for k_{obs} were obtained from the slopes of the linear plots in Figure 1A.

Reaction with Hydroxylamine. Hydroxylamine has been shown in certain cases to restore activity to DEPC-inactivated enzymes (*25*−*28*). The effect of hydroxylamine on both DEPC-inactivated and unmodified KDO 8-P synthase was examined. Treatment of DEPC-inactivated KDO 8-P synthase with hydroxylamine did not restore activity but instead resulted in even greater reduction of enzyme activity. The enzymatic activities of KDO 8-P synthase treated with either 0.26 or 0.68 mM DEPC for 20 min at 4 °C were 42% and 9% of control, respectively. These activities were further reduced to 12% and 2%, respectively, following a 30 min incubation in the presence of 20 mM hydroxylamine. Treatment of unmodified KDO 8-P synthase with 20 mM hydroxylamine resulted in the loss of 36% enzymatic activity. The inactivation of unmodified enzyme by hydroxylamine was found to be concentration-dependent (data not shown). These results indicate that KDO 8-P synthase is directly inactivated by hydroxylamine.

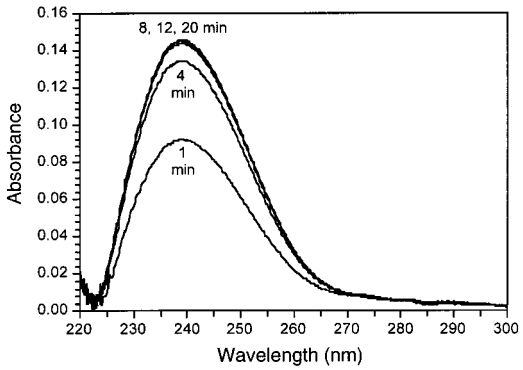


FIGURE 2: Difference spectra of DEPC-modified KDO 8-P synthase. KDO 8-P synthase (15 μM) in 20 mM Tris-HCl (pH 7.6) was incubated with DEPC (1 mM) at 25 °C. At the indicated times, spectra were acquired versus an identical reference sample consisting of only enzyme and buffer.

Correlation between Enzyme Activity and Histidine Residues Modified by Diethyl Pyrocarbonate. The modification of the histidine residues in KDO 8-P synthase was monitored by absorbance changes in the UV region. The difference spectrum of DEPC-treated enzyme versus unmodified enzyme at pH 7.6 revealed only a single peak at 240 nm, which is characteristic of an *N*-carbethoxyimidazole derivative (Figure 2.). Modification of either a cysteine or tyrosine residue by DEPC was not detected since no change in the difference spectrum at 230 nm (for cysteine modification) and/or 278 nm (for tyrosine modification) was observed (*29*, *30*).

This increase in absorbance at 240 nm upon treatment with DEPC was used to estimate the number of histidine residues modified within the protein. KDO 8-P synthase contains six histidine residues at positions 40, 67, 97, 202, 241, and 246 in its primary structure as predicted from its DNA sequence (*kdsA*) and as determined from the total amino acid analysis performed on the recombinant KDO 8-P synthase (data not shown). The number of histidine residues modified by DEPC as determined by the difference spectra at 240 nm correlates with the loss of enzyme activity (Figure 3). Sequential modification of histidine residues was achieved by the addition of increasing amounts of DEPC. The plot of residual enzyme activity versus the number of histidine residues modified by DEPC (Figure 3) shows that the random modification of three out of six histidine residues is sufficient to completely inactivate the enzyme.

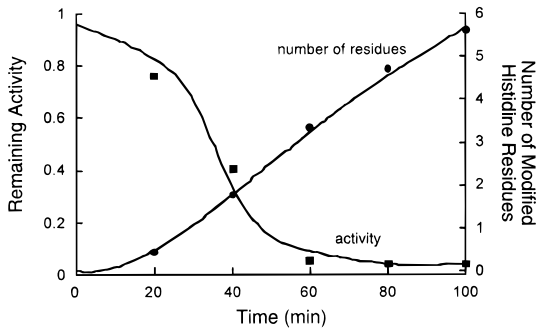


FIGURE 3: Relationship between the extent of histidine modification and the loss of catalytic activity of KDO 8-P synthase upon reaction with DEPC. A sample of KDO 8-P synthase (12.5 μM) in 20 mM Tris-HCl (pH 7.6) was sequentially treated with incremental amounts of DEPC (five in total, between 0.03 and 3 mM final concentration) for 20 min at each of the respective concentrations of reagent. At the end of the 20 min incubation and prior to the addition of the next increment of DEPC, the number of modified histidine residues was determined by measuring the absorbance increase of the sample at $\lambda = 240$ nm (●). An aliquot was removed and assayed for residual enzyme activity (■) by method 1.

