Substrate and Metal Complexes of 3-Deoxy-D-manno-octulosonate-8-phosphate Synthase from *Aquifex aeolicus* at 1.9-Å Resolution

IMPLICATIONS FOR THE CONDENSATION MECHANISM*

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3-Deoxy-D-manno-octulosonate-8-phosphate synthase (KDO8PS) from the hyperthermophilic bacterium *Aquifex aeolicus* differs from its *Escherichia coli* counterpart in the requirement of a divalent metal for activity (Duewel, H. S., and Woodard, R. W. (2000) J. Biol. Chem. 275, 22824–22831). Here we report the crystal structure of the *A. aeolicus* enzyme, which was determined by molecular replacement using *E. coli* KDO8PS as a model. The structures of the metal-free and Cd\(^{2+}\) forms of the enzyme were determined in the uncomplexed state and in complex with various combinations of phosphoenolpyruvate (PEP), arabinose 5-phosphate (A5P), and erythrose 4-phosphate (E4P). Like the *E. coli* enzyme, *A. aeolicus* KDO8PS is a homotetramer containing four distinct active sites at the interface between subunits. The active site cavity is open in the substrate-free enzyme or when either A5P alone or PEP alone binds, and becomes isolated from the aqueous phase when both PEP and A5P (or E4P) bind together. In the presence of metal, the enzyme is asymmetric and appears to alternate catalysis between the active sites located on one face of the tetramer and those located on the other face. In the absence of metal, the asymmetry is lost. Details of the active site that may be important for catalysis are visible at the high resolution achieved in these structures. Most notably, the shape of the PEP-binding pocket forces PEP to assume a distorted geometry at C-2, which might anticipate the conversion from sp\(^2\) to sp\(^3\) hybridization occurring during intermediate formation and which may modulate PEP reactivity toward A5P. Two water molecules are located in van der Waals contact with the *si* and *re* sides of C-2\(^{\text{PEP}}\), respectively. Abstraction of a proton from either of these water molecules by a protein group is expected to elicit a nucleophilic attack of the resulting hydroxide ion on the nearby C-2\(^{\text{PEP}}\), thus triggering the beginning of the catalytic cycle.

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1 The abbreviations used are: KDO8PS, 3-deoxy-D-manno-octulosonate-8-phosphate synthase; KDO8PS1, 3-deoxy-D-manno-octulosonate 8-phosphate; PEP, phosphoenolpyruvate; A5P, arabinose 5-phosphate; E4P, erythrose 4-phosphate; DAH7P, 3-deoxy-3-arabino-heptulosonate 7-phosphate; DAH7PS, 3-deoxy-3-arabino-heptulosonate-7-phosphate synthase; L, loop.

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structure of \( A. \) \( aeolicus \) KDO8PS in the presence of various combinations of substrates and metal. Results from this study support the previous hypothesis (11) that the reaction of KDO8P synthesis proceeds through the formation of a linear intermediate and point to the essential role played by a surface loop in isolating the active site from bulk solvent and by the geometry of the active site in influencing the electronic configuration of the substrates during turnover.

**EXPERIMENTAL PROCEDURES**

**Protein Purification and Crystallization—**Recombinant \( A. \) \( aeolicus \) KDO8PS was isolated from \( E. \) \( coli \) BL21(DE3) cells harboring plasmid pAakdsA, as described previously (16). A concentrated KDO8PS solution (30 mg/ml in \( \sim \) 15 mM Tris-Cl) was obtained from purified enzyme that had been dialyzed against 5 mM Tris-Cl (pH 7.5), lyophilized, and then reconstituted with distilled water. Lyophilization does not affect the activity of the reconstituted enzyme. Crystals were obtained by vapor diffusion in hanging drops. For this purpose, the concentrated enzyme solution (30 mg/ml) was mixed 1:1 with a reservoir solution containing 100 mM sodium acetate (pH 4.8–4.9), 21% polyethylene glycol 4000, 16% glycerol, and 5% ethylene glycol.

The concentrated solution of \( A. \) \( aeolicus \) KDO8PS as well as crystals of this enzyme in the original crystallization drops have a slight pink color. As discussed by Duewel et al. (15, 16), this color is probably due to the presence of substoichiometric amounts of bound iron in the enzyme produced in \( E. \) \( coli \). For these studies, we have opted to collect diffraction data from crystals that were either completely depleted of any metal or in which the iron had been replaced with \( \text{Cd}^{2+} \), the ion that confers the highest activity in solution (15). Incubation of \( A. \) \( aeolicus \) KDO8PS crystals in a cryoprotectant liquor containing 100 mM sodium acetate (pH 4.8–4.9) and 5–6% polyethylene glycol 4000. The crystallization trays were incubated at 4°C for 1–2 weeks. Crystals (typically 0.12–0.15 mm\(^3\)) were harvested and maintained in a cryoprotectant holding solution consisting of 100 mM sodium acetate (pH 4.8–4.9), 21% polyethylene glycol 4000, 16% glycerol, and 5% ethylene glycol.

