3-Deoxy-d-manno-octulosonate 8-phosphate synthase (KDO8P) synthase catalyzes the condensation of phosphoenolpyruvate (PEP) with arabinose 5-phosphate (A5P) to form KDO8P and inorganic phosphate. KDO8P is the phosphorylated precursor of 3-deoxy-D-octulosonate 8-phosphate (KDO8P synthase, EC 4.1.2.16) plays a key role in the biosynthesis of KDO. This enzyme catalyzes the aldol-type condensation of phosphoenolpyruvate (PEP) with arabinose 5-phosphate (A5P) to form KDO8P (precursor to KDO) and inorganic phosphate (Fig. 1) (3). Dephosphorylation of KDO8P to KDO and synthase of CMP-KDO (from CTP and KDO) occur prior to insertion of the sugar into LPS (2). Strains of Salmonella have been isolated with mutations in KDO8P synthase that confer temperature-sensitive growth (4, 5). Such strains fail to synthesize KDO at the nonpermissive temperature, which leads to the inhibition of LPS biosynthesis and, as a consequence, to the arrest of cell growth. These studies indicate that KDO8P synthase provides an essential function for bacterial homeostasis.

Earlier studies have determined that the reaction of KDO8P synthase is a sequential process in which the binding of PEP precedes the binding of A5P and the release of inorganic phosphate precedes the release of KDO8P (6). The condensation step of the reaction is stereospecific, involving the addition of the si face of C9PEP to the re face of the A5P carbonyl (7, 8). It has also been established that phosphate release occurs by cleavage of the C-O bond of PEP (9, 10) and that the anomeric oxygen of the product KDO8P originates from bulk solvent (10).

However, the mechanistic details of the condensation reaction remain unclear and very little is known about the residues of the enzyme that are involved in catalysis.

As an aid to understanding the mechanism of KDO8P synthase, we have determined the crystal structure of the Escherichia coli enzyme at 2.4 Å resolution. The structure provides a wealth of information on the organization of the active site and on the interactions between the enzyme subunits. A reaction very similar to KDO8P synthesis is the formation of 3-deoxy-d-arabino-heptulosonate 7-phosphate (DAH7P) catalyzed by DAH7P synthase (11). This reaction is the first step of the shikimate pathway for the biosynthesis of aromatic amino acids. Analysis of the structural and evolutionary relationships between KDO8P synthase and DAH7P synthase provides additional insight into the mechanism of both enzymes and strongly suggests that the syntheses of both KDO8P and DAH7P proceed through the formation of a linear rather than a cyclic intermediate.

**EXPERIMENTAL PROCEDURES**

**Protein Purification and Crystallization**—The E. coli KDO8P synthase (KDO8PS) is encoded by the kdsA gene (12). The translated
monomeric product contains 284 amino acids with a calculated Mr of 30,833. KDO8P synthase was purified from E. coli BL21(DE3) harboring the kdsA gene in the plasmid pT7-7 by a modification of the procedure described by Dotson et al. (10). Ten ml from a small scale growth of the overexpressing strain were transferred to two flasks each containing 1 liter of Luria-Bertani medium supplemented with 100 μg ml⁻¹ ampicillin, and the culture was maintained under shaking at 37 °C until mid-log phase. At this point, expression of KDO8P synthase was induced with 60 μM isopropyl-β-D-thiogalactopyranoside, and the cells were grown for 4 h. Cells were harvested by centrifugation, washed twice with 25 mM potassium phosphate buffer (pH 7.4), and lysed with two passes through a French press. The lysate was centrifuged at 100,000 x g for 1 h, passed through a 0.45-μm filter, and loaded directly onto a DEAE-Sepharose column (200 ml bed volume) equilibrated with 25 mM potassium phosphate, pH 7.4. The column was first washed with two column volumes of 25 mM potassium phosphate and then eluted with a two volume gradient from 25 to 130 mM potassium phosphate. Fractions containing active KDO8P synthase were pooled, and solid ammonium sulfate was added to a final concentration of 0.5 M. The protein solution was then applied to a phenyl-Sepharose column (100-ml bed volume) equilibrated with 25 mM potassium phosphate, 0.5 M ammonium sulfate and eluted with a gradient in one column volume from 25 mM potassium phosphate, 0.5 M ammonium sulfate to 25 mM potassium phosphate. Fractions containing the active enzyme were pooled and concentrated by pressure filtration. The concentrated enzyme (80 mg ml⁻¹) in 25 mM potassium phosphate was flash frozen and stored in liquid nitrogen in small aliquots until crystallization. Crystallization was performed by vapor diffusion in hanging drops. Cubic crystals (space group I23, a = 228.6 Å) were obtained from drops containing 400 mM potassium phosphate, pH 7.5, 1.4 M ammonium sulfate, 6% ethylene glycol, 4 mM PEP, 4 mM ATP, and 30 mg ml⁻¹ KDO8PS, equilibrated at 23 °C against 400 mM potassium phosphate, 6% ethylene glycol, and 1.7–1.9 M ammonium sulfate. Crystals were harvested and maintained in a cryo-protectant holding solution containing 400 mM potassium phosphate, pH 7.5, 2.4 M ammonium sulfate, 6% ethylene glycol, 10% glycerol.