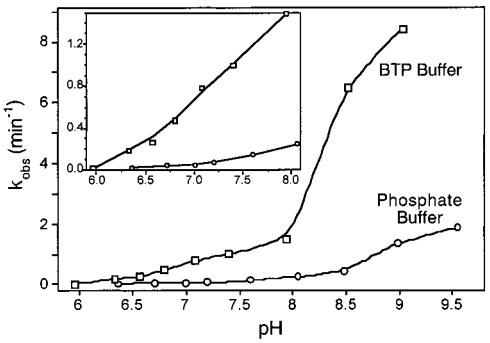


FIGURE 4: Inactivation of KDO 8-P synthase as a function of pH. KDO 8-P synthase (3.4 μM) was incubated with DEPC (1 mM) at various pH values in 100 mM BTP buffers (□) or 100 mM potassium phosphate buffers (○). At various time intervals, aliquots were removed and assayed for residual enzyme activity by method 1 to obtain the pseudo-first-order rate constants of inactivation (k_{obs}) at each pH. Inset: An expanded view of the KDO 8-P synthase DEPC titration in BTP (□) and phosphate (○) buffers in the pH range 6−8.

To calculate the number of "essential" histidine residues in KDO 8-P synthase, the method of Tsou (*31*) was utilized where (remaining activity)$^{1/\mu}$ is plotted versus the number of modified histidine residues. In this plot, the whole number μ is varied in value from $\mu = 1$ to n, where n equals the possible number of modifiable groups. The value of μ that produces the highest correlation coefficient when the data are fit to a straight line provides an estimate of the essential number of modified residues (*31*, *32*). KDO 8-P synthase was treated with DEPC, and over the course of the modification, the number of histidine residues modified and the remaining activity at a given time were simultaneously determined. When the data were analyzed as described above, the following correlation coefficients were obtained: 0.980, 0.990, 0.988, and 0.985 for μ values of 1, 2, 3 and 4, respectively. Within error, μ values of 2 and 3 gave the highest and essentially equivalent correlation coefficients. When DEPC modification of KDO 8-P synthase was performed in the presence of A 5-P and the data were analyzed as above, correlation coefficients of 0.991, 0.981, and 0.975 were obtained for μ values of 1, 2 and 3, respectively. In this case, the highest correlation coefficient was obtained for a value of $\mu = 1$. These results indicate that two or three histidine residues in KDO 8-P synthase are "essential". In the presence of A 5-P, the modification of only one histidine residue results in the inactivation of the enzyme.

pH Dependence of Inactivation. The inactivation of KDO 8-P synthase by DEPC was examined in BTP buffers over the pH range of 6−9. The pseudo-first-order rate constants of inactivation (k_{obs}) at various pH values were obtained from plots of residual activity versus effective time. A plot of the experimentally determined k_{obs} values as a function of pH indicates that DEPC inactivation of KDO 8-P synthase is pH-dependent (Figure 4). The dependence of k_{obs} on pH features two steps in which k_{obs} increases gradually between pH 6 and 8 and then rises sharply above pH 8 to a maximum observed experimental value of 8.4 min^{-1} at pH 9.

The dependence of inactivation on pH can be evaluated in greater detail according to

$$k_{obs} = k_{obs(max)}/(1 + [H^+]/K_a) \quad (2)$$

Equation 2 can be rearranged to the following linear form:

$$k_{obs}[H^+] = K_a\, k_{obs(max)} - K_a k_{obs} \quad (3)$$

where K_a is the acidic dissociation constant of the reacting group and $k_{obs(max)}$ is the pseudo-first-order rate constant of the unprotonated reacting group (*33*). When the dependence of k_{obs} over the pH range of 6−8 in the BTP buffers was analyzed according to eq 3 (from a plot of $k_{obs}[H^+]$ versus k_{obs}), a pK_a value of 7.3 and a $k_{obs(max)}$ value of 1.9 min^{-1} are obtained. As discussed previously, the UV difference spectrum of DEPC-treated KDO 8-P synthase versus untreated enzyme at pH 7.6 and $\lambda = 240$ nm (Figure 2) is indicative of a protein-bound *N*-carbethoxyhistidine. These results suggest that DEPC inactivation of KDO 8-P synthase between pH 6 and 8 results from modification of a histidine residue with a pK_a of 7.3 or from modification of one or more histidine residues with closely related pK_as.

The increased inactivation of KDO 8-P synthase by DEPC at pH > 8, as reflected in the marked increase in k_{obs} values (Figure 4), might suggest the modification of another residue-(s) that is essentially protonated below pH 8. The UV difference spectrum of enzyme modified with DEPC at pH 9.2, however, did not exhibit the characteristic changes expected for the simple modification of a histidine, cysteine or tyrosine residue (data not shown). Therefore, the basis of the increased rate of inactivation by DEPC at pH > 8 is not clear. It is possible that pH-dependent conformational changes in enzyme structure occur that either are directly deleterious to enzyme activity or increase the accessibility of DEPC to the reactive residue(s). Although the data in the second region of the profile at pH > 8 could be analyzed according to eq 3, producing a pK_a value of 9.0 and $k_{obs(max)}$ value of 20 min^{-1}, the importance of these values must await further studies.