The concentrated solution of \( A. \) \( aeolicus \) KDO8PS crystals in a cryoprotectant liquor containing 100 mM sodium acetate (pH 4.8–4.9), 21% polyethylene glycol 4000, 16% glycerol, and 5% ethylene glycol.

The asymmetric unit of \( A. \) \( aeolicus \) KDO8PS crystals contains a homodimer. Two crystal forms of \( E. \) \( coli \) KDO8PS previously reported by this laboratory contain instead a homotetramer of the enzyme with 222 local symmetry (9, 11). A third crystal form reported by Wagner et al. (10) contains only one chain in the asymmetric unit, but application of crystal symmetry generates a tetramer essentially identical to that present in the other two crystal forms. Application of one of the operations of crystallographic symmetry (\( x = y, y = x, z = -z \)) of space group \( P3_121 \) produces a tetrameric assembly of \( A. \) \( aeolicus \) KDO8PS that can be superimposed on the \( E. \) \( coli \) enzyme with a root mean square deviation of only 1.22 Å for 696 aligned C-\( \alpha \) atoms. In line with this finding, comparative analysis of the \( A. \) \( aeolicus \) (\( M_r 30,833 \)) and \( E. \) \( coli \) (\( M_r 29,734 \)) KDO8PSs by analytical size-exclusion chromatography reveals that the two enzymes have almost identical elution profiles (16). The enzyme from \( S. \) \( typhimurium \) also demonstrates analogous chromatographic behavior (20). These observations suggest that the native form of KDO8PS might be a tetramer in all Gram-negative bacteria.

As in the case of \( E. \) \( coli \) KDO8PS, the \( A. \) \( aeolicus \) enzyme adopts a \( (\beta/\alpha)_{8} \)-barrel topology (Fig. 2). Each monomer of \( A. \) \( aeolicus \) KDO8PS differs from the \( E. \) \( coli \) counterpart mainly at
The N terminus, as the E. coli enzyme has an additional β-hairpin that seals the N-terminal end of the barrel. Consequently, the first residue of the thermophilic KDO8PS corresponds to residue 16 of the E. coli sequence. The enzyme active sites are located at the C-terminal end of the β-barrel of each subunit, at the interface with the adjacent subunit. Three long loops, L2, L7, and L8, control access to the active site cavity (Fig. 2). L2 and L8 were both disordered in the E. coli enzyme (11). L8 is always ordered in A. aeolicus KDO8PS; L7 becomes ordered under particular conditions (see below).

Structure of the Substrate-free Enzyme—The structure of substrate-free A. aeolicus KDO8PS was determined for the both the Cd²⁺ and metal-free forms of the enzyme. The Cd²⁺ form (Table I, Cd²⁺ column) was intentionally pursued because Cd²⁺ is the most effective activator of KDO8PS (15). In the active site of the enzyme, the Cd²⁺ ion displays a distorted octahedral coordination: the thiolate of Cys-11 and the nitrogen of His-185 provide the equatorial coordination (Fig. 3). The water molecule that acts as equatorial ligand is also stabilized by a hydrogen bond to the N-nitrogen of Lys-46. These features of the active site of A. aeolicus KDO8PS are particularly reminiscent of the homologous enzyme DAH7PS. The recently reported crystal structure of the E. coli phenylalanine-regulated DAH7PS (8) shows a lead ion (Pb²⁺) bound in the active site and interacting with PEP. The lead ion, which was included in the crystallization for the purpose of determining the structure by multiple wavelength anomalous diffraction, but is itself an extremely poor activator of DAH7PS (8), is believed to occupy the same active site location as other metals that are good activators (13). As expected, several residues in the coordination sphere of the lead ion in DAH7PS (Cys-61, His-268, Glu-302, and Asp-326) have counterparts in the active site of A. aeolicus KDO8PS (Cys-11, His-185, Glu-222, and Asp-233).

In the substrate-free Cd²⁺ form of A. aeolicus KDO8PS, a phosphate ion (herein designated as PO₄⁻₁) occupies the position corresponding to the phosphate moiety of PEP in DAH7PS (8) or to the SO₄⁻₂ site described in the E. coli enzyme (11) (Fig. 3). A second phosphate ion (herein designated as PO₄⁻₂), corresponding to the SO₄⁻₂ ion of the E. coli enzyme, is located ~10 Å from PO₄⁻₁ in a raised position close to the opening of