Assays—KDO8PS activity was determined by measuring the amount of KDO8P produced using the periodate-thiobarbituric acid assay (13). Protein concentration was determined by the method of Lowry (14).

Structure Determination and Refinement—A native data set was collected at 100 K with a Raxis IV image plate detector at the CuKα wavelength. The structure determination of the enzyme employed the anomalousdiffraction at multiple wavelengths (MAD) of a mercury derivative (Table 1) obtained by soaking crystals for 24 h in a holding solution containing 9 mM mercurichrome (2',7'-dibromo-4'-[hydroxymercurio]-fluorescein) and sodium sulfate in place of ammonium sulfate. Anomalous diffraction at the absorption edge of mercury and at a remote wavelength were collected at beamline X12C, Brookhaven National Laboratories, with a charge-coupled device detector. Oscillation data were processed with HKL (15). Three heavy atom sites were identified with SOLVE (16), which was also used for MAD phasing in combination with SHARP (17). Density modification and 2-fold noncrystallographic symmetry averaging with DM (18) and SOLOMON (18) gave an interpretable map at 3.2 Å. Phases were improved and extended stepwise (0.15 Å increments) to 2.4 Å (resolution of the native data set). The model for subunits A and B was fit into the density using O (19). Subunits C and D were generated by application of the local symmetry. Model refinement was carried out with CNS version 0.5 (20) using cross-validated maximum likelihood as the target function (21). Solvent molecules were added only during the final stages of refinement after the protein model had stabilized. Residues 206–217 (L7) and 245–252 (L8) of subunits A, B, and C, and residues 206–217 and 245–252 of subunit D could not be identified unambiguously and were not included in the final model. The global and local validity of the model were assessed with the programs SFCHECK (22) and DDQ (23).

Sequence and Structure Alignments—Multiple sequence alignments were performed with the program ClustalW (24). Structure superposition and structure-based sequence alignment were carried out using the program LSQMAN (25).

RESULTS

Overall Structure—The asymmetric unit of the crystal contains a homotetramer of KDO8P synthase (subunits ABCD) with 222 local symmetry (three 2-fold axes intersecting in the tetramer center) (Fig. 2); each monomer has the fold of a typical β/αβ barrel (eight-stranded parallel β-barrel surrounded by eight helices) as observed in the structure of triose-phosphate isomerase (TIM) (26). The secondary structure elements of the E. coli KDO8P synthase (284 residues) and TIM from various sources (247–250 residues) superimpose well (not shown) with the main differences occurring at the N terminus, where KDO8P synthase has an additional β-hairpin that seals the N-terminal end of the barrel, and at the C terminus, where helix H8 of KDO8P synthase has a random coil extension (Fig. 2). Despite the significant structural kinship between KDO8P synthase and TIM, neither a direct sequence comparison nor a structure based alignment reveals any sequence similarity between the two proteins.

