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Enzymatic Synthesis of 3-Deoxy-D-*manno*-octulosonate 8-Phosphate, 3-Deoxy-D-*altro*-octulosonate 8-Phosphate, 3,5-Dideoxy-D-*gluco(manno)*-octulosonate 8-Phosphate by 3-Deoxy-D-*arabino*-heptulosonate 7-Phosphate Synthase

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Abstract: The phenylalanine-sensitive 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAH 7-P) synthase (phe), a key enzyme involved in the biosynthesis of the aromatic amino acid phenylalanine, expressed by the *Escherichia coli* gene *aro*G, which catalyzes the condensation of D-erythrose 4-phosphate with phospho*enol*pyruvate (PEP) to give DAH 7-P, was cloned into the expression vector pT7-7 for overexpression in *E. coli*. Purified enzyme from this expression system was used to demonstrate that DAH 7-P synthase (phe) also catalyses the aldol-type condensation of PEP with the 5-carbon analogues D-arabinose 5-phosphate, D-ribose 5-phosphate, and 2-deoxy-D-ribose 5-phosphate to yield 3-deoxy-D-*manno*-octulosonate 8-phosphate, 3-deoxy-D-*altro*-octulosonate 8-phosphate, and 3,5-dideoxy-D-*gluco(manno)*-octulosonate 8-phosphate, respectively, as determined by ^{1}H NMR and other standard analytical methods. The kinetic parameters, K_m and V_{max}, for these reactions were determined. The 3- and 6-carbon phosphorylated monosaccharides, D,L-glyceraldehyde 3-phosphate and D-glucose 6-phosphate, as well as the nonphosphorylated 5-carbon analogues D-arabinose 5-phosphate, D-ribose 5-phosphate, and 2-deoxy-D-ribose 5-phosphate were not substrates.

1. Introduction

The enzyme 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAH 7-P) synthase (phe), a key enzyme involved in the biosynthesis of phenylalanine, expressed by the *Escherichia coli* gene *aro*G, catalyzes the condensation of D-erythrose 4-phosphate (E 4-P, **1**) with phospho*enol*pyruvate (PEP) to give DAH 7-P (**2**) (Figure 1). Alternately, 3-deoxy-D-*manno*-octulosonate 8-phosphate (KDO 8-P) synthase, a key enzyme in the biosynthesis of the lipopolysaccharide (LPS) region of G-negative organisms, expressed by the *E. coli* gene *kds*A, catalyzes the condensation of D-arabinose 5-phosphate (A 5-P, **4**) with PEP to give KDO 8-P (**7**) (Figure 2). Sequence alignments of the two genes from *E. coli* reveal minimal significant regions of either identity or homology;[1] however, both enzymes catalyze an identical aldol-type condensation in which a "carbanion" generated on the *si* face of PEP attacks the *re* face of the carbonyl of the respective phosphorylated monosaccharide.[2−5] Both of the enzymes are also members of a unique class of

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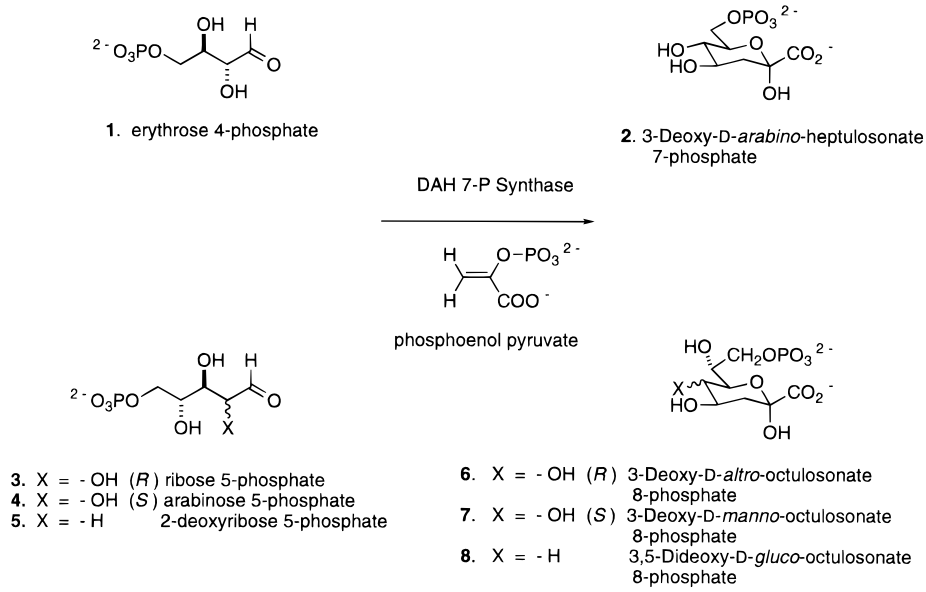

Figure 1. Alternate substrates utilized by *E. coli* DAH 7-P synthase (phe).

