Stereochemistry of 3-Deoxyoctulosonate 8-Phosphate Synthase[†,‡]

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ABSTRACT: (*Z*)- and (*E*)-[3-^{2}H]phosphoenolpyruvate were prepared chemically by the reductive deuteration of (*Z*)- and (*E*)-3-bromophosphoenolpyruvate, respectively, and were converted into 3-deoxyoctulosonate 8-phosphates deuterated at the C-3 position by incubation with unlabeled D-arabinose 5-phosphate in the presence of the enzyme, 3-deoxyoctulosonate 8-phosphate synthase (EC 4.1.2.16) purified from *Escherichia coli* K-12 containing the plasmid pMW101. Analysis of the stereochemistry of the two 3-deoxyoctulosonate 8-phosphates deuterated at the C-3 position by ^{1}H NMR showed that the (*Z*)-[3-^{2}H]phosphoenolpyruvate had produced [3-^{2}H]-3-deoxyoctulosonate 8-phosphate of predominantly the 3*S* configuration and that the *E* isomer had given predominantly (3*R*)-[3-^{2}H]-3-deoxyoctulosonate 8-phosphate. The 3-deoxyoctulosonate 8-phosphate synthase reaction is therefore stereospecific with respect to the C-3 of phosphoenolpyruvate. The results indicate a *si* face attack from the C-3 of phosphoenolpyruvate, a result identical to that reported for 3-deoxyheptulosonate 7-phosphate synthase (EC 4.1.2.15), an enzyme catalyzing an identical aldol-type condensation, except that it takes place between phosphoenolpyruvate and D-erythrose 4-phosphate. The stereochemistry with respect to the face of the carbonyl of the attacked aldehyde, in both 3-deoxyoctulosonate 8-phosphate synthase and 3-deoxyheptulosonate 7-phosphate synthase, is *re*. On the basis of the results of the studies reported herein, the presence of a transient methyl group at the C-3 of phosphoenolpyruvate as part of the reaction mechanism seems unlikely.

The outer membrane of the Gram-negative bacteria cellular envelope is composed of an unsymmetrical bilayer of phospholipid (interior) and lipopolysaccharide (LPS, exterior) (Raetz, 1990). This LPS layer, which is responsible for the pathophysiological phenomena associated with Gram-negative infections, contains the unique eight-carbon acidic sugar 3-deoxy-D-*manno*-octulosonic acid (KDO)[1] (Ray et al., 1983). KDO is a site-specific molecule found only in Gram-negative bacteria and has been shown to be required for lipid A maturation and cellular growth (Rick & Osborn, 1977). Thus, inhibition of KDO biosynthesis and/or its incorporation into lipid A are considered viable targets for chemotherapeutic intervention.

The incorporation of KDO into the elongating nonrepeating oligosaccharide, designated the core region, is accomplished by way of the activated nucleotide, CMP–KDO. CMP–KDO is formed enzymatically through condensation of CTP and KDO by the enzyme, CMP–KDO synthetase (cytidine 5′-triphosphate:cytidine 5′-monophosphate–3-deoxy-D-*manno*-octulosonate cytidylyl transferase) (Ray & Benedict, 1982b). The KDO portion originated from KDO 8-P available through the action of the enzyme KDO 8-P phosphatase (Ray & Benedict, 1982a) on KDO 8-P. The KDO 8-P is formed in an enzymatic aldol-type condensation of the C-3 of phosphoenolpyruvate (PEP) with the C-1 of D-arabinose 5-phosphate (A 5-P) by KDO 8-P synthase in the first committed step of the KDO pathway. Therefore, C-1–C-3 of KDO 8-P are derived from PEP, while C-4–C-8 are derived from A 5-P. This enzymatic condensation is similar to that catalyzed by 3-deoxyheptulosonate 7-phosphate (DAH 7-P) synthase in which PEP is condensed with D-erythrose 4-phosphate (E 4-P) to give DAH 7-P (Floss et al., 1972). In order to contribute to the understanding of the biochemical reaction mechanism operative in the enzyme KDO 8-P synthase, we decided to investigate the stereochemistry of the reaction. In this article, we report the steric course for the formation of KDO 8-P from A 5-P and stereospecific, isotopically labeled PEP.

EXPERIMENTAL PROCEDURES

Materials. The chemicals used were reagent grade or of the highest purity commercially available and were not further purified. HA-Ultrogel, Q Sepharose, D-arabinose 5-phosphate, phosphoenolpyruvate (unlabeled), and 3-deoxyoctulosonic acid were purchased from Sigma Chemical Company (St. Louis, MO).

