Essential Cysteines in 3-Deoxy-D-*manno*-octulosonic Acid 8-Phosphate Synthase from *Escherichia coli*: Analysis by Chemical Modification and Site-Directed Mutagenesis†

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ABSTRACT: The enzyme 3-deoxy-D-*manno*-octulosonic acid 8-phosphate synthase (EC 4.1.2.16) (KDO 8-P synthase) that catalyzes the condensation of D-arabinose 5-phosphate (A 5-P) with phosphoenolpyruvate (PEP) to give 3-deoxy-D-*manno*-octulosonic acid 8-phosphate (KDO 8-P) and inorganic phosphate (P_i) was inactivated by the thiol-modifying reagents 5,5-dithiobis(2-nitrobenzoate) (DTNB) and methyl methanethiosulfonate (MMTS). Reaction of cloned native KDO 8-P synthase with DTNB correlated with modification of two of the four cysteine sulfhydryls per monomer of enzyme and total loss of enzymatic activity which could be partially restored by treatment with dithiothreitol (DTT). Cyanolysis of the DTNB-inactivated enzyme with KCN led to the elimination of 2 equiv of 5-thio-2-nitrobenzoate and partial recovery of activity. The presence of either substrate(s) or product(s) provided no protection against inactivation nor affected the number of cysteines modified, indicating that the cysteines modified are most likely not at the active site of KDO 8-P synthase. Titration of denatured enzyme with DTNB resulted in the modification of all four cysteines. After treatment of native enzyme with MMTS, no cysteines could be titrated with DTNB and no enzymatic activity could be detected. Treatment of the MMTS-inactivated KDO 8-P synthase with DTT resulted in restoration of enzymatic activity and the presence of two DTNB-titratable cysteine residues. Based on these observations and a report that KDO 8-P synthase is inactivated in a time-dependent manner with 3-bromopyruvate and that the substrate PEP protects against this inactivation, all four cysteines (38, 166, 206, and 249) were individually mutated to alanines via a modified PCR methodology. The C206A and C249A mutants were both enzymatically active with K_m and V_{max} values approximately identical to those of wild-type KDO 8-P synthase, and both native mutants reacted with DTNB to modify only one of the three remaining cysteine sulfhydryls per monomer of enzyme. Titration of denatured C206A and C249A mutants resulted in the modification of three cysteines. The C38A and C166A mutants were both for the most part enzymatically inactive. Titration of native C38A and C166A with DTNB resulted in modification of two cysteines while titration of the denatured mutant protein resulted in modification of the three remaining cysteines. Circular dichroism measurements of wild-type KDO 8-P synthase and the four C→A mutants indicate modest but significant changes in the structure of the mutants. These results indicate that C206 and C249 in native KDO 8-P synthase are readily accessible to the modification reagent DTNB and therefore inactivation may result from structural changes in the DTNB-modified KDO 8-P synthase or blockage of access of substrates to the active site. The C38 and C166 in native KDO 8-P synthase are inaccessible to the modification reagent DTNB, indicating that they are located in the interior of KDO 8-P synthase, and loss of activity in the C38A and C166A mutants suggests their essentiality in the KDO 8-P synthase reaction.

The enzyme 3-deoxy-D-*manno*-octulosonic acid 8-phosphate (KDO 8-P)[1] synthase (EC 4.1.2.16) catalyzes the condensation of D-arabinose-5-phosphate (A 5-P) with phosphoenolpyruvate (PEP) to yield inorganic phosphate (P_i) and KDO 8-P (Figure 1). KDO is a site-specific molecule found only in G− organisms and is required for lipid A, part of the lipopolysaccharide (LPS) region of Gram-negative (G−) bacteria, maturation and cellular growth. The inhibition of KDO 8-P synthase would be an excellent chemotherapeutic approach (Ray et al., 1980) since mutants producing incomplete LPS are more susceptible to antibiotics and less pathogenic.