PEP-utilizing enzymes in which loss of the phosphate moiety of PEP occurs through cleavage of the C−O bond rather than through the more common mode in which the O−P bond is broken.[6,7] DAH 7-P synthase (phe) is tetrameric, requires the presence of a divalent metal, and is inhibited by L-phenylalanine.[8] KDO 8-P synthase, on the other hand, is trimeric, does not require a metal, nor has any known feedback inhibitor.[9]

Interest in the utilization of enzymes in the synthesis of natural and unnatural monosaccharides has prompted numerous investigations into determining the substrate specificity profiles for various enzymes involved in carbon−carbon bond formation, in particular those catalyzed by aldolases.[10] Broad substrate specificity or substrate "ambiguity" has been shown by Sugai et al.[11] for the KDO forming enzyme, KDO aldolase [E.C. 4.1.2.23] (not KDO 8-P synthase [E.C. 4.1.2.16]). KDO aldolase catalyzes the aldol condensation of pyruvate (not PEP) with a large number of nonphosphorylated monosaccharides including trioses, tetroses, pentoses, and hexoses, especially those that possess the 3*R*-configuration, in addition to its natural substrate D-arabinose. The condensation is *re*-face specific with respect to the monosaccharide as is the condensation catalyzed by both of the enzymes, DAH 7-P and KDO 8-P synthases. This study suggests that the KDO 8-P synthase may also tolerate alternate substrates, and indeed several studies have indicated this to be the case. In particular, the phosphonate analogue of A 5-P, 5,6-dideoxy-6-phosphono-D-*arabino*-hexose,[12] and 4-deoxy-D-arabinose 5-phosphate are alternate substrates for *E. coli* KDO 8-P synthase.[13] Ray et al.[14] tested a number of other phosphorylated monosaccharides, including E 4-P, as alternate substrates for purified KDO 8-P synthase from *E. coli*, utilizing the Aminoff method[15] and found no alternate substrates and only one inhibitor, ribose 5-phosphate (R 5-P) (K_i = 5 mM). Other more recent reports suggesting substrate ambiguity by KDO 8-P synthase, isolated from crude extracts of several plants as well as from *Neisseria gonorrheae*, have appeared.[1,16] However, these studies, unlike those reported by Sugai et al.,[11] for KDO aldolase failed to adequately characterize either the enzyme responsible for the transformation or the saccharide product obtained, to firmly establish substrate ambiguity.

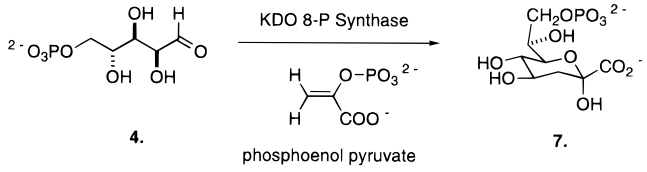

Figure 2. Reaction catalyzed by *E. coli* KDO 8-P synthase.

Broad substrate specificity has also been reported for the pyruvate aldolases, 2-keto-3-deoxy-6-phospho-gluconate (KDPG) aldolase [E.C. 4.1.2.14][17] and *N*-acetyl-neuraminic aldolase [E.C. 4.1.3.3].[17] KDPG aldolase, dissimilar to KDO aldolase, catalyses the *si*-face condensation of pyruvate to the aldehyde functionality of D-erythrose to give 3-deoxy-D-*ribo*-2-heptulosonate (DRH). These studies suggest that DAH 7-P synthase may show broad substrate specificity. Indeed, for *E. coli* DAH 7-P synthase (phe), the two phosphonate analogues of E 4-P, 4-deoxy-4-phosphono-D-*erythro*-tetrose and 4,5-dideoxy-5-phosphono-D-*erythro*-pentose have been shown to be alternate substrates.[6,18] The 3-carbon monosaccharide, D-glyceraldehyde 3-phosphate, however, has been shown to be neither a substrate nor an inhibitor.[19] It has been recently reported that purified overexpressed *E. coli* DAH 7-P synthase (trp) utilizes R 5-P as an alternate substrate; however, no kinetic data was reported,

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Table 1. DAH 7-P Synthase(phe) Kinetics with Alternate Substrates[a]

monosaccharide	K_m^{PEP} (μM)	$K_m^{Monosaccharide}$ (μM)	V_{max} (μmol/mg/min)
G 3-P	NS	NS	
E 4-P	5.3	141	16.3
erythrose	NS	NS	
A 5-P	5	30	0.085
R 5-P	10	6000	1.13
2-deoxyR 5-P	15	6800	0.73
arabinose	NS	NS	
ribose	NS	NS	
2-deoxyribose	NS	NS	
G 6-P	NS	NS	
A 5-P[b]	6	23.9	5.9
R 5-P[b]		5000 (K_i)[c]	

[a] Values determined by the method of Schoner and Herrmann.[22] NS, not a substrate. [b] Values for KDO 8-P synthase (*E. coli*) and determined by all three spectrophotometric methods.[22,24,25] [c] Value given is the K_i determined by method 3;[15] the abbreviations used are: A 5-P, D-arabinose 5-phosphate; DAH 7-P, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; E 4-P, erythrose 4-phosphate; 2-deoxyR 5-P, 2-deoxyribose 5-phosphate; G 6-P, D-glucose 6-phosphate; G 3-P, D,L-glyceraldehyde 3-phosphate; R 5-P, ribose 5-phosphate.

nor was the product from the condensation reaction isolated.[20] Recently, substrate ambiguity of DAH 7-P synthases from crude plant extracts has been explored; however, no analytical data supporting the identity of the products from the incubation was presented.[21] Besides these few reports, there is no exhaustive study of alternate monosaccharides (or their phosphorylated derivatives) as substrates for purified *E. coli* DAH 7-P synthase (phe) or the other two *E. coli* isozymes (*aro*H and *aroF*).