Expression Vector. The expression vector, designated pMW101, utilized in this study was engineered and kindly provided by Professor Gregor Högenauer (Woisetschlager & Högenauer, 1986). Briefly, the vector contained a 1.5 kilobase *Pvu*II fragment selected by complementation with a *kds*A mutant. The open reading frame, *kds*A, expressing KDO

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[1] Abbreviations: A 5-P, D-arabinose 5-phosphate; CMP–KDO, cytidine 5′-monophosphate–3-deoxy-D-*manno*-octulosonate; CTP, cytidine 5′-triphosphate; DAH 7-P, 3-deoxyheptulosonate 7-phosphate; DTT, dithiothreitol; E 4-P, D-erythrose 4-phosphate; KDO 8-P, 3-deoxyoctulosonate 8-phosphate; LPS, lipopolysaccharide; NMR, nuclear magnetic resonance; PEP, phosphoenolpyruvate; SDS, sodium dodecyl sulfate; TBA, thiobarbituric acid; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)-aminomethane.

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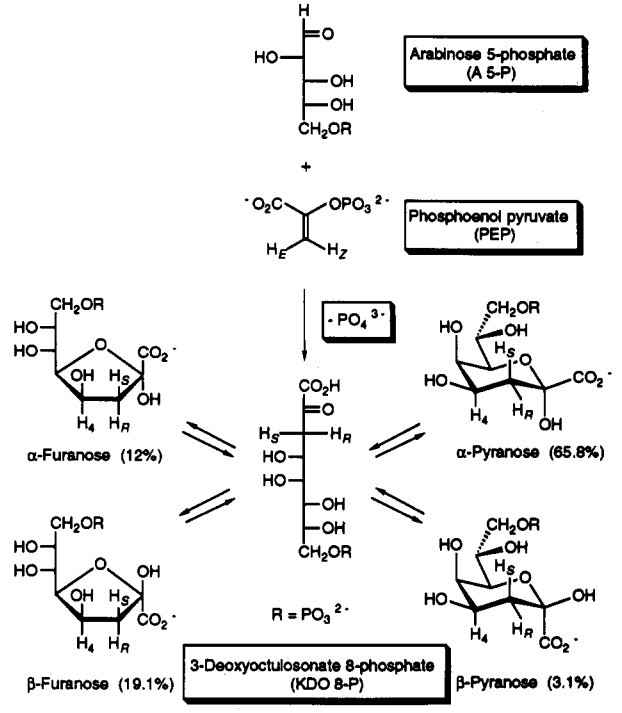

FIGURE 1: Reaction catalyzed by KDO 8-P synthase and the distribution of product conformers.

8-P synthase, mapped to the Kohara recombinant λ phage 4D10 (Woisetschlager et al., 1988).

Preparation and Purification of Recombinant KDO 8-P Synthase. (a) Cell Culture. Escherichia coli K-12 harboring pMW101 was grown overnight to stationary phase (OD_{600} = 6.0) in 5 mL of 2× TY media. The preparative media (2× TY) was inoculated with 100 μL of an overnight culture per 500 mL of media. The cultures were incubated at 37 °C with vigorous shaking (300 rpm) in 2-L baffled flasks (500 mL/flask) overnight (OD_{600} = 8.0).

(b) Preparation of Crude Extract. The following modifications of the reported isolation procedure (Ray, 1980) were used to purify the KDO 8-P synthase. Cells from the above culture were harvested by centrifugation (4500*g*, 15 min), and the total wet weight of the cells was measured. The cell pellets were suspended in 20 mM Tris-HCl (pH 7.4) buffer (1 g/5 mL). This step and all subsequent purification steps were performed at 4 °C, and all buffers contained 0.2 mM DTT. The cells were disrupted by 2 min of sonication, with cooling (4 × 30-s pulses with a 30-s delay between pulses), and centrifuged at 25000*g* for 20 min. The supernatant was removed, the pellet was resuspended in Tris-HCl buffer as described above, and the whole process was repeated.

(c) Acid Precipitation. Protamine sulfate (2.2%, pH 7) was added to the combined supernatants dropwise with magnetic stirring to bring the final protamine concentration to 0.26%. Stirring was continued for 15 min, and the suspension was subjected to centrifugation at 35000*g* for 30 min. The pH of the supernatant was slowly and carefully adjusted to pH 5.0 by the subsurface addition of 0.5 M acetic acid. The thick suspension was stirred slowly for an additional 30–45 min to ensure a stable pH. After centrifugation of the suspension at 20000*g* for 30 min, the supernatant was dialyzed overnight against 5 mM potassium phosphate buffer (pH 7.4) containing 75 mM KCl (3 × 1000 mL).