Previous biochemical studies indicated that the E. coli KDO8P synthase is a trimer of identical subunits (13). However, in the cubic crystals of KDO8P synthase there are no intersubunit contacts along the directions of the crystallographic 3-fold axes that would support a trimeric architecture. A tetramer is also contained in the asymmetric unit of a different crystal form of KDO8P synthase belonging to a monoclinic space group. Together these observations suggest that the tetramer probably represents the native form of the enzyme. The four monomers of KDO8P synthase are very similar with root mean square deviations not exceeding 1.0 Å for all atoms and 0.5 Å for Ca atoms in all pairwise comparisons. The contact surface between subunits A and B (or C and D) is characterized by both polar and hydrophobic interactions occurring primarily between helices H6, H7, and H8 of one subunit and the same helices of the other subunit (Fig. 2). Two loops play a key role in completing the assembly of the tetramer: loop L2 (residues 58–72) of subunit A contacts helices H4 and H5 of subunit C and loop L6 (residues 170–182) of subunit A contacts the same loop of subunit C at the center of the tetramer. Also, the C-terminal coil extension of each subunit extends over helices H7 and H8 of the neighboring subunit (Fig. 2). Because of the 222 symmetry of the tetramer, the interactions described above (A to C) occur again between the same subunits in reverse (C to A) and between the other two subunits (B to D and D to B). An interesting feature of KDO8P synthase crystals is that the three-dimensional lattice is sparsely populated (70% solvent content) with only subunits A, B, and C involved in crystal contacts. This characteristic packing may be responsible for the fact that the temperature factors of subunit D are about 30% higher than those of the other three subunits of the enzyme.

Attempts were made to visualize the substrates PEP and A5P by incubating crystals of KDO8P synthase in the presence of a large excess (4 x 10⁻⁴-fold) of either of these compounds over their Kₘ values (KₘPEP = 6 μM; KₘA5P = 30 μM; Ref. 6). Unfortunately, neither PEP nor A5P were visible under these conditions, probably because of the high ionic strength of the holding solution (2.4 M ammonium sulfate). Attempts to transfer crystals of KDO8P synthase to a low ionic strength holding solution were not successful.

Although the active site of KDO8P synthase could not be identified directly, its location could be derived by comparison with the structures of other enzymes that adopt a TIM barrel topology. In these enzymes the active site is always located in...
X-ray Structure of KDO8P Synthase

TABLE I
Data collection, MAD phasing, and model refinement statistics

|                | Native | Mercurochrome |
|----------------|--------|---------------|
| Data collection|        |               |
| Wavelength (Å) | 1.5418 | 0.970002 (remote) | 1.007524 (edge) |
| Resolution range (Å) | 23–2.4 | 29–3.2 | 30–3.2 |
| Measurements | 1,582,716 | 1,279,795 | 1,261,629 |
| Unique reflections | 76,684 | 33,324 | 33,252 |
| Redundancy | 38.4 | 37.9 | 37.9 |
| Completeness (%) | 99.3 (94.6) | 99.8 (100.0) | 99.8 (99.9) |
| <I>/<I>-Rmerge | 22.3 (2.3) | 27.5 (6.8) | 26.1 (5.9) |
| Rmerge (%) | 8.6 (56.4) | 13.7 (55.8) | 14.5 (59.3) |
| Mean figure of merit | 0.52 (0.48) | 47.8 (53.7) | 83.8 (97.7) |
| Refinement statistics | | | |
| Rcryst (%) | 19.9 | | 1.54 (0.77) |
| Rfree (%) | 23.9 | | 2.26 (1.13) |
| Amino acids | 1062 | | 0.83 (0.43) |
| Water molecules | 446 | | |
| <B> (Å²) protein | 49.5 | | |
| <B> (Å²) waters | 49.9 | | |
| rmsd bond lengths (Å) | 0.012 | | |
| rmsd bond angles (deg) | 1.574 | | |
| rmsd impropers (deg) | 1.012 | | |
| ϕ/ψ angles (%) | 90.6 | | |
| Most favored | 90.6 | | |
| Allowed | 9.4 | | |

Values in parentheses refer to the highest resolution bin (0.15 Å wide).

Phasing power (dispersive) $= \frac{1}{\langle I \rangle} - \langle I \rangle$ for <I>-Rmerge, where $\langle I \rangle$ is the phase-integrated lack of closure and $F_{\text{calc}}$ are the structure factors at the remote and edge wavelengths, respectively. $R_{\text{merge}}$ is the ratio between the anomalous lack of closure and the anomalous difference.

