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New Insights into the Mechanism of CDP-D-Tyvelose 2-Epimerase: An Enzyme-Catalyzing Epimerization at an Unactivated Stereocenter

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Abstract: Tyvelose is a 3,6-dideoxyhexose found in the *O*-antigen of *Yersinia pseudotuberculosis* IVA and is the only member of this class of sugars to be produced directly from another 3,6-dideoxyhexose, paratose. The C-2 epimerization required for this conversion has been proposed to be catalyzed by CDP-D-tyvelose 2-epimerase. This enzyme is intriguing since it belongs to a group of epimerases, including the well-studied UDP-D-galactose 4-epimerase, that can invert unactivated stereocenters. To study the mechanism of this enzyme, we have cloned and expressed the *tyv* gene that encodes CDP-D-tyvelose 2-epimerase. The purified tetrameric protein contains approximately one equivalent of bound NAD^+ per monomer and a small fraction of NADH. Four possible mechanisms involving NAD^+ can be proposed for this enzyme; two involve oxidation at C-2 of the substrate, while the other two require oxidation at C-4. In a previous contribution, we presented preliminary data that supported a retro-aldol-type mechanism initiated by C-4 oxidation. However, this mechanism was refuted by further investigations, which revealed that the 4-fluoro analogue of CDP-D-paratose could be turned over by the enzyme. More importantly, the direct transfer of a deuterium from C-2 of the labeled substrate to the enzyme-bound NAD^+ was observed by mass spectrometry. These results suggest that epimerization is in fact initiated by oxidation at C-2, followed by the transfer of the hydride from the transiently formed NADH to the opposite side of the 2-hexulose intermediate.

Epimerization is a common biological transformation performed by a great variety of enzymes that operate on a diverse array of substrates including carbohydrates, amino acids, and fatty acids. Nature has in fact evolved epimerases and racemases which operate by distinct mechanisms to catalyze this important transformation.[1] For instance, many enzymes involved in the interconversion of L- and D-amino acids rely on using pyridoxal phosphate (PLP) to transform the sp^3 reaction center to an sp^2 carbon via an enamine intermediate. It is the non-stereospecific reprotonation from either side of this planar intermediate that leads to racemization.[1,2] The need for any cofactor is apparently obviated by a number of other amino acid racemases. In these cases, the reaction typically involves two basic groups in the active-site: one for deprotonation and the other for reprotonation of the resulting intermediate from the opposite side.[1,2] A similar mechanism is found for acyl-CoA α-epimerases which catalyze the epimerization of α-branched-acylthioesters.[3] However, in the case of fatty acid β-epimerase, the inversion is achieved by the breaking and reforming of the β-carbon−oxygen bond of

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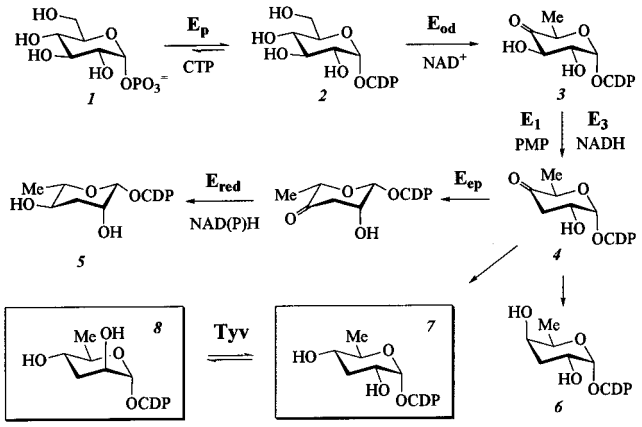
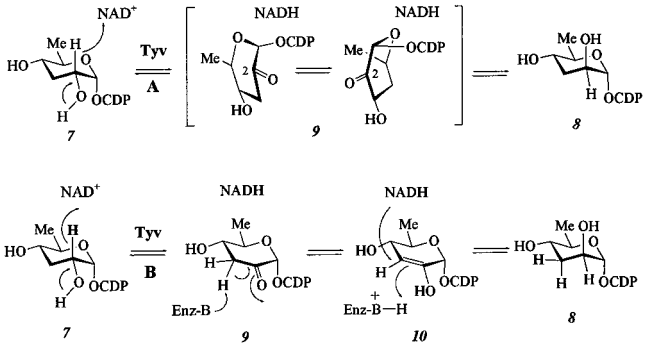
Scheme 1