(d) Anion Exchange Chromatography. The chromatography was performed on a modified ISCO low-pressure system. The pH of dialyzed enzyme was adjusted to 7.4 with 1 M Tris base and filtered through a 0.22-μm sterile filter. The supernatant was divided into three portions. One-third of the supernatant was applied to an anion exchange column (Bio-Rad 1.5 × 70 cm Econo column packed with Q Sepharose) equilibrated with 20 mM Tris-HCl (pH 7.4) buffer containing 75 mM KCl. The column was first washed with 200 mL of equilibration buffer and then eluted with 1 L of a linear gradient of 100–400 mM KCl in 20 mM Tris-HCl (pH 7.4) buffer. The flow rate was kept at 1 mL/min. Fractions (14 mL) containing KDO 8-P synthase, determined by the thiobarbiturate assay (Ray, 1980) and SDS–PAGE, were pooled and subjected to concentration via lyophilization. The concentrated enzyme solutions from three individual runs were combined and dialyzed overnight against 5 mM potassium phosphate buffer (pH 7.2; 3 × 1000 mL).

(e) Hydroxyapatite Chromatography. The dialyzed enzyme solution from the Q Sepharose column was applied to a hydroxyapatite column (Bio-Rad 2.5 × 20 cm Econo column packed with HA-Ultrogel) equilibrated with 5 mM potassium phosphate buffer (pH 7.2). The column was washed initially with 200 mL of equilibration buffer and then eluted with 1 L of a linear gradient of 10–200 mM potassium phosphate buffer (pH 7.2). The flow rate was kept at 1 mL/min. Fractions (5 mL) containing KDO 8-P synthase, determined by the thiobarbiturate assay and SDS–PAGE, were pooled and subjected to concentration via lyophilization.

Assay Procedures. The production of KDO 8-P was monitored using the periodate–TBA assay reported for KDO 8-P synthase by Ray (1982). The assay mixture contained 0.1 M Tris–acetate (pH 7.5), 3 mM PEP, 3 mM A 5-P, and enzyme in a final volume of 150 μL. After incubation for 10 min at 37 °C, the reaction was terminated by the addition of 150 μL of 10% TCA and centrifuged to remove the protein. An aliquot of 100 μL was treated with 200 μL of 0.025 M $NaIO_4$ in 0.125 N H_2SO_4 for 10 min at room temperature. The oxidation reaction was quenched with 400 μL of 2% $NaAsO_2$ in 0.5 N HCl. When the yellow color disappeared, 2.0 mL of 0.36% thiobarbituric acid (pH 9.0) was added, and the solution was heated in a heat block at 110 °C for 10 min. Immediately after heating, 2.0 mL of DMSO was added to stabilize the pink chromophore. The solution was allowed to cool for 3–5 min, and the absorbance was read at 549 nm. The amount of KDO 8-P produced is determined from a standard curve generated using KDO synthesized by a modification of the Cornforth reaction (Cornforth et al., 1958) or from commercially available KDO (Ray, 1982). One unit of activity is defined as 1 μmol of KDO 8-P released per min at 37 °C.

Protein Assays. The protein concentrations of enzyme fractions were determined using the Bio-Rad protein assay (Bio-Rad, Richmond, CA) with bovine serum albumin as standard.

Polyacrylamide Gel Electrophoresis. All electrophoretic analyses were performed on a Bio-Rad Mini-Protean II. For denaturing gels, 5 μL of protein sample or protein standards (Bio-Rad, low molecular weight), mixed with 20 μL of denaturing gel loading buffer [62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.00125% bromophenol blue], were heated in a dry heat block (110 °C) for 5 min. Samples were subjected to electrophoresis on 12% denaturing gels (prepared according to the Bio-Rad protocol) using the following discontinuous Laemmli buffer: 5 g/L Tris base, 14.4 g/L glycine, and 1 g/L SDS (pH 8.3). Electrophoresis was performed at a constant 200 V following the Bio-Rad protocols (Bio-Rad, Richmond, CA). Gels were

stained using the Coomassie blue staining procedure described in the Bio-Rad manual (Bio-Rad, Richmond, CA).

Preparation of (Z)- and (E)-[3-^{2}H]Phosphoenolpyruvate. (*Z*)- and (*E*)-[3-^{2}H]phosphoenolpyruvate were prepared chemically via the reductive deuteration of (*Z*)- and (*E*)-3-bromophosphoenolpyruvate, respectively, as previously reported by this laboratory (Gore et al., 1990). In brief, ethyl (*Z*)- and (*E*)-3-bromoPEP were prepared via treatment of ethyl 3,3-dibromopyruvate with trimethyl phosphite under Perkow-type reaction conditions followed by separation into their separate geometric isomers utilizing a medium-pressure silica gel chromatograph. Reductive deuteration was achieved using a zinc/silver couple doped with ^2H_2O. Deprotection of the acid functions first, by treatment with trimethylsilyl bromide, followed by KOH and neutralization on a Dowex-50Wx8 cation exchange column led to the title compounds.

