Substrate Ambiguity of 3-Deoxy-D-*manno*-Octulosonate 8-Phosphate Synthase from *Neisseria gonorrhoeae* Revisited

GALINA YA SHEFLYAN, APPAVU K. SUNDARAM, WILLIAM P. TAYLOR,
AND RONALD W. WOODARD*

*Interdepartmental Program in Medicinal Chemistry, College of Pharmacy,
University of Michigan, Ann Arbor, Michigan 48109-1065*

Homogeneous, recombinant 3-deoxy-D-*manno*-octulosonate 8-phosphate synthase from *Neisseria gonorrhoeae* is shown to catalyze the formation of 3-deoxy-D-*manno*-octulosonate 8-phosphate from phosphoenolpyruvate and D-arabinose 5-phosphate as determined from ^{1}H-nuclear magnetic resonance analysis of the product. This enzyme does not catalyze the condensation of D-erythrose 4-phosphate and phosphoenolpyruvate to form 3-deoxy-D-*ribo*-heptulosonate 7-phosphate, as was previously reported (P. S. Subramaniam, G. Xie, T. Xia, and R. A. Jensen, J. Bacteriol. 180:119–127, 1998).

3-Deoxy-D-*manno*-octulosonate 8-phosphate (KDO8P) synthase and 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAH7P) synthase (phenylalanine repressible) catalyze similar aldol-type condensations between phosphoenolpyruvate (PEP) and the monosaccharides D-arabinose 5-phosphate (A5P) and D-erythrose 4-phosphate (E4P), respectively (Fig. 1). Both enzymes from *Escherichia coli* are tetrameric, and although their quaternary structures are dissimilar, the monomeric subunits that comprise each enzyme are nearly superimposable (9). As one might expect from this degree of subunit similarity, the facial selectivities of both enzymes from *E. coli* have been shown to be *re*-face with respect to the phosphorylated monosaccharide and *si*-face with respect to PEP, resulting in an (*R*) configuration at the C-4 position of each product (2, 4, 7, 8). Reports from this laboratory have shown that *E. coli* DAH7P synthase (encoded by *aroG*) is capable of utilizing arabinose 5-phosphate, ribose 5-phosphate, and 2-deoxyribose 5-phosphate to synthesize the corresponding eight carbon monosaccharides (14), while *E. coli* KDO8P synthase is unable to utilize E4P as a substrate (D. L. Howe, G. Y. Sheflyan, and R. W. Woodard, unpublished data). The facial selectivity of DAH7P synthase with regard to both PEP and the alternate substrates listed above is the same as with the natural substrates (16a).

It has recently been reported not only that KDO8P synthase from *Neisseria gonorrhoeae* utilizes E4P as an alternate substrate but also that the facial selectivity of the enzyme with respect to this monosaccharide is *si*-face, resulting in an (*S*) configuration at the C-4 position of the product (16). This suggests that the enzyme from *N. gonorrhoeae* is distinct from all other reported KDO8P synthases, in terms of both its substrate ambiguity and its stereoselectivity. However, these unusual results were obtained using enzyme isolated directly from *N. gonorrhoeae* that was neither rigorously purified nor thoroughly biochemically characterized (16). The product of the enzymatic reaction, C$_7$-X (original authors' designation), was neither isolated nor purified, and its stereochemistry was assigned solely on the intensity and rapid production of the chromophore produced in the classic Aminoff assay (periodate-thiobarbituric acid assay) for 3-deoxy-monosaccharides

(1, 10). This assay is known to be sensitive to the stereochemistry of the hydroxyl groups of the monosaccharide (11, 18) but is also known to give false-positive results with compounds such as shikimic acid, guanosine, and adenosine, as well as with DNA (6). Since studies in our laboratory are aimed at the elucidation of the similarities and differences between KDO8P and DAH7P synthase, we were intrigued by the reported use of E4P by a KDO8P synthase and decided to further investigate this unusual finding.