KDO 8-P synthase is a member of a family of PEP-utilizing enzymes including 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAH 7-P) synthase (EC 4.1.2.15) and *N*-acylneuraminate 9-phosphate (NAN 9-P) synthase (EC 4.1.3.20) that catalyzes the condensation of PEP with a phosphorylated sugar to produce a new phosphorylated 3-deoxy-α-keto sugar acid three carbons longer. Mechanistic studies of the first two of these enzymes reveal that the condensation step is stereospecific in that the attack of the

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[1] Abbreviations: A 5-P, D-arabinose 5-phosphate; DTNB, 5,5-dithiobis(2-nitrobenzoate); DTT, dithiothreitol; EPTase, UDP−*N*-acetylglucosamine enolpyruvate transferase; E 4-P, D-erythrose 4-phosphate; KDO 8-P, 3-deoxy-D-*manno*-octulosonate 8-phosphate; LPS, lipopolysaccharide; MMTS, methyl methanethiosulfate; PEP, phosphoenol pyruvate; P_i, inorganic phosphate; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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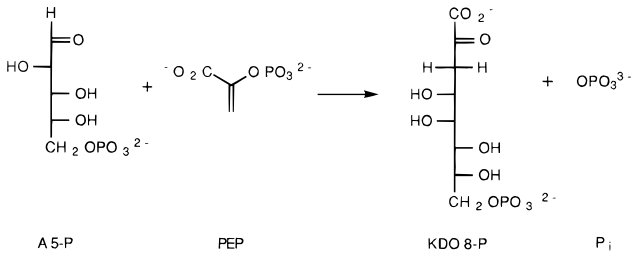

FIGURE 1: The KDO 8-P synthase reaction.

si face of the C3 of PEP is on the *re* face of the carbonyl carbon of the sugar substrate (Floss et al., 1972; Dotson et al., 1993; Kohen et al., 1993) and the reaction is essentially irreversible with the breaking of the C−O−P bond occurring between the C and O as opposed to between the P and O as occurs in other PEP-utilizing enzyme reactions studied to date (Hedstrom & Abeles, 1988; Dotson et al., 1995). It has been shown for the phenylalanine-sensitive isozyme of DAH 7-P synthase that Cys-61 is essential for catalytic activity as well as for metal binding while Cys-328 was shown to be nonessential for catalytic activity but mutation of C-328 had significant negative effects on V_{max} and K_m for PEP and Mn (Stephens & Bauerle, 1992). All DAHP synthases appear to require at least one cysteine sulfhydryl for enzyme activity (Huisman & Kosuge, 1974; Nagano & Zalkin, 1970; Staub & Denes, 1969). Two other PEP-utilizing enzymes that catalyze the addition of the intact carboxyvinyl portion of PEP concurrent with the breaking of the C−O−P bond between the C and O, EPSP (5-enolpyruvylshikimate 3-phosphate) synthase and UDP−*N*-acetylglucosamine enolpyruvate transferase (EPTase), have recently received considerable attention. While it has been demonstrated that the cysteines in EPSP synthase are nonessential (Padgette et al., 1988), it has been shown by three separate groups (Brown et al., 1994; Dotson & Woodard, 1994; Wanke & Amrhein, 1993) that the enzyme EPTase involves the formation of a bond between Cys-115 and PEP to form some type of thiolactoyl intermediate. It has been shown that all three isozymes of DAH 7-P synthase require metal ions for catalysis. Neither EPSP synthase, EPTase, nor KDO 8-P synthase requires metal ions for catalysis. It has also been reported that KDO 8-P synthase undergoes a time-dependent inactivation with 3-bromopyruvate (Hedstrom, 1986). Since the substrate PEP, but not A 5-P, protects against this inactivation, it was suggested that 3-bromopyruvate is an active-site-directed affinity label.

In this paper, we present evidence that in KDO 8-P synthase two cysteines may be proximal to the active site and may be essential for the KDO 8-P synthase reaction. Of the four cysteines in *Escherichia coli* KDO 8-P synthase, C38 and C166 are essential for the condensation of PEP and A 5-P but are inaccessible to the thiol-modifying DTNB while both C206 and C249 are reversibly modified by DTNB. The DTNB modification of C206 and C249 leads to inactivation of KDO 8-P synthase which can be recovered by treatment with thiol reagents.

EXPERIMENTAL PROCEDURES

Materials. The chemicals used were of reagent grade or of the highest purity commercially available and were not further purified. Q Sepharose, D-arabinose 5-phosphate, phosphoenolpyruvate, 3-deoxyoctulosonic acid, DTT, and Tris were purchased from Sigma Chemical Co. The bacterial purine−nucleoside phosphorylase (EC 2.7.4.4) purchased from Sigma Chemical Co. (N 8654) was further purified on Mono Q anion exchange chromatography. Restriction and DNA modifying enzymes were from Boehringer Mannheim and New England Biolabs. The *E. coli* strains BL 21 and BL (DE 3) were obtained from Novagen. The thermal cycling was performed using an MJR Research Thermal cycler. The 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) was synthesized by the method of Broom and Milne (1974). Oligonucleotides were synthesized by the University of Michigan Biomedical Research Resources Core Facility. DNA sequencing, N-terminal protein sequencing, and total amino acid content as well as electrospray mass spectral analysis were performed by the University of Michigan Biomedical Research Resources Core Facility. The GeneAmp kit was from Perkin-Elmer/Cetus except recombinant Vent DNA polymerase (New England Biolabs) was substituted for *Taq* DNA polymerase. Promega DNA and PCR purification kits were utilized.

