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Mechanistic divergence of two closely related aldol-like enzyme-catalysed reactions†

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The analysis of the interaction of threose 4-phosphate and 2-deoxyerythrose 4-phosphate with 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (DAH7PS) reveals previously unrecognised mechanistic differences between the DAH7PS-catalysed reaction and that catalysed by the closely related enzyme, 3-deoxy-D-*manno*-octulosonate 8-phosphate synthase (KDO8PS).

3-Deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (DAH7PS) and 3-deoxy-D-*manno*-octulosonate 8-phosphate synthase (KDO8PS) are two functionally unrelated enzymes that share many mechanistic and structural features. DAH7PS (EC 2.5.1.54) catalyses the first committed step of the shikimate pathway, responsible for the biosynthesis of aromatic compounds.[1] This enzyme catalyses an aldol-like condensation reaction between phosphoenolpyruvate (PEP) and D-erythrose 4-phosphate (E4P) to generate DAH7P and inorganic phosphate. KDO8PS (EC 2.5.1.55) catalyses an analogous reaction using D-arabinose 5-phosphate (A5P) in place of E4P, giving rise to the eight carbon phosphorylated sugar KDO8P (Fig. 1). This reaction is a key step in the biosynthesis of cell wall lipopolysaccharide in Gram-negative bacteria.[2] As both pathways are found in microorganisms but not in animals, the enzymes of these pathways have attracted interest as targets for the development of novel antibiotics.[1,3]

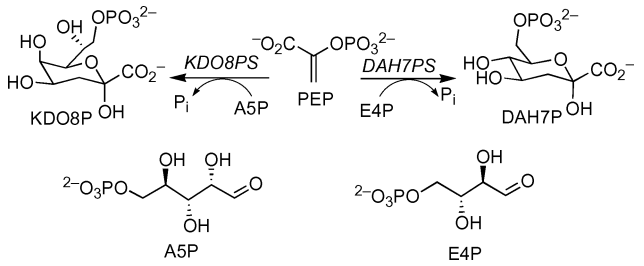

Fig. 1 KDO8PS and DAH7PS reactions.

Despite low sequence similarity between DAH7PS and KDO8PS many key mechanistic similarities have been shown. Both enzymes catalyse the condensation of PEP with a phosphorylated aldose by a similar ordered-sequential mechanism where PEP binds first and phosphate is released last.[4,5] Both reactions involve the cleavage of the C–O bond of PEP,[6,7] and are highly stereospecific with the *si* face of PEP coupling with the *re* face of their respective sugar substrates.[8,9] Additionally, X-ray crystal structures of DAH7PSs (from *Escherichia coli*, *Thermotoga maritima*, *Pyrococcus furiosus* and *Saccharomyces cerevisiae*) have been found to be remarkably similar to those of KDO8PSs (*Aquifex aeolicus*, *E. coli*).[10–15]

Based on these similarities the phylogenetic relationship between DAH7PS and KDO8PS has recently received attention.[16–18] Two types of DAH7PS have been identified based on molecular mass and sequence.[19] The type I enzymes are a broad family of 3-deoxyald-2-ulosonate phosphate synthases that includes the KDO8PSs. This group is also divided into two subfamilies (Iα and Iβ).[16] Currently only DAH7PSs are known in subfamily Iα, whereas subfamily Iβ contains both DAH7PSs ($I\beta_D$) and KDO8PSs ($I\beta_K$). It has been proposed that the ancestor of the type I enzymes was a DAH7PS of the $I\beta_D$ subfamily.[16,17]

While the similarities are clear, two key differences in these enzyme-catalysed reactions have intrigued us. Firstly, whereas all known DAH7PSs require a divalent metal for catalysis, both metal-dependent and metal-independent KDO8PSs have been characterised.[20] Recent studies have shown that metal-dependent KDO8PSs can be converted to metal-independent enzymes following mutation of the metal-binding Cys to Asn (the "natural" substitution found in metal-independent enzymes).[21,22] Secondly, the substrate specificity with respect to the configuration at C2 of the aldose phosphate substrate differs; and it has been reported that the C2 epimer of A5P (D-ribose 5-phosphate, R5P) with E4P-like C2 configuration is not a substrate for KDO8PSs from *E. coli* and *A. aeolicus*.[2,23,24]