An open reading frame corresponding to the KDO8P synthase gene (*kdsA*) was retrieved via 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) using a fragment of *E. coli* KDO8P synthase. Standard PCR methodologies were employed to amplify this gene sequence from *N. gonorrhoeae* DNA. The amplified product was isolated, restricted, and ligated into the expression vector pT7-7 (17). The ligation mixture was used to transform competent *E. coli* XL1-Blue cells (Stratagene). Plasmids isolated from several transformants were subjected to DNA sequencing, and one plasmid with the expected sequence was isolated (pT7-7-NGKDO). In our sequence, position 119 is threonine; this residue is absolutely conserved in all known KDO8P synthase sequences (M. R. Birck and R. W. Woodard, submitted for publication).

KDO8P synthase from *N. gonorrhoeae* was overexpressed in *E. coli* BL21(DE3) cells harboring pT7-7-NGKDO and purified, as previously reported, using anion-exchange chromatography (14; W. P. Taylor and R. W. Woodard, submitted for publication). The resultant protein was determined to be >95% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. The apparent molecular mass was determined by size-exclusion chromatography and was found to be 88.2 kDa. This value is similar to those reported for the KDO8P synthases from *E. coli*, *Aquifex aeolicus*, and *Salmonella enterica* serovar Typhimurium (3, 12–14; Taylor and Woodard, submitted).

The *N. gonorrhoeae* KDO8P synthase-catalyzed condensation of A5P and PEP was monitored by observing the disappearance of the α,β-unsaturated carbonyl absorbance ($\lambda = 232$ nm, $\varepsilon = 2,840$ M^{-1} cm^{-1}) of PEP (14). All reactions were performed in 100 mM Tris-acetate, pH 7.4, at 37°C. The kinetic parameters were determined from progress curves of substrate consumption, as previously described (Taylor and
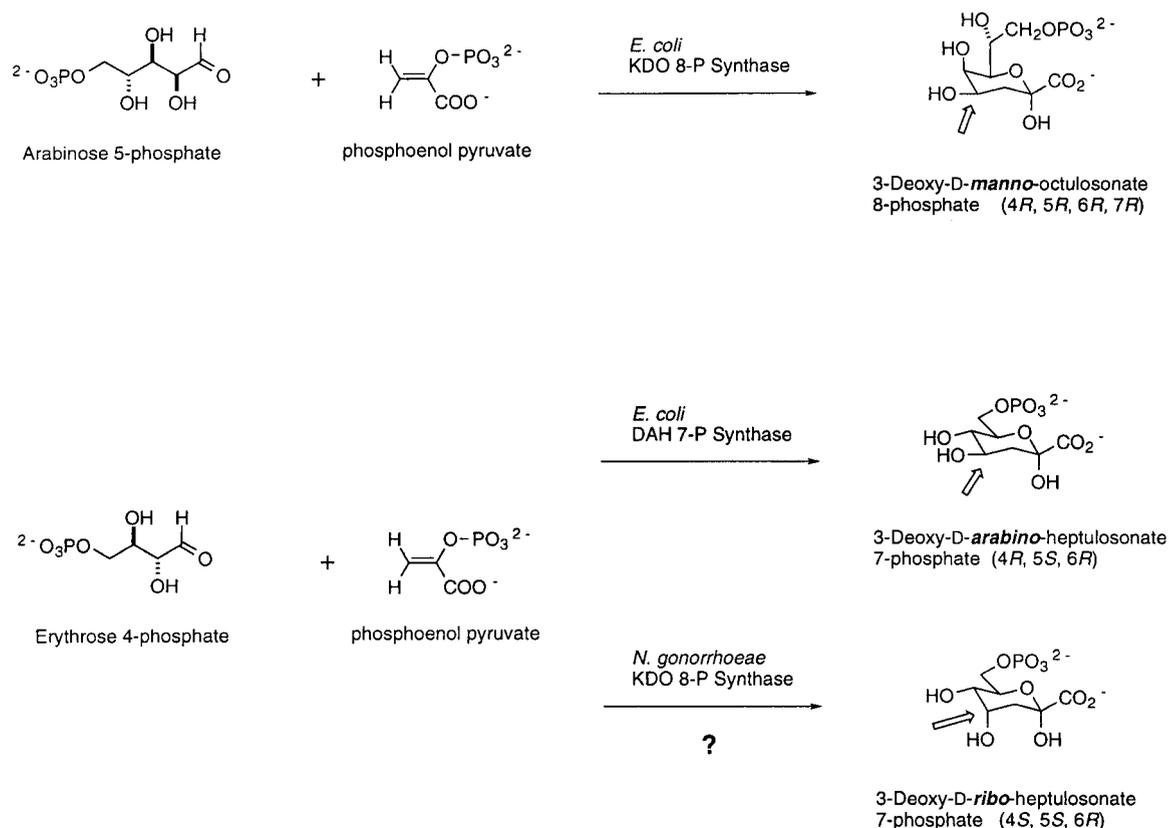