the substrate which is a CoA-bound β-hydroxylthioester.[1,4] Such a dehydration/rehydration mechanism is analogous to that observed with crotonases.[5] Interestingly, just like the acyl-CoA α-epimerase and the crotonases, many of the sugar epimerases act on stereocenters adjacent to the keto moiety of their substrates. Epimerization of their substrate, the ketohexoses, is achieved by the deprotonation of the activated α-H and reprotonation at α-C from the opposite side.[1] However, the most intriguing carbohydrate epimerases are those that execute their actions at unactivated stereocenters in their substrates. In this category there are as yet only three enzymes that have been studied in detail. These include UDP-galactose 4-epimerase,[6] L-ribulose-5-phosphate 4-epimerase,[7] and UDP-*N*-acetylglucosamine 2-epimerase.[8] This work describes a new member to this exclusive group of carbohydrate epimerases, CDP-D-tyvelose 2-epimerase,[9] a novel enzyme found in the biosynthetic pathway of 3,6-dideoxyhexoses.

The biosynthetic pathway of 3,6-dideoxyhexoses starting from glucose-1-phosphate (**1**) is depicted in Scheme 1,[10] illustrating that four different 3,6-dideoxyhexoses are produced from one common intermediate, **4**. Out of these four sugars (drawn as the cytidylyldiphosphate derivatives)—ascarylose (**5**), abequose (**6**), paratose (**7**), and tyvelose (**8**)—tyvelose is the only 3,6-dideoxyhexose derived from another 3,6-dideoxyhexose, paratose. As delineated in Scheme 1, in the final stage of the biosynthesis of CDP-D-tyvelose (**8**), the $\Delta^{3,4}$-glucoseen intermediate **4** is converted to CDP-D-paratose (**7**) by CDP-D-paratose synthase (Prt) in a NADPH-dependent reduction.[11] The subsequent inversion of the C-2 hydroxyl group catalyzed by CDP-D-tyvelose 2-epimerase (Tyv) converts **7** to CDP-D-tyvelose (**8**).

Scheme 2



The epimerization performed by CDP-D-tyvelose 2-epimerase is intriguing since it occurs at a stereogenic center that is not activated by an adjacent keto or other electron-withdrawing groups. Examination of the translated sequence of the *tyv* gene from *Yersinia pseudotuberculosis* IVA led to the identification of a possible NAD-binding motif near the N terminus,[12] suggesting that Tyv may be a pyridine nucleotide-dependent catalyst.[9] The highly studied UDP-galactose 4-epimerase is an example of an epimerase whose catalysis is also NAD^+-dependent.[6] The NAD^+ cofactor in this enzyme plays a key role in the formation of a 4-ketosugar intermediate. Rotation of the hexulose about the P_β of UDP and the glycosyl oxygen bond followed by NADH reduction allows the return of the hydride to either face of the pyranose ring, resulting in epimerization at C-4. It is possible that an analogous mechanism could be operative with CDP-D-tyvelose 2-epimerase, as illustrated by Mechanism A in Scheme 2. However, it is difficult to envision that a similar rotation in the case of Tyv could properly align C-2 of the putative 2-ketohexose intermediate **9** to the coenzyme in order to allow the internal hydride return to C-2. Therefore, three other possible mechanisms have been proposed for the epimerization catalyzed by CDP-D-tyvelose 2-epimerase.[9]

As depicted in Scheme 2, Mechanism B also incorporates a C-2 oxidation step as in Mechanism A. In this case, however, the acidity of the C-3 proton is exploited to allow keto−enol tautomerization to give **10**. Subsequent reduction at C-3 should be suprafacial, whereas protonation at C-2 could occur from either face of the pyranose, resulting in the return of H-2 to C-3 and the racemization of the C-2 configuration. As depicted in Scheme 3, Mechanism C involves a reversible dehydration process made possible by an initial oxidation at C-4 to generate **11**. The addition of water to either face of the enone **12** gives a mixture of C-2 epimers. The final step is the reduction at C-4 to afford **8** and to regenerate the NAD^+ coenzyme. The fourth mechanism, D, relies on the oxidation at C-4 to activate the hexose to **11** for retro-aldol ring cleavage at the C-2/C-3 bond. Rotation about the C-1/C-2 bond of **13** followed by aldol ring reclosure and then reduction at C-4 produces the two epimers **7** and **8**. Our preliminary evidence, presented in a previous contribution,[9] appeared to support Mechanism D, a retro-aldol process, for this epimerization. In this work, we provide a full