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**Table I**

Data collection and refinement statistics

|                | Cd²⁺ | PEP | Cd²⁺/PEP | Cd³⁺/Asp | PEP/Asp | Cd²⁺/PEP/Asp | Cd³⁺/PEP/PE/4P |
|----------------|------|-----|----------|----------|---------|--------------|----------------|
| Resolution range (Å) | 43 to 2.06 | 26 to 1.8 | 25 to 1.94 | 24 to 1.9 | 43 to 1.89 | 26 to 1.8 | 27 to 1.85 | 25 to 1.9 |
| Measurements | 362,497 | 475,864 | 394,602 | 613,584 | 381,111 | 407,194 | 763,003 | 556,519 |
| Unique reflections | 34,118 | 54,713 | 46,302 | 47,213 | 45,480 | 56,785 | 51,825 | 46,313 |
| Redundancy | 10.6 | 8.7 | 8.5 | 13.0 | 8.4 | 7.17 | 14.7 | 12.0 |
| Completeness (%) | 81.7 | 83.9 | 94.2 | 90.2 | 84.7 | 91.8 | 91.4 | 88.4 |
| (h/α)(f) | 17.5 | 13.6 | 28.6 | 18.4 | 29.2 | 22.3 | 17.3 | 14.4 |
| Rmerge (%) | 8.8 | 9.7 | 4.3 | 6.0 | 8.0 | 11.0 | 8.8 | 12.0 |

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The Cα trace and side chains of the Cd²⁺ enzyme are shown with salmon and white bonds, respectively. The Cα trace and side chains of the metal-free enzyme are shown with transparent light-blue bonds. The coordination of the Cd³⁺ ion is shown as transparent light-green bonds. Phosphate ions are labeled PO₄⁻₁ and PO₄⁻₂. Blue: nitrogen; red: oxygen; yellow, sulfur, pale blue, phosphorus; cyan, cadmium. WAT: water.
the position of the imidazole ring of His-185, which undergoes a rotation of ~40° around the C-β–C-γ bond. Although the position of PO₄⁻¹ is essentially unchanged, PO₄⁻² is not present, possibly as a consequence of the lack of stabilizing interactions provided by L7. This loop is completely disordered in both subunits of the metal-free enzyme between Val-187 and Met-200.

Structure of the Enzyme in Complex with PEP—Crystals of the metal-free or Cd²⁺ enzyme were incubated in the presence of 5 mM PEP (K_m(PEP) = 40 μM at 60 °C), and their structures were determined (Table I, PEP and Cd²⁺/PEP columns, respectively). As predicted from the analysis of the structure of E. coli KDO8PS, PEP binds at the bottom of the active site cavity of the A. aeolicus enzyme, with its phosphate and carboxylate moieties stabilized by a network of hydrogen bonds and salt bridges (Fig. 4A). The phosphate group is anchored by salt bridges to the side chains of Lys-124 and Arg-154 and by a hydrogen bond to the backbone amide of Ala-102. The carboxylate group forms salt bridges with Lys-41, Lys-46, and Lys-124 and a hydrogen bond with the hydroxyl of Ser-43. Finally, Lys-124 also forms a hydrogen bond with the bridging oxygen of PEP. PEP binds in a similar fashion in the active site of the
enzyme both in the presence and absence of a divalent metal. The most notable difference between the two structures is in the position of the Glu-222 side chain. If Cd\(^{2+}\) is present, Glu-222 is a metal ligand, whereas in the absence of the metal, its side chain moves slightly away from the vacant metal site (data not shown). Interestingly, the water molecule that, in the presence of Cd\(^{2+}\), is one of the metal ligands is retained even in the absence of Cd\(^{2+}\). This water is located on the si face of PEP in van der Waals contact with the substrate C-2 and C-3. The presence of PEP must have a significant stabilizing role, as this water molecule is not visible in the structure of the substrate-and metal-free enzyme. A water molecule is also in van der Waals contact with the re side of PEP (Fig. 4A). The potential role in catalysis of the water molecules located on both the si and re sides of PEP is discussed below.

A striking feature of PEP bound in the active site of KDO8PS is that the geometry of the molecule at C-2 deviates appreciably from the planarity observed in the crystal structures of free PEP in various ionization states (reviewed in Ref. 21): the magnitude of the distortion is provided by the value of the angle between the plane defined by C-2, C-1, and C-3 and the plane defined by C-1, C-3, and O-2, which is \(-12^\circ\) instead of the expected value of 0\(^\circ\) (Fig. 4B). This deviation of the PEP molecule from perfect planarity appears to be imposed by the particular geometry of the active site and may be indicative of the fact that the enzyme modifies the electronic configuration of the substrate to favor a nucleophilic attack at C-2\(^{\text{PEP}}\) (see below).