Phasing power = $<F_H/E>$, where $F_H$ is the calculated heavy atom structure factor. $R_{\text{cryst}} = \sum |F_{\text{obs}} - F_{\text{calc}}| / \sum |F_{\text{obs}}|$. $R_{\text{free}}$ was calculated on 10% of the data omitted from refinement. Stereochemistry was assessed with PROCHECK (18).

A cavity at the C-terminal end of the barrel (27). A similar motif is also observed in KDO8P synthase, where an elongated depression at the interface between subunits opens into a deeper cavity that is likely to represent the active site. Two cavities are visible on each face of the tetramer, one between subunit A and C and one between subunit B and D (Figs. 2 and 3A). Most of the residues of the putative active site are contributed by the C-terminal end of the barrel and by the loops of one subunit (Fig. 3A, yellow shading) with a small contribution originating from helices H4 and H5, and loops L4 and L5 of another subunit (Fig. 3C, cyan shading).

Anion Binding Sites—A sulfate ion (SO4–1), stabilized by salt bridges to the side chains of Lys-138 and Arg-168 and by a hydrogen bond to the backbone amide of Ala-116, is located in the most recessed part of the active site cavity (Fig. 3B). The pattern of noncovalent interactions around this ion is very similar to that observed in the structure of yeast enolase from which the crystals are maintained, it is possible that a sulfate ion also substitutes for PEP in the active site of KDO8P synthase. Additional evidence that the position of SO4–1 represents the binding site of the phosphate moiety of PEP derives from the observation that PEP, but not A5P, protects His-202 (wherein in 4.7 Å from one of the oxygen atoms of SO4–1, Fig. 3B) from chemical modification by diethylpyrocarbonate (29).

A second sulfate ion (SO4–2) is located approximately 13 Å from SO4–1, in a raised position above the opening of the barrel and beneath the very long L2 loop (residues 58–72) (Fig. 3B). This sulfate ion is stabilized by a salt bridge to the side chain of Arg-63 and by hydrogen bonds to both the side chain and the backbone of Ser-64. The distance between the aldehyde carbon (C1) and the phosphate moiety of A5P is approximately 7.5 Å, and the distance between C3 and the phosphate group of PEP is approximately 3.5 Å. The sum of these distances (11 Å) matches closely the distance of 13 Å between SO4–1 and SO4–2, which suggests that PEP and A5P may bind with their phosphate moieties occupying the sites of these sulfate ions and with C3PEP in close proximity of C1A5P. The proposed positions of PEP and A5P, with PEP located more deeply in the active site and A5P closer to the opening, are consistent with the observation that the synthesis of KDO8P is an ordered reaction in which the binding of PEP precedes the binding of A5P (6).

In subunit B and C only, a third sulfate ion (SO4–3) is bound near the outer rim of the active site cavity, in a region that includes contributions from L1 and H1 (Figs. 2 and 3). This sulfate ion is stabilized by a salt bridge to Arg-31 and possibly by the positive charge of the N-terminal end of helix H1. The fact that only two of four potential SO4–3 sites are occupied, despite equal accessibility of Arg-31 in all four subunits of the enzyme, suggests that the initial binding of sulfate to one face of KDO8P synthase (subunits B and C in Figs. 2 and 3A) introduces an asymmetry in the tetramer that may affect the binding of sulfate to the other face. The physiological function of the SO4–3 site remains uncertain. However, it is of interest that inorganic phosphate is a noncompetitive inhibitor of the E. coli KDO8P synthase (6). Thus, the SO4–3 site identified in the x-ray structure of the enzyme may correspond to the inhibitory phosphate binding site predicted from kinetic measurements. In this context, it is of note that the binding of sulfate at the SO4–3 site of subunit B and C coincides with several small structural changes in the side chains of Asp-32, Arg-36, Glu-245, and Pro-252, which appear to propagate from Arg-31 in the direction of the active site cavity.