FIG. 1. The reactions catalyzed by *E. coli* KDO8P and DAH7P synthases with their respective substrates and the proposed reaction for the *N. gonorrhoeae* KDO8P synthase-catalyzed condensation of E4P and PEP. Open arrow, location of newly formed C-C bond.

Woodard, submitted), and are as follows: for PEP, $K_m = 3.1 \pm 0.2$ μM; for A5P, $K_m = 8.5 \pm 0.1$ μM; for both PEP and A5P, $k_{cat} = 1.0 \pm 0.1$ s^{-1}. The Michaelis constants for both PEP and A5P are very similar to those reported for both *E. coli* and *S. enterica* serovar Typhimurium KDO8P synthase, although the value for k_{cat} was reduced approximately three- to sixfold (12; Taylor and Woodard, submitted). The effect of divalent metal ions on enzymatic activity was investigated by quantitating the amount of KDO8P produced using the periodate-thiobarbituric acid assay (1, 10, 14). Inclusion of Mn^{2+} or Zn^{2+} (1 mM) in the assay mixture had no effect on activity. Preincubation of the enzyme with a 10 mM concentration of either metal prior to reaction led to a small decrease in enzyme activity. Prolonged treatment of purified enzyme with 5 mM EDTA resulted in no loss of enzymatic activity.

Purified KDO8P synthase (12 μM) was incubated with 24.3 mM A5P, 23.7 mM PEP, 0.5 mM MnCl$_2$, and 1 mM dithiothreitol in 100 mM Tris-acetate buffer (pH 7.5) in a final volume of 2.0 ml. Even though it is shown in the present study that *N. gonorrhoeae* KDO8P synthase does not require any metal ion for activity, MnCl$_2$ was included in the reaction mixture in order to mimic the reaction conditions reported by Subramaniam et al. (16). Tris-acetate was substituted for phosphate buffer in the present study since inorganic phosphate has been shown to inhibit *E. coli* KDO8P synthase (5). Incubation of KDO8P synthase from *N. gonorrhoeae* with E4P and PEP in 75 mM phosphate buffer (pH 7.0), however, gave no indication of deoxymonosaccharide formation as determined by periodate-thiobarbituric acid analysis. The reaction mixture was incubated at 37°C for 5 h,

and then the reaction was quenched by the addition of 10% trichloroacetic acid (2.0 ml). The 3-deoxy-α-keto sugar acid component of the reaction mixture was purified by anion-exchange chromatography as previously described (14). The 500 MHz ^{1}H-nuclear magnetic resonance (NMR) spectrum of the product isolated from the above reaction is shown in Fig. 2 and is in complete agreement with that reported previously for 3-deoxy-D-*manno*-octulosonic acid 8-phosphate obtained from the *E. coli* KDO8P synthase-catalyzed condensation of A5P and PEP (Fig. 2) (2). In a parallel experiment, 33 mM E4P was substituted for A5P and the mixture was subjected to the reaction conditions and purification protocol described above. Periodate-thiobarbituric acid analysis of the quenched enzymatic reaction mixture before chromatography as well as analysis of all column fractions after purification yielded no indication of a keto-deoxy-monosaccharide acid product. ^{1}H-NMR analysis of the column fractions where acidic phosphorylated monosaccharides normally elute (fractions 30 to 40) gave no signals representative of the condensation product (14). Starting materials were recovered in fractions 23 to 27 (E4P) and fractions 35 to 39 (PEP). Thus, we found that KDO8P synthase from *N. gonorrhoeae* exhibits the same reaction stereochemistry and substrate selectivity observed for *E. coli* KDO8P synthase (2, 4).