Structure of the Enzyme in Complex with A5P—The structure of A. aeolicus KDO8PS in complex with A5P was determined by soaking crystals of the Cd\(^{2+}\) enzyme in the presence of 10 mM A5P (K\(_{m}\) for A5P = 8 \(\mu\)M at 60 °C) (Table I, Cd\(^{2+}\)/A5P column). As predicted by Radaev et al. (11), A5P binds with its phosphate moiety coincident with the position occupied by PO\(_4\)-2 in the substrate-free enzyme (see above). The position of PO\(_4\)-1 is unchanged with respect to the substrate-free enzyme. The phosphate moiety of A5P is stabilized by a salt bridge to Arg-49 and by hydrogen bonds to the backbone and side chain of Ser-50 (Fig. 5A). In this structure, L7 is not ordered and therefore does not appear to contribute significantly to A5P binding. The carbon tail of A5P is stabilized by a network of hydrogen bonds directed to the substrate hydroxyls and to the aldehyde moiety, originating from Asp-233, Asn-48, and PO\(_4\)-1 and from several water molecules (Fig. 5, A and B). In particular, C-2–OH\(_{\text{a}}\) is only 2.9 Å from Cd\(^{2+}\) and can be considered a weak ligand of the metal. In this fashion, the substrate C-2–OH appears to replace the water molecule that is a ligand of Cd\(^{2+}\) in other structures of the enzyme. Polar interactions of A5P with the active site residues are listed in Table II.

A5P binds only to one of the two molecules of KDO8PS present in the asymmetric unit. The active site of the other molecule is filled by PO\(_4\)-1 and PO\(_4\)-2. Moreover, if crystallographic symmetry is applied to generate the enzyme tetramer, it becomes apparent that the two active sites in which A5P binds are located on the same “face” of the enzyme. This observation is reminiscent of the asymmetry observed in the E. coli enzyme with regard to the binding of phosphate ions at a surface site (11).

Structure of the Enzyme in Complex with PEP plus A5P—A. aeolicus KDO8PS has optimal activity at 95 °C and no detectable activity at 4 °C (16). As crystals of this enzyme are obtained and maintained at 4 °C, it was deemed possible to bind both substrates PEP and A5P without turnover. Both metal-free and Cd\(^{2+}\) crystals of A. aeolicus KDO8PS were incubated in the presence of 5 mM PEP and 10 mM A5P, and the structure of the enzyme was determined under these conditions (Table I, PEP/A5P and Cd\(^{2+}\)/PEP/A5P columns, respectively). The two substrates bind in the active site at the same positions they occupy in the structures of the enzyme in complex with PEP alone or A5P alone, respectively. However, in the Cd\(^{2+}\) enzyme, A5P binds to only one of the two active sites contained in the asymmetric unit (as observed in the structure of the Cd\(^{2+}\) enzyme incubated with A5P alone), and PO\(_4\)-2 fills the second active site. In the metal-free enzyme, A5P binds to both active sites (Fig. 6). In all the cases in which A5P and PEP bind simultaneously, L7 becomes ordered and isolates the active site from the external environment. This loop stabilizes the phosphate moiety of A5P via hydrogen bonds to the backbone and side chain of Ser-50 (Fig. 5A). Interestingly, the electron density associated with L7 is better ordered in the metal-free enzyme, some residual electron density associated with L7 is better ordered in the metal-free enzyme, and some residual electron density associated with L7 is better ordered in the metal-free enzyme, thus confirming the presence of a metal site.

### Table II

| Polar interactions between active site residues and PEP or A5P |
|-----------------|-----------------|
| Cd\(^{2+}\) enzyme/A5P | Metal-free enzyme |
| Lys-41 N\(\text{C}\) | O-1 (2.74) |
| Ser-43 O\(\gamma\) | O-1 (3.08) |
| Lys-46 N\(\varepsilon\) | O-1 (2.99) |
| Glu-99 O\(e-1\) | O-2 (3.0) |
| Lys-124 N\(\varepsilon\) | O-2 (2.92) |
| Lys-124 N\(\zeta\) | O-2 (3.14) |
| Lys-124 N\(\zeta\) | O-2 (3.87) |
| Arg-154 N\(\varepsilon\) | O-3P (2.92) |
| Arg-154 N\(\eta\) | O-2P (2.79) |
| A5P O\(\varepsilon\) | O-2P (2.64) |
| Ala-102 N | O-1P (2.85) |
| Glu-188 O\(e\) | OH-4 (3.02) |
| Asp-233 O\(\beta\) | OH-4 (2.68) |
| Asp-233 O\(\varepsilon\) | OH-4 (2.98) |
| Lys-46 N\(\zeta\) | OH-2 (3.19) |
| Asn-48 O\(\varepsilon\) | O-1 (2.68) |
| Asn-48 N\(\varepsilon\) | O-1 (2.05) |
| Arg-49 N\(\varepsilon\) | O-1 (2.42) |
| Arg-49 N\(\eta\) | OP-3 (3.59) |
| Arg-49 N\(\varepsilon\) | OP-1 (2.83) |
| Ser-50 N | OP-1 (2.81) |
| Arg-106 N\(\eta\) | OP-1 (2.77) |
| Ser-197 O\(\gamma\) | OP-2 (2.86) |
| Ser-197 N | OP-2 (2.89) |
| Ser-50 O\(\gamma\) | OP-2 (2.52) |
| PO\(_4\)-1 O\(\gamma\) | OH-2 (2.88) |
| PO\(_4\)-1 O\(\varepsilon\) | O-1 (2.80) |
| Cd\(^{2+}\) | OH-2 (2.93) |
| Cd\(^{2+}\) | OH-3 (3.13) |

\(\text{*This residue belongs to a different chain.}\)
In the other active site, the water molecule is present, but the density of A5P is not continuous between C-2 and C-4 (Fig. 6, A and B). Polar interactions of PEP and A5P with the active site residues are listed in Table II.