Our interest in elucidating the details of the mechanisms of DAH 7-P and KDO 8-P synthases led us to more carefully examine the substrate specificity of each enzyme for the monosaccharide substrate in a systematic manner. Our goals were to isolate and fully characterize the product(s) from the enzymatic condensations of diverse monosaccharides and PEP by these two enzymes and to determine the kinetic parameters of the process. The substrate diversity of purified *E. coli* DAH 7-P synthase (phe) is reported herein.

Results

To explore the active site topology and to investigate the substrate specificity of DAH 7-P synthase for the monosaccharide, a number of 3-, 5- and 6-carbon monosaccharides were used as probes along with the natural 4-carbon monosaccharide substrate, E 4-P.

The progress of the condensation of PEP with the monosaccharides listed in Table 1 in the presence of 95% pure overexpressed DAH 7-P synthase (phe) was monitored by three different methodologies, (1) the continuous ultraviolet spectral assay originally described by Schoner and Herrmann[22] for DAH 7-P synthase to monitor the disappearance of the absorption at $\lambda = 232$ nm ($\epsilon = 2800$) that is due to the double bond of PEP, (2) a purine nucleotide phosphorylase-coupled continuous assay to detect the phosphate released during the condensation[23−25] and (3) the classic discontinuous colorimetric Aminoff (thiobarbituric acid) assay to detect the presence of a 2-deoxymonosaccharide.[15,26] The ability of a monosaccharide to act as a substrate for DAH 7-P synthase was first established by demonstrating the loss of the double bond of PEP by method 1 and then correlating this loss with the concomitant formation of an equivalent quantity of phosphate by method 2. Finally, the presence of a 2-deoxymonosaccharide product was verified via the Aminoff methodology (method 3).

Utilizing this series of kinetic methods, we have established that DAH 7-P synthase utilized not only its natural 4-carbon monosaccharide phosphate (E 4-P) to form DAH 7-P but also the three 5-carbon monosaccharide phosphate analogues, ribose 5-phosphate (R 5-P), 2-deoxyribose 5-phosphate (deoxyR 5-P), and arabinose 5-phosphate (A 5-P), the natural substrate for KDO 8-P synthase. If the divalent metal, Mn(II), was omitted from the incubation mixture or if L-phenylalanine, the feedback inhibitor of the reaction, was included in the assays, no turnover was observed by any of the assay methodologies, indicating that the transformation being observed is indeed due to DAH 7-P synthase (phe).

The K_m and V_{max} for both PEP and the respective monosaccharide phosphate analogues were determined and are given in Table 1. DAH 7-P synthase (phe) binds A 5-P tighter than R 5-P or 2-deoxyR 5-P and 4 times tighter than it binds its natural substrate, E 4-P. The rate of turnover for A 5-P, however, is the slowest of the three 5-carbon monosaccharide phosphate analogues, which is 200 times slower than the rate of turnover for E 4-P. The K_m values for PEP with all of the substrate monosaccharides, as compared to E 4-P, were practically the same.

To unequivocally establish the identity of the reaction products of the DAH 7-P synthase (phe)-catalyzed enzymatic condensation of the various monosaccharides with PEP, a large scale enzymatic reaction was performed with 95% pure cloned enzyme. The reaction was quenched with trichloroacetic acid, and the reaction mixture was subjected to anion-exchange chromatography. The fractions containing the product deoxymonosaccharide, as determined by the Aminoff method, were pooled, freeze-dried, and dissolved in D_2O for ^1H NMR analysis. The identity of the monosaccharide product as well as the stereospecificity of the condensation reaction, with respect to the aldehyde carbon of the monosaccharide (i.e., the stereochemistry at C-4 of the new product monosaccharide), was established by comparing the ^1H NMR of the product with the ^1H NMR of known KDO derivatives.[11] The ^1H NMR spectra (Figure 3) clearly demonstrate that DAH 7-P synthase has catalyzed the condensation of PEP with R 5-P, deoxyR 5-P, and A 5-P to form 3-deoxy-D-*altro*-octulosonate 8-phosphate, 3,5-dideoxy-D-*gluco(manno)*-octulosonate 8-phosphate, and 3-deoxy-D-*manno*-octulosonate 8-phosphate, respectively. The stereochemistry, at the newly created C-4 position of each of the KDO 8-P analogues, formed from their respective 5-carbon analogues, indicated that the attack of the PEP moiety was onto the *re* face of the carbonyl function, which is identical to the stereospecificity observed with E 4-P as the substrate. Further characterization of the reaction products by mass spectroscopy and optical rotation measurement substantiated the structural assignments.