Recombinant Wild-Type KDO 8-P Synthase Preparation and Purification. KDO 8-P synthase was prepared from *E. coli* BL 21 (DE 3) harboring the plasmid pT7-7/*kdsA* as previously described by this laboratory (Dotson et al., 1995), except that the concentrated (Centriprep-10) fast flow Q column fractions were dialyzed against 20 mM Tris-HCl, pH 7.6 (buffer A), and further purified on a Mono Q HR 10/10 (Pharmacia) using a linear gradient of 0−0.25 KCl in buffer A. The KDO 8-P synthase-containing Mono Q fractions were concentrated and stored at −80 °C.

Assay Procedures. (1) KDO 8-P Synthase Assay. (A) The KDO 8-P synthase activity was assayed by measuring either the amount of KDO 8-P produced using the periodate−TBA assay (Ray, 1980) or the amount of P_i produced using the method of Lanzetta et al. (1979). One unit of activity is defined as 1 μmol of KDO 8-P or 1 μmol of P_i released per minute at 37 °C. (B) 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 (1992), was used to determine kinetic parameters. The standard assay mixture contained 300 μM PEP, 300 μM A 5-P, 40 mM Tris−acetate, pH 7.6, 100 μM MESG, PNPase, and KDO 8-P synthase in 1 mL. The first five reagents were mixed and incubated at 37 °C for 5 min. The assay, initiated by the addition of the KDO 8-P synthase, was monitored for 5 min at $\lambda = 360$ nm for an increase in absorption. ($\epsilon = 11\,000$ M^{-1} cm^{-1} for the newly formed purine.) The initial rates were determined by least-squares fitting of the first portion of the progress curve (50−80 s, depending on the initial concentration of PEP) to a straight line. Best fits of K_m and V_{max} values were determined utilizing the PC-IBM version of Enzfitter (1987).

(2) Protein Assay. The protein concentrations of enzyme fractions were determined using the Bio-Rad protein assay (Bio-Rad, Richmond, CA) with bovine serum albumin as standard. Based on numerous total amino acid composition analyses, the Bio-Rad protein assay overestimates KDO 8-P synthase by a factor of 1.3.

(3) Polyacrylamide Gel Electrophoresis. Electrophoretic analyses were performed utilizing 12% denaturing gels in the discontinuous Laemmli buffer system (Laemmli, 1970) with a Bio-Rad Mini-Protean II. Gels were stained using 0.25% Coomassie Brilliant Blue R-250.

DTNB Inactivation Experiments. KDO 8-P synthase, freshly purified or previously frozen at −80 °C, isolated as

described above was treated with DTT (5 mM) in buffer A for 30 min at 4 °C. The thiol reagent was removed by passage through a G-25 column using deoxygenated buffer A. The solvent reservoir was maintained under a constant blanket of nitrogen. Reaction mixtures containing this KDO 8-P synthase (± ligands) in 100 mM Tris-HCl, pH 7.5, (buffer B) were preincubated for 5 min at 25 °C and treated with 0.20 mM freshly degassed DTNB (in 50 mM HEPES, pH 7.0), to give a final volume of 0.8−1.0 mL. The increase in absorbance at $\lambda = 412$ nm was monitored as a function of time against a buffer control containing the ligands and reagents. The thiol concentration was determined using ϵ_{412} 13 600 cm^{-1} M^{-1} for the released 5-thio-2-nitrobenzoate (TNB) chromophore. For the denatured KDO 8-P synthase titrations, the above procedure was followed except that the final concentration of urea in the reaction mixture was 4 M.