A $[F_o - F_c]$ difference map of the active sites in the metal-free enzyme shows clearly three small positive densities surrounding the sulfur of Cys-11. The most plausible interpretation of these densities is that, during the course of the prolonged incubation of the enzyme first with EDTA to remove any traces of metal and then with PEP and A5P, the sulfhydryl group (-SH) of Cys-11 was oxidized to a sulfonic group (-SO$_3$). This interpretation is supported by other studies carried out with the soluble enzyme in which it was shown that, in the absence of metal, Cys-11 becomes prone to oxidation.

Structure of the Enzyme in Complex with PEP plus E4P—It has been previously reported that E4P, the substrate of DAH7PS, does not serve as a substrate for $A$. aeolicus KDO8PS (15, 16). Furthermore, in the E. coli enzyme, E4P acts as a competitive inhibitor with respect to A5P. Therefore, in view of the mechanistic and structural similarities between KDO8PS and DAH7PS, it might be expected that E4P binds in the active site of KDO8PS in the same position as A5P, but that such binding does not lead to a condensation product with PEP because E4P is one carbon shorter than A5P. To verify this hypothesis, crystals of $A$. aeolicus Cd$^{2+}$ KDO8PS were incubated in the presence of 5 mM PEP and 10 mM E4P, and the structure of the enzyme was determined (Table I, Cd$^{2+}$/PEP/E4P column). Under these conditions, PEP binds to both subunits, but E4P binds only to one subunit. If a tetramer is generated by application of crystal symmetry, binding of E4P takes place in the two active sites that are visible from the same side of the enzyme. In these sites, the phosphate moiety of E4P occupies the same position assumed by the phosphate moiety of A5P. The electron density for the bridging oxygen and C-4 are well defined; however, unambiguous assignment of the configuration assumed by the remaining part of E4P in the active site is not possible. A plausible interpretation of the electron density is that the aldehyde carbonyl is hydrogen-bonded to the water molecule coordinating Cd$^{2+}$ and to one of the hydroxyls of the phosphate moiety of PEP; C-3–OH$^{2+}$ and C-2–OH$^{2+}$ are also in hydrogen bond distance from the hydroxyl of Ser-197 and from the amide moiety of Asn-48, respectively (Fig. 7). As in the case of A5P and PEP, L7 becomes well ordered when E4P and PEP are both bound and isolates the active site from the external environment.

**DISCUSSION**

KDO8PS and DAH7PS catalyze similar condensation reactions between PEP and A5P and between PEP and E4P, respectively. Two mechanisms have been proposed to explain these reactions. According to the first hypothesis (22) a water molecule attacks at C-2 of PEP, whereas C-3 of PEP is added to the aldehyde of A5P or E4P; this process would yield a linear intermediate (Fig. 8, MECHANISM I). According to the second hypothesis (5, 23), condensation of C-3 of PEP with the carbonyl carbon of A5P or E4P is concurrent with an attack by C-3–OH of the monosaccharide on C-2 of PEP; this process would lead to the formation of a cyclic intermediate (Fig. 8, MECHANISM II). We speculated that two sulfate ions bound in the active site of E. coli KDO8PS occupy the positions of the phosphate moieties...
of PEP and A5P and posited that the distance of 13 Å between these positions is consistent with a mechanism that proceeds through the formation of a linear intermediate (11). However, direct experimental confirmation of this hypothesis was not possible since attempts to visualize the substrates PEP and A5P by incubating crystals of *E. coli* KDO8PS in the presence of these compounds were hampered by the high ionic strength of the holding solution, which prevented binding of either substrate. The crystal structure of DAH7PS, although providing the location for PEP in the active site, did not contain E4P, hence also could not provide direct experimental confirmation of our hypothesis.

In contrast, crystals of the *A. aeolicus* enzyme are stable in a holding solution of low ionic strength, which allowed the determination of the structure of the enzyme both in its substrate-free form and in the presence of bound PEP and A5P (or E4P). The distance between the phosphate moieties of PEP and A5P (or E4P) in *A. aeolicus* KDO8PS is only 10 Å, which is the same distance observed in the structure of the *E. coli* DAH7PS (8) between the phosphate moiety of PEP and a lone sulfate (or phosphate) ion believed to occupy the position of the phosphate group of E4P. A distance of 10 Å between two sulfate ions bound in the active site has been reported also by Wagner *et al.* (10) for a crystal form of *E. coli* KDO8PS in which only one chain is present in the asymmetric unit. We had originally postulated that the distance of 13 Å between the two sulfate ions bound in the active site of *E. coli* KDO8PS (versus 10 Å in DAH7PS) reflected the fact that A5P, the substrate of KDO8PS, is one carbon longer than E4P, the substrate of DAH7PS. This element of our hypothesis is clearly not supported by the observation that, in *A. aeolicus* KDO8PS, both A5P and E4P are accommodated in the active site without a change in the distance between the phosphate moieties of these substrates and that of PEP. The experimentally observed positions of PEP and A5P (Fig. 6) or of PEP and E4P (Fig. 7) are, however, clearly consistent with the formation of a linear reaction intermediate because the distance between C-2PEP and C-3-OHSA5P or between C-2PEP and C-2-OHE4P is >5 Å. If the reaction took place via formation of a cyclic intermediate, these two atoms would have to be much closer than 5 Å for a bond to be formed between them, without invoking large conformational changes in the active site.

