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Neisseria gonorrhoeae 3-Deoxy-D-*arabino*-heptulosonate 7-Phosphate Synthase Does Not Catalyze the Formation of the *ribo* Analogue

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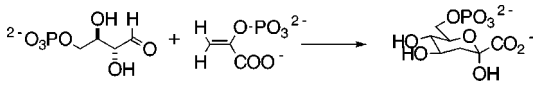
ABSTRACT



Neisseria gonorrhoeae **3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAH 7-P) synthase catalyzes an aldol-type condensation between D-erythrose 4-phosphate and phospho*enol*pyruvate (PEP) to form 3-deoxy-D-*arabino*-heptulosonate 7-phosphate and *not* 3-deoxy-D-*ribo*-heptulosonate 7-phosphate. Similar to the *Escherichia coli* enzyme, *N. gonorrhoeae* DAH 7-P synthase condenses D-arabinose 5-phosphate with PEP to give 3-deoxy-D-*manno*-octulosonate 8-phosphate. Therefore, the stereochemistry of the reaction catalyzed by *N. gonorrhoeae* DAH 7-P synthase at C1 of the phosphorylated monosaccharide is the same as that for the *E. coli* enzyme, namely, *re* face attack.**

The Shikimate pathway in microorganisms and plants leads to the biosynthesis of aromatic amino acids and other aromatic cofactors essential for cellular functions.[1] The formation of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAH 7-P) from D-erythrose 4-phosphate (E 4-P) and phospho*enol*pyruvate (PEP),[2] the first committed step in the Shikimic acid pathway, is catalyzed by the enzyme DAH 7-P synthase (Figure 1). In *Escherichia coli*, three isozymes of DAH 7-P synthase have been identified.[3] Each isozyme responds to feedback regulation by one and only one of the aromatic amino acids. These isozymes are denoted as phenylalanine-sensitive (encoded by the gene *aroG*), tyrosine-sensitive (encoded by the gene *aroF*) and tryptophan-sensitive (encoded by the gene *aroH*) DAH 7-P synthases. Since in *E. coli* the DAH 7-P synthase (phe) accounts for about 80% of the total DAH 7-P synthase activity,[4] it has been the main focus of research efforts to date.

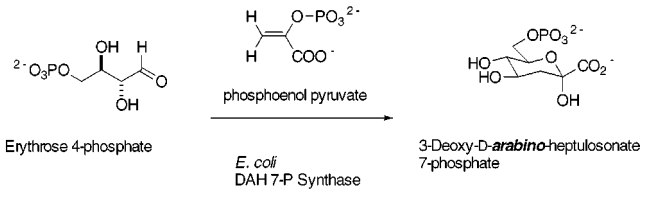


Figure 1. Reaction catalyzed by *E. coli* DAH 7-P synthase.

3-Deoxy-D-*manno*-octulosonate 8-phosphate (KDO 8-P) synthase, which catalyzes a similar aldol-type condensation except between PEP and D-arabinose 5-phosphate (A 5-P) to form KDO 8-P (Figure 2), is an important enzyme involved in the biosynthesis of lipopolysaccharide portion of all Gram-negative bacteria.[5] Both DAH 7-P synthase (phe)

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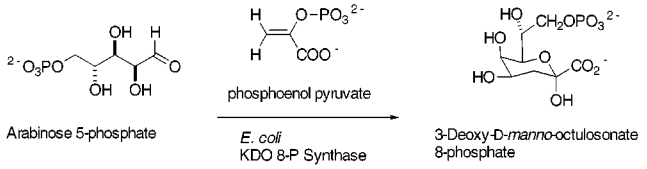

Figure 2. Reaction catalyzed by *E. coli* KDO 8-P synthase.

and KDO 8-P synthase from *E. coli* have been well characterized, and it has been demonstrated that both enzymes catalyze the addition of the *si* face of C3 of PEP on to the *re* face of C1 of the phosphorylated monosaccharide to give the respective condensation product with an *R* configuration at C4.[6] It has also been shown that DAH 7-P synthase (phe) from *E. coli* can utilize A 5-P, D-ribose 5-phosphate, and 2-deoxy-D-ribose 5-phosphate, all phosphorylated monosaccharides that are one carbon unit longer than the natural substrate E 4-P, as alternate substrates.[7] The corresponding KDO 8-P analogues have the *R* configuration at C4, indicating that the stereochemistry of the condensation reaction is the same as that observed for the natural substrate. On the other hand, the substrate specificity of *E. coli* KDO 8-P synthase for the phosphorylated monosaccharide is more stringent.[8]