The pH dependency of DEPC modification of KDO 8-P synthase was also examined in potassium phosphate buffer. The pH profile obtained in phosphate buffer also demonstrated the two major steps as observed in the BTP buffers; however, inactivation proceeded at a much slower rate in the phosphate buffer (Figure 4). Analysis of the rate versus pH data in the phosphate buffers according to eq 3 produced values for $k_{obs(max)}$ of 0.3 and 3.1 min^{-1} and pK_a values of 7.6 and 9.2 for the two steps of the profile, respectively.

Table 2: Deconvoluted CD Data for Wild-Type and Mutant KDO 8-P Synthases[a]

mutant	α-helix (%)	β-sheet (%)	β-turn (%)	random coil (%)	relative activity[b]
wild-type	23.4	23.6	25.9	25.7	100
H40A	25.1 (+1.7)[c]	22.7 (−0.9)	25.4 (−0.5)	26.6 (+0.9)	100
H67G	27.2 (+3.8)	19.0 (−4.6)	25.9 (0.0)	26.9 (+1.2)	112
H97G	23.9 (+0.5)	23.9 (+0.3)	26.5 (+0.6)	26.1 (+0.4)	50
H202G	20.3 (−3.1)	27.4 (+3.8)	26.7 (+0.8)	25.4 (−0.3)	0.2
H241G	24.8 (+1.4)	25.2 (+1.6)	28.7 (+2.8)	23.1 (−2.6)	25
H246G	27.1 (+3.7)	18.9 (−4.7)	23.8 (−2.1)	26.0 (+0.3)	110

[a] Determined by the method of Sreerama and Woody (*24*). [b] Determined by method 1. [c] Values in parentheses indicate the change relative to the wild-type enzyme.

The pK_a values determined in the BTP (pK_as of 7.3 and 9.2) and phosphate (pK_as of 7.6 and 9.2) buffers differ slightly from each other and could reflect differences in the properties of the two buffers. The lower rates of inactivation in the phosphate buffer as compared to the BTP buffer potentially indicate that inorganic phosphate, a product of the KDO 8-P synthase-catalyzed reaction, inhibits or protects the enzyme from modification with DEPC.

Histidine Mutagenesis of KDO 8-P Synthase. The chemical modification studies indicated that histidine residues play an important role in KDO 8-P synthase. To specifically identify the potential critical residue(s), an individual mutagenic scan of all six histidines was performed. The KDO 8-P synthase mutants H40A, H67G, H97G, H202G, H241G, and H246G were constructed with the QuikChange mutagenesis kit from Stratagene Cloning Systems (*19*, *20*). In each case, the template for the PCR mutagenesis was plasmid pT7-7/*kdsA*, which contains the wild-type KDO 8-P synthase nucleotide sequence. Mutagenic primers were designed to either introduce or remove a specific endonuclease restriction site in the target plasmid DNA, permitting positive mutants to be identified by restriction analysis. The mutagenic primers are listed in Table 1 along with the restriction sites used for the initial screen. Following the initial restriction screen, the KDO 8-P synthase gene for each mutant was subjected to DNA sequencing to confirm the site-specific mutation and to ensure that no other mutations had been introduced. Each of the recombinant mutant proteins was purified to >95% homogeneity by the same procedure as for the wild-type enzyme (*11*).

The KDO 8-P synthase histidine mutants were designed having glycine (or alanine in the case of H40A) as the replacement residue in order to allow chemical rescue experiments to be performed. Mutants containing alanine replacements of important functional amino acids have been successfully rescued by the addition of exogenous reagents to replace the missing functionality of the mutated amino acid (*34*−*36*). However, several reports have indicated that if a histidine residue is replaced by glycine rather than alanine, the mutant is more prone to be chemically rescued due to the larger cavity created by the glycine mutation (*37*−*39*). The H40A mutant was constructed since none of the H40G mutant sequences permitted the addition or deletion of an endonuclease site for restriction screening purposes. Therefore, for the initial mutagenic scan, H40A and not H40G was studied.

Circular Dichroism Studies. The conformational integrity of the mutant KDO 8-P synthases was probed by circular dichroism spectroscopy and the deconvoluted spectral data are presented in Table 2. The data suggest minor to no change in the global structure of the mutant proteins. The greatest changes in secondary structure composition occur in the H67G and H246G mutants, in which both enzymes exhibit small changes in both the α-helix (<4%) and β-sheet (<5%) content as compared to the wild-type enzyme. As will be discussed below, these apparent changes do not appear to affect the activity of these mutants. Very modest differences in secondary structure for the H202G and H241G mutants are also suggested by the data, primarily in the α-helix and β-sheet content of H202G (<4%) and the β-turn and random coil content of H241G (<3%). The deconvoluted data for the H40A and H97G mutants are essentially unchanged compared to that of the wild-type enzyme.