Cyanolysis of DTNB-Inactivated KDO 8-P Synthase. The DTNB-inactivated native KDO 8-P synthase reactions, prepared as described above, were passed through a G-25 column to remove the excess DTNB. The Centricon-concentrated fractions containing the modified KDO 8-P synthase were treated with freshly degassed 10 mM KCN in 100 mM Tris-HCl (7.1) at 25 °C. The release of the 5-thio-2-nitrobenzoate (TNB) chromophore was monitored at $\lambda = 412$ nm as above. The KCN-treated sample was desalted on a G-25 column and Centricon-concentrated, and the enzyme activity was determined (periodate−TBA assay) versus a sample of KDO 8-P synthase treated identically except that it was not treated with DTNB initially.

MMTS Inactivation Experiments. Reaction mixtures containing wild-type recombinant native KDO 8-P synthase in 100 mM Tris-HCl, pH 7.5, were treated with 0.20 mM freshly degassed MMTS (in 50 mM HEPES, pH 7.0) for 30 min at 4 °C. The excess MMTS was removed by passage through a Sephadex G-25 column, and the protein-containing fractions were combined and concentrated (Centricon-10). One-fourth of this sample was assayed for enzyme activity by both the periodate−TBA assay and the Lanzetta phosphate assay. One-fourth of the sample was subjected to DTNB titration (both native and urea-denatured) as described above. The remaining portion of the sample was treated with DTT passed through a Sephadex G-25 column to remove small molecular weight compounds, concentrated, assayed for activity, and subjected to titration with DTNB.

Construction of Mutant Clones. The *E. coli kdsA* mutant genes were prepared by a modified PCR method (Chen & Przybyla, 1994) using the following mutagenic primers (designated Pm): C38A, 5′-CGATGCGCATT**GCG**GAG-CACTACG-3′; C166A, 5′-CGAAAAAGTGATTCTT**GCG** GATCGCGG-3′; C206A, 5′-CCACGCA CTGCAA**GCG**-CGCGATCC-3′; C249A, 5′-GCGGATGC ACCATCAGC-TTT **CGC**ATGTT CC-3′ (non-reading frame). The nucleotides replaced from the wild-type *kdsA* gene are in bold. The forward primer used upstream of the *kds*A N-terminus was the commercially available T7 sequencing primer, 5′-TAATACGACTCACT ATAGGG-3′ (designated P1). The general reverse primer (non-reading frame) that was used downstream of the *kdsA* C-terminus was 5′-GCATTGG-TAACTGTCAGACC-3′ (designated P2). The synthetic mutagenic primer (Pm) containing the desired mutagenic change and P2 for the three mutants C38A, C166A, and C206A and P1 for mutant C249A were used for the first round of PCR amplification using wild-type *kds*A as template and Vent DNA polymerase. The amplification was performed for 25 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1.5 min. Following the last cycle, the reaction mixture was held at 72 °C for 5 min. The proper base-sized fragments from the first round of PCR were purified by electrophoresis on 1% low-melting agarose gel using 0.5× TAE buffer throughout. The excised bands (minimum amount of gel) were used directly as primers (in the double-stranded form) together with the alternate primer (P1 for the first three mutants and P2 for C249A) to amplify a second round of DNA synthesis. The wild-type *ksd*A was again used as template with Vent DNA polymerase. The inclusion of additional $MgSO_4$ to compensate for the EDTA carried over from the LM agarose gel greatly improved the yields in the second amplification step. The newly amplified DNA was purified by LM agarose gel electrophoresis and eluted from the gel. The purified DNA was digested with *Nde*1 and *Hin*DIII and ligated into the expression plasmid pT7-7 digested with the same two restriction enzymes. The ligation mixture was used to transform chemically competent BL 21 *E. coli* cells. The plasmid obtained from the mutant clones was characterized by either restriction digestion and/or DNA sequencing. DNA containing the proper mutagenic sequence was used to transform chemically competent BL 21 (DE 3) *E. coli* cells for the expression of the desired mutant protein. An overnight culture of the transformed cells (1 mL) was mixed with 500 μL of 50% glycerol and stored at −85 °C.

Mutant KDO 8-P Synthase Preparation and Purification. The four C→A mutants were grown, isolated, and purified by the methods described for the recombinant wild-type KDO 8-P synthase. The activities of these mutants were determined as described above. The K_m and V_{max} values were determined for C206A and C249A but not for the inactive C38A and C166A mutants.