Recently, *Neisseria gonorrhoeae* KDO 8-P synthase has been cloned into the pT7-7 vector system for overexpression in *E. coli* BL21(DE3) cells.[9] Utilizing this purified recombinant *N. gonorrhoeae* KDO 8-P synthase, it was shown that *N. gonorrhoeae* KDO 8-P synthase utilizes A 5-P but not E 4-P as the phosphorylated monosaccharide substrate and that the stereochemistry of the reaction is the same as that for *E. coli* KDO 8-P synthase.[9] This is in contradiction to the original report by Subramaniam et al.[10] that *N. gonorrhoeae* KDO 8-P synthase can utilize E 4-P as an alternate substrate and that the facial selectivity at C1 (carbonyl carbon) of the monosacharide is *si* and not *re*, as is observed with both the *E. coli* KDO 8-P synthase and DAH 7-P synthase catalyzed condensations. It is possible that *N. gonorrhoeae* DAH 7-P synthase, potentially present as an impurity in the preparation of *N. gonorrhoeae* KDO 8-P synthase by Subramaniam et al., may have catalyzed the condensation of E 4-P and PEP and therefore may be responsible for the reported substrate ambiguity as well as the unusal stereochemistry of the product (3-deoxy-D-*ribo*-heptulosonate 7-phosphate). To test the hypothesis that *N. gonorrhoeae* DAH 7-P synthase exhibits this unusual stereochemistry, we have cloned *N. gonorrhoeae* DAH 7-P synthase into the expression vector pT7-7, overexpressed it in *E. coli* cells, isolated, and utilized the purified enzyme in the substrate specificity studies. The focus of this Letter is to report the cloning and overexpression of *N. gonorrhoeae* DAH 7-P synthase as well as the results from preliminary susbtrate specificity studies and stereochemical characterization of the condensation reactions catalyzed by this purified recombinant *N. gonorrhoeae* DAH 7-P synthase.

Cloning the *N. gonorrhoeae* DAH 7-P synthase gene into pT7-7 vector for expression in *E. coli* involved three steps. First, a tBLASTn search of the *N. gonorrhoeae* FA 1090 databank at the University of Oklahoma Advanced Center for Genome Technology (http://www.genome.ou.edu/gono_blast.html) utilizing the entire sequence of *E. coli* DAH 7-P synthase (phe) retrieved an open reading frame corresponding to the *E. coli aroG* gene.[11] On the basis of the sequence of this open reading frame (located in contig #126), the 5′ forward primer (5′-CCCATCAGGAAAGATGCCATATGACACACCATACCCC-3′) and the 3′ reverse primer (5′-GACATTCTGC GTCAGGATCCTCAACCTGCGCGCGCCATACG-3′) were synthesized and employed, utilizing standard PCR protocols, to amplify the gene from a genomic sample of *N. gonorrhoeae* DNA.

Second, the amplified gene sequence was restricted with *Nde* I and *Bam*H I (restriction sites are underlined in the above primers) and ligated into the expression vector pT7-7, also restricted with *Nde* I and *Bam* H I, via standard molecular biology protocol.[12] Overexpression in the vector pT7-7 is controlled by the isopropyl β-D-thiogalactopyranoside (IPTG)-inducible T7-RNA polymerase. Chemically competent *E. coli* XL1-Blue cells (Stratagene) were transformed using the above ligation mixture and several plasmids isolated from the transformants were restricted with appropriate enzymes to verify the presence of the correct insert.

Third, chemically competent *E. coli* BL21(DE3) cells were transformed using a plasmid containing the correct insert. Plasmids isolated from several transformants were then verified for the presence of the correct insert (pT7-7-*NgaroG*) by DNA sequencing (using ABA model 373A DNA sequencers at the University of Michigan Biomedical Research Resources Core Facility). A glycerol stock of the *E. coli* Bl21(DE3) cells harboring the pT7-7-*NgaroG* was prepared and stored at −80 °C for future use.