While the present report demonstrates that E4P is not a substrate for *N. gonorrhoeae* KDO8P synthase, the question remains as to the actual identity of the C$_7$-X compound identified by Subramanian et al. (16). Based on the reported rate of oxidation as well as the fact that C$_7$-X is not a substrate for

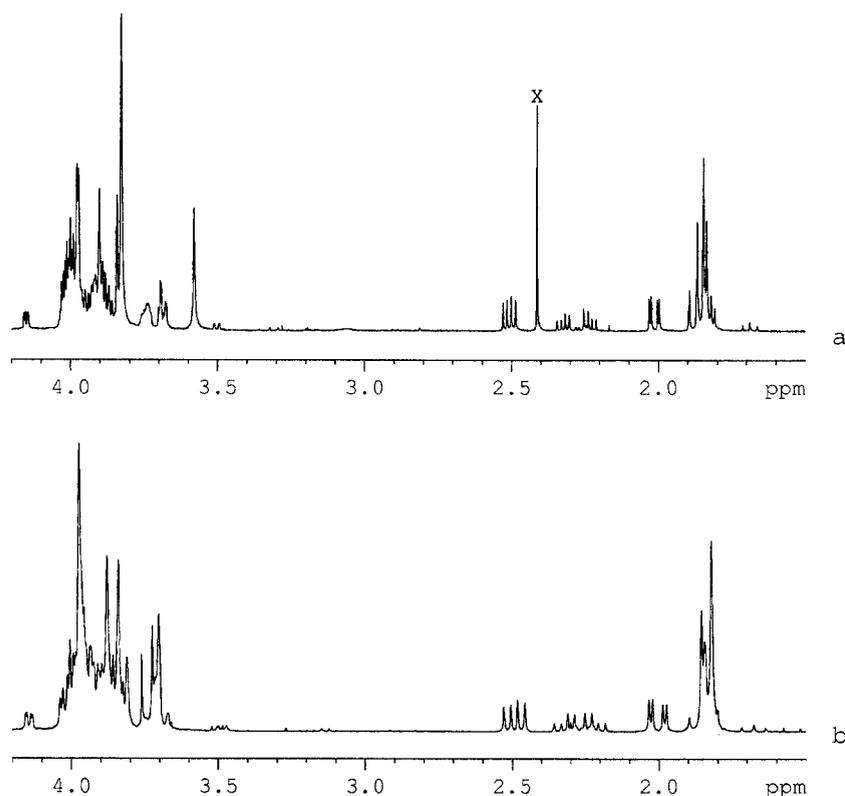FIG. 2. 500-MHz ^{1}H-NMR spectra of the product derived from the condensation of A5P and PEP as catalyzed by *E. coli* KDO8P synthase (X, peak from buffer) (a) and *N. gonorrhoeae* KDO8P synthase (b).

dehydroquinate synthase, it is tempting to suggest that this compound is indeed 3-deoxy-D-*ribo*-heptulosonate 7-phosphate. It is known that 2-keto-3-deoxy-6-phosphogluconate aldolase catalyzes the aldol-like condensation of E4P and pyruvic acid to form 3-deoxy-D-*ribo*-heptulosonate 7-phosphate (15). Alternatively, a minor DAH7P synthase contaminant could prove to be the source of C$_7$-X formation, although this would also require addition to the *si*-face of E4P, in contrast to the stereochemistry of all other known DAH7P synthase-catalyzed reactions. Efforts are underway in our laboratory to clone, overexpress, and purify *N. gonorrhoeae* DAH7P synthase in order to address this latter possibility.