Table 3: Kinetic Constants of Wild-Type KDO 8-P Synthase and Mutants

enzyme	K_M^{PEP} (μM)	$K_M^{A\,5-P}$ (μM)	k_{cat} (s^{-1})
wild type	19 ± 4	29 ± 3	6.8 ± 0.5
H40A	20 ± 5	33 ± 6	6.2 ± 0.4
H67G	16 ± 3	32 ± 1	8.4 ± 0.6
H97G	32 ± 4	194 ± 22	3.2 ± 0.3
H202G	nd[a]	nd	0.016 ± 0.007
H241G	241 ± 28	250 ± 22	1.7 ± 0.1
H246G	15 ± 1	31 ± 2	7.3 ± 0.4

[a] nd, not determined.

Kinetic Analysis of the Histidine Mutants. Each of the six histidine mutants of KDO 8-P synthase was characterized kinetically and the results are presented in Table 3. The kinetic constants for H40A, H67G, and H246G are essentially equivalent to those determined for the wild-type enzyme, suggesting that the histidine residues at these positions are not necessary to maintain enzyme activity. In contrast, kinetic analysis of the H97G, H241G, and H202G mutants indicate their importance to enzyme function. The H97G mutant exhibits an approximately 7-fold increase in K_M for A 5-P and a 2-fold reduction in k_{cat}, while the K_M for PEP increased slightly. The H241G mutant exhibits an approximately 10-fold increase in K_M for both substrates and a 4-fold lowering of k_{cat}. Analysis of the H202G mutant indicated an almost complete loss of catalytic activity. Due to the extremely low activity of the H202G mutant and the consequently high concentrations of enzyme required to accurately measure activity, it was not possible to obtain rate data at low (<50 μM) concentrations of substrates and maintain pseudo-first-order conditions (i.e. [S] ≫ [E]). However, rate information with higher concentrations of substrates could be measured under steady-state conditions and fit to a hyperbola, allowing a reliable estimate of V_{max}. By use of this information, a k_{cat} value of 0.016 s^{-1} was determined, a value 400-fold lower than that for the wild-type enzyme.

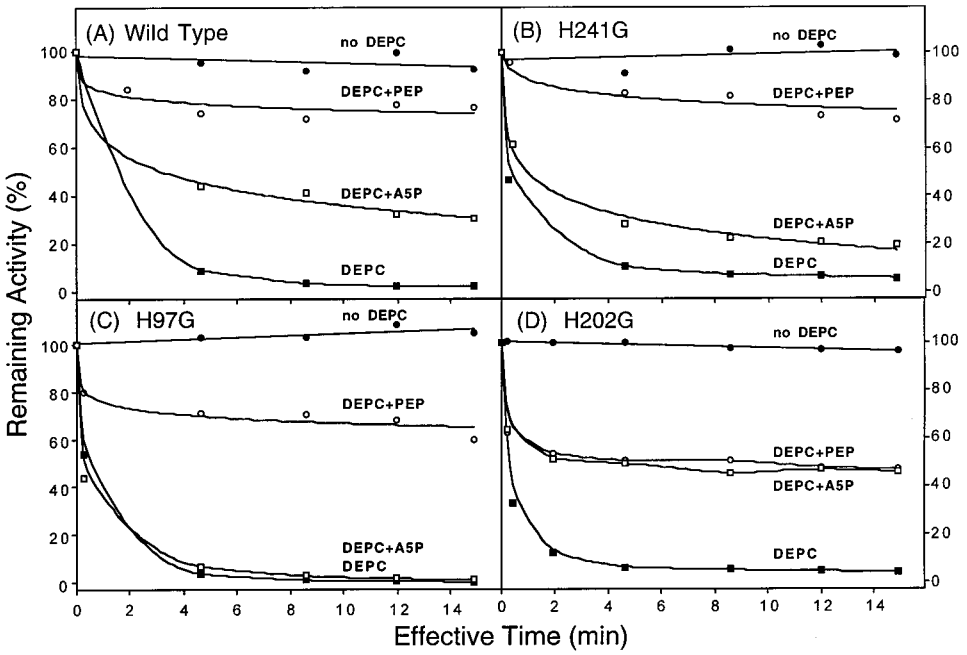


FIGURE 5: Substrate protection against DEPC inactivation of KDO 8-P synthase wild type and mutants H97G, H202G, and H241G. Wild-type KDO 8-P synthase (**A**) or the H241G (**B**), H97G (**C**), and H202G (**D**) mutants (A−C, 3.4 μM; D, 35 μM) were incubated in 20 mM Tris-HCl (pH 7.6) at 4 °C without DEPC (●), in the presence of 3.5 mM DEPC (■), or in the presence of both 3.5 mM DEPC and 3 mM PEP (○) or both 3.5 mM DEPC and 3 mM A 5-P (□). Over time, aliquots were removed and assayed for KDO 8-P synthase activity. Activities are expressed as the percentage of activity remaining compared to the initial activity of the untreated enzyme.