E. coli BL21(DE3) cells harboring the pT7-7-*NgaroG* plasmid were grown in 2xYT medium at 37 °C. Expression of protein was induced by addition of IPTG (to a final concentration of 0.4 mM) to this culture at mid-logarithmic phase (0.6 OD at 600 nm). The cells were harvested 4 h after induction by centrifugation at 10 000*g* for 15 min at 4

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(11) The *N. gonorrhoeae* genomic DNA sequence presently available, unlike the *E. coli* sequence, does not seem to contain the sequences that correspond to the *aroF* (tyrosine-sensitive) or *aroH* (tryptophan-sensitive) gene. Therefore, the sequence corresponding to the predicted *aroG* gene was used in the present study.

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°C. The cell pellet was resuspended in 10 mM 1,3-bis[tris-(hydroxymethyl)methylamino]propane (BTP) buffer (pH 6.8) and sonicated at 4 °C, using Sonicator W220 (Heat Systems Ultrasonics), by applying three 30 s pulses with an 1 min delay between pulses. Cell debris was removed by centrifugation at 15 000*g* for 15 min at 4 °C. *N. gonorrhoeae* DAH 7-P synthase was isolated and purified by anion exchange chromatography using a High Q Cartridge column (Bio-Rad) as reported previously for the *E. coli* enzyme.[7] Fractions containing *N. gonorrhoeae* DAH 7-P synthase as identified by protein assay (Bio-Rad Protein assay) and DAH 7-P synthase activity assay (using standard assay conditions developed for *E. coli* DAH 7-P synthase) were then pooled together and stored at −80 °C.[13] Analysis of the resulting protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown) showed it to be greater than 95% pure.

Since *E. coli* DAH 7-P synthase requires a divalent metal ion for its activity, the metal requirement of *N. gonorrhoeae* DAH 7-P synthase was determined. Metal-free enzyme was prepared by treating the purified *N. gonorrhoeae* DAH 7-P synthase solution with ethylenebis(oxyethylenenitrilo)tetraacetic acid (EDTA) (to a final concentration of 10 mM) for 1 h at 4 °C followed by removal of EDTA and EDTA−metal complex by size exclusion chromatography. The metal-free enzyme thus obtained was essentially inactive (less than 5% of the maximum activity exhibited in the presence of 500 μM Mn^{2+}) in the absence of an exogenously added divalent metal ion. Inclusion of 0.5 mM Mn^{2+} ions in the assay mixture restored activity to the metal-free enzyme solution. Therefore, similar to the *E. coli* enzyme, *N. gonorrhoeae* DAH 7-P synthase also requires a divalent metal ion for its activity.

The DAH 7-P synthase activity of the *N. gonorrhoeae* enzyme was assayed using a continuous spectrophotometric method that monitors the disappearance of the α,β-unsaturated carbonyl absorbance at $\lambda = 232$ nm ($\epsilon = 2840$ M^{-1} cm^{-1}).[7,13] The standard assay mixture contained 50 mM BTP buffer, pH 6.8, 0.5 mM $MnCl_2$, 0.15 mM PEP, and 1 mM E 4-P in a total volume of 1 mL. The reaction mixture was incubated at 37 °C for 3 min, and the reaction was initiated by addition of 5 μL of the enzyme solution (1 mg/mL). Kinetic parameters were obtained as previously described, by maintaining the concentration of one substrate (PEP) at an apparent saturating concentration while varying the concentration of the other substrate (E 4-P) and vice versa.[13] The K_m^{PEP} for *N. gonorrhoeae* DAH 7-P synthase was found to be 60 μM while the $K_m^{E\ 4-P}$ was found to be 95 μM. *N. gonorrhoeae* DAH 7-P synthase has a k_{cat} value of 6 s^{-1}. The kinetic parameters obtained for *N. gonorrhoeae* DAH 7-P synthase are similar to those reported for DAH 7-P synthase from *E. coli*.[13]