In this study we have probed these two key differences by mutation at the metal-binding site of the $I\beta_D$ enzyme from *P. furiosus*, and by investigation of D-threose 4-phosphate (T4P), and 2-deoxy-D-erythrose 4-phosphate (2-deoxyE4P) as substrates for DAH7PS, and R5P and 2-deoxy-D-ribose 5-phosphate (2-deoxyR5P) as substrates for KDO8PS. Our analysis illuminates significant and previously unrecognised differences in the catalytic mechanisms of DAH7PS and KDO8PS.

P. furiosus DAH7PS is the most closely related DAH7PS to KDO8PS yet characterised.[15] Unlike other DAH7PSs (and like KDO8PSs) it is not subject to allosteric inhibition by shikimate pathway end products, and its structure reveals a basic catalytic $(\beta/\alpha)_8$-barrel with no significant extensions.[15,25] It has also been shown to be relatively ambiguous with respect to substrate selection with an expanded ability to accept the five-carbon phosphorylated sugars A5P and R5P.[15] Therefore we chose this enzyme to investigate the dispensability of metal-dependency in DAH7PSs. The *P. furiosus* DAH7PS Cys31Asn mutant was constructed and purified following established procedures.[26] This enzyme appeared to be identical to wild-type by native and SDS-PAGE. Comparison of the UV-visible spectrum of the Cys31Asn mutant in the presence of Cu^{2+} to that obtained with wild-type enzyme indicated that this mutant enzyme was unable to bind metal ions.[27] In contrast to the observations made with the equivalent mutations in the metal dependent KDO8PSs from both *Aquifex pyrophilis* and *A. aeolicus*,[21,22] the Cys31Asn mutant of *P. furiosus* DAH7PS showed no detectable enzymic activity with or without EDTA or added Mn^{2+}.

T4P was synthesised from D-diethyl tartrate in seven steps with an overall yield of 25% (Scheme 1). D-Diethyl tartrate was benzylated and reduced. The resulting diol was then monophosphorylated and oxidised using Dess–Martin periodinane, and the aldehyde functionality was protected as the dimethyl acetal. Hydrogenolysis and cleavage of the acetal gave rise to T4P. For the preparation of 2-deoxyE4P (*S*)-β-hydroxy-γ-butyrolactone was benzylated and then reduced to the lactol (Scheme 2). Following protection of the aldehyde functionality, the C4

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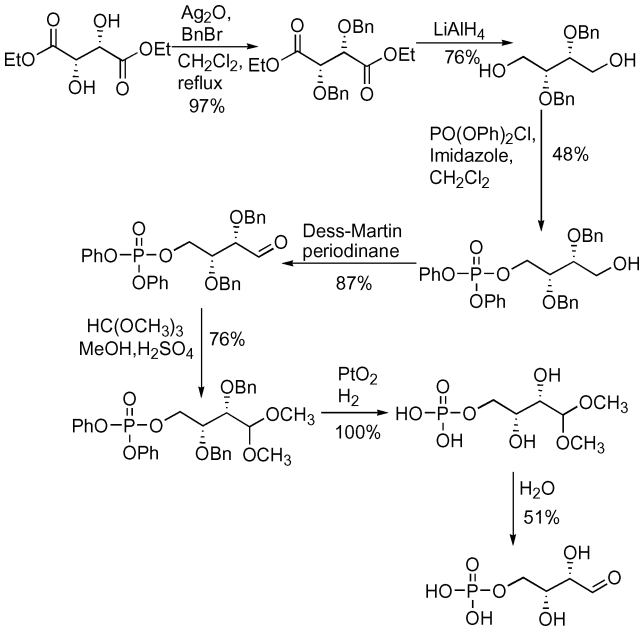
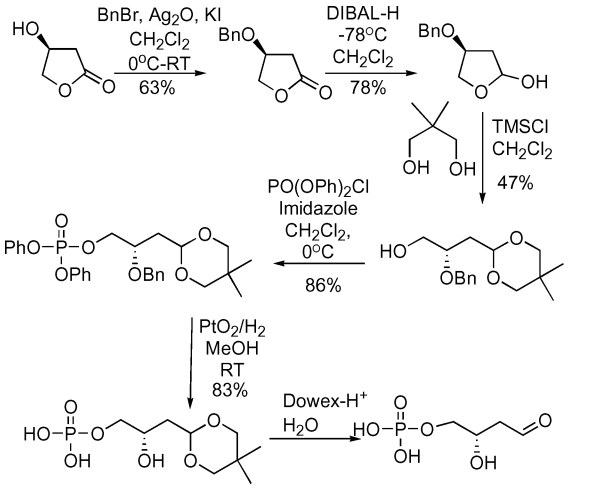