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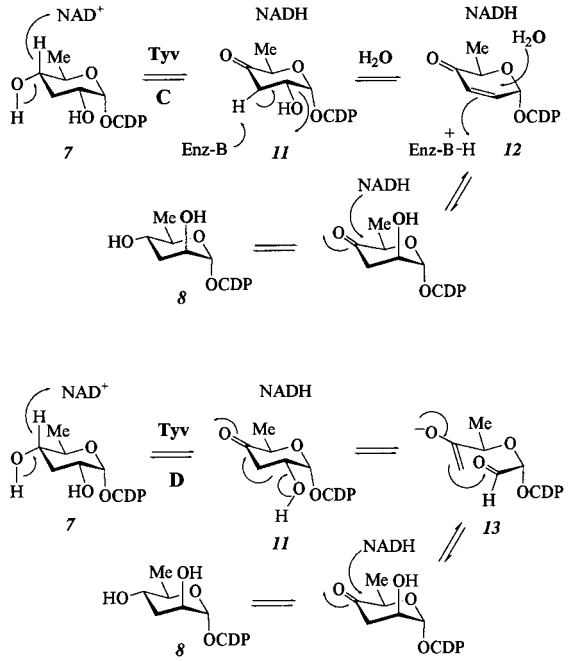
Scheme 3



account of our study on this enzyme that ultimately indicates mechanism A and not D is operative in CDP-D-tyvelose 2-epimerase.

Experimental Section

General. Protein concentrations were determined according to Bradford[13] using bovine serum albumin as the standard. The NMR spectra were acquired on a Varian Unity 300 or 500 spectrometer, and chemical shifts (δ in ppm) are given relative to those for Me_4Si (for ^1H and ^{13}C), $CFCl_3$ (external, for ^{19}F), and aqueous 85% H_3PO_4 (external, for ^{31}P), with coupling constants reported in hertz (Hz). Electronic absorption spectra were recorded on a Beckman DU650 spectrophotometer. N-terminal sequencing of purified CDP-D-tyvelose 2-epimerase, synthesis of oligonucleotide primers for polymerase chain reaction (PCR), and amino acid analysis were all performed by the Microchemical Facility in the Institute of Human Genetics at the University of Minnesota. Electrospray mass spectrometry was carried out by the Mass Spectrometry Consortium for the Life Sciences at the University of Minnesota-St. Paul, and other high-resolution mass spectrometry was performed either by the Resource for Biomedical and Bio-organic Mass Spectrometry at Washington University or by the Department of Chemistry Mass Spectrometry Facility at the University of Minnesota-Minneapolis. Flash chromatography was performed on Lagand Chemical silica gel (230−400 mesh) by elution with the specified solvents. Analytical thin-layer chromatography (TLC) was carried out on Polygram Sil G/UV_{254} plates (0.25 mm). TLC spots were visualized by heating the plate previously stained with a solution of phosphomolybdic acid (5% in EtOH). All chemicals were products of Aldrich Co. (Milwaukee, WI). Bio-Gel P2 resin was purchased from Bio-Rad Laboratories (Hercules, CA). The bacterial strain *Y. pseudotuberculosis* IVA was a generous gift from Professor Robert Brubaker at Michigan State University.

Gene Amplification and Cloning. The sequence of the *tyv* gene (previously known as the *rfbE* gene)[14] in *Y. pseudotuberculosis* IVA has been reported,[12b] allowing the design of oligonucleotide primers to be used in the amplification of the *tyv* gene from the genomic DNA by PCR. The genomic DNA of *Y. pseudotuberculosis* IVA was isolated by alkali lysis of the cells and ethanol precipitation. Amplification of *tyv* proved to be difficult using primers that contained added restriction sites necessary for cloning. Therefore, initial PCR was performed with primers designed to exactly match the sequence at each end of the gene without added restriction sites. The start primer, 5′-CGG TTG GTT GTG CCA ATA T-3′, was complementary to the sequence 63 base pairs upstream of the start codon of *tyv*. The halt primer, 5′-GGT ACC CGC AAT TAA TT-3′, was complementary to the sequence 26 base pairs downstream of the halt codon. The resulting PCR product was used in subsequent amplification reactions that contained primers with the appropriate restriction sites. The start primer, 5′-GCG C**GA ATT C**TA AGG AAT ATA AAT GAA ACT GCT GAT TAC TGG TGG TTG T-3′, contained an *Eco*R1 restriction site (in bold), the ribosomal binding sequence and the AT rich region found just prior to the start codon, and the codons for the first eight amino acid residues. Underlined base pairs were modified to match codons of higher bias in *Escherichia coli*. The halt primer, 5′-GCG CG**C TCG AG**A ACA GTT TCA ACC CAA T-3′, introduced a *Xho*I restriction site (in bold) immediately downstream of the last codon. The stop codon was omitted, and the necessary codons for a histidine tag were incorporated. The PCR-amplified DNA fragment was purified, digested with *Eco*R1 and *Xho*I, and ligated into the *Eco*R1/*Xho*I sites of the transcription vector, pET-24+ (Novagen) to give the recombinant plasmid pTHEp-10. This plasmid was used to transform *E. coli* HB101. Positive clones were identified by digestion of the plasmid DNA with *Eco*R1 and *Xho*I, and visualization of the excised insert, by staining an agarose gel with ethidium bromide after electrophoresis. The plasmid DNA from positive clones was used to transform *E. coli* BL21(DE3). The general methods and protocols for recombinant DNA manipulations were as described by Sambrook et al.[15]