Homology between KDO8P Synthase and DAH7P Synthase—A reaction similar to KDO8P synthesis constitutes the first step in the metabolic pathway for the biosynthesis of...
KDO8P synthase (data not shown) nor is any metal observed in the x-ray structure of the enzyme. These differences might be interpreted as representative of the fact that, although the two enzymes catalyze very similar reactions, they are not evolutionarily related. Surprisingly, the recently reported crystal structure of the E. coli phenylalanine-regulated DAH7P synthase (31) shows that both the tertiary and quaternary structures of this enzyme are remarkably similar to those of KDO8P synthase. Like KDO8P synthase, DAH7P synthase is a homotetramer with 222 symmetry. A superposition of the monomers of the E. coli KDO8P and DAH7P synthases is shown in Fig. 5. The core structure, represented by the elliptical (βα)8 barrel (colored in yellow for KDO8PS and blue for DAH7PS), is almost identical in the two enzymes. However, several important structural features are unique to each protein. The N terminus of DAH7P synthase contains an extension of 50 amino acids that includes one short β-strand and two helices (colored in cyan in Fig. 5). The second of the two helices seals the N-terminal end of the barrel and thus occupies a position corresponding to that of the N-terminal β-hairpin in KDO8P synthase (colored in orange in Fig. 5, see also Fig. 2). The L2 loop, which in KDO8PS is involved in the binding of SO4−2, is substantially longer and convoluted in DAH7PS (colored in green in Fig. 5). In DAH7P synthase the connection between helix H5 and strand S6 is provided by a β-hairpin (strands S6a and S6b, colored in pink in Fig. 5) that extends outside of the barrel. This hairpin forms a three-stranded antiparallel β-sheet with the short N-terminal strand of a neighboring monomer and thus is important for the assembly of the DAH7PS tetramer. Finally, the L8 loop (colored in magenta in Fig. 5) is longer in DAH7PS than in KDO8PS; however, in both enzymes this loop is partially disordered.

A comparison of the binding interactions of ions and substrates in the active sites of KDO8P and DAH7P synthases provides insight into the mechanism and evolution of these enzymes. KDO8PS and DAH7PS adhere almost perfectly to the canonical characteristics of enzymes with a TIM barrel topology (27). Their active site is located at the C-terminal end of the barrel (Figs. 2 and 3), and important functional groups are contributed almost every βα unit of the barrel (Fig. 6). In the active site of DAH7P synthase, PEP is bound with its phosphate moiety occupying a position analogous to that of SO4−1 in KDO8P synthase, whereas a sulfate ion occupies a position similar to that of SO4−2 in KDO8P synthase (Fig. 6). The distance between the sulfate ion and the phosphate moiety of PEP (11 Å) in DAH7PS is, however, less than the distance between SO4−1 and SO4−2 (13 Å) in KDO8PS. The amino acid side chains involved in the coordination of PEP and of the sulfate ion have counterparts in KDO8P synthase; in general it is found that equivalent residues of the two enzymes occupy almost identical positions in the two structures (Table II, Fig. 6). Lys-55, Lys-60, and Lys-138 in KDO8P synthase and Arg-92, Lys-97, and Lys-186 in DAH7P synthase are expected to stabilize the α-carboxylate of PEP in its ionized form via ion pairing. Ala-116 (backbone amide), Arg-168, and Lys-138 in KDO8P synthase and Ala-164 (backbone amide), Arg-234, and Lys-186 in DAH7P synthase bind the phosphate moiety of PEP. Interestingly, because the sulfate ion of DAH7P synthase is shifted by ~2 Å with respect to the SO4−2 of KDO8P synthase, the L2 loop of DAH7P synthase is stretched to accommodate the different position of the sulfate ion. As a consequence, the equivalent residues of the loop, Arg-99 and Thr-100 in DAH7P synthase and Arg-63 and Ser-64 in KDO8P synthase, do not occupy identical positions in the active sites of the two enzymes (Fig. 6).

