Mechanistic Studies of the Biosynthesis of Tyvelose: Purification and Characterization of CDP-D-tyvelose 2-Epimerase

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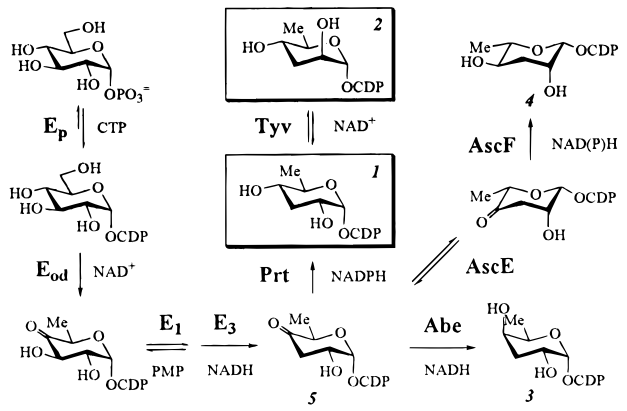
The 3,6-dideoxyhexoses, found almost exclusively at the nonreducing end of the *O*-antigen of the lipopolysaccharide (LPS), have gained notoriety for their influence on the pathogenicity of many Gram-negative bacteria.[1] Recognition of their importance as immunodominant sugars and the potential for controlling or inhibiting their formation have prompted investigations into their biosynthesis.[2] With the exception of colitose,[3] four of the five naturally occurring 3,6-dideoxyhexoses are known to be synthesized via a complex enzymatic series starting from CDP-D-glucose.[2] As delineated in Scheme 1, the biosynthetic formation of paratose (**1**), abequose (**3**), and ascarylose (**4**) is completed by C-4 reduction of the corresponding CDP-4-ketosugar precursor. However, CDP-D-tyvelose (**2**) is produced from CDP-D-paratose (**1**) via C-2 epimerization, making it the only member of this class produced directly from another 3,6-dideoxyhexose.

The C-2 epimerization of **1** to **2** has been proposed to be catalyzed by CDP-D-tyvelose 2-epimerase.[4] Unlike most other sugar epimerases that catalyze stereochemical inversions adjacent to a carbonyl or a carboxylate,[5] CDP-D-tyvelose 2-epimerase is intriguing because it epimerizes a stereocenter that bears no acidic proton and, thus, cannot utilize a deprotonation−reprotonation strategy. The recent identification and sequencing of the gene encoding CDP-D-tyvelose 2-epimerase, *tyv*,[6] in *Salmonella typhi*[4b] and *Yersinia pseudotuberculosis* IVA[7] has revealed a Rossmann fold (G**G**C**G**FL**G**)[8] in the translated sequence. This binding motif

Scheme 1



suggests the presence of a NAD^+ in the active site and, hence, the possible involvement of NAD^+ in the epimerization reaction.

Indeed, a group of epimerases are known to be able to invert unactivated stereocenters using NAD^+ as their coenzyme.[9] The best-studied example is UDP-D-galactose 4-epimerase that catalyzes the interconversion between UDP-D-glucose and UDP-D-galactose.[10] The tightly bound NAD^+ in this enzyme acts as a hydride carrier and directly participates in the reversible dehydrogenation at C-4 of the substrate via a 4-ketosugar intermediate. The UDP group of the substrate serves as a binding anchor while the 4-keto-hexopyranosyl moiety rotates around the P_β of UDP and the glycosyl oxygen bond. Such a rotation allows the hydride of the resulting NADH to be returned to either face of the 4-ketohexose ring, achieving epimerization. However, an analogous mechanism would be more difficult for CDP-D-tyvelose 2-epimerase, because this type of rotation would alter the juxtaposition of C-2 of the required 2-ketohexose intermediate to the coenzyme and, hence, prohibit the internal hydride return. Clearly, gaining insight into the CDP-D-tyvelose 2-epimerase reaction presents a great challenge but also offers an excellent opportunity to explore this unusual catalysis.