Growth of *Escherichia coli* (pTHEp-10) Cells. An overnight culture of *E. coli* BL21(DE3)/pTHEp-10, grown in Luria-Bertani (LB) medium supplemented with kanamycin (35 μg/mL) at 37 °C was used to inoculate six 1 L cultures of the same medium and antibiotic. These cultures were incubated at 37 °C until the OD_{600} reached 0.6, followed by induction with 0.2 mM isopropyl β-D-thiogalactoside (IPTG) and 4 h of additional incubation at 37 °C. The cells were harvested by centrifugation (6000*g*, 10 min) and stored at −20 °C.

Enzyme Purification. Thawed cells were resuspended in 100 mL of binding buffer consisting of 50 mM potassium phosphate buffer (pH 8.0), 300 mM NaCl, 10% glycerol, and 10 mM imidazole. The cells were disrupted by sonication in five 1-min bursts with a 1-min break between each blast. Cell debris was removed by centrifugation (11000*g*, 20 min), and the supernatant was mixed by slow agitation with 2 mL of packed Ni-NTA agarose resin (Qiagen) for 2 h at 4 °C. The resin was collected by centrifugation (6000*g*, 3 min) and resuspended in 10 mL of binding buffer containing 10 mM imidazole. The slurry was poured into a small column (1 cm diameter), followed by washing with 60 mL of binding buffer that contained 20 mM imidazole. Elution of nonbinding proteins was monitored at A_{280}, and once the absorbance reached the background level, the target protein was eluted with binding buffer containing 250 mM imidazole. The desired fractions, as detected at A_{280} and confirmed by SDS-PAGE, were pooled and dialyzed against 1 L of 20 mM Tris-HCl (pH 7.5) containing 10% glycerol. The purified enzyme was stored at −80 °C.

Polyacrylamide Gel Electrophoresis. The purity of the gene product was assessed by SDS-polyacrylamide gel electrophoresis. Electrophoresis was carried out in the discontinuous buffer system of Laemmli,[16] and the separating gel and stacking gel were 12 and 4% polyacrylamide, respectively. Prior to electrophoresis, protein samples were boiled for 5 min in 62.5 mM Tris-HCl buffer (pH 6.8) containing 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.0025% bromophenol blue. Electrophoresis of the heated samples was run in 25 mM Tris-HCl, 192 mM glycine, and 0.1% SDS (pH 8.3) at 25 mA. Gels were stained with Coomassie blue and destained with acetic acid/ethanol/water (15:20:165 by volume).

Molecular Mass Determination. The native molecular mass of CDP-D-tyvelose 2-epimerase was determined by gel filtration performed

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on a Pharmacia FPLC equipped with a Superdex 200 HR 10/30 column. The proteins were eluted with 50 mM potassium phosphate (pH 7.0) and 0.15 M NaCl at a flow rate of 0.7 mL/min. The system was calibrated with protein standards (Sigma): β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa). The data were analyzed by the method of Andrews.[17] The subunit molecular mass was estimated by SDS-PAGE as described by Laemmli.[16] Protein standards included α-lactalbumin (14 kDa), trypsin inhibitor (20 kDa), trypsinogen (24 kDa), carbonic anhydrase (29 kDa), glyceraldehyde-3-phosphate dehydrogenase (35 kDa), egg albumin (45 kDa), and bovine serum albumin (66 kDa).

Enzyme Assay. CDP-D-tyvelose 2-epimerase activity was determined by a discontinuous HPLC assay using a Spherisorb S5 SAX column (0.46 × 25 cm) with a linear gradient from 50 mM potassium phosphate (pH 3.5) to 140 mM potassium phosphate buffer (pH 3.5) over 20 min. The substrate and product ratios were calculated from the integration of the corresponding peaks from the HPLC chromatogram. A typical reaction contained 10 μM CDP-D-paratose and ∼0.1 μg of CDP-D-tyvelose 2-epimerase in 100 μL of 50 mM potassium phosphate (pH 7.5). The solution was incubated at room temperature for 5 min, followed by heating at 85 °C for 3 min to inactivate the enzyme. Assays with inhibitors were carried out in a similar manner except that 45 μM CDP-D-paratose and 0.27 μg of epimerase were used with a variety of inhibitor concentrations.