REFERENCES

1. **Aminoff, D.** 1961. Methods for the quantitative estimation of *N*-acetylneuraminic acid and their application to hydrolysates of sialomucoids. Biochem. J. **81:**384–392.
2. **Dotson, G. D., P. Nanjappan, M. D. Reily, and R. W. Woodard.** 1993. Stereochemistry of 3-deoxyoctulosonate 8-phosphate synthase. Biochemistry **32:**12392–12397.
3. **Duewel, H. S., G. Y. Sheflyan, and R. W. Woodard.** 1999. Cloning, overexpression and purification of a thermostable 3-deoxy-D-*manno*-octulosonic acid 8-phosphate synthase from *Aquifex aeolicus*. Biochem. Biophys. Res. Commun. **263:**346–351.
4. **Kohen, A., R. Berkovich, V. Belakhov, and T. Baasov.** 1993. Stereochemistry of the KDO8P synthase. An efficient synthesis of the 3-fluoro analogues of KDO8P. Bioorg. Med. Chem. Lett. **3:**1577–1582.
5. **Kohen, A., A. Jakob, and T. Baasov.** 1992. Mechanistic studies of 3-deoxy-D-*manno*-2-octulosonate 8-phosphate synthase from *Escherichia coli*. Eur. J. Biochem. **208:**443–449.
6. **Millican, R. C.** 1963. A thiobarbituric acid assay for shikimic acid. Anal. Biochem. **6:**181–192.
7. **Onderka, D. K., and H. G. Floss.** 1969. Stereospecificity of the 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthetase reaction. Biochem. Biophys. Res. Commun. **35:**801–804.
8. **Onderka, D. K., and H. G. Floss.** 1969. Steric course of the chorismate synthetase reaction and the 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthetase reaction. J. Am. Chem. Soc. **91:**5894–5896.
9. **Radaev, S., P. Dastidar, M. Patel, R. W. Woodard, and D. L. Gatti.** 2000. Structure and mechanism of 3-deoxy-D-*manno*-octulosonate 8-phosphate synthase. J. Biol. Chem. **275:**9476–9484.
10. **Ray, P. H.** 1980. Purification and characterization of 3-deoxy-D-*manno*-octulosonate 8-phosphate synthetase from *Escherichia coli*. J. Bacteriol. **141:**635–644.
11. **Rothe, G. M., W. Maurer, E. Bohrisch, and P. Reichert.** 1980. Automated test systems for the determination of deoxy sugars and 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase. Anal. Biochem. **106:**213–222.
12. **Salleh, H. M., M. A. Patel, and R. W. Woodard.** 1996. Essential cysteines in 3-deoxy-D-*manno*-octulosonic acid 8-phosphate synthase from *Escherichia coli*: analysis by chemical modification and site-directed mutagenesis. Biochemistry **35:**8942–8947.
13. **Sheflyan, G. Y., H. Duewel, and R. W. Woodard.** 1999. Identification of essential histidine residues in 3-deoxy-D-*manno*-octulosonic acid 8-phosphate synthase: analysis by chemical modification with diethylpyrocarbonate and site-directed mutagenesis. Biochemistry **38:**14320–14329.
14. **Sheflyan, G. Y., D. L. Howe, T. L. Wilson, and R. W. Woodard.** 1998. 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. J. Am. Chem. Soc. **120:**11027–11032.
15. **Shelton, M. C., I. C. Cotterill, S. T. A. Novak, R. M. Poonawala, S. Sudar-**

shan, and E. J. Toone. 1996. 2-Keto-3-deoxy-6-phosphogluconate aldolases as catalysts for stereocontrolled carbon-carbon bond formation. J. Am. Chem. Soc. **118:**2117–2125.

16. **Subramaniam, P. S., G. Xie, T. H. Xia, and R. A. Jensen.** 1998. Substrate ambiguity of 3-deoxy-D-*manno*-octulosonate 8-phosphate synthase from *Neisseria gonorrhoeae* in the context of its membership in a protein family containing a subset of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthases. J. Bacteriol. **180:**119–127.

16a.**Sundaram, A. K., and R. W. Woodard.** Probing the stereochemistry of *E. coli* 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (phenylalanine-sensitive) catalyzed synthesis of KDO 8-P analogs. J. Org. Chem., in press.

17. **Tabor, S.** 1990. Expression using the T7 RNA polymerase/promoter system, p. 16.2.1–16.2.11. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. Greene Publishing and Wiley-Interscience, New York, N.Y.

18. **Waravdekar, V. S., and L. D. Saslaw.** 1959. A sensitive colorometric method for the estimation of 2-deoxy sugars with the use of the malonaldehyde thiobarbituric acid reaction. J. Biol. Chem. **234:**1945–1950.