Substrate Protection of Wild-Type and Mutant KDO 8-P Synthases against DEPC Inactivation. The ability of the substrates, PEP and A 5-P, to protect KDO 8-P synthase against inactivation by DEPC was evaluated and the results are shown in Figure 5A. PEP provides a high level of protection for KDO 8-P synthase against DEPC inactivation. These results suggest that PEP interacts with a histidine(s) at or near the active site of KDO 8-P synthase or that PEP binding induces a conformational change that prevents histidine modification by DEPC. A 5-P also afforded protection against inactivation, although to a lesser extent (Figure 5A).

The three KDO 8-P synthase mutants that exhibit altered kinetic parameters were also subjected to substrate protection studies to provide further information on the role of the mutated histidine residues. The pattern of substrate protection observed for the H241G mutant (Figure 5B) is essentially the same as that for the wild-type enzyme with the exception that the protection afforded by A 5-P has been marginally reduced. These results suggest that H241 does not participate in substrate binding or is located in an area of the protein that is removed from the active site. However, the kinetic analysis of the H241G mutant nonetheless indicates the importance of H241 for enzyme activity.

Substrate protection studies with the H97G mutant enzyme (Figure 5C) indicate that unlike the partial protection afforded by A 5-P to the wild-type enzyme, A 5-P no longer protects the H97G mutant against DEPC inactivation. These results strongly suggest that it is the interaction between A 5-P and H97 in KDO 8-P synthase that is responsible for the observed protection against modification and that H97 may participate in binding the phosphorylated monosaccharide. The increase in K_M observed for A 5-P in the H97G mutant is also consistent with this hypothesis. The protection provided by PEP against inactivation of H97G was only slightly lower than that provided to the wild-type enzyme. This could indicate that H97 may also be in proximity to the PEP binding site or interacts indirectly with this substrate, which would be consistent with the modest increase in the K_M for PEP observed with the H97G mutant.

Although the activity of the H202G mutant was extremely low, it was still possible to perform substrate protection studies. The level of protection provided by PEP and A 5-P to the H202G mutant against inactivation by DEPC were the lowest and highest, respectively, observed for any of the enzymes examined (Figure 5D). If interactions between PEP and H202 are exclusively responsible for the observed protection afforded to the wild-type enzyme by PEP, then PEP would no longer be expected to protect a mutant lacking this histidine but containing other modifiable residues. The reduced protection of the H202G mutant by PEP compared to the wild-type enzyme provides evidence in favor of the close proximity of H202 to the PEP binding site. However, the substantial protection still afforded to the H202G mutant by PEP indicates that another residue besides H202 is also involved in PEP binding. This residue is most likely H97. The considerable protection furnished by A 5-P to H202G suggests that this substrate does not interact with H202 but instead binds in proximity to and protects H97.

Imidazole Rescue Studies. The ability of exogenously added imidazole to substitute for the missing imidazole ring of the mutated histidine moiety in the H97G, H202G, and H241G mutants, and thus restore catalytic activity, was investigated by two different methods. Preincubation of the mutants in 50 mM imidazole, followed by assaying under standard conditions (method 1 with 0.3 mM and 0.6 mM A 5-P in the assay mixture) in which the final concentration of imidazole was diluted to 5 mM in the assay mixture, did not result in any significant restoration of activity; however, the inclusion of 50 mM imidazole in the assay mixture did

provide restoration of activity but to a varying degree in each mutant. No detectable change in activity for the H241G was observed in the presence of imidazole. The activity of H202G was increased from <1% to 4% of wild-type activity. The most significant results were obtained with H97G, in which the activity increased from 20% to approximately 60% of wild-type KDO 8-P synthase. This dramatic increase in activity was further investigated by preincubation of H97G with 50 mM imidazole in the presence of each substrate. The presence of PEP and imidazole did not affect the recovered activity as compared to only imidazole. Interestingly, the presence of both A 5-P and imidazole in the preincubation mixture resulted in a 20% decrease in recovered activity as compared to preincubating H97G in imidazole alone. This observation indicates that preincubation of the mutant H97G with A 5-P reduces the ability of imidazole to restore activity to the H97G mutant and may suggest that A 5-P and imidazole, in the absence of PEP, form a stable but nonproductive complex with the enzyme.

DISCUSSION

A combination of chemical modification and site-directed mutagenesis studies has been utilized to identify functionally important histidine residues in *E. coli* KDO 8-P synthase.

Several reports describe the utilization of DEPC to explore the role of histidine residues in a number of enzymes (*26*, *27*, *30*, *33*, *40*−*44*). Chemical modification studies have shown that KDO 8-P synthase is inactivated by DEPC in a time-dependent manner. Kinetic analysis of the inactivation reveals that the reaction between DEPC and KDO 8-P synthase is bimolecular and follows pseudo-first-order kinetics (Figure 1). A second-order rate constant of 340 M^{-1} min^{-1} was determined for the inactivation of KDO 8-P synthase by DEPC. This value falls in the range of rate constants obtained for the inactivation of other proteins containing important histidine residues, in which values between 0.67 and 368 M^{-1} min^{-1} have been reported (*26*, *40*−*43*, *45*, *46*).