Scheme 1 Synthesis of T4P.



Scheme 2 Synthesis of 2-deoxyE4P.

hydroxyl group was phosphorylated. Deprotection with H_2 over platinum followed by dissolution in water gave 2-deoxyE4P.

T4P and 2-deoxyE4P were tested as an alternative substrate for DAH7PSs from both *E. coli* (Iα) and *P. furiosus* ($I\beta_D$). Both DAH7PSs were able to accept T4P and 2-deoxyE4P as alternative substrates to E4P (Table 1). Intriguingly, for *P. furiosus* DAH7PS, 2-deoxyE4P was preferred as a substrate to the "natural" substrate E4P, and T4P was utilised with a comparable efficiency. For *E. coli* DAH7PS a significant (but similar) increase in the K_m value was recorded with both alternative substrates. For both T4P and 2-deoxyE4P the seven carbon phosphorylated sugar products of the enzymatic reactions were isolated and characterised by both NMR and HRMS and were identified as the expected 3-deoxy-D-*lyxo*-heptulosonate 7-phosphate[28] and 5-deoxyDAH7P[29] respectively.

Commercially available R5P and 2-deoxyR5P were tested as substrates for the KDO8PS from *Neisseria meningitidis*[31] (Table 2). 2-DeoxyR5P was a poor substrate, and no evidence of substrate activity was observed for R5P, even at high substrate and enzyme concentrations. These observations parallel those made for the KDO8PS from *E. coli*.[23] Neither E4P, T4P or 2-deoxyE4P were able to act as alternative substrates for *N. meningitidis* KDO8PS. However, DAH7PS from both *E. coli* and *P. furiosus* were able to utilise the five carbon phosphorylated sugars A5P, R5P and 2-deoxyR5P.[15,32]

What then can account for differences in metal requirement and sugar substrate C2 configuration between DAH7PS and KDO8PS as detailed in these studies? On one hand, DAH7PSs appear to show an absolute requirement for metal for catalytic function, whereas for KDO8PSs the metal-binding is dispensable. On the other hand, DAH7PSs show an ambivalence to sugar substrate C2 configuration, yet KDO8PSs have a strict requirement for the correct (and opposite) stereochemistry at this position.

Careful comparison of the active sites of DAH7PS and KDO8PS reveals three absolutely conserved substitutions to residues that interact directly with either PEP or the aldose phosphate substrate[10–15,24] (Fig. 2): an Arg binds the PEP carboxylate in DAH7PS, whereas Lys is found in KDO8PS; secondly, an Arg in the PEP phosphate binding site in DAH7PS is substituted by Phe in KDO8PS; and, thirdly, in the aldose phosphate binding site, a Pro to AlaAsn substitution is found in the absolutely conserved Lys**Pro**ArgThr motif of DAH7PS (creating an equivalent conserved Lys**AlaAsn**ArgSer motif in KDO8PS). We propose that these latter two differences account for both the altered substrate specificity and metal requirement and give rise to different catalytic mechanisms.