Circular Dichroism Studies. The circular dichroism spectra of the wild-type and the mutant enzymes were obtained by scanning the sample solutions in a 1 mm stoppered strain-free quartz cuvette, over the far-UV range (250−185) at 25 °C using an Aviv circular dichroism spectrophotometer Model 62 DS containing a water jacket attached to a circulating water bath. All protein samples were desalted into 2 mM potassium phosphate buffer (pH 7.5) using a Sephadex G-25 column, the protein levels were determined as described above, and the concentration was adjusted to 0.1 mg/mL. Scans were collected at 1 nm intervals with a band width of 1.5 nm and a dwell time of 1 s. The dynode voltage remained below 600 V for all samples. A total of five readings for each 1 nm measured were averaged for each scan, and a total of five scans were collected for each protein solution; the signal was averaged, calibrated to remove the background of the buffer, and smoothed using the program supplied with the spectrometer (smoothing program gave a value which was similar to the *Q* test @ 99% and 95% CL). The estimated percentages of secondary structure were calculated from the CD spectra utilizing the self-consistent method (SELCON program) described by Sreerama and Woody (1993).

RESULTS AND DISCUSSION

KDO 8-P Synthase Continuous Spectrophotometry Assay. Webb (1992) has recently reported a continuous spectrophotometric assay for the measurement of phosphate release that uses purine−nucleoside phosphorylase (EC 2.4.2.1) and the unusual guanosine analogue, 2-amino-6-mercapto-7-

Table 1: Kinetic Constants of Mutant KDO 8-P Synthase

enzyme[a]	V_{max} [μmol/(min·mg)]	K_M^{PEP} (μM)	$K_M^{A\ 5-P}$ (μM)
wild-type[b]	3.7	6	20
wild-type[c]	5.0	7.6	26
wild-type[d]	9	6.5	9.1
C38A	0.1	nd	nd
C166A	0.4	nd	nd
C206A	7	3.5	9.9
C249A	6.5	7.9	29.5

[a] The mutant enzymes are designated by the identity of the wild-type residue followed by the position of the residue, then the single amino acid code of the mutant residue. Kinetic values obtained from: [b] The thiobarbiturate assay (Ray, 1980). [c] The continuous assay based on the disappearance of the PEP chromophore at $\lambda = 232$ nm ($\epsilon = 2840\ M^{-1}\ cm^{-1}$) (Kohen et al., 1992), and the coupled PNPase assay reported herein. nd = not determined.

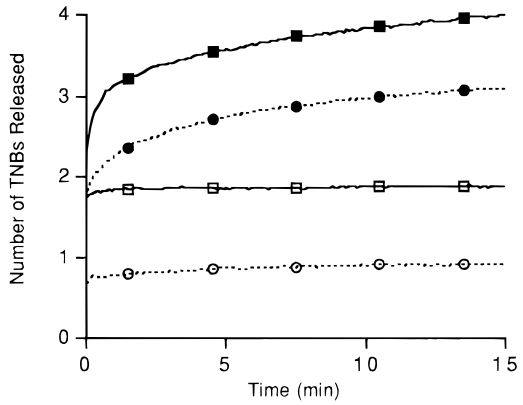

FIGURE 2: Representative DTNB titration curves: ○, C206A native; □, wild-type KDO 8-P synthase native; ●, C206A denatured; ■, wild-type KDO 8-P synthase denatured.

methylpurine ribonucleoside (MESG). The phosphorylase utilizes free inorganic phosphate released by the enzyme to be assayed to catalyze the hydrolysis of the MESG into its purine component and ribose 1-phosphate. The assay is based on the difference in absorbance at 360 nm between the unstable methylated 6-thiopurine nucleoside, and the purine base product formed by attack of the phosphate at C-1 of the ribose portion of MESG ($\epsilon = 11\ 000\ M^{-1}\ cm^{-1}$ for the newly formed purine).

The method is reported to be linear from 2 to 25 μM phosphate. The K_m for A 5-P and PEP and V_{max} values for native recombinant KDO 8-P synthase obtained by this method are in agreement with values previously reported (see Table 1). The previously reported continuous assay for KDO 8-P synthase (Kohen et al., 1992), a modification of the method reported for the analysis of DAH 7-P synthase (Schoner & Herrmann, 1976), is based on the absorbance difference at 232 nm between PEP ($\epsilon = 2840\ M^{-1}\ cm^{-1}$) and the other substrates and products ($\epsilon < 60\ M^{-1}\ cm^{-1}$) under assay conditions. While both continuous assay procedures provide comparable values, the present assay has been found to be less problematic in our hands.