Equilibrium Constant. The equilibrium constant, K_{eq}, was calculated from the ratio of product to substrate at equilibrium. The product and substrate ratios were determined for both the forward and reverse directions by integration of the corresponding peaks from the HPLC chromatogram. For the forward direction, 8.6 nmol of CDP-D-paratose was mixed with ∼1 mg of CDP-D-tyvelose 2-epimerase in 100 μL of 50 mM potassium phosphate buffer (pH 7.5). The incubation was allowed to reach equilibrium as determined by the constant product/substrate ratio, which was monitored by SAX HPLC described under "Enzyme Assay". The same procedure was repeated for the reverse direction with 7 nmol of CDP-D-tyvelose and ∼1 mg of enzyme in 100 μL of 50 mM potassium phosphate buffer (pH 7.5).

Determination of Kinetic Parameters. Assays were carried out as described above. The kinetic parameters were deduced by fitting the data to the Michaelis−Menten equation (eq 1) where K_m is the Michaelis−Menten constant, V is the maximum rate, and S is the substrate concentration. Data for deuterium isotope effects were fitted to eq 2 where F_i is the fraction of deuterium in the deuterated substrate, E_V and $E_{V/K}$ are the isotope effects minus 1 on V and V/K_m, respectively.

$$v = \frac{VS}{K_m + S} \quad (1)$$

$$v = \frac{VS}{K_m(1 + F_iE_{V/K}) + S(1 + F_iE_V)} \quad (2)$$

Quantitation of Enzyme Bound NAD(P)$^+$ and NAD(P)H. Quantitation of bound NAD(P)$^+$ and NAD(P)H was determined as described by Klingenberg.[18] Bound NAD(P)$^+$ was extracted from 250 μL of enzyme (0.75 mg) using 1.25 mL of 0.6 N $HClO_4$. The denatured protein was removed by centrifugation, and 0.75 mL of the supernatant was mixed with 0.15 mL of 1 N K_2HPO_4. Concentrated KOH (10 N) was used to neutralize the mixture, and the resulting precipitate was removed by centrifugation. For determination of NAD$^+$, 0.25 mL of the supernatant was mixed with 0.25 mL of 0.1 N $Na_4P_2O_7$ buffer containing 45 mM semicarbazide-HCl (pH 8.8) and 10 μL of ethanol. The fluorescence (F_1) was measured with an excitation wavelength of 365 nm and an emission wavelength of 460 nm. The slit widths for excitation and emission were 5 and 15 nm, respectively. Alcohol dehydrogenase from yeast (2 units) was used to convert the NAD$^+$ to NADH, and the fluorescence was measured again (F_2). Another fluorescence reading (F_3) was taken after addition of 10 μL of 80 μM NAD$^+$ as an internal standard. The concentration of NAD$^+$ in this assay solution was calculated from the change of fluorescence emission at 460 nm due to NADH formation: $[F_2 - F_1]$[concentration of NAD$^+$ internal standard]/$[F_3 - F_2]$. For the determination of NADP$^+$, 12 μL of 0.2 M glucose-6-phosphate and 3 μL of 1 M $MgSO_4$ were combined with 500 μL of the neutralized supernatant from the original $HClO_4$ enzyme extraction. The fluorescence of this solution was measured as described above. Glucose-6-phosphate dehydrogenase from yeast (4 units) was used to convert NADP$^+$ to NADPH. The internal standard was 10 μL of 80 μM NADP$^+$.

Determination of bound NADH and NADPH was performed by first boiling the enzyme for 3 min to release the coenzyme. After removing the precipitated protein by centrifugation, the fluorescence of the supernatant (500 μL) was measured directly as described above. The fluorescence was recorded again after addition of 10 μL of 80 μM NAD(P)H as an internal standard.