Table 1 Kinetic parameters for E4P, T4P and 2-deoxyE4P with DAH7PS

DAH7PS Source[a]	Substrate	E4P	T4P	2-DeoxyE4P
E. coli	K_m/μM	39 ± 4	390 ± 13	410 ± 40
	k_{cat}/s^{-1}	26 ± 3	2.5 ± 0.2	25 ± 3
	k_{cat}/K_m/s^{-1} μM^{-1}	0.7 ± 0.1	0.006 ± 0.001	0.06 ± 0.01
P. furiosus	K_m/μM	9 ± 1[15]	21 ± 1	6 ± 1[15]
	k_{cat}/s^{-1}	1.4 ± 0.1[15]	2.4 ± 0.1	3.0 ± 0.1[15]
	k_{cat}/K_m/s^{-1} μM^{-1}	0.16 ± 0.03	0.11 ± 0.01	0.5 ± 0.1

[a] DAH7PS from both sources were assayed in accordance with previously reported procedures.[15,30]

Table 2 Kinetic parameters for A5P, R5P and 2-deoxyR5P with *N. meningitidis* KDO8PS

	Substrate	A5P	R5P	2-DeoxyR5P
N. meningitidis KDO8PS[31]	K_m/μM	12 ± 1	Not a substrate	230 ± 20
	k_{cat} /s^{-1}	2.7 ± 0.6		0.13 ± 0.01
	k_{cat}/K_m/s^{-1} μM^{-1}	0.23 ± 0.05		0.0006 ± 0.0001

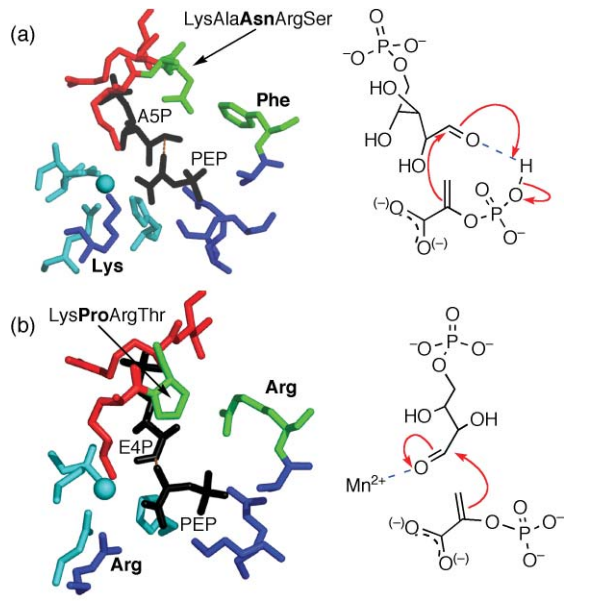


Fig. 2 Comparison of active sites and proposed (partial) reaction mechanisms for (a) KDO8PS (*A. aeolicus*, PDB 1FWW[12]) (b) DAH7PS (*P. furiosus* with E4P modelled, PDB 1ZCO[15]). E4P has been modelled into this structure based on the observed binding of glycerol 3-phosphate to *S. cerevisiae* DAH7PS[13] and the proposed binding of E4P to *T. maritima* DAH7PS.[14] The key changes discussed in the text are highlighted in green. Metal and metal ligands are in cyan and PEP ligands are shown in blue. Substrates, PEP and A5P (or E4P) are shown in black.