Inactivation of Recombinant Wild-Type KDO 8-P Synthase with Sulfhydryl Reactive Reagents. Treatment of native wild-type KDO 8-P synthase with DTNB resulted in the instantaneous release of two 5-thio-2-nitrobenzoate (TNB) chromophores and an inactive enzyme (see Figure 2 and Table 2). Incubation for an additional 2 h resulted in no further benzoate release (data not shown). The majority of activity could be restored by treatment of the DTNB-modified KDO 8-P synthase with the thiol reagent DTT (see Table 3).

Table 2: Reaction of KDO 8-P Synthase with DTNB

sample	equiv of TNB released	act. remaining[a] (%)
1. wild (native)	1.9	0
2. wild (denatured)	4	nd
3. wild (native) + PEP	1.9	0
4. wild (native) + A 5-P	2.1	0
5. Wild (native) + P_i	1.9	0
6. wild (native) + KDO 8-P	2.2	0
7. C38A	1.7	0
8. C38A (denatured)	2.8	nd
9. C166A	2	0
10. C166A (denatured)	2.8	nd
11. C206A	0.9	0
12. C206A (denatured)	3	nd
13. C249A	0.85	0
14. C249A (denatured)	3	nd

[a] Determined by the thiobarbiturate assay method after removal of excess reagents by G-25 Sephadex. Fraction is based on comparison to a sample of untreated wild-type KDO 8-P synthase. For samples 3−6 the KDO 8-P synthase was first preincubated with either the substrate or product listed (60 mM) for 5 min at 25 °C before reaction with DTNB. nd = not determined

Table 3: Activity Recovery of DTNB-Inactived KDO 8-P Synthase

inactivation reagent[a]	reactivation reagent[b]	no. of TNBs released	fraction of act. recovered[c] (%)
1. DTNB	none	none	nd
2. DTNB	DTT	2.1	76
3. DTNB	KCN	1.7	63

[a] Wild-type KDO 8-P synthase (0.75−1 mg of protein) was inactivated with DTNB as described in the Experimental Procedures and then desalted on a G-25 Sephadex column. [b] DTNB-inactivated wild-type KDO 8-P synthase was treated with the reactivation reagent listed in this column to determine the number of TNB chromaphores released. [c] Value determined, after treatment with reactivation reagent and removal of liberated masking groups, by the thiobarbiturate assay method. The % activity recovered is based on comparison to a sample of wild-type treated in a similar manner except the original KDO 8-P synthase sample was not treated with any inactivation reagent. nd = not determined

Preincubation with either substrate PEP or A 5-P or either product KDO 8-P or phosphate had no effect on the number of released TNB moieties, nor did it provide protection from inactivation (see Table 2). When urea-denatured recombinant wild KDO 8-P synthase was treated with DTNB all four cysteines, as predicted by the nucleotide sequence, sulhydryl groups of KDO 8-P synthase reacted. These results indicate that there are not cystine-type linkages in KDO 8-P synthase. Reaction of a DTNB-free solution of DTNB-modified KDO 8-P synthase with KCN resulted in the release of two bound TNB molecules and the formation of the S-cyanolated enzyme with partially restored catalytic activity (see Table 3). The lack of activity in the TNB-modified protein could be due to either an increased steric bulk brought about by modification which would limit the access of substrates to the active site or the essentiality of the sulhydryl group of the cysteine modified for the reaction catalyzed by KDO 8-P synthase. The latter scenario is eliminated by the observation that enzymatic activity was regained when the TNB moiety of the inactivated cysteine was replaced by the smaller cyano group. The cyanolysis of TNB-inactivated proteins has been used previously by a number of workers to demonstrate the role of the thiol group of reactive cysteines (Birchmeier et al., 1973; Degani et al., 1974; Padgette et al., 1988). The failure of both substrates and products to provide protection against DTNB inactivation would also

Table 4: Activity Recovery of DTNB/MMTS-Inactived KDO 8-P Synthase

inactivation reagent[a]	reactivation reagent	no. of TNBs released	act. recovered[d] (%)
1. MMTS	none	0.1[b]	0
2. MMTS (denatured)	none	0.2[b]	nd
3. MMTS	DTT	1.7[c]	71

[a] Wild-type KDO 8-P synthase (1.5 mg of protein) was inactivated with MMTS as described in the Experimental Procedures and then desalted on a G-25 Sephadex column. [b] MMTS-inactivated KDO 8-P synthase both native and urea-denatured was treated with DTNB. [c] The desalted MMTS-inactivated KDO 8-P synthase was first treated with DTT, desalted, and then reacted with DTNB. [d] Value determined, after treatment with reactivation reagent and removal of small molecules, by the thiobarbiturate assay method. The % activity recovered is based on comparison to a sample of wild-type treated in a similar manner except the original KDO 8-P synthase sample was not treated with MMTS. nd = not determined.