DAH 7-P synthase (phe) was not able to utilize either the 6-carbon or 3-carbon phosphorylated monosaccharides, D-glucose 6-phosphate or D,L-glyceraldehyde 3-P,[19] respectively,

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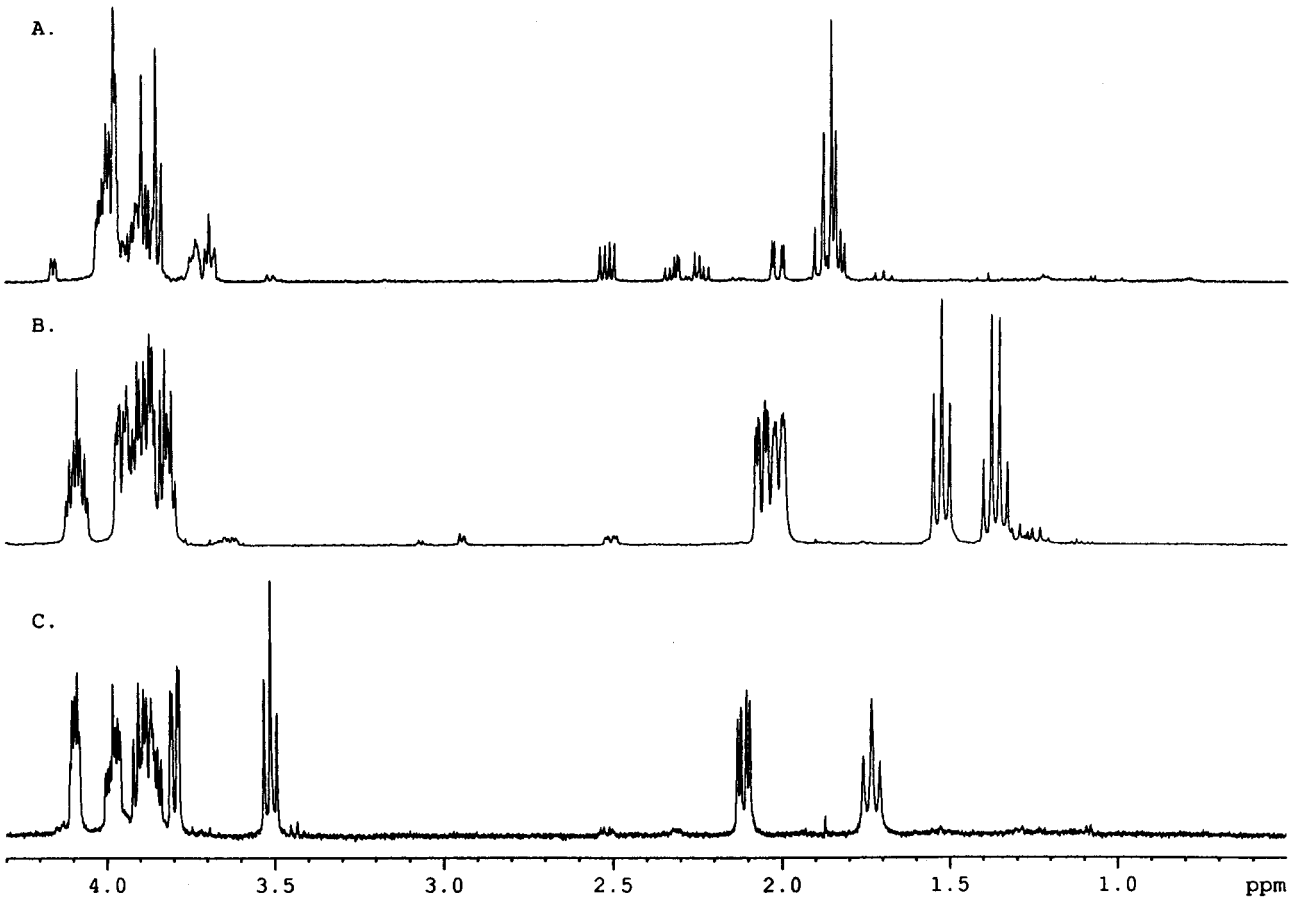

Figure 3. ^{1}H NMR spectra at 500 MHz of the purified small molecule component isolated from the incubation of DAH 7-P Synthase (phe) and PEP with: (A) arabinose 5-phosphate, (B) 2-deoxyribose 5-phosphate, and (C) ribose 5-phosphate.

at concentrations as high as 3 mM (see Table 1) as determined by any of the assays described. Furthermore, the presence of the phosphate group is also necessary for catalytic activity.

Discussion

In the present work, we have demonstrated by three different kinetic methodologies that DAH 7-P synthase is able to catalyze the aldol condensations with PEP of three 5-carbon phosphorylated monosaccharides, A 5-P, R 5-P, and deoxyR 5-P, but not their corresponding nonphosphorylated derivatives. The 6-carbon, D-glucose 6-phosphate, or 3-carbon, D,L-glyceraldehyde 3-phosphate, monosaccharides were not substrates even at high concentrations. Thus, the active site topology of DAH 7-P synthase can accommodate a substrate that is 1 carbon longer than the natural substrate E 4-P but not 1 carbon shorter or 2 carbons longer. Utilization of 5-carbon monosaccharides by DAH 7-P may suggest a similarity in mechanism with KDO 8-P synthase which utilizes a 5-carbon monosaccharide as substrate. In addition, the phosphate group on the monosaccharide appears to be absolutely essential for activity. The phosphate group may be one of the binding determinants helping to position the monosaccharide into the proper location in the active site, thus orienting the distal carbonyl functionality into the proper position for attack by the "carbanion-like" species generated at C-3 of PEP.