Production and Characterization of CDP-D-tyvelose. The substrate for CDP-D-tyvelose 2-epimerase was prepared from CDP-D-glucose (**2**) using four enzymes previously overexpressed in our laboratory.[11,19−21] A 1-mL reaction contained 29 μmol of **2** and 6 mg of CDP-D-glucose 4,6-dehydratase (E_{od}, S.A. = 125 μmol h^{-1} mg^{-1})[22] in 50 mM potassium phosphate buffer, pH 7.5, was incubated for 2 h at room temperature. The extend of conversion to the product, CDP-6-deoxy-D-*glycero*-L-*threo*-4-hexulose (**3**), was determined spectrophotometrically at 320 nm under alkaline conditions.[22,23] To this solution was added 40 μmol of NADH, 12 mg of CDP-6-deoxy-D-*glycero*-L-*threo*-4-hexulose 3-dehydrase (E_1, S.A. = 100 nmol min^{-1} mg^{-1}) and 0.6 mg of E_1 reductase (E_3, S.A. = 40 μmol min^{-1} mg^{-1}), followed by incubation for 2 h at room temperature.[19,24] The extent of conversion of **3** to **4** was analyzed by SAX HPLC using conditions described in "Enzyme Assay". The product, CDP-3,6-dideoxy-D-*glycero*-D-*glycero*-4-hexulose (**4**, ∼29 μmol), without purification was incubated with 40 μmol of NADPH and 2 mg of CDP-D-paratose synthase (S.A. = 90 μmol min^{-1} mg^{-1}) in 1 mL of 50 mM potassium phosphate buffer (pH 7.5) for 2 h at room temperature.[11] The conversion was monitored by the consumption of NADPH at 340 nm, and once the conversion was complete, the protein was removed with a Centricon 10 microconcentrator (Amicon). The resulting CDP-D-paratose (**7**) was purified by FPLC using a MonoQ HR (10/10) column with a linear gradient from 0 to 150 mM NH_4HCO_3 over 30 min, followed by a 5 min wash with 500 mM NH_4HCO_3. The purified product was lyophilized, and its identity was verified by ^{1}H NMR.[11] CDP-D-paratose (**7**, 1.3 μmol) was then incubated with 80 μg of CDP-D-tyvelose 2-epimerase (S.A. = 250 nmol min^{-1} mg^{-1}) in 200 μL of 50 mM potassium phosphate buffer (pH 7.5) for 1.5 h at room temperature, and the conversion to CDP-D-tyvelose (**8**) was followed by SAX HPLC as described under "Enzyme Assay". The desired product was purified by FPLC using the identical conditions for the isolation of CDP-D-paratose. The purified CDP-D-tyvelose (**8**) was lyophilized, and the identity was confirmed by ^{1}H NMR. ^{1}H NMR (^2H_2O, 300 MHz) 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, 5″-H), 7.82 (1H, d, J = 7.5 Hz, 6″-H). ^{13}C NMR (^2H_2O, 75 MHz) δ 16.8, 32.5, 64.4 (d, J = 4.5 Hz), 66.7, 67.6 (d, J = 9.8 Hz), 69.0, 70.5, 74.2, 82.6 (d, J = 9.8 Hz), 89.2, 94.9 (d, J = 7.5 Hz), 96.5, 141.3, 160.2, 166.1.

Synthesis of CDP-D-[2-^{2}H]paratose. The 2-^{2}H-labeled substrate for CDP-D-tyvelose 2-epimerase was enzymatically synthesized from D-[2-^{2}H]glucose. The 4-mL incubation mixture contained 20 μmol of D-[2-

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^{2}H]glucose (Cambridge Isotope Laboratories), 150 μmol of $MgCl_2$, 72 μmol of ATP, and 60 units of hexokinase in 50 mM potassium phosphate buffer (pH 7.5). After an incubation of 1 h at room temperature, 33 μmol of CTP, 25 units of inorganic pyrophosphatase, 70 units of rabbit muscle phosphoglucomutase, 1 μmol of glucose 1,6-diphosphate, and 8 mg of α-D-glucose-1-phosphate cytidylyltransferase (E_p, S.A. = 10 μmol h^{-1} mg^{-1}) were added, and the incubation was continued for 2 h at room temperature. To this mixture was then added 370 units of E_{od}, and incubation was allowed to proceed for another 2 h at room temperature. After the conversion to **3** was complete, 6 mg of E_1, 1 mg of E_3, and 30 μmol of NADH were added with another 2 h of incubation at room temperature. The final transformation to make CDP-D-paratose (**7**) was accomplished by the addition of 25 μmol of NADH and 0.5 mg of CDP-D-paratose synthase to the above reaction mixture, followed by 1 h of incubation at room temperature. The proteins were removed by a Centricon 10 microconcentrator (Amicon), and the resulting CDP-D-[2-^{2}H]paratose was purified by FPLC as described under "Production and Characterization of CDP-D-tyvelose". After lyophilization, the sample was analyzed by ^{1}H NMR to confirm and quantify the incorporation of deuterium at C-2.