tend to argue against the latter idea that the cysteines inactivated do not seem to be either near the active site or involved with the catalytic process. The modification of native KDO 8-P synthase with the smaller thio-modifying reagent MMTS resulted in the modification of all four cysteines since titration of the denatured MMTS-modified KDO 8-P synthase with DTNB resulted in the release of no TNB. The activity of MMTS-inactivated native KDO 8-P synthase could be restored by treatment with DTT (see Table 4). The reactivated enzyme reacted with DTNB to release two TNBs and resulted in an inactive enzyme.

Site-Directed Mutagenesis of KDO 8-P Synthase. The four cysteine to alanine mutants were constructed using a modification of a three-primer, two-step PCR-based mutagenesis procedure reported by a number of workers. The overexpression vector pT7-7/*kdsA* (wild-type) was used as the template. Vent DNA polymerase was used for amplification since it lacks terminal transferase activity and therefore adds no extra nucleotides (usually A's) onto the 3′ ends. The success of this procedure absolutely requires the formation of blunt ends for the first PCR product. Our choice of the first two PCR primers is generally, but not always, governed by a desire to produce the longest PCR product in the first PCR reaction. This choice of primers required that, for the production of mutant C249A, the first outside primer corresponded to the forward reading frame sequence and the mutagenic primer corresponded to the nonreading frame sequence. Since the frequency of wild-type production in this methodology is determined by the amount of carry-over or contamination of the outside primers into the second PCR reaction, the first PCR reaction is purified by low-melting gel electrophoresis which allows for the complete removal of both the mutagenic and the first outside primer. The excised band corresponding to the proper base size DNA is used directly, in its double-stranded form, as a primer in the second PCR reaction. Additional $MgSO_4$ is added to the second PCR reaction mixture to compensate for the EDTA present in the low-melting gel. The second PCR reaction mixture was purified by electrophoresis in low-melting agarose gel. The desired DNA fragment, separated from the gel, was restricted with *Nde*1 and *Bam*H1 and ligated into the similarly restricted expression vector, pT7-7. The DNA sequence of the various mutants was confirmed by DNA sequencing. The various cysteine to alanine mutants were overproduced from the pT7-7-based vectors containing the desired DNA mutagenic sequence as previously described for the overproduction of wild-type recombinant KDO 8-P synthase, and purified to >95% homogeneity, as assessed by SDS−polyacrylamide gel electrophoresis. The mutants were isolated in the soluble portion of the cellular extract and purified on anion exchange resin at the same salt concentration as wild KDO 8-P synthase, indicating little gross change in overall charge and structure. Both C206A and C249A, 75% [6.935 μmol/(mg·min)] and 70% [6.33 μmol/(mg·min)] of wild [9.34 μmol/(mg·min)], respectively, had activities similar to that of wild KDO 8-P as assayed by both the periodate−TBA assay and the Lanzetta phosphate assay, whereas C38A had only residual wild-type KDO 8-P synthase activity carried over from the host BL21(DE3) *E. coli* (not harboring an expression vector) and C166A had only 3% activity above wild-type host activity as compared to wild-type recombinant KDO 8-P synthase. A major problem with defining the role of the cysteine mutants is a technical one in that *E. coli* has an absolute requirement for KDO 8-P synthase; therefore, our mutant enzyme preparations are all contaminated with a very small amount of the wild-type KDO 8-P synthase. The K_m and V_{max} parameters, as determined by the PNPase KDO 8-P synthase-coupled assay, for the wild-type, C206A, and C249A are given in Table 1.

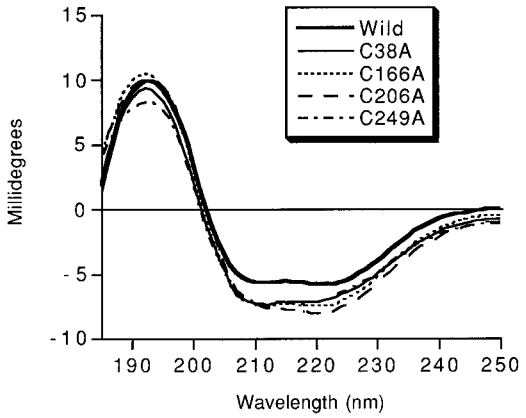

FIGURE 3: CD spectra: wild-type KDO 8-P synthase (bold line) and the four mutants (thin and dashed lines).