Synthetic scale incubation of the three 5-carbon monosaccharide phosphate analogues, R 5-P, deoxyR 5-P, and A 5-P, with PEP in the presence of large quantities of purified enzyme allowed the isolation and characterization of the KDO 8-P analogue products, 3-deoxy-D-*altro*-octulosonate 8-phosphate, 3,5-dideoxy-D-*gluco(manno)*-octulosonate 8-phosphate, and 3-deoxy-D-*manno*-octulosonate 8-phosphate, respectively. Intrinsic in this observation is the fact that each of these KDO analogues have the same stereochemistry at C-4 (formerly C-1 of the monosaccharide) indicating that *all* three of the condensation reactions occurred with *re*-face selectivity, with respect to the aldehyde of the monosaccharides, which is identical to the face selectivity observed for E 4-P with DAH 7-P synthase and for A 5-P with KDO 8-P synthase. These results strongly suggest commonality in the geometry of binding of the carbonyl portion of these alternate substrates in the active site of DAH 7-P synthase as well as commonality in the carbonyl binding motifs of both enzymes despite their poor primary amino acid sequence homology.

Since the stereochemistry of *all* three of the 5-carbon monosaccharides [A 5-P (2*S*, 3*R*, 4*R*), R 5-P (2*R*, 3*R*, 4*R*), 2-deoxyR 5-P (3*S*, 4*R*)] is identical at C-3 and C-4 (the descriptor change for the deoxy is due to a change in priorities, not in absolute stereochemistry at C-3), the stereochemistry at the C-2, which is different for each monosaccharide, must account for the profound differences observed in the K_m and the rate of the reaction. A 5-P has the same stereochemistry at C-2 as does the product of the normal reaction catalyzed by DAH 7-P synthase between E 4-P and PEP, namely 2*S* for A 5-P and 4*R* for DAH 7-P (4*R*, 5*S*, 6*R*) (note the change in priority and elongation of the carbon chain by 3 carbon atoms due to the condensation with PEP). It, therefore, may not be surprising that A 5-P has a lower K_m than E 4-P (Table 1) since the C-2 of A 5-P may mimic the "C-4" of the transition state (or postreaction intermediate) of the condensation of E 4-P with

PEP. While this may add positively to the K_m, it may have a negative effect on the rate since the geometry of the metal, the carbonyl of A 5-P, and the C-3 "carbanion" of PEP may no longer be optimal for facile condensation. At C-2, R 5-P is "*R*", whereas deoxyR 5-P, having two hydrogens at C-2, is prochiral. DeoxyR 5-P could also be thought of as deoxyA 5-P; thus, the differences in K_m between A 5-P and deoxyR 5-P may only be due to the loss of the binding energy associated with the loss of the C2−OH hydrogen bonding. While a K_m in the millimolar range is not impressive, it is interesting to note that the K_m for R 5-P with DAH 7-P synthase is very similar to the K_i reported for R 5-P with KDO 8-P synthase. Again, it is tempting to infer some active-site structural similarities between these two enzymes from these values, but further studies are needed.

Other factors that affect the rates of enzymes that utilize monosaccharides are the equilibrium between the acyclic and cyclic form and the distribution of the various cyclic forms. Since E 4-P, the natural substrate for DAH 7-P synthase, exists only in the acyclic form, the active site of DAH 7-P has most likely evolved to accommodate a linear (acyclic) phosphorylated monosaccharide. It has been clearly demonstrated for KDO 8-P synthase that the phosphorylated monosaccharide reacts at the active site in the acyclic form.[13] ^{13}C NMR data[27] suggest that at equilibrium the concentration of the acyclic form of A 5-P, both the hydrate and free carbonyl form, is 4 times higher than that for R 5-P. Thus, an additional contributing factor to help explain the difference in the K_m between A 5-P and R 5-P for DAH 7-P synthase may be the concentration of acyclic monosaccharide present in the enzymatic reaction.

We have demonstrated unequivocally that purified overexpressed *E. coli* DAH 7-P synthase (phe) catalyses the condensation of PEP with R 5-P, deoxyR 5-P and A 5-P to yield 3-deoxy-D-*altro*-octulosonate 8-phosphate, 3,5-dideoxy-D-*gluco(manno)*-octulosonate 8-phosphate, and 3-deoxy-D-*manno*-octulosonate 8-phosphate, respectively. While the kinetic parameters suggest that these analogues probably do not act as natural substrates for DAH 7-P synthase, their reactivity and kinetic parameters are important factors in mapping common topologies and amino acid residues at the active sites as well as assisting in the potential elucidation of the mechanism of this very important enzyme.

The results from a similar study describing *E. coli* KDO 8-P synthase's monosaccharide substrate specificity will appear elsewhere, as will a report detailing the stereochemistry of both enzyme reactions with respect to the face selectivity of PEP during the aldol-type condensation.