*A. aeolicus* KDO8PS, like all known DAH7PSs, requires a divalent cation for activity. Moreover, the coordination of the metal in the *A. aeolicus* enzyme is very similar to that observed for the metal present in the structure of *E. coli* DAH7PS. What is the function of the metal in these enzymes? One possibility is that it serves a structural function such as that of correctly orienting PEP, A5P (or E4P), or the reaction intermediate in the active site. With regard to this point, we have not observed significant changes in the position of PEP in *A. aeolicus* KDO8PS in the absence or presence of metal. However, changes in the position of PEP might occur during catalysis. For example, in the crystal structure of the *E. coli* DAH7PS in complex with Mn$^{2+}$ and the PEP analog 2-phosphoglycolate, the octahedral coordination of the metal is completed by the carboxylate moiety of 2-phosphoglycolate, which substitutes for PEP in the active site (24). The binding mode of 2-phosphoglycolate might reflect the fact that a direct coordination of PEP with the active site metal occurs during turnover. Likewise, a direct interaction between A5P and Cd$^{2+}$ might occur at some stage during catalysis. An example of this kind of interaction was observed in the structure of the Cd$^{2+}$-enzyme in complex with A5P (Fig. 5). Thus, two different conformations of A5P have been observed in the various complexed states of *A. aeolicus* KDO8PS. In one conformation, a water molecules coor-

dinates Cd$^{2+}$ and is also in hydrogen bond distance from both C-2-OHSA5P and C-3-OHSA5P (Fig. 6). In the second conformation, C-2-OHSA5P replaces water as a ligand of Cd$^{2+}$ (Fig. 5). Thus, although the active site holds PEP very tightly, it allows significant conformational flexibility to A5P. It is unlikely that such an active site would have been retained during evolution without a specific reason. An interesting possibility is that the two conformations of A5P reflect the affinity of the enzyme for A5P (or for the chemical groups originating from A5P) at different stages of catalysis. At the start of the reaction, water may be necessary as a simultaneous ligand of the metal and A5P to favor the formation of a bond between PEP and A5P (see below). Once the reaction intermediate is formed, then that part of the molecule that originated from A5P could be stabilized via a direct interaction with the metal. The requirement for water at the beginning of the reaction may be rationalized by noticing that the postulated mechanism of formation of a linear intermediate predicts that a water molecule attacks C-2PEP (Fig. 8, MECHANISM I) and that this attack would be favored by abstraction of a proton from this water by a base. The water molecule that acts as one of the Cd$^{2+}$ ligands in *A. aeolicus* KDO8PS is in van der Waals contact with the si face of C-2PEP. A water molecule is also one of the equatorial ligands of the metal in the structure of DAH7PS in complex with Mn$^{2+}$ and 2-phosphoglycolate (24). Coordination of water to a divalent cation is expected to lower its pK$_a$ and to favor its deprotonation to a hydroxide ion. However, if the function of the metal in *A. aeolicus* KDO8PS (and in DAH7PS) is that of activating a catalytic water, how can *E. coli* KDO8PS catalyze the same reaction without metal? In this context, it is probably important that, in the *E. coli* enzyme, a water molecule is present in almost the same position as the water coordinating Cd$^{2+}$ in the *A. aeolicus* enzyme. In the *E. coli* enzyme, this water is stabilized by hydrogen bonds to two bases, the ζ-nitrogen of Lys-60 and the ε-nitrogen of His-202, that might favor its deprotonation.

If the water molecule that coordinates Cd$^{2+}$ is involved in
attacking the *si* face of C-2PEP, then the formation of the linear intermediate would be the result of a *syn* addition of water to C-2PEP and of C-1A5P to C-3PEP. However, a *syn* addition to PEP is unprecedented. Therefore, it is worthwhile to examine the alternative possibility of a water attack on the *re* side of C-2PEP.