To study the mechanism of CDP-D-tyvelose 2-epimerase, the encoding gene, *tyv*,[6] which was previously identified and sequenced by Hobbs and Reeves,[7] was amplified by polymerase chain reaction (PCR) from *Y. pseudotuberculosis* IVA. This gene was cloned into a pET-24(+) vector, and the resulting construct, pTHEp-10, was used to transform *Escherichia coli* BL21(DE3) cells. The expressed C-terminal His-tagged protein (Tyv) was purified to near homogeneity by a Ni-NTA column (Qiagen), and its identity was confirmed by N-terminal amino acid sequencing.[11] Judging from a M_r of 147 kDa estimated by gel filtration and a calculated mass of 37 987 Da per monomer, this enzyme must exist as a tetramer in its native form. The purified Tyv contains approximately one bound NAD^+ per monomer with a small percentage of NADH that varies among different enzyme preparations.[12] Incubation of this enzyme with CDP-D-paratose (**1**)[13] gave rise to a new product that was identified as CDP-D-tyvelose (**2**)

(1) (a) Lüderitz, O.; Staub, A. M.; Westphal, O. *Bacteriol. Rev.* **1966**, *30*, 192−248. (b) Ashwell, G.; Hackman, J. *Microb. Toxins* **1971**, *4*, 235−266. (c) Williams, N.; Wander, J. In *The Carbohydrates: Chemistry and Biochemistry;* Pigman, W., Horton, D., Eds.; Academic Press: New York, 1980; Vol. 1B, pp 761−798. (d) Bishop, C. T.; Jennings, H. J. In *The Polysaccharides*; Aspinall, G. O., Ed.; Academic Press: New York, 1982; Vol. 1, pp 291−330. (e) Lindberg, B. *Adv. Carbohydr. Chem. Biochem.* **1990**, *48*, 279−318. (f) Raetz, C. R. H. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd ed.; Neidhardt, F. C., Curtiss, R., Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M., Umbarger, H. E., Eds.; ASM Press: Washington, D. C, 1996; Vol. 1, pp 1035−1063.

(2) (a) Liu, H.-w.; Thorson, J. S. *Annu. Rev. Microbiol.* **1994**, *48*, 223−256. (b) Kirschning, A.; Bechthold, A. F.-W.; Rohr, J. In *Bioorganic Chemistry: Deoxysugars, Polyketides & Related Classes: Synthesis, Biosynthesis, Enzymes*; Rohr, J., Ed.; Springer: Berlin, 1997; pp 1−84. (c) Johnson, D. A.; Liu, H.-w. In *Comprehensive Chemistry of Natural Products Chemistry*; Barton, D., Nakanishi, K., Meth-Cohn, O.; Eds.; Elsevier Science: Oxford; 1999; pp 311−365.

(3) Heath, E. C.; Elbein, A. D. *Proc. Natl. Acad. Sci. U.S.A.* **1962**, *48*, 1209−1216.

(4) (a) Matsuhashi, S.; Strominger, J. L. *Biochem. Biophys. Res. Commun.* **1965**, *20*, 169−175. (b) Verma, N.; Reeves, P. *J. Bacteriol.* **1989**, *171*, 5694−5701.

(5) Tanner, M. E.; Kenyon, G. L. In *Comprehensive Biological Catalysis*; Sinnott, M., Ed.; Academic Press: San Diego, 1998; Vol. II, pp 7−39.

(6) The gene was originally known as *rfbE*.[4b] According to a new nomenclature for genes involved in the biosynthesis of sugars found in the LPS, the *rfbE* gene should now be designated as *tyv* (Reeves, P. R.; Hobbs, M.; Valvano, M. A.; Skurnik, M.; Whitfield, C.; Coplin, D.; Kido, N.; Klena, J.; Maskell, D.; Raetz, C. R. H.; Rick, P. D. *Trends Microbiol.* **1996**, *4*, 495−503).

(7) Hobbs, M.; Reeves, P. R. *Biochim. Biophys. Acta* **1995**, *1245*, 273−277.

(8) McKie, J. H.; Douglas, K. T. *FEBS Lett.* **1991**, *279*, 5−8. (b) Wierenga, R. K.; De Maeyer, M. C. H.; Hol, W. G. J. *Biochemistry* **1985**, *24*, 1346−1357. (c) Wierenga, R. K.; Drenth, J.; Schulz, G. E. *J. Mol. Biol.* **1983**, *167*, 725−739.

(9) Tanner, M. E. In *Comprehensive Biological Catalysis*; Sinnott, M., Ed.; Academic Press: San Diego, 1998; Vol. III, pp 76−82.

(10) (a) Frey, P. A. *FASEB* **1996**, *10*, 461−470. (b) Bauer, A. J.; Rayment, I.; Frey, P. A.; Holden, H. M. *Proteins* **1992**, *12*, 372−381. (c) Burke, J. R.; Frey, P. A. *Biochemistry* **1993**, *32*, 13220−13230.

(11) N-terminal amino acid sequencing confirmed that the first 10 residues (MKLLITGGCG) of this protein are identical to those of the translated *tyv* sequence.

(12) Quantitation of bound NAD^+ and NADH was determined as described by Klingenberg, M. In *Methods of Enzymatic Analysis*; Bergmeyer, H. U., Ed.; Verlag Chemie: New York, 1984; pp 2045−2059, with the exception that the NADH was released by boiling for 3 min.