4. Experimental Section

Materials. Restriction and DNA-modifying enzymes were from Boehringer Mannheim, New England Biolabs, and Gibco BRL. The Promega DNA purification kit and the 5 prime → 3 prime Perfectprep Plasmid DNA Kit were utilized for plasmid isolation and purification. The *E. coli* strain BL 21(DE 3) was obtained from Novagen. The XL-Blue 1 super competent cells and the QuikChange Mutagenesis kit were obtained from Stratagene Cloning Systems. *Vent* DNA polymerase was purchased from New England Biolabs and was substituted for the recombinant *Pfu* DNA polymerase provided in the QuikChange Mutagenesis kit. The thermal cycling was performed using an MJR Research Thermal cycler. The mutagenic oligonucleotides were synthesized by the University of Michigan Biomedical Research Resources Core Facility using β-cyanoethyl phosphoramidite chemistry on polystyrene support columns. DNA sequencing was performed by the University of Michigan Biomedical Research Resources Core Facility using ABI model 373A DNA sequencers. Phospho*enol*pyruvate mono(cyclohexyl)ammonium salt and manganese (II) chloride were obtained from the Sigma Chemical Company. The 1,3-bis[tris-(hydroxymethyl)methylamino]propane was purchased from Calbiochem. Erythrose-4-phosphate was prepared in our laboratory by lead tetraacetate oxidative degradation of glucose-6-phosphate.[28]

Cloning of *aro*G. Two primers were constructed to correspond to the 5′ and 3′ ends of the open reading frame identified as the *aro*G (DAHP-phe) gene (16.94 min of the *E. coli* map). The 5′ sequence was 5′-GATTCTAGAATTCATATGAATTATCAGAACGACG-3′. The 3′ sequence was 5′-GATTCTGAATTCGGATCCTTACCCGCGAC-GCGCTTTTACTGC-3′. The primers incorporated an *Eco*RI (underlined) for the forward primer and a *Bam*HI (underlined) site for the reverse primer for cloning into the expression vector, pT7-7.[29] The *aro*G gene was amplified from Kohara λ phage fragment miniseries #179 by standard PCR methodologies.[30] The annealing temperature was 45 °C. After 35 cycles, the amplified DNA was purified and subcloned into pT7-7 that had been digested with *Eco*RI and *Bam*HI. The resulting plasmid contained a 15 bp fragment 5′ to the ATG codon of *aroG* beginning with the ATG codon provided by pT7-7. This inframe fragment would result in the expression of DAH 7-P synthase with five additional N-terminal amino acid residues. To delete this fragment, the single internal *Nde*I restriction site was eliminated utilizing the Stratagene QuikChange Mutagenesis Kit with the following mutagenic primers: 5′ sequence (forward) was 5′-CTGATTAAC-GATCCGCACATGGATAATAGCTTCCAG-3′ and the 3′ sequence (reverse) was 5′-CTGGAAGCTATTATCCATGTGCGGATCGT-TAATCAG-3′. The resulting plasmid was restricted with *Nde*I, releasing the 15 bp fragment, and then religated. The correct length pT7-7/aro*G* was placed into BL 21 (DE 3) cells for overexpression.

Overexpression of DAH 7-P Synthase. *E. coli* BL 21 (DE 3) cells harboring the plasmid pT7-7/*aroG* were grown in 2×TY medium at 37 °C. IPTG was added to the culture at the mid logarithmic phase ($A_{600} = 0.6$) to a final concentration of 400 μM to induce expression. The cells were harvested 4 h postinduction by centrifugation, resuspended in 10 mM BTP buffer (pH 6.8), and sonicated (sonicator W220, Heat Systems Ultrasonics) for 2 min at 4 °C (4 × 30-s pulses with 1 min delay between pulses). Cell debris was removed by centrifugation at 12000*g* for 20 min at 4 °C. All other manipulations of the enzyme were performed at 4 °C unless otherwise stated. DAH 7-P synthase (phe) was purified by anion-exchange chromatography on a High Q Cartridge column (BioRad). The protein on the column was first washed with 45 mL of 10 mM BTP buffer (pH 6.8) followed by a linear gradient of 0−0.5 M KCl (100 mL total volume) in 10 mM BTP buffer (pH 6.8) at a flow rate of 1.7 mL/min. The fractions (4 mL each) exhibiting DAH 7-P synthase activity as determined by the PEP disappearance assay were pooled, concentrated to 4 mg/mL, and stored at −80 °C. The protein was determined to be >95% pure by SDS−PAGE analysis. Results of analytical analysis such as total amino acid composition and molecular weight of the DAH 7-P protein were consistent with that predicted from the DNA sequence. The frozen enzyme retained activity for several months.