Enzymatic Conversion of Unlabeled, (Z)-, and (E)-[3-^{2}H]Phosphoenolpyruvates to Their Corresponding 3-Deoxyoctulosonate 8-Phosphates. KDO 8-P synthase (4 units) was added to a 50-mL Erlenmeyer flask containing the following: phosphoenolpyruvate [PEP, (*E*)- or (*Z*)-[3-^{2}H]-PEP] monocyclohexylamine salt (0.012 g, 0.045 mmol), A 5-P disodium salt (0.012 g, 0.045 mmol), Tris–acetate (pH 7.5, 1.5 mmol), and H_2O (final volume, 10 mL). This reaction mixture was incubated at 37 °C for 2 h in a shaking water bath. The progress of the enzymatic reaction was monitored by removing 100-μL aliquots every 15 min and analyzing for the KDO 8-P present as described in the Assay Procedures section. The reaction mixture, unquenched, was loaded directly onto a 1.5 × 27 cm anion exchange column (Bio-Rad AG-MP1, Cl^- form), washed with 100 mL of H_2O, and eluted with a linear gradient of 500 mL of 0–400 mM KCl. Fractions of 8 mL were collected, and 100 μL of each fraction was assayed for KDO 8-P by oxidation with $NaIO_4$ followed by condensation with TBA, as described in the Assay Procedures section. Two periodate–TBA-positive peaks were obtained, the first KDO and the second KDO 8-P. The fractions (8 mL) containing the KDO 8-P were pooled, freeze-dried, reconstituted in 5 mL of H_2O, and desalted on a 2 × 60 cm Bio-Gel P2 column. The periodate–TBA-positive, AgCl-negative fractions (8 mL) were pooled and freeze-dried to give the desired KDO 8-Ps.

^{1}H NMR Analysis of Unlabeled, (Z)-, and (E)-[3-^{2}H]-Phosphoenolpyruvates and Their Corresponding Enzymatically Derived 3-Deoxyoctulosonate 8-Phosphates. Nuclear magnetic resonance spectra were recorded on a Bruker AMX500 spectrometer (11.75 T) at a probe temperature of 298 K using 5-mm high-resolution NMR tubes. Sample volumes were 500 μL at a concentration of 10 mg/mL in deuterium oxide (100 atom %) (pH 7). Spectra were obtained with a 1.3-s relaxation delay and 16 384 complex points in the time domain using simultaneous detection of real and imaginary components. The phosphorus-decoupled ^{1}H spectra were obtained using a WALTZ 16-pulse sequence for phosphorus decoupling with the X-transmitter output centered on the ^{31}P spectrum and the output power attenuated by 31 dB for an effective continuous wave output power level of ca. 0.75 W (Shaka et al., 1983). Sodium 3-(trimethylsilyl)-propionate-*2,2,3,3-d*$_4$ (final concentration 1 mM) was added for referencing. The 90° proton pulse was 8.3 μs, the acquisition time was 1.622 s, and the spectral width was 5050.5 Hz. For 1D proton spectra, the number of transients was 4 for the ^{31}P-decoupled spectra and 64 for all others. [COSY spectra (not shown) were acquired into 512 t_1 experiments of 64 scans and 1024 complex points each. The 2D spectra were processed with zero-filling to 1K and sine-bell multiplied in each direction.]

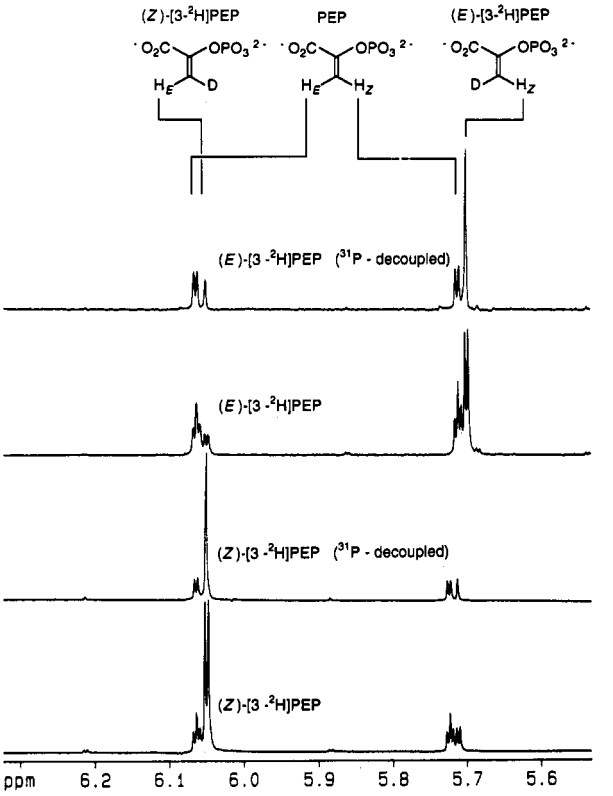

FIGURE 2: 500-MHz ^{1}H NMR spectra and phosphorus-decoupled ^{1}H NMR spectra of synthetic (*E*)- and (*Z*)-[^{2}H]PEP.