Such an event might indeed occur in *A. aeolicus* KDO8PS because a water molecule is also present on the *re* side of PEP in van der Waals contact with C-2PEP (Figs. 4, 6, and 7). This water is stabilized by a network of hydrogen bonds that includes one of the hydroxyls of the phosphate moiety of PEP, Asp-81, and His-83 (Fig. 9). A chain of hydrogen bonds could be involved in transferring a proton from this water molecule via Asp-81 to His-83, which is a more likely final acceptor than the aspartic acid. Asp-81 and His-83 are conserved in all known KDO8PSs, and a glutamic acid is present at the equivalent position of Asp-81 in DAH7PSs (see multiple alignment of Radaev *et al.* (11)). However, a residue equivalent to His-81 that could act as a final accepting base is not present in DAH7PSs. On the other hand, the electronic environment of the active site in DAH7PS might raise the *pK*_a* of the glutamic acid carboxylate sufficiently for it to act as a base. Therefore, as water molecules potentially capable of attacking C-2 are located on both the *si* and *re* sides of PEP, a direct discrimination between a *syn* and an *anti* attack on PEP is not possible at this time.

An additional observation allowed by the high resolution of the structures reported in this study deserves special attention. In all the cases in which PEP is bound in the active site of *A. aeolicus* KDO8PS, it appears to be distorted to optimally fit in the binding pocket. In particular, the geometry of C-2PEP is intermediate between trigonal and tetrahedral (Fig. 4B). This distortion might be preparing C-2PEP for the conversion from *sp*² to *sp*³ hybridization that will take place during the formation of the linear intermediate. Although the direction of the distortion is such that C-2 bulges out of the *si* face (Fig. 4B), it is difficult to predict on theoretical grounds whether this would favor a water attack from the *si* side or from the *re* side. An additional effect of the distortion is that the dihedral angle between the C-2–C-3 double bond and the C=O double bond of the carboxylate moiety of PEP is different from 0°. It has been theorized that the relative orientations of the two planes in which the C=C and C=O double bonds reside control the reactivity of C-3PEP toward C-1A5P (25), and *ab initio* calculations indicate that such reactivity should be maximal when the angle between the two planes is 90°. However, nothing is known yet about how solvation effects and the active site mi-

croenvironment might change the intrinsic properties and reactivity of PEP as derived from quantum chemistry calculations. Undoubtedly, additional theoretical and experimental work will be necessary to clarify these points.

One of the key observations resulting from our study is that binding of substrates controls the opening or closing of the active site to the surrounding environment. When both A5P (or E4P) and PEP are bound simultaneously, L7 is well ordered and isolates the active site from bulk solvent. If A5P alone or PEP alone binds, the loop is not ordered, suggesting that it might be alternating between an open and a closed conformation. Thus, *A. aeolicus* KDO8PS is structured in such a way that its active site remains open until both substrates are bound sequentially. This is particularly important in view of the fact that the active site is shaped like a funnel, with PEP bound in the active site of KDO8PS is viewed edge-on, with its *si* face pointing up and to the left and its *re* face pointing down and to the right. The water (WAT) molecule located on the *re* side of C-2PEP could be activated by transfer of a proton to a hydrogen bond chain ending with His-83.

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**Fig. 8. Proposed mechanisms of KDO8PS synthesis.** MECHANISM I, a linear tetrahedral intermediate is formed after a water molecule attacks C-2PEP and C-3PEP is added to C-1A5P. MECHANISM II, a cyclic intermediate is formed via nucleophilic attack by C-3–OH* or* on C-2PEP followed by condensation of C-3PEP with the carbonyl carbon of A5P. Although both mechanisms are shown as *syn* addition, the exact mode of addition is not known.

**Fig. 9. Solvent localization on the *si* and *re* sides of PEP.** PEP bound in the active site of KDO8PS is viewed edge-on, with its *si* face pointing up and to the left and its *re* face pointing down and to the right. The water (WAT) molecule located on the *re* side of C-2PEP could be activated by transfer of a proton to a hydrogen bond chain ending with His-83.
active site becomes isolated from bulk solvent during catalysis. This may be needed to create a microenvironment in which the \( pK_a \) values of catalytic waters and specific amino acid groups are different from the values normally expected in bulk phase equilibria. In view of all of these mechanistic requirements, there must exist a way by which information on the occupancy of the two substrates in the active site controls the conformation of L7. In this context, it may be of relevance that His-185, located at the very beginning of L7, is one of the Cd\(^{2+}\) ligands. The \( \varepsilon \)-nitrogen of His-185 is between 3.3 and 3.6 Å from C-2–OH\(^{\text{ASP}}\) (in the two conformations observed for this substrate) and is also in hydrogen bond distance of the water molecule sitting on the si side of PEP. Also at the beginning of L7, on the carboxyl side of His-185, Gln-188 is hydrogen-bonded to C-4–OH\(^{\text{ASP}}\) (in the conformation of A5P observed in the metal-free enzyme with PEP also bound). Thus, it is possible that His-185 and Gln-188 sense disturbances in the delicate network of hydrogen bonds and hydrophobic interactions surrounding the substrates and act as transducers of information from the active site to L7.