Kinetic Methods

1. Loss of Absorbance at 232 nm Due to the Loss of PEP's Double Bond. The determination of K_m for PEP and A 5-P (or other phosphorylated monosaccharides) utilized a spectrophotometric assay based on the absorption of the PEP double bond ($\epsilon^{232} = 2840$ M^{-1} cm^{-1}) at $\lambda = 232$ nm. The final reaction mixture contained PEP, A 5-P, 100 mM Tris-acetate (pH 7.5), 1 mM $MnCl_2$ and ∼100 nM DAH 7-P synthase in 1 mL total volume. K_m^{PEP} was determined with A 5-P at saturating levels and PEP concentrations varying from $^1/_{10}$ K_m to 10 K_m. Similarly, $K_m^{A\ 5-P}$ was determined with PEP at saturating

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concentration and the A 5-P concentration varying from $^1/_{10}K_m$ to $10K_m$. The reaction mixture, excepting DAH 7-P synthase, was incubated at 25 °C for 5 min. The reaction was initiated by the addition of enzyme, and absorbance data were collected at $\lambda = 232$ nm. The initial rate of reaction was determined by a linear fit of the initial rate data (typically 5−35 s). The concentration of substrate was plotted versus initial rate, and the data were fit to the Michaelis−Menten equation using the least-squares method. KaleidaGraph (v 3.08d for Power Macintosh) was used for all data manipulation.

2. Phosphate Liberation Assay. The determination of K_m for PEP and A 5-P (or other phosphorylated monosaccharides) utilized a spectrophotometric assay based on the change in absorbance between 7-methylinosine (m^7-Ino) and 7-methylhypoxanthine (5.091 $\Delta A/\mu M_{P_i}$ at $\lambda = 280$ nm or 2.3334 $\Delta A/\mu M_{P_i}$ at $\lambda = 291$ nm). In a total volume of 1 mL, the reaction mixture contained PEP, A 5-P, 100 mM Tris-acetate (pH 7.5), 1 mM $MnCl_2$, 100 μM m^7-Ino, ∼200 nM recombinant bacterial purine nucleoside phosphorylase (PNPase), and ∼100 nM DAH 7-P synthase. The reaction mixture, excepting DAH 7-P synthase, was incubated at 25 °C for 5 min to allow for the reaction of any inorganic phosphate present from the chemical degradation of substrates. The enzymatic reaction was initiated by the addition of enzyme, and absorbance data were collected at either $\lambda = 280$ or 291 nm. The kinetic parameters were determined as described above.

3. Aminoff Method: Formation of 3-Deoxymonosaccharides. In a standard colorimetric fixed-point assay, DAH 7-P synthase (1 pmole) was added to a reaction mixture of 3.0 mM phospho*enol*pyruvate, 3.0 mM A 5-P (or test monosaccharide), 3 mM $MnCl_2$, and 100 mM Tris-acetate (pH 7.5) (or BTP) buffer for a total volume of 150 μL. This mixture was incubated at 37 °C for 10 min and then quenched with 10% trichloroacetic acid to a final volume of 300 mL. The concentration of KDO 8-P (or product monosaccharide) was determined by subjecting 100 μL of the enzymatic reaction mixture, in a 10 mL glass tube, to total oxidation with 200 μL of $NaIO_4$ (25 mM in 0.125 N H_2SO_4) at 20° for 10 min for the potential 8-carbon products (60° for 10 min for all other deoxymonosaccharides). The excess oxidizing reagent was reduced by the addition of 400 μL of $NaAsO_2$ (2 wt %/vol in 0.5 N HCl). Following the disappearance of the yellow color, 2 mL of a thiobarbituric acid solution (0.36 wt %/vol, pH = 9 adjusted with NaOH) was added and the tube heated to 100 °C for 10 min. After the samples were cooled to room temperature, the optical density of the samples was measured at $\lambda = 549$ ($\epsilon = 1.03 \times 10^5$ for the chromophore formed between α-formylpyruvate and thiobarbiturate; calculated with purified KDO 8-P as standard). Due to low yields in the oxidation step, the concentration of DAH or DAH 7-P is underestimated by 70% as determined with purified DAH 7-P as standard.

Instrumental Methods. The ^{1}H NMR was measured on a Bruker Advance DRX 500 (1H, 500.13235 MHz) with a 5-mm multinuclear inverse gradient probe using the water suppression program, Watergate gradient suppression. The molecular weights were obtained on an HPLC-MS System utilizing a HP Series 1100 pump with Gilson 215 autosampler and Micromass Platform LC detector. The $[\alpha]^{25}_D$'s were acquired on a Jasco DIP-370 Digital Polarimeter.

Enzymatic Synthesis of KDO 8-P Analogues. DAH 7-P synthase (2 mg, 53 nmoles) was added to a 10-mL plastic centrifuge tube containing the following: phospho*enol*pyruvate monocyclohexylamine salt (7 mg, 0.026 mmol), monosaccharide analogue (13.0 mg for each analogue, 0.061 mmol for 2-deoxy-R 5-P and 0.047 mmol for A 5-P and R 5-P), BTP (1,3-bis[tris(hydroxymethyl)-methylamino]propane) pH 6.8 (0.075 mmol), $MnCl_2$ (1.5 μmol), and H_2O in a final volume of 1.5 mL. This reaction mixture was incubated at 37 °C for 2 h in a water bath. The reaction mixture was quenched by the addition of 0.3 mL of 10% trichloroacetic acid (w/v), centrifuged to remove precipitated protein, and immediately loaded onto a 5-mL Econo-Pac High Q Anion-Ion Exchange Cartridge (BioRad) in the chloride form preequilibrated with water. After loading, the column was washed with 20 mL of water at a flow rate of 1 mL/min and then eluted with a linear gradient of 0 mM to 500 mM LiCl (60 min at 1 mL/min). Two milliliter fractions were collected. One major periodate-TBA positive peak was obtained at 200 mM LiCl for the KDO 8-P and analogues except for the 2-deoxy-R 5-P for which two peaks were obtained. Some small amounts of the dephosphorylated KDO and KDO analogues were observed in an earlier fraction for all three analogues but were <5%. The fractions containing the KDO 8-P or KDO 8-P analogue were pooled, freeze-dried, and reconstituted in 0.5 mL of D_2O for analysis.