RESULTS

The study of the stereochemical fate of the C-3 vinylic carbon of PEP during the enzymatic aldol-type condensation with A 5-P to give the C-3 of KDO 8-P was divided into four segments: (a) synthesis of (*E*)- and (*Z*)-[3-^{2}H]PEP; (b) isolation and purification of sufficient quantities of KDO 8-P synthase; (c) enzymatic conversion of both (*E*)-[3-^{2}H]PEP with A 5-P and (*Z*)-[3-^{2}H]PEP with A 5-P into their respective labeled KDO 8-P's; and (d) isolation and purification followed by ^{1}H NMR analysis of the labeled KDO 8-P's.

Synthesis of (E)- and (Z)-[3-^{2}H]PEP. The study of the stereochemistry of the unusual aldol-type condensation catalyzed by KDO 8-P synthase required the synthesis of both (*Z*)- and (*E*)-[3-^{2}H]phosphoenolpyruvate. This entailed the preparation of the two corresponding (*Z*)- and (*E*)-bromophosphoenolpyruvates, previously described (Gore et al., 1990), from commercially available ethyl bromopyruvate. The bromo analogues were individually reduced, utilizing a zinc/silver couple doped with ^2H_2O followed by deprotection, to give the desired (*Z*)- and (*E*)-[3-^{2}H]PEPs. The ^{1}H NMR and ^{31}P-decoupled ^{1}H NMR spectra of (*Z*)- and (*E*)-[3-^{2}H]-PEP are shown in Figure 2. The desired labeled compounds should each contain a single deuterium at the C-3 position; therefore, the remaining vinyl proton signal should appear as a doublet shifted upfield by 8–19 parts per billion (ppb) due to the isotopic nuclear shielding properties of the C-3 deuterium (Hansen, 1988). The ^{1}H NMR spectra of the two labeled PEPs confirm that each compound, however, contains, in addition to the major monodeuterated isomer, a small quantity of the opposite geometric isomer as well as some [3,3-^{1}H$_2$]-PEP (diprotio species). In the ^{1}H NMR spectra of both (*Z*)- and (*E*)-[3-^{2}H]PEP, the diprotio species appears as a pair of

pseudotriplets[2] resonating slightly downfield of the deuterium-shifted doublets of the major and minor isotopically labeled [3-^{2}H]PEPs. In the ^{31}P decoupled ^{1}H NMR spectra, the former doublets due to the monodeuterated PEPs now appear as singlets upfield of the doublets due to the remaining diprotio PEP. The integrals of the ^{1}H NMR spectra of (*E*)-[3-^{2}H]-PEP reveal 18% unlabeled PEP and 17% (*Z*)-[3-^{2}H]PEP, while the (*Z*)-[3-^{2}H]PEP contains 18% unlabeled PEP and 13% (*E*)-[3-^{2}H]PEP.

Isolation and Purification of KDO 8-P Synthase. The specific activity of the crude extract obtained from the *E. coli* K-12 containing the construct pMW101 was 0.1 unit/mg versus the wild-type at 0.017 unit/mg. KDO 8-P synthase that was >90% pure as judged by polyacrylamide gel electrophoresis and had a specific activity of 4 units/mg after the hydroxyapatite chromatography step was utilized for the present studies.

Production and Purification of Labeled KDO 8-P. Incubation of PEP with A 5-P, (*Z*)-[3-^{2}H]PEP with A 5-P, and (*E*)-[3-^{2}H]PEP with A 5-P each in the presence of KDO 8-P synthase yielded sufficient quantities of the various KDO 8-P's for NMR analysis. The KDO 8-P from each of the above reaction mixtures was separated by anion exchange chromatography. The freeze-dried fractions containing KDO 8-P were desalted by gel filtration.

^{1}H NMR Analysis of the Labeled and Unlabeled KDO 8-P's. In the ^{1}H NMR spectra of the two enzymatic products obtained from (*Z*)- and (*E*)-[3-^{2}H]PEP, one would like to observe the disappearance of the C-3 ^{1}H resonance of KDO 8-P corresponding to the deuterated position and the collapse of the remaining C-3 ^{1}H resonance to a doublet resulting from the disappearance of the geminal coupling. The coupling to the C-4 ^{1}H will remain, and the magnitude of the coupling constant would be diagnostic of the geometric relationship between the remaining C-3 ^{1}H and the C-4 ^{1}H. Unfortunately, in aqueous solution, KDO 8-P, like KDO, exists in an equilibrium of four conformers, the α- and β-anomers of both the pyranose and furanose ring forms. The isomer equilibrium distribution for KDO 8-P has been reported to be 65.8% α-pyranose, 3.1% β-pyranose, 12.0% α-furanose, and 19.1% β-furanose (McNicholas et al., 1986; Baasov & Jakob, 1990) and that for KDO as 60% α-pyranose, 10% β-pyranose, 18% α-furanose, and 12% β-furanose (Cherniak et al., 1979; Brade et al., 1984). On the basis of previous literature assignments and 2D NMR studies from our laboratory (COSY, not shown), the resonances of the C-3 ^{1}H's of all four conformers of KDO 8-P appear between 1.5 and 2.5 ppm (see Figure 3). Since the C-3 ^{1}H resonances of the β-furanose isomer do not overlap with other resonances and can be seen as two distinct doublets of doublets [H_{3S}: δ 1.90 (dd, J_{HCH} = 14.5 Hz, J_{HCCH} = 3.0 Hz); H_{3R}: δ 2.42 (dd, J_{HCH} = 14.5 Hz, J_{HCCH} = 7.5 Hz] in the nonisotopically labeled enzymatic product (Figure 3a), it was chosen as the major point for analysis.