(13) Hallis, T. M.; Lei, Y.; Que, N. L. S.; Liu, H.-w. *Biochemistry* **1998**, *37*, 4935−4945.

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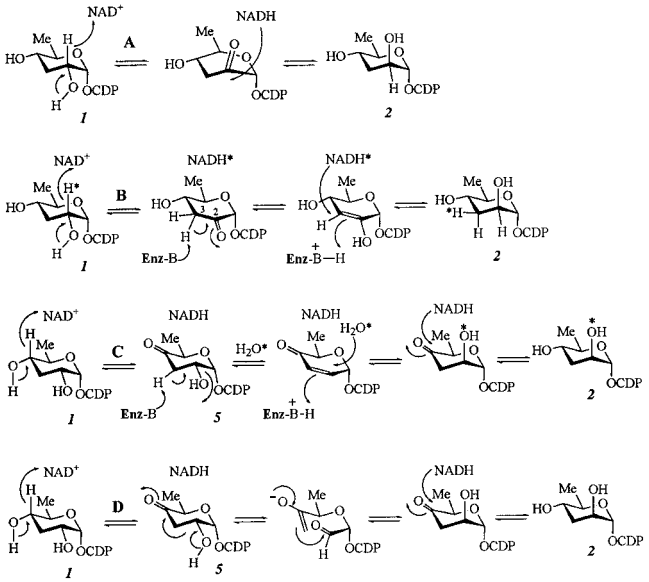
Scheme 2



by ^{1}H NMR.[14] This result provided the first biochemical evidence substantiating the catalytic function of Tyv as CDP-D-tyvelose 2-epimerase. As expected, the C-2 epimerization catalyzed by Tyv is a reversible process with an equilibrium constant, K_{eq}, of 1.22.[15]

Scheme 2 illustrates four possible mechanisms for the epimerization in which NAD^+ is the recycled cofactor, initially oxidizing either the C-2 or C-4 hydroxyl before returning the hydride to the transient ketohexose. Mechanism A is analogous to that of UDP-D-galactose 4-epimerase. In mechanism B, oxidation of the C-2 hydroxyl by NAD^+ lowers the pK_a of the C-3 proton, allowing deprotonation by an active-site base. Suprafacial reduction of the ensuing enolate (or enol) at C-3 followed by protonation at C-2 from either face of the pyranose returns H-3 to C-2 and racemizes the C-2 configuration. Mechanism C proceeds by oxidation at C-4, and the subsequent enolization facilitates reversible dehydration at C-2 so that epimerization is achieved by addition of water to either face of the enolate. The fourth mechanism also involves oxidation at C-4, but in this case the C-2/C-3 bond is cleaved by a retro-aldol process. Rotation about the C-1/C-2 bond prior to aldol ring reclosure produces the two epimers.

We speculated that if either mechanism C or D were operative, epimerase containing bound NADH instead of NAD^+ may be able to convert the 4-ketohexose intermediate **5** to CDP-D-tyvelose (**2**) and CDP-D-paratose (**1**). The required 4-ketohexose **5** was readily available as the product from the E_1/E_3 catalysis,[13] Converting the bound NAD^+ to NADH was accomplished by reduction of purified Tyv with $NaBH_4$. HPLC analysis after incubation revealed the appearance of two new peaks that corresponded to CDP-D-paratose and CDP-D-tyvelose. Neither of these peaks was detected in the controls lacking either Tyv or **5**. This result demonstrates that the 4-ketosugar **5** is likely an intermediate in Tyv catalysis, and therefore, C-4 oxidation of CDP-D-paratose or CDP-D-tyvelose may be a prerequisite for initiating the C-2 epimerization. Further investigation of the mechanism by incubating CDP-D-paratose (**1**) with the epimerase in 100 mM potassium phosphate buffer, pH 7, prepared with either ^2H_2O or $H_2{}^{18}O$, revealed no incorporation of ^{18}O into the hexose moiety by FAB-MS and no ^2H on the sugar backbone by ^1H NMR. Unless the accessibility of the active site toward bulk solvent is strictly limited, these results do not support mechanisms B and C. In addition, when [2-$^2H_1$]-labeled **1**[16] was used in the incubation, the deuterium isotope at C-2 was retained in both CDP-D-paratose and CDP-D-tyvelose, negating a hydride transfer from C-2 to C-3 as depicted in mechanism B.