A lead ion (Pb2+) is bound in the active site of DAH7P...
**Fig. 3.** The active site of KDO8P synthase. **A**, stereo image of the molecular surface of the tetramer viewed from the same angle as in Fig. 2. Two of the four active sites are visible from this side of the enzyme. A central elongated depression, located at the interface between subunits, gives access to the active site cavities. In this view of the enzyme the larger contribution to the active site surface originates from subunits B and C (yellow). The smaller contribution originating from subunits A and D is colored in cyan. The SO4–3 site is colored in blue. Sulfate ions are represented as ball-and-stick models and colored according to atom type (sulfur, white; oxygen, red). **B**, stereo image of the active site cavity showing the interactions between the sulfate ions and the protein. The backbone nitrogen atoms of Ala-116 and Ser-64 are shown as isolated blue balls. The strands of the β-barrel, loops L1 and L2, and helices H1 and H2 of the subunit that provides the largest contribution to the active site are shown in beige. Loop L4 and helix H4 of the neighboring subunit are shown in dark cyan. A was generated with GRASP (43).

**DISCUSSION**

KDO8P synthase and DAH7P synthase catalyze two very similar condensation reactions between PEP and A5P and PEP and E4P, respectively. We have determined the crystal structure of KDO8P synthase, and based on both sequence and three-dimensional alignments we propose that the latter is a structural and mechanistic homolog of DAH7P synthase. One interesting difference between the two enzymes is observed in the N-terminal extension that seals the amino end of the barrel. This extension adopts the form of a β-hairpin in KDO8P synthase and of a helix in DAH7P synthase. A possible explanation of this difference is that the two enzymes evolved independently from a common ancestor in which both ends of the barrel were open. Clearly, it must be important for the function of these synthases that the active site remains accessible only from one side of the barrel. Thus, it is conceivable that when this requirement emerged during the evolution of the two enzymes, two different strategies, a hairpin and a helix, were adopted to achieve the same goal.

Two mechanisms have been proposed to explain the reaction of KDO8P synthase. According to one hypothesis (9) a water molecule attacks at C9PEP, coincident with the addition of C3PEP to the electrophilic aldehyde of A5P, to yield an open chain intermediate (Fig. 8, Mechanism I). According to the second hypothesis (10, 33), an initial nucleophilic attack by C3-OH₅A⁵P onto C2PEP is followed by condensation of C3PEP with the carbonyl carbon of A5P, forming a cyclic intermediate (Fig. 8, Mechanism II). Attempts to isolate and analyze the
putative reaction intermediates have not been successful (34). Inhibitor studies were also unable to discriminate between the two models. For example, the two best known inhibitors of the enzyme, both with \( K_i \) around 4.0 \( \mu \)M, are the amino phosphonate analog of the putative linear intermediate (35) and the C2 phosphonate analog of the putative cyclic intermediate (33). The structural information presented here does not reconcile well with Mechanism II. The formation of a cyclic intermediate demands that PEP and A5P bind side by side such that the distance between C2PEP and C3-OH\(_{\text{A5P}}\) be less than 3 Å. Instead, the distance between C2\(_{\text{PEP}}\) and C3-OH\(_{\text{A5P}}\) is predicted to be −6.0 Å if PEP and A5P bind with their phosphate moieties at the positions of SO4−1 and SO4−2. The binding configuration of the two substrates proposed in this report is supported by the observation that PEP binds in the active site of the \( E. \) coli DAH7P synthase with its phosphate moiety in a position analogous to that of SO4−1 in KDO8P synthase (Fig. 6). Likewise, in DAH7P synthase a sulfate ion occupies the position predicted for the phosphate moiety of E4P. It is also of note that in KDO8P synthase the distance between the two sulfate ions is approximately equal to the sum of the length of PEP (defined as the distance between P and C3) and of the length of A5P (defined as the distance between C1 and P), whereas in DAH7P synthase the distance between the sulfate ion and the phosphate group of PEP is approximately equal to the sum of the length of PEP and E4P. Altogether, the conclusions derived from the structures of KDO8P synthase and DAH7P synthase are complementary and support the view that the two substrates bind in the active site in such a way that C3_{\text{PEP}} is less than 3 Å from C1_{\text{E4P}} and the phosphate moieties are far apart from each other. This binding mode is expected to lead to the formation of a linear diphosphate inter-
mediate as postulated by Mechanism I (Fig. 8). Interestingly, the E. coli DAH7P synthase can synthesize KDO8P from A5P and PEP at modest rates (36), whereas KDO8P synthase cannot catalyze the synthesis of DAH7P from E4P and PEP.5 Clearly, the active site of DAH7P synthase must be able to accommodate the longer A5P. In contrast, the binding of E4P and PEP in the active site of KDO8P synthase would leave a gap of approximately 3.5 Å between C1E4P and C3PEP, decreasing the probability of forming a bond between these two atoms.