Although DEPC is generally considered to selectively modify histidine residues in the pH range of 5.5−8.5 (*46*), the functional groups of other amino acids may react with DEPC. The UV difference spectrum of DEPC-treated KDO 8-P synthase versus untreated KDO 8-P synthase in 20 mM Tris-HCl (pH 7.6) shows an increase in absorbance only at 240 nm (Figure 2), which is a characteristic of protein-bound *N*-carbethoxyhistidine (*30*, *46*). The possible modification of either a tyrosine or cysteine residue by DEPC was not detected in the spectrum. Except for the pH dependency studies, the modification studies of KDO 8-P synthase and its mutants were performed at pH 7.6. At this pH, the evidence suggests only the modification of histidine residues. Although unlikely at this pH, these data do not preclude the modification of lysine residues.

To obtain additional experimental evidence implicating the modification of histidine residues in KDO 8-P synthase by DEPC, the effect of hydroxylamine on the modified enzyme was examined. Hydroxylamine has been utilized to restore catalytic activity to a number of DEPC-inactivated enzymes (*25*−*28*). The treatment of DEPC-modified KDO 8-P synthase with hydroxylamine does not restore catalytic activity but rather leads to further reduction in enzymatic activity. Unmodified wild-type KDO 8-P synthase is inactivated by hydroxylamine in a concentration-dependent manner. Thus, it is not surprising that treatment of DEPC-inactivated KDO 8-P synthase with hydroxylamine does not restore catalytic activity but rather leads to an additional loss in activity.

The complete modification of all six histidine residues in KDO 8-P synthase by DEPC can be achieved (Figure 3) after prolonged reaction in the presence of an excess of DEPC. The correlation between the number of residues modified and enzyme activity was assessed by the method of Tsou (*31*), revealing that either two or three histidine residues appear to be essential for activity. The results from this analysis cannot be used to unambiguously distinguish between these two values. In the presence of A 5-P, only one histidine residue appears to be essential. Therefore, it is assumed that the other essential histidine(s) is protected by A 5-P from DEPC modification. The analogous study with PEP as the protecting substrate could not be performed since PEP has an absorbance maximum at $\lambda = 232$ nm, which overlaps with the absorbance maximum of the carbethoxylated histidine moiety at $\lambda = 240$ nm.

The rate of inactivation of KDO 8-P synthase by DEPC in BTP buffer is dependent on pH (Figure 4) and a value of 7.3 was calculated for the pK_a of the modified residue. Analysis of the enzyme inactivation profile in phosphate buffer (Figure 4) suggests a pK_a value of 7.6 for the modified residue, similar to the pK_a value obtained in BTP buffer. In addition, it was observed that the DEPC inactivation of KDO 8-P synthase occurs at significantly slower rates in phosphate buffer than in a BTP buffer system. This observation strongly suggests that inorganic phosphate, a product of the condensation reaction catalyzed by KDO 8-P synthase, may bind at or near the histidine residues that are subject to DEPC inactivation. Preliminary studies indicate that the activity of KDO 8-P synthase in phosphate buffer is substantially lower than in other buffer systems over a wide pH range (data not shown), which is consistent with the observation that inorganic phosphate is a noncompetitive inhibitor of the enzyme (*47*). Under such high concentrations of phosphate, it is likely that phosphate could also bind to either or both substrate's phosphate binding sites.

Both A 5-P and PEP protect KDO 8-P synthase from inactivation by DEPC suggesting that the substrate binding sites are located near important histidine residues. The observed protection could also indicate that, upon substrate binding, conformational changes occur within KDO 8-P synthase that limit the accessibility of DEPC to these residues. The protection afforded by PEP is significantly greater than that provided by A 5-P (Figure 5A). The enhanced protection by PEP could reflect the formation of a tight enzyme−PEP complex that results in more effective shielding of the histidine residues from inactivation by DEPC than the enzyme complex formed with A 5-P or that PEP interacts with more histidine residues than does A 5-P. In all likelihood, the enzyme conformation adopted in the presence of the individual substrates differs from that in the ternary complex, and the substrate binding pockets in the former may not be fully defined.

Chemical modification studies have demonstrated the importance of histidine residues in KDO 8-P synthase. To identify and more fully characterize the role of these residues, each of the six histidine residues of KDO 8-P synthase was

individually mutated and the resulting mutants were analyzed by standard initial rate studies. This method of analysis allows a simple comparison of the various kinetic parameters of the wild-type and mutant KDO 8-P synthases. The kinetic data (Table 3) indicates that mutation of three of the six histidine residues does not have a significant effect on the catalytic properties of the enzyme (H40A, H67G, and H246G). Hence, histidine residues H40, H67, and H246 most likely do not play an important role in enzyme function. Interestingly, the mutants with the highest specific activity (H67G and H246G) seem to have undergone the most significant structural changes as a result of the mutagenesis, as determined from their CD spectra (Table 2).