Synthesis of CDP-D-[4-^{2}H]paratose. Labeling of CDP-D-paratose at C-4 with deuterium was accomplished by using (4*S*)-[4-^{2}H]NADPH[25] in the reduction of CDP-3,6-dideoxy-D-*glycero*-D-*glycero*-4-hexulose (**4**) by CDP-D-paratose synthase. To make the labeled coenzyme, a mixture of 20 μmol of $NADP^+$, 100 μmol of D-[1-^{2}H]glucose (Cambridge Isotope Laboratories, Inc.), and 12 units of glucose dehydrogenase from *Bacillus megaterum* in 3 mL of 50 mM potassium phosphate buffer (pH 7.5) was incubated at room temperature for 1 h. The progress of the reaction was monitored at 340 nm. After the glucose dehydrogenase was removed with a Centricon 10 microconcentrator (Amicon), the filtrate was mixed with 9 μmol of **4** and 0.5 mg of CDP-D-paratose synthase, and the reaction was incubated for 1 h at room temperature. The deuterated CDP-D-paratose was purified by HPLC with a Spherisorb S10 SAX semiprep column (1.0 × 25 cm) using a gradient from 50 mM to 118 mM potassium phosphate, pH 3.5, followed by a 5 min wash with 500 mM potassium phosphate, pH 3.5. The flow rate was 3 mL/min, and the detector was set at 270 nm. The collected sample was neutralized with 5 N KOH and lyophilized. The salts were removed by dissolving the sugar product in 70% ethanol, pipetting the supernatant from the settled precipitate, and subjecting the supernatant to rotary evaporation to remove the ethanol. The sample was dissolved in ^2H_2O and lyophilized again to ensure the removal of all ethanol before analysis by ^{1}H NMR to confirm and quantify the incorporation of deuterium at C-4.

Incubation in Buffer Made with ^2H_2O. A 2-mL reaction contained approximately 0.3 mg of CDP-D-tyvelose 2-epimerase, 3 μmol of CDP-D-paratose, and 20 mM Tris-HCl buffer, pD 7.5, made with >99% deuterated water (Cambridge Isotope Laboratories). The reaction was incubated at room temperature for 2 h, and the protein was removed with a Centricon 10 microconcentrator. The product/substrate mixture was purified by FPLC as described under "Production and Characterization of CDP-D-tyvelose". The collected peaks were lyophilized and analyzed for deuterium incorporation by ^{1}H NMR.

Incubation in Buffer Made with $H_2{}^{18}O$. To a lyophilized sample of 0.45 μmol of CDP-D-paratose and 0.1 μmol of NAD^+ were added 0.08 mg of CDP-D-tyvelose 2-epimerase (20 μL), 20 μL of 1 M Tris-HCl buffer (pH 7.6), and 1 g of $H_2{}^{18}O$ (Isotec Inc., 96.8%). The mixture was incubated at room temperature for 1 h, and a control incubation was performed concurrently in regular buffer. The product and substrate from each incubation were purified by FPLC as described under "Production and Characterization of CDP-D-tyvelose". The collected peaks were lyophilized and analyzed by high-resolution FAB MS.

Incubation with CDP-D-[2-^{2}H]paratose. The reaction contained 3.6 μmol of CDP-D-[2-^{2}H]paratose and approximately 0.3 mg of CDP-D-tyvelose 2-epimerase in 0.8 mL of 50 mM potassium phosphate buffer, pH 7.5. The progress of the reaction was monitored by SAX HPLC as described under "Enzyme Assay". After 2 h of incubation at room temperature, the sample was purified by SAX HPLC as described under "Synthesis of CDP-D-[4-^{2}H]paratose". The isolated CDP-D-paratose and CDP-D-tyvelose were further purified by FPLC as described under "Production and Characterization of CDP-D-tyvelose". The position of the deuterium in the purified samples was determined by ^{1}H NMR.

Reduction of Enzyme Bound NAD^+ with $NaBH_4$. A 500-μL reaction containing 1.5 mg of CDP-D-tyvelose 2-epimerase in 20 mM Tris-HCl buffer, pH 7.5 was treated with approximately 1 mg of solid $NaBH_4$. After the foaming subsided, the solution was dialyzed against 500 mL of 20 mM Tris-HCl buffer (pH 7.5) overnight. Reduction of the bound NAD^+ was confirmed by observing the increase at 350 nm after treatment with $NaBH_4$.

Incubation of CDP-3,6-dideoxy-D-*glycero*-D-*glycero*-4-hexulose and Enzyme Reduced with $NaBH_4$. Three incubations were performed. The first sample contained approximately 12 nmol of CDP-3,6-dideoxy-D-*glycero*-D-*glycero*-4-hexulose (**4**) and 148 μg of reduced CDP-D-tyvelose 2-epimerase in 150 μL of 20 mM Tris-HCl buffer (pH 7.5), while the second contained only enzyme and the third contained only **4**. After incubation for 2 h at room temperature, all reactions were spun through a Microcon microconcentrator to remove protein. The reactions were then analyzed by SAX HPLC as described in "Enzyme Assay". Another sample containing a mixture of CDP-D-tyvelose and CDP-D-paratose was used as a control for comparison during the HPLC analysis.