3-Deoxy-D-*manno*-octulosonate 8-phosphate. The title compound, derived from the incubation of PEP with A 5-P in the presence of DAH 7-P synthase, was obtained in 46% yield as determined by the Aminoff method with PEP as the limiting reagent. ^{1}H NMR (D_2O) for α-pyranose form δ 1.836 (H-3eq, $J_{3eq,3ax} = 13.5$ Hz, $J_{3eq,4} = 6.2$ Hz, $J_{3eq,5} = 1.0$ Hz), 1.892 (H-3ax, $J_{3ax,4} = 13.0$ Hz); for β-pyranose form δ 2.226 (H-3eq, $J_{3eq,3ax} = 13.2$, $J_{3eq,4} = 7.0$, $J_{3eq,5} = 1.0$ Hz), 1.700 (H-3ax, $J_{3ax,4} = 12.4$ Hz); for furanose form δ 2.238 (H-3eq, $J_{3eq,3ax} = 13.5$, $J_{3eq,4} = 7.0$ Hz), 2.327 (H-3ax, $J_{3ax,4} = 7.5$ Hz); for lactone form δ 2.015 (H-3eq, $J_{3eq,3ax} = 14.3$, $J_{3eq,4} = 3.2$ Hz), 2.519 (H-3$_{ax}$, $J_{3ax,4} = 7.3$ Hz); $[\alpha]^{25}_D = +39.7°$ (*c* 0.38, water).

3-Deoxy-D-*altro*-octulosonate 8-phosphate. The yield for the title compound, derived from the incubation of PEP with R 5-P in the presence of DAH 7-P synthase, was 60% as determined by the Aminoff method with PEP as the limiting reagent. ^{1}H NMR (D_2O) δ 1.734 (1 H, dd, $J_{3ax,4} = 12.0$ Hz, $J_{3ax,3eq} = 12.8$ Hz, H-3ax), 2.112 (1 H, dd, $J_{3eq,4} = 5.1$ Hz, $J_{3ax,3eq} = 12.6$ Hz, H-3eq), 3.514 (1 H, dd, $J_{5,4} = 9.0$ Hz, $J_{5,6} = 9.8$ Hz, H-5), 3.982 (1 H, ddd, $J_{8,7} = 6.8$ Hz, $J_{8,8'} = 10.9$ Hz, H-8), 4.096 (1 H, ddd, $J_{8',7} = 6.8$ Hz, $J_{8',8} = 10.9$ Hz, H-8′), 3.799 (1 H, dd, $J_{6,7} = 2.8$ Hz, $J_{6,5} = 10.0$ Hz, H-6) H-4 and H-7 is a complex multiplet at δ 3.882; $[\alpha]^{25}_D = +39.7°$ (*c* 0.28, water).

3,5-Dideoxy-D-*gluco*(*manno*)-octulosonate 8-phosphate. Since the oxidation of the title KDO derivative, derived from 2-deoxy-R 5-P as the monosaccharide, with periodate to give α-formylpyruvate, would at best be very poor (the oxidation works best with gem diols although oxidation can occur at isolated -CH_2OH-'s), the yield (94%) was calculated based on the amount of 2-deoxy-R 5-P remaining at the end of the reaction, again based on PEP as the limiting reagent. The amount of 2-deoxy-R 5-P was quantitated by the Aminoff method except at $\lambda = 532$ nm and $\epsilon_{532} = 8.3 \times 10^3$ M^{-1} cm^{-1}. ^{1}H NMR (D_2O) δ 1.365 (1 H, ddd, $J_{5ax,4} = 11.8$ Hz, $J_{5ax,4} = 11.9$ Hz, $J_{5ax,5eq} = 12.2$ Hz, H-5ax), 1.526 (1 H, dd, $J_{3ax,4} = 12.2$ Hz, $J_{3ax,3eq} = 12.7$ Hz, H-3ax), 2.011 (1 H, dddd, $J_{5eq,3eq} = 1.7$ Hz, $J_{5eq,6} = 2.4$ Hz, $J_{5eq,4} = 4.4$, $J_{5eq,5ax} = 12.2$ Hz, H-5eq), 2.062 (1 H, ddd, $J_{3eq,5eq} = 1.8$ Hz, $J_{3eq,4} = 4.6$ Hz, $J_{3eq,3ax} = 12.7$ Hz, H-3eq), 4.088 (1 H, ddddd, $J_{4,3eq} = 4.7$ Hz, $J_{4,5eq} = 4.7$ Hz, $J_{4,5ax} = 11.5$, $J_{4,3ax} = 11.5$ Hz, H-4), H-6, H-7, H-8, and H-8′ is a complex multiplet at δ 3.884; $[\alpha]^{25}_D = +25.1°$ (*c* 0.72, water).

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