Table 5: Deconvoluted CD Data for KDO 8-P Synthase and C→A Mutants[a]

mutant	α-helix (%)	β-sheet (%)	β-turn (%)	random coil	act. (% of wild)
wild	22.2	27.4	21.8	19.2	100
C38A	23.5	29	25.8	20.6	1.4
C166A	27.9	20.3	24	22.1	4.7
C206A	29.2	20.7	23.5	23.9	74
C249A	29.3	22.8	24.1	22.3	68

[a] Determined by the method of Sreerama and Woody (1993).

Circular Dichroism Studies. The conformational integrity of the mutant proteins was probed by circular dichroism spectroscopy. The CD spectra of the wild-type KDO 8-P synthase and the four mutants (see Figure 3) indicate modest but significant conformational change in the mutant enzymes. Deconvolution of the spectral data (see Table 5) by the self-consistency method of Sreerama and Woody (1993) suggests the conformational change may be due to an increase in the helix and random coil content of the mutants C166A, C206A, and C249A as compared to wild-type KDO 8-P synthase and the C38A mutant with a concomitant decrease in the β-sheet content. Analysis by several other deconvolution programs resulted in similar comparisons (data not shown).

Inactivation of KDO 8-P Synthase C→A Mutants with DTNB. Treatment of native C206A and C249A KDO 8-P synthase with DTNB resulted in the liberation of only one TNB moiety, while titration of the urea-denatured proteins resulted in the liberation of 3 mol of TNB, indicating that both cysteine 206 and 249 are readily accessible to the outside surface of KDO 8-P synthase (see Figure 2 for a typical titration of C206A). Titration of native C38A and C166A with DTNB produced 2 equiv of TNB and a third when the titration was repeated with denatured mutants. Therefore, cysteines 38 and 166 must be in the interior of KDO 8-P synthase and inaccessible to the DTNB molecule.

The sensitivity of KDO 8-P synthase to thiol-inactivating reagents such as bromopyruvate (98% irreversible inactivation) or 1.0 mM Hg^{2+} (reversible by the addition of 1 mM dithiothreitol), and to a lesser extent iodoacetamide (9% inactivation at 10 mM) and iodoacetic acid (29% inactivation at 10 mM), raised the question of whether an essential cysteine residue participates in catalysis as an active nucleophile, in substrate binding, or as a general acid. In view of the lack of in-depth information on the reactivity of cysteine residues in KDO 8-P synthase, the studies reported in this paper were undertaken. While KDO 8-P synthase shares a number of mechanistic similarities to other PEP-utilizing enzymes, there is no sequence homology between KDO 8-P synthase and DAH 7-P synthase (all three isozymes), EPSP synthase, or EPTase. As discussed in the introduction, each of these PEP-utilizing enzymes has different requirements and/or roles for the thiol group of their active site cysteine(s). In the present work, we have established that cysteines 38 and 166 play some important role(s) in the KDO 8-P synthase reaction since their replacement with alanine residues resulted in an inactive enzyme. The results from the DTNB titrations of wild-type and mutant KDO 8-P synthase suggest that cysteines 38 and 166 are located in the interior of KDO 8-P synthase since they seem inaccessible for reaction with DTNB. The exact role of these cysteines whether mechanistic, necessary for substrate binding, or structural is at present unclear, but based on the previous stereochemical mechanistic studies, it is unlikely that the cysteine thiol(s) forms a covalent bond with PEP as is the case of EPTase since there was no scrambling of isotopic labels found in either previous study. In addition, unlike EPTase, in which addition of UDP-GlcNAC to EPTase results in formation of some product, no detectable KDO 8-P is formed by treating KDO 8-P synthase with A 5-P in the absence of PEP. Additional mutagenic studies involving replacing cysteine residues 38 and 166 with serine are ongoing to determine if the hydroxyl group of a serine can substitute for the thiol group of cysteine in whatever function the thiol group is performing. Further studies to determine which active site cysteine reacts with bromopyruvate are in progress. Once that has been determined, the pK_a of the acceptor cysteine residue will be determined to help further elucidate its role in the KDO 8-P synthase reaction. This should prove to be quite informative since it has recently been postulated that an acidic active residue is potentially needed to protonate the leaving phosphate group at C-2 of the postulated diphosphate intermediate in order gain the necessary rate enhancement seen in the KDO 8-P synthase reaction (Baasov & Kohen, 1995).

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