Since the (*Z*)- and (*E*)-[3-^{2}H]PEPs each contained some [3,3-^{1}H$_2$]PEP (18%) and either (*E*)-[3-^{2}H]PEP (13%) or (*Z*)-[3-^{2}H]PEP (17%), respectively, one would expect, assuming that the KDO 8-P synthase catalyzed condensation is stereospecific, that the product KDO 8-P's would contain the three corresponding species of the sugar phosphate, namely, a [3,3-^{1}H$_2$]KDO 8-P, the major chiral [^{2}H]KDO 8-P, and either a small amount of contaminating (3*S*)-[^{2}H]- or (3*R*)-[^{2}H]KDO 8-P. The final complication in the interpretation of the ^{1}H NMR spectra of the enzymatically derived KDO 8-P's, as was observed in the ^{1}H NMR spectra with the deuterated PEP analogues used as substrates in this study, is that the 3*R* and 3*S* ^{1}H signals of 3*S*- and 3*R*-deuterated KDO 8-P, respectively, will be isotopically shifted upfield due to geminal deuterium ^{1}H nuclear shielding. The ^{1}H nuclear shieldings observed for the geminal sp^3 deuterium effect are usually 8–19 ppb for the monodeuterated compounds (Hansen, 1988).

Figure 4b depicts the ^{1}H NMR spectrum of the product isolated from the enzymatic reaction utilizing (*Z*)-[3-^{2}H]PEP as a substrate. Using the β-furanose resonances as the focal point of analysis, it can be seen that the resonance originally centered at δ 2.42 (H_{3R}) in Figure 4a has both collapsed to

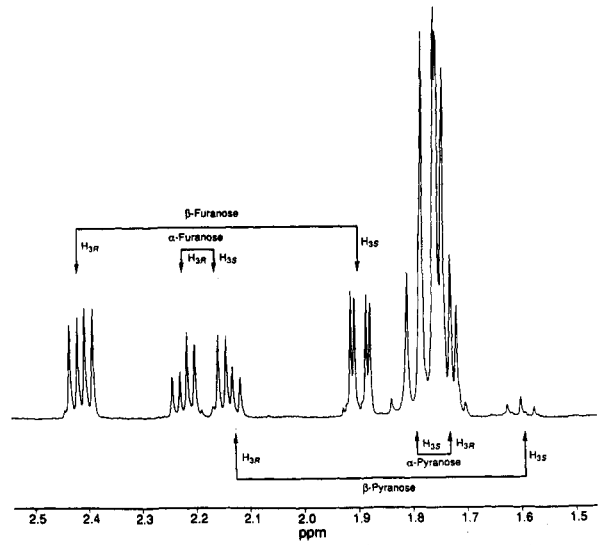

FIGURE 3: C-3 proton assignments of the 500-MHz ^{1}H NMR spectrum of the α- and β-isomers of the furanose and pyranose forms of KDO 8-P.