To study the stereochemical course of the reaction catalyzed by *N. gonorrhoeae* DAH 7-P synthase, preparative scale reactions were run to obtain sufficient quantities of the products for analysis by ^{1}H NMR spectroscopy. *N. gonorrhoeae* DAH 7-P synthase (500 μL of 1 mg/mL solution) was added to reaction mixtures containing 20 mM PEP and 1 mM $MnCl_2$ in 100 mM BTP buffer and either 18 mM A 5-P or 20 mM E 4-P (pH 6.8) in a final volume of 1.5 mL. The reaction mixtures were incubated at 37 °C for 2 h and quenched by the addition of 0.5 mL of a 10% trichloroacetic acid solution. The precipitated protein was removed by centrifugation, the supernatant was loaded on an anion exchange column (High Q Econo-Pac 5 mL Cartridge), and the condensation product was eluted using a LiCl gradient as described previously.[7] The ^{1}H NMR spectrum (Figure 3)

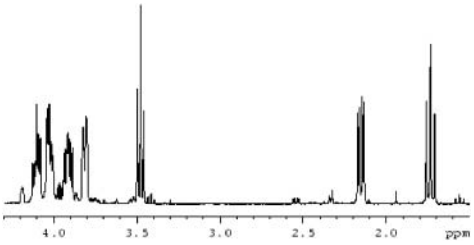

Figure 3. Reaction catalyzed by *E. coli* KDO 8-P synthase.

recorded for the 3-deoxy-α-keto sugar acid product obtained from condensation of E 4-P and PEP is identical to that reported for DAH 7-P obtained by the *E. coli* DAH 7-P synthase catalyzed condensation of E 4-P and PEP.[7] This ^{1}H NMR spectrum is clearly much different from that reported for 3-deoxy-D-ribo-2-heptulosonate obtained from *E. coli* 2-keto-3-deoxy-6-phosphogluconate aldolase catalyzed condensation of D-erythrose and pyruvate.[14] Therefore, *N. gonorrhoeae* DAH 7-P synthase catalyzes the condensation of E 4-P and PEP to give only 3-deoxy-D-*arabino*-heptulosonate 7-phosphate and not 3-deoxy-D-*ribo*-heptulosonate 7-phosphate. Similarly, the ^{1}H NMR spectrum (Figure 4) recorded for the product obtained from condensation of A 5-P and PEP is identical to that reported for KDO 8-P obtained from both the *E. coli* KDO 8-P synthase and

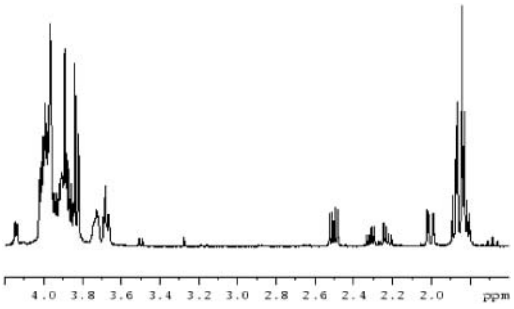

Figure 4. ^{1}H NMR spectrum of KDO 8-P formed by *N. gonorrhoeae* DAH 7-P synthase catalyzed condensation of A 5-P and PEP.

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the *E. coli* DAH 7-P synthase catalyzed condensations of A 5-P and PEP.[6b,7] Therefore, *N. gonorrhoeae* DAH 7-P synthase exhibits a broad substrate specificity and the same stereochemistry as that of *E. coli* DAH 7-P synthase (phe), at least with respect to C1 of the phosphorylated monosaccharide.

The results of the present study clearly demonstrate that *N. gonorrhoeae* DAH 7-P synthase can utilize A 5-P as a substrate as well as the natural substrate, E 4-P. The results further indicate that, irrespective of the phosphorylated monosaccharide substrate tested, the C3 of PEP attacks only on the *re* face of the C1 of the aldehyde of the monosaccharide, forming products with the *R* configuration at C4. Therefore, the stereochemistry of the condensation step in the reaction catalyzed by *N. gonorrhoeae* DAH 7-P synthase, at C1 of the phosphorylated monosaccharide, is the same as that observed for the *E. coli* enzyme. Now that both DAH 7-P synthase and KDO 8-P synthase from *N. gonorrhoeae* have been shown to exhibit the same stereochemical course as their counterparts from *E. coli*, the origin of 3-deoxy-D-*ribo*-heptulosonate 7-phosphate remains unexplained.

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