Thus, the current data appear to be more compatible with mechanism D.[17] It is noteworthy that according to a BLAST search, CDP-D-tyvelose 2-epimerase exhibits the highest sequence homology to enzymes which catalyze C-4 oxidation of a hexose substrate, such as TDP-glucose 4,6-dehydratase from *S. typhi* (29% identity, 45% similarity), GDP-mannose 4,6-dehydratase from *E. coli* (25% identity, 43% similarity), and UDP-galactose 4-epimerase from *S. typhi* (25% identity, 41% similarity). However, oxidation at C-2 still remains a possible option since epimerase containing NADH may be able to reduce the 4-ketohexose **5**, thereby generating epimerase with NAD^+ and CDP-D-paratose (**1**), which could then follow mechanism A to produce the epimer. Because hydride transfer from NADH to either the C-4 or C-2 position of the hexose ring would require considerable flexibility on the part of the enzyme, we still favor a retro-aldol process, as outlined in mechanism D. While a similar retro-aldol mechanism has been suggested for another sugar epimerase, L-ribulose-5-phosphate 4-epimerase, which interconverts L-ribulose-5-phosphate and D-xylulose-5-phosphate, its catalysis is cofactor-independent and only requires a divalent metal ion.[18,19] Therefore, a prerequisite C-4 oxidation/reduction, as suggested by this preliminary study, would make CDP-D-tyvelose 2-epimerase a unique catalyst that is capable of inverting an unactivated stereocenter by C−C bond cleavage. An unequivocal determination of the mechanism of this enzyme, however, must await further study.

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(14) Spectral data of **2**: ^{1}H NMR (D_2O) δ 1.06 (3H, d, J = 6.3 Hz, 5″-Me), 1.70 (1H, ddd, J = 3.0, 11.2, 14.0 Hz, 3″$_{ax}$-H), 1.84 (1H, ddd, J = 3.6, 4.6, 14.0 Hz, 3″$_{eq}$-H), 3.40 (1H, ddd, J = 4.6, 9.6, 11.2 Hz, 4″-H), 3.70 (1H, dq, J = 6.3, 9.6 Hz, 5″-H), 3.84 (1H, bs, 2″-H), 4.00−4.20 (5H, m, 2′-H, 3′-H, 4′-H, 5′-Hs), 5.12 (1H, d, J = 7.5 Hz, 1″-H), 5.81 (1H, d, J = 4.2 Hz, 1′-H), 5.95 (1H, d, J = 7.5 Hz, 7-H), 7.82 (1H, d, J = 7.5 Hz, 6-H). Ribose hydrogens are denoted with a (′) and hexose hydrogens are denoted with a (″).

(15) The equilibrium constant, K_{eq}, was determined from two separate Tyv incubations; the first contained CDP-D-tyvelose, and the other contained CDP-D-paratose. Each reaction was allowed to reach equilibrium as determined by HPLC analysis using a Spherisorb S5 SAX column (0.46 × 25 cm) with a gradient from 50 mM potassium phosphate buffer, pH 3.5, to 140 mM potassium phosphate buffer, pH 3.5, over 20 min. The substrate and product ratios were determined by integrating the corresponding peaks from the HPLC chromatogram.

(16) [2-$^2H_1$]glucose was incubated with hexokinase (yeast), $MgSO_4$, and ATP in 100 mM potassium phosphate buffer, pH 7.5 to give [2-$^2H_1$]glucose 6-phosphate. Further incubation with CTP, inorganic phosphatase (yeast), phosphoglucomutase (rabbit muscle), and 1,6-bisphosphate glucose provided [2-$^2H_1$]glucose 1-phosphate, which was converted to CDP-D-[2-$^2H_1$]paratose as described in ref 13.

(17) It should be noted that a 1,2-elimination/addition mechanism, similar to that catalyzed by UDP-*N*-acetylglucosamine 2-epimerase (Morgan, P. M.; Sala, R. F.; Tanner, M. E. *J. Am. Chem. Soc.* **1997**, *119*, 10269−10277), is also possible; however, this mechanism is NAD^+-independent.

(18) Johnson, A. E.; Tanner, M. E. *Biochemistry* **1998**, *37*, 5746−5754.

(19) Another interesting example also believed to follow a retro-aldol process is the conversion of UDP-D-glucuronate to UDP-apiose catalyzed by UDP-apiose synthetase, a NAD^+-dependent enzyme. The catalytic sequence includes formation of a 4-hexulose intermediate, elimination of the C-6 carboxyl group, cleavage of the C-2/C-3 bond, ring contraction upon aldol ring closure, and reduction at the branched carbon atom (see Frey, P. A. In *Pyridine Nucleotide Coenzymes (B)*; Dolphin, D., Poulson, R., Avramovic, O., Ed.; Wiley: New York, 1987; Part B, p 461−511).