In contrast to these results, mutants H97, H202, and H241 appear to be necessary for proper enzyme function. Not surprisingly, sequence alignments (not shown) reveal that H97, H202, and H241 in *E. coli* KDO 8-P synthase are fully conserved among all known KDO 8-P synthases, while H40, H67, and H246 are not (*48*).

The mutation of histidine 202 to glycine renders the enzyme virtually inactive. The value of k_{cat} determined for the H202G mutant implies that H202 may participate in a catalytic manner. The partial loss of PEP's ability to protect the H202G mutant against DEPC inactivation (Figure 5) indicates some degree of interaction between H202 and PEP. PEP still provides some protection against DEPC inactivation for the H202G mutant, indicating an additional interaction between PEP and another histidine. The H241G mutant is protected by PEP against DEPC inactivation to the same extent as the wild type enzyme, while the protection afforded to H97G by PEP is slightly lower, thus suggesting an interaction between H97 and PEP. In contrast, the protection studies with H202G and A 5-P imply that A 5-P does not bind near H202.

Several observations lead to the conclusion that H97 is directly involved in the binding of A 5-P. The results from the kinetic studies of H97G (Table 3) reveal a elevated K_M for A 5-P with only a modest change in the K_M value for PEP. The 2-fold reduction in k_{cat} observed for the H97G mutant indicates that H97 may be required to correctly position A 5-P in the active site for efficient catalysis to occur. Both observations imply an important interaction between this histidine residue and A 5-P. Furthermore, A 5-P no longer provides protection against DEPC inactivation as it does for the wild-type, H202G, and H241G enzymes (Figure 5). The kinetic and substrate protection experiments indicate that H97 is an important binding determinant for A 5-P. In addition, the moderate yet detectable decrease in the protection provided by PEP against modification of the H97G mutant also suggests that H97 may be in the vicinity of the PEP binding region.

The K_Ms for the H241G mutant of KDO 8-P synthase for both substrates are increased an order of magnitude relative to those for the wild-type enzyme, while k_{cat} is reduced 4-fold (Table 3). These findings strongly demonstrate the importance of H241 for proper enzyme function. The substrate protection pattern observed for the H241G mutant suggests that neither A 5-P nor PEP binds in the vicinity of this residue. These results could indicate that histidine H241 plays an important structural role in maintaining the correct *local* environment at the active site since no global structural changes in H241G was observed in the CD analysis.

A combination of chemical modification and site-directed mutagenesis studies on KDO 8-P synthase has revealed that three histidines play an important role in the aldol-type condensation catalyzed by KDO 8-P synthase. We believe that histidine 241 plays an important structural role, albeit in a local sense. Histidine 97 plays an essential role in A 5-P binding. The role of H202, while not fully elucidated, appears to be involved in PEP binding; however, a second histidine residue is also affected by PEP binding. The extreme loss of activity in the H202 mutant may indicate an important catalytic function, perhaps as a general acid or base. Studies utilizing other mutants of H202 are in progress to further define the function of histidine 202.

ACKNOWLEDGMENT

This investigation was supported by U.S. Public Health Service Grant GM 53069. We thank M. Birck and W. Taylor for their critical reading and discussion of the manuscript and C. Woodard for the careful reading of the manuscript. The contents of this paper are solely the responsibility of the authors and do not necessarily reflect the official views of NIGMS.