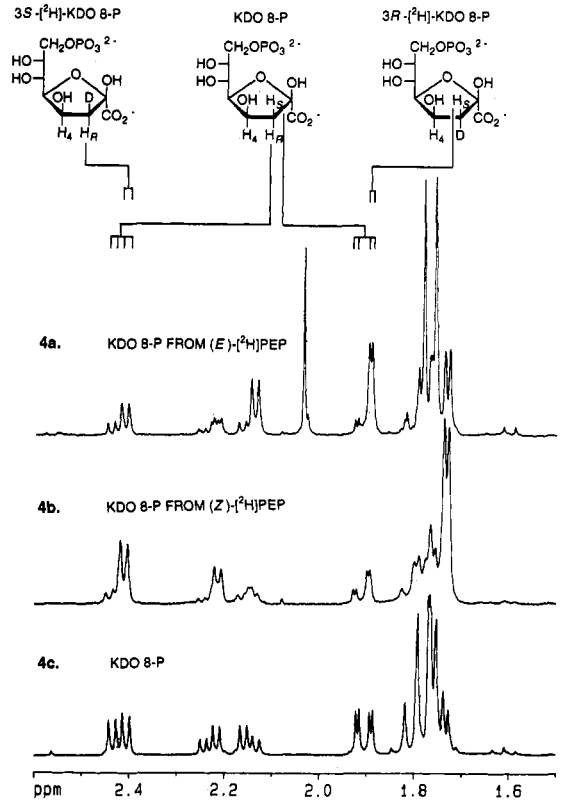

FIGURE 4: 500-MHz ^{1}H NMR spectra of enzyme-derived KDO 8-P's from PEP and (*E*)- and (*Z*)-[^{2}H]PEPs.

[2] Doublet of doublets with ^{1}H–^{1}H vinylic coupling (J = 2.16 Hz) and ^{31}P–^{1}H coupling (J_{cis} = 1.71 Hz and J_{trans} = 2.08 Hz).

a doublet (J = 7.5 Hz) and been shifted upfield by 15 ppb, while the resonance originally centered at δ 1.90 (H_{3S}) in 4a has also collapsed to a doublet shifted upfield by 15 ppb; however, the intensity is diminished in comparison to H_{3R}. Portions of the nonisotopically labeled KDO 8-P resonances can be seen slightly downfield of each of these signals, with the upfield portion of the doublet of doublets masked by the deuterium-shifted resonance of the isotopically labeled diastereomers. These results indicate that it is the H_{3S} position that is deuterated. The ^{1}H NMR spectrum of the isolate from the enzymatic reaction utilizing (*E*)-[3-^{2}H]PEP as a substrate is shown in Figure 4c. In this case, it is the signal originally centered at δ 2.42 that is diminished while the signal originally centered at δ 1.90 (J = 3.0 Hz) is major, indicative of deuteration of the H_{3R} position. Integration of the peaks due to the β-furanose anomer in spectrum 4b, after compensation for the amount of diprotio KDO 8-P present,[3] reveals that the (3*S*)-[^{2}H]-KDO 8-P makes up 79% of the deuterated sugar phosphates, which represents 66.4% of the total KDO 8-P formed in the enzymatic reaction. In spectrum 4c, integration of the same peaks reveals that the (3*R*)-[^{2}H]KDO 8-P makes up 81% of the labeled KDO 8-P's representing 64.7% of the total KDO 8-P formed in the enzymatic reaction. Each isotopic sugar phosphate mixture contains 18% nondeuterated KDO 8-P. These values represent only a 2–8% loss in stereospecificity in the KDO 8-P synthase reaction with no apparent loss of deuterium! The C-3 protons of the isolated deuterium-labeled or the unlabeled KDO 8-P's showed no solvent exchange with ^2H_2O after storage at –8 °C for 6 months, as evidenced by integration of the ^{1}H NMR spectra of the samples.

These results are further confirmed by examining the ^{1}H NMR spectrum of the α-pyranose resonance centered at δ 1.77 (Figure 4a), which has collapsed to a major doublet in both Figure 4b,c. The signal in Figure 4b is centered at δ 1.75 (J = 5.0 Hz), while that in Figure 4c is centered at δ 1.78 (J = 12.0 Hz). It has been previously demonstrated (Baasov & Jakob, 1990) that the largest vicinal coupling constant in the α-pyranose is the trans-diaxial coupling between the proton on C-4 and the H_{3S}. Since the larger coupling constant is not seen with the major doublet in Figure 4b, H_{3S} must be the deuterated position, while in Figure 4c the larger coupling constant is observed with the major doublet, indicating that H_{3R} carries the deuterium label. These results parallel those of the β-furanose.

DISCUSSION

During the reaction catalyzed by KDO 8-P synthase, the C-1 (carbonyl carbon) of A 5-P is bonded to the C-3 (vinyl carbon) of PEP. In the product KDO 8-P, the C-1 carbonyl carbon of A 5-P has become the chiral C-4 of KDO 8-P and possesses the *R* configuration. This stereochemistry at the C-4 of KDO 8-P indicates that the attack by PEP has occurred upon the *re* face of the carbonyl carbon of A 5-P. The facial designation of the C-3 of PEP is described by that of the neighboring C-2 face (Hanson, 1966). Therefore, the sp^2 C-3 of PEP also has both a *re* and a *si* face, either of which could condense with A 5-P to become the C-3 methylene carbon of KDO 8-P, depending on the binding orientation of the PEP in the active site. If the enzymatic aldol-type condensation