Substitution of positively charged Arg to hydrophobic Phe in KDO8PS eliminates a salt bridge to the PEP phosphate that is found in DAH7PS, and increases the hydrophobicity in the vicinity of the PEP phosphate group in KDO8PS. Modelling studies suggest that this allows the aldehyde functionality of A5P in KDO8PS to be positioned differently, and PEP to be bound to KDO8PS in its dianionic rather than the trianion form. Therefore, in KDO8PS the phosphate moiety of PEP may hydrogen-bond to the aldehyde oxygen of A5P. The second key substitution (Lys**AlaAsn**ArgSer rather than Lys**Pro**ArgThr) provides an additional binding contact for A5P ensuring correct placement of the aldehyde moiety in KDO8PS close to the PEP phosphate moiety.

The key chemical event in these condensation reactions is attack by C3 of PEP on the aldehyde group of co-substrate E4P or A5P. In DAH7PS, structural and modelling studies are consistent with activation of the aldehyde by the metal[13,14] (Lewis acid catalysis), meaning that the divalent metal plays an essential and indispensable role. In contrast, for KDO8PS, the metal (when present) is too far from the aldehyde functionality (~6 Å) to be involved in electrophilic activation,[12] and activation is by protonation (Brønsted acid catalysis). In KDO8PS, activation and positioning of the aldehyde moiety is more delicately choreographed. For KDO8PS the C2 hydroxyl group plays a critical role, as *via* coordination to metal or an Asn side chain (most likely *via* an intermediate water), the dihedral angle about the C1–C2 bond of A5P is controlled. Consequently, altering the configuration of C2 of A5P is catalytically catastrophic. DAH7PS on the other hand is tolerant to changes in the C2 position, with the *P. furiosus* accepting T4P or 2-deoxyE4P, with similar or greater efficiency than the natural substrate. This alternative placement of the aldehyde moiety means that despite the different C2 configuration in A5P and E4P, attack by C3 of PEP on its aldose co-substrate follows the Felkin-Anh model in both condensation reactions.

For either enzymic reaction, formation of the oxycarbenium ion intermediate (or transition state) will be followed by the attack of water on C2 of PEP. A water molecule located on the *re* face of PEP, observed in both DAH7PS and KDO8PS structures, is the likely candidate, giving overall (favourable) *anti* addition to PEP. Studies with enzyme mutants and substrate analogues are currently underway to test further these proposed mechanisms.

It should be noted that the structure of *A. aeolicus* KDO8PS in complex with R5P has also been solved.[24] Binding of R5P appeared to disrupt the coordination of a water molecule proposed at that time to act as the nucleophilic water in the catalytic mechanism. However, a direct catalytic role for the divalent metal ion in water activation has been largely discounted as metal-independent KDO8PS enzymes arise due to a single Cys to Asn mutation.[21,22] What is clear from these structures however, is that the carbonyl functionality in R5P adopts a significantly different orientation, entirely consistent with our analysis where we predict that appropriate interaction with the C2 hydroxyl is vital for addition of C3 of PEP to the carbonyl of A5P. The ability of 2-deoxyR5P to act as an alternative, yet poor substrate for KDO8PS noted in this study and by others[23] is consistent with a greater likelihood of this analogue accessing the reactive conformation. Likewise, this analysis would also appear to account for the recently reported disparity in behaviour with 3-fluoroPEP.[33] In this study the existence of significant differences between the PEP subsites of KDO8PS and DAH7PS have been proposed in order to account for the different abilities of the enzymes to process the two 3-fluoroPEP diastereoisomers.

Identification of key mechanistic similarities, the discovery of metal-dependent KDO8PSs, and phylogenetic analysis has led to the assumption that a common mechanism applies to these two related enzyme-catalysed reactions. This present study, together with a reinterpretation of existing substrate specificity and structural data, suggests that the evolutionary process that led to altered substrate specificity also gave rise to different mechanisms of catalysis. Consequently the divalent metal on which some KDO8PSs rely for catalytic activity, plays an altered and dispensable role in the enzyme-catalysed reaction. Its presence, however, in some enzymes, albeit as an evolutionary carry-over, provides further evidence for a common DAH7PS-like ancestor for this enzyme family.

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