REFERENCES

1. Ray, P. H. (1980) *J. Bacteriol. 141*, 635−44.
2. York, W. S., Darvill, A. G., McNeil, M., and Albersheim, P. (1985) *Carbohydr. Res. 138*, 109−126.
3. Doong, R. L., Ahmad, S., and Jensen, R. A. (1991) *Plant Cell Environ. 14*, 113−120.
4. Yamada, H., Hirano, M., and Kiyohara, H. (1991) *Carbohydr. Res. 219*, 173−92.
5. Shin, K. S., Kiyohara, H., Matsumoto, T., and Yamada, H. (1997) *Carbohydr. Res. 300*, 239−49.
6. Ray, P. H., Kelsey, J. E., Bigham, E. C., Benedict, C. D., and Miller, T. A. (1983) *ACS Symp. Ser. 231*, 141−169.
7. Onderka, D. K., and Floss, H. G. (1969) *Biochem. Biophys. Res. Commun. 35*, 801−4.
8. Onderka, D. K., and Floss, H. G. (1969) *J. Am. Chem. Soc. 91*, 5894−6.
9. Dotson, G. D., Nanjappan, P., Reily, M. D., and Woodard, R. W. (1993) *Biochemistry 32*, 12392−7.
10. Kohen, A., Berkovich, R., Belakhov, V., and Baasov, T. (1993) *Bioorg. Med. Chem. Lett. 3*, 1577−1582.
11. Dotson, G. D., Dua, R. K., Clemens, J. C., Wooten, E. W., and Woodard, R. W. (1995) *J. Biol. Chem. 270*, 13698−705.
12. Hedstrom, L., and Abeles, R. (1988) *Biochem. Biophys. Res. Commun. 157*, 816−20.
13. Walsh, C. T., Benson, T. E., Kim, D. H., and Lee, W. J. (1996) *Curr. Biol. 3*, 83−91.
14. Woisetschlager, M., and Hogenauer, G. (1986) *J. Bacteriol. 168*, 437−9.
15. Woisetschlager, M., and Hogenauer, G. (1987) *Mol. Gen. Genet. 207*, 369−73.
16. Woisetschlager, M., Hodl-Neuhofer, A., and Hogenauer, G. (1988) *J. Bacteriol. 170*, 5382−4.
17. Salleh, H. M., Patel, M. A., and Woodard, R. W. (1996) *Biochemistry 35*, 8942−7.
18. Rieger, C. E., Lee, J., and Turnbull, J. L. (1997) *Anal. Biochem. 246*, 86−95.
19. Weiner, M. P., and Costa, G. L. (1994) *PCR Methods Appl. 4*, S131−6.
20. Weiner, M. P., Costa, G. L., Schoettlin, W., Cline, J., Mathur, E., and Bauer, J. C. (1994) *Gene 151*, 119−23.
21. Aminoff, D. (1961) *Biochem. J. 81*, 384−392.
22. Webb, M. R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 4884−7.
23. Melchior, W. B., Jr., and Fahrney, D. (1970) *Biochemistry 9*, 251−258.

24. Sreerama, N., and Woody, R. W. (1993) *Anal. Biochem. 209*, 32−44.
25. Gomi, T., and Fujioka, M. (1983) *Biochemistry 22*, 137−143.
26. Bhattacharyya, D. K., Bandyopadhyay, U., and Banerjee, R. K. (1993) *J. Biol. Chem. 268*, 22292−22298.
27. Lemaire, M., Schmitter, J. M., Issakidis, E., Miginiac-Maslow, M., Gadal, P., and Decottignies, P. (1994) *J. Biol. Chem. 269*, 27291−27296.
28. Huynh, Q. K. (1987) *Arch. Biochem. Biophys. 258*, 233−9.
29. Miles, E. W. (1977) *Methods Enzymol. 47*, 431−442.
30. Saluja, A. K., and McFadden, B. A. (1980) *Biochem. Biophys. Res. Commun. 94*, 1091−1097.
31. Tsou, C. (1962) *Sci. Sin. 11*, 1535−1558.
32. Deka, R. K., Kleanthous, C., and Coggins, J. R. (1992) *J. Biol. Chem. 267*, 22237−42.
33. Takeuchi, M., Asano, N., Kameda, Y., and Matsui, K. (1986) *J. Biochem. (Tokyo) 99*, 1571−1577.
34. Viladot, J. L., de, R. E., Durany, O., and Planas, A. (1998) *Biochemistry 37*, 11332−42.
35. Huang, S., and Tu, S. C. (1997) *Biochemistry 36*, 14609−15.
36. Boehlein, S. K., Walworth, E. S., Richards, N. G., and Schuster, S. M. (1997) *J. Biol. Chem. 272*, 12384−92.
37. Jung, Y. S., Vassiliev, I. R., Qiao, F., Yang, F., and Bryant, D. A. (1996) *J. Biol. Chem. 271*, 31135−44.
38. Rynkiewicz, M. J., and Seaton, B. A. (1996) *Biochemistry 35*, 16174−9.
39. den Blaauwen, T., Vandekamp, M., and Canters, G. W. (1991) *J. Am. Chem. Soc. 113*, 5050−5052.
40. Cheng, K., & Nowak, T. (1989) *J. Biol. Chem. 264*, 19666−19676.
41. Church, F. C., Lundblad, R. L., and Noyes, C. M. (1985) *J. Biol. Chem. 260*, 4936−4940.
42. Fukumori, F., and Hausinger, R. P. (1993) *J. Biol. Chem. 268*, 24311−24317.
43. Lundblad, R. L., and Noyes, C. M. (1984) in *Chemical Reagents for Protein Modification*, pp 105−125, CRC Press, Boca Raton, FL.
44. Saluja, A. K., and McFadden, B. A. (1982) *Biochemistry 21*, 89−95.
45. Fukumori, F., and Hausinger, R. P. (1993) *J. Bacteriol. 175*, 2083−2086.
46. Miles, B. W., Mareya, S. M., Post, L. E., Post, D. J., Chang, S. H., and Raushel, F. M. (1993) *Biochemistry 32*, 232−240.
47. Kohen, A., Jakob, A., and Baasov, T. (1992) *Eur. J. Biochem. 208*, 443−9.
48. Subramaniam, P. S., Xie, G., Xia, T. H., and Jensen, R. A. (1998) *J. Bacteriol. 180*, 119−127.

BI9829884