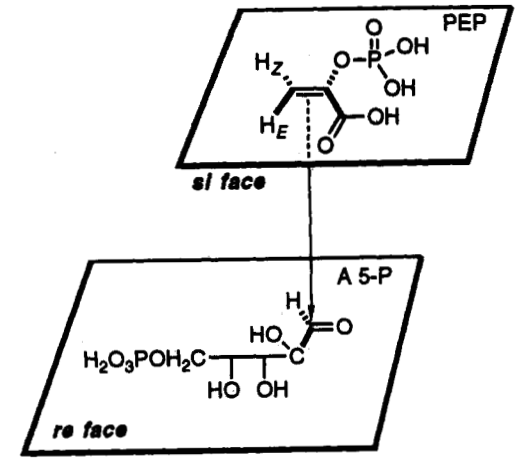

FIGURE 5: Steric course of the KDO 8-P synthase reaction.

reaction, catalyzed by KDO 8-P synthase, occurs directly, i.e., the mechanism of the reaction involves an attack of a nucleophile (either solvent, the C-2 or C-3 hydroxyl group of A 5-P, or enzymatic) at the C-2 of PEP with the concomitant formation of a "transient" carbanion-type intermediate without the formation of a freely rotating methyl group which then attacks the *re* face of the C-1 of A 5-P followed by cleavage of the C-2 oxygen bond, then one would predict that one of the two vinylic hydrogen atoms of the C-3 of PEP will become one of the diastereotopic hydrogens of the C-3 of KDO 8-P and the other vinylic hydrogen will become the other diastereotopic hydrogen of KDO 8-P. Specifically, if the enzymatic condensation proceeds via attack of the *si* face of PEP upon the *re* face of A 5-P, then we would expect the H_E and H_Z of PEP to become the H_{3R} and H_{3S} of KDO-8P, respectively. If, however, the *re* face of PEP condensed upon the *re* face of A 5-P, then the opposite diastereotopic pairing would result. Our ability to distinguish between and assign which of the diastereomeric protons is which via ^{1}H NMR has allowed us to determine the overall facial stereochemical orientation of the enzymatic condensation reaction with respect to the C-3 of PEP and C-1 of A 5-P.

The KDO 8-P synthase reaction is stereospecific with respect to the facial orientation of the C-3 of PEP and C-1 of A 5-P (see Figure 5). The ^{1}H NMR spectrum of the KDO 8-P synthesized from (*Z*)-[3-^{2}H]PEP shows predominantly [3-^{2}H]-KDO 8-P of the 3*S* configuration, while that synthesized from (*E*)-[3-^{2}H]PEP shows predominantly [3-^{2}H]KDO 8-P of the 3*R* configuration. This indicates condensation of the *si* face of PEP with the *re* face of A 5-P, an orientation analogous to that seen for the similar aldehyde lyase, DAH 7-P synthase (Floss et al., 1972). DAH 7-P synthase, which catalyzes the condensation of PEP and E 4-P, is one of the key enzymes in the aromatic amino acid biosynthetic pathway. As pointed out by Floss and co-workers (1972), the attack of the *si* face of PEP in DAH 7-P synthase is common to a number of other enzymes that utilize PEP, such as phosphopyruvate carboxylases (from four sources) and pyruvate kinase, which also catalyzes the addition of an electrophile (H^+) to the *si* face of the C-3 of PEP. A notable exception to this stereochemical trend is enzyme I of the *E. coli* phosphoenolpyruvate-dependent phosphotransferase system, which protonates (*Z*)-phospho-[^{2}H]enolbutyrate from the 2*re*,3*si* face (this is the equivalent of *re* face protonation of PEP) (Hoving et al., 1983). When taken together, the results presented here and those previously observed strongly suggest a common PEP binding motif. However, there appears to be no primary protein sequence homology between KDO 8-P synthase and any of the three

[3] The amount of diprotio KDO 8-P present in the isotopically labeled samples was determined by integration of the visible downfield portion of the diprotio's doublet of doublets at δ 1.92 and 2.43. These values were subtracted from the integration of the isotopically shifted doublets of the deuterated species.

E. coli DAH 7-P synthase isoenzymes. This seems unusual since both KDO 8-P synthase and DAH 7-P synthase catalyze a similar condensation between PEP and homologous phosphorylated D-sugars, A 5-P versus E 4-P, which differ by only one HCOH unit, and past and present stereochemical studies that imply that both enzymes must bind their respective substrates in similar geometries at their active sites. Neither enzyme, however, will accept the sugar phosphate substrate of the other as either substrate or inhibitor.

Mechanistically, the results of this study strongly suggest the absence of a transient methyl group at the C-3 of phosphoenolpyruvate as part of the reaction mechanism, since the same proton used to protonate the C-3 of PEP before, during, or after nucleophilic attack at the C-2 of PEP would have to be the one abstracted. Protonation/deprotonation of the same proton would imply that either the half-life of the intermediate methyl group would have to be extremely short or that once formed, the methyl group would be restricted from rotation by a group(s) at the active site of KDO 8-P synthase. Both of these possibilities seem rather unlikely.

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