In DAH7P synthase, the si face of PEP is in van der Waals contact with the imidazole ring of His-268. This residue appears to be also involved in binding the active site metal. Although in the active site of KDO8P synthase there is no metal, a histidine residue (His-202) occupies the same position as His-268 in DAH7P synthase. Mutation of this residue to glycine or its modification with diethylpyrocarbonate completely inactivates KDO8P synthase (29). The H268G mutant of DAH7P synthase is also completely inactive.5 Thus, it is tempting to speculate that this residue might be intimately involved in the catalytic mechanism. For example, if KDO8P synthesis occurs according to the pathway described by Mechanism I, the stereochemistry of the reaction will be determined by the initial attack of a water molecule at C2PEP. This attack can occur from either side of the planar molecule of PEP, with each leading to a different disposition of groups around the newly formed chiral center. Because His-202 (His-268 in DAH7P synthase) is located on the si face of PEP, it is equally plausible that its imidazole ring might either protect the si side of PEP from water or act as a general base extracting a proton from a water molecule attacking at C2PEP. The current level of detail in the structures of both KDO8P and DAH7P synthase does not allow an unambiguous discrimination between these two possibilities. In this context it is of note that whereas several water molecules can be identified in KDO8P synthase in the region of the active site that would face the re side of PEP, there are no basic side chains that could participate in the activation of these solvents for a nucleophilic attack onto PEP.

Interest for pursuing the studies presented here derives in part from the fact that inhibition of the enzymes involved in the
synthesis and incorporation of KDO into LPS is detrimental to the survival and growth of Gram-negative bacteria (4, 5). The inhibition of KDO synthesis is of particular importance because it may also produce a decrease in pathogenicity because of the accompanying loss of the endotoxin component of LPS (38, 39). Of added significance is the fact that the homologous DAH7P synthase serves a crucial role in the metabolism of both bacteria and fungi by catalyzing the first step in the biosynthesis of KDO8P synthase and the equivalent residues of DAH7P synthase are shaded in light gray. The two residues involved in the binding of SO4–2 in KDO8P synthase and of the only sulfate ion in DAH7P synthase are in bold face to highlight the shift in sequence corresponding to the positional shift of the two sulfate ions. The dark gray boxes below the alignment represent regions conserved (identities plus conservative substitutions) in both KDO8PS and DAH7PS.

**FIG. 7.** Structure-based sequence alignment of the *E. coli* DAH7P and KDO8P synthases. The regions of the two sequences that were used for the structure alignment are boxed; these are 205 residues of the two superimposed structures whose Ca atoms are within 3.8 Å of each other. The numbering of the two sequences is shown above (KDO8P synthase) and below (DAH7P synthase) the alignment. Residues involved in the architecture of the active site of KDO8P synthase and the equivalent residues of DAH7P synthase are shaded in light gray. The two residues involved in the binding of SO4–2 in KDO8P synthase and of the only sulfate ion in DAH7P synthase are in bold face to highlight the shift in sequence corresponding to the positional shift of the two sulfate ions. The dark gray boxes below the alignment represent regions conserved (identities plus conservative substitutions) in both KDO8PS and DAH7PS.

**FIG. 8.** Proposed mechanisms of KDO8P synthesis. Mechanism I, PEP and A5P bind head to tail. A linear tetrahedral intermediate is formed after a water molecule attacks C2PEP and the nucleophilic C3PEP is added to C1A5P. Mechanism II, PEP and A5P bind side by side. A cyclic intermediate is formed via nucleophilic attack by C3-OHA5P onto C2PEP followed by condensation of C3PEP with the carbonyl carbon of A5P. Whereas both mechanism have been depicted as syn-addition, the exact mode of addition is not known.
aromatic amino acids. Inhibition of this pathway should be equally effective in a large spectrum of microorganisms. Thus, it is hoped that the findings reported here will help provide the structural basis not only for the elucidation of the mechanism of KDO8PS and DAH7PS but also for the rational design of compounds with inhibitory efficacy against both enzymes.

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