Of particular mechanistic interest is the observation that, in the Cd\(^{2+}\) enzyme, A5P (or E4P) binds only to one of the two active sites of the dimer contained in the asymmetric unit of the crystal. Application of crystal symmetry to generate the tetramer reveals that binding of A5P occurs at the active sites located on one face of the enzyme, whereas at the active sites located on the opposite face (see structure of the metal-free enzyme in complex with PEP and A5P or E4P). However, in the absence of metal, information on the occupancy of the active sites of one face is no longer transferred to the other face. The consequence of this lack of communication is that the active sites on both faces of the enzyme act independently and bind A5P simultaneously (see structure of the metal-free enzyme in complex with both PEP and A5P). Thus, altogether, the crystallographic studies of KDO8PS point to the existence of a mechanism of alternating site (or face) catalysis that has been overlooked by previous kinetic studies.

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REFERENCES

1. Levin, D. H., and Racker, E. (1959) J. Biol. Chem. 234, 2532–2539
2. Raetz, C. R. (1990) Annu. Rev. Biochem. 59, 129–170
3. De Leo, A. B., and Sprinson, D. B. (1968) Biochim. Biophys. Res. Commun. 32, 873–877
4. Kohen, A., Berkovitch, R., Belakhov, V., and Baasov, T. (1993) Bioorg. Med. Chem. Lett. 3, 1577–1582
5. Dotson, G. D., Dua, R. K., Clemens, J. C., Wooten, E. W., and Woodard, R. W. (1995) J. Biol. Chem. 270, 13698–13705
6. Dotson, G. D., Nanjappan, P., Reily, M. D., and Woodard, R. W. (1993) Biochemistry 32, 12392–12397
7. Floss, H. G., Onerka, D. K., and Carrol, M. (1972) J. Biol. Chem. 247, 736–744
8. Shumilin, I. A., Kretsinger, R. H., and Bauerle, R. H. (1999) Struct. Fold. Des. 7, 865–875
9. Radaev, S., Dastidar, P., Patel, M., Woodard, R. W., and Gatti, D. L. (2000) Acta Crystallogr. Sect. B Biol. Crystallogr. 56, 516–519
10. Wagner, T., Kretsinger, R. H., Bauerle, R., and Tolbert, W. D. (2000) J. Mol. Biol. 301, 233–238
11. Radaev, S., Dastidar, P., Patel, M., Woodard, R. W., and Gatti, D. L. (2000) J. Biol. Chem. 275, 9476–9484
X-ray Structure of Aquifex aeolicus KDO8PS

12. Stephens, C. M., and Bauerle, R. (1992) J. Biol. Chem. 267, 5762–5767
13. Stephens, C. M., and Bauerle, R. (1991) J. Biol. Chem. 266, 20810–20817
14. Birck, A., and Woodard, R. (2003) J. Mol. Evol., in press
15. Duewel, H. S., and Woodard, R. W. (2000) J. Biol. Chem. 275, 22824–22831
16. Duewel, H. S., Sheflyan, G. Y., and Woodard, R. W. (1999) Biochem. Biophys. Res. Commun. 263, 346–351
17. Otwinowski, Z., and Minor, W. (1997) Methods Enzymol. 276, 307–326
18. Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. Sect. D Biol. Crystallogr. 54, 905–921
19. Adams, P. D., Pannu, N. S., Read, R. J., and Brunger, A. T. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 5018–5023
20. Taylor, W. P., Sheflyan, G. Y., and Woodard, R. W. (2000) J. Biol. Chem. 275, 32141–32146
21. Souhassou, M., Schaber, P. M., and Blessing, R. H. (1996) Acta Crystallogr. Sect. B 52, 865–875
22. Hedstrom, L., and Abeles, R. (1988) Biochem. Biophys. Res. Commun. 157, 816–820
23. Baasov, T., Sheffer-Dee-Noor, S., Kohen, A., Jakob, A., and Belakhov, V. (1993) Eur. J. Biochem. 217, 991–999
24. Wagner, T., Shumilin, I. A., Bauerle, R., and Kretsinger, R. H. (2000) J. Mol. Biol. 301, 389–399
25. Li, Y., and Evans, J. N. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4612–4616
26. Kohen, A., Jakob, A., and Baasov, T. (1999) Eur. J. Biochem. 268, 443–449
27. Liang, P. H., Lewis, J., Anderson, K. S., Kohen, A., D’Souza, F. W., Benenson, Y., and Baasov, T. (1998) Biochemistry 37, 16390–16399
28. Kleywegt, G. J., and Jones, T. A. (1997) Methods Enzymol. 277, 525–545
29. Kraulis, P. J. (1991) J. Appl. Crystallogr. 24, 946–950
30. Esnouf, R. M. (1997) J. Mol. Graph. 15, 132–134
31. Merritt, E. A., and Murphy, M. E. P. (1994) Acta Crystallogr. Sect. D Biol. Crystallogr. 50, 869–873
32. Nichols, A., Sharp, K. A., and Henig, B. (1991) Proteins Struct. Funct. Genet. 11, 281–290