Investigation of CDP-D-glucose and CDP-6-deoxy-D-glucose as Substrates. A 100-μL incubation containing 20 μg of CDP-D-tyvelose 2-epimerase, 20 nmol of CDP-D-glucose (**2**), and 50 mM potassium phosphate buffer, pH 7.5 was carried out in parallel with a control reaction without enzyme. After incubation for 20 min at room temperature, the enzyme was denatured by heating at 85 °C for 3 min. A similar incubation was also performed with CDP-6-deoxy-D-glucose. The formation of new products was analyzed by SAX HPLC under conditions described in "Enzyme Assay".

Large Scale Incubation of CDP-6-deoxy-D-glucose. A reaction containing 15 μmol of CDP-6-deoxy-D-*glycero*-L-*threo*-4-hexulose (**3**), 20 μmol of NADPH, and 3 mg of CDP-D-paratose synthase in 1 mL of 50 mM potassium phosphate buffer, pH 7.5 was incubated at room temperature. The formation of CDP-6-deoxy-D-glucose was followed by the consumption of NADPH at A_{340}. After the conversion was complete, the reaction was mixed with 1.2 mg of CDP-D-tyvelose 2-epimerase, and incubation was continued at room temperature for 1 h. The protein was removed with a Centricon 10 microconcentrator, and the product was purified by HPLC with a Spherisorb S10 SAX semiprep column (1.0 × 25 cm) as described under "Synthesis of CDP-D-[4-^{2}H]paratose". The purified product, CDP-6-deoxy-D-mannose, was lyophilized and analyzed by ^{1}H NMR. ^{1}H NMR (^2H_2O, 300 MHz) δ 0.96 (3H, d, J = 6 Hz, 5-Me), 3.13 (1H, dd, J = 9.6, 9.6 Hz, 4-H), 3.54 (1H, dd, J = 3.4, 9.6 Hz, 3-H), 3.58 (1H, m, 5-H), 3.72 (1H, dd, J = 1.8, 3.4 Hz, 2-H), 3.82−4.05 (5H, m, 2′-H, 3′-H, 4′-H, 5′-Hs), 5.12 (1H, dd, J = 1.8, 7.5 Hz, 1-H), 5.68 (1H, d, J = 3.9 Hz, 1′-H), 5.85 (1H, d, J = 7.5 Hz, 5″-H), 7.70 (1H, d, J = 7.5 Hz, 6″-H).

Synthesis of CDP-4-deoxy-4-fluoro-D-paratose (14). Methyl 2-*O*-Benzyl-3,6-dideoxy-α-D-*ribo*-hexopyranoside (17). A solution of methyl 3,6-dideoxy-α-D-*ribo*-hexopyranoside **16**[26] (6.0 g, 37 mmol) and tributyltin oxide (40 mL, 75 mmol) in dry toluene (250 mL) was refluxed for 23 h under N_2 in a flask equipped with a Dean−Stark separator. After the toluene was removed by distillation, the residue was mixed with benzyl bromide (27 mL, 220 mmol), heated at 95 °C for 24 h, cooled to room temperature, and purified by flash chromatography on silica gel. Tributyltin oxide and excess benzyl bromide were removed first with hexanes, followed by elution with hexanes: ethyl acetate (5:1 to 1:1), to give the benzyl ether **17** (4.2 g, 45% yield) as a colorless oil.^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.21 (3H, d, J = 6.3 Hz, 5-Me), 1.74 (1H, br s, OH), 1.80 (1H, m, 3-H_{ax}), 2.16 (1H, ddd, J = 11.4, 4.8, 4.8 Hz, 3-H_{eq}), 3.21 (1H, ddd, J = 11.4, 9.3, 4.8 Hz, 4-H), 3.40 (3H, s, OMe), 3.51 (2H, m, 2-H, 5-H), 4.55 and 4.62 (2H, ABq, J = 12.3 Hz, $-CH_2Ph$), 4.60 (1H, d, J = 2.4 Hz, 1-H), 7.32 (5H, m, PhHs). ^{13}C NMR ($CDCl_3$, 50 MHz) δ 17.4 (C-6), 33.5 (C-3), 54.8 (OMe), 68.5 (C-5), 71.0 (C-4)*, 71.1 (benzylic-C)*, 73.9 (C-2), 93.1

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(C-1), 127.8, 128.4, 128.5, 138.1. HRMS (CI) calcd for $C_{14}H_{24}NO_4$ ($M + NH_4^+$) 270.1705; found 270.1695.

Methyl 2-*O*-Benzyl-4-fluoro-3,4,6-trideoxy-α-D-*ribo*-hexopyranoside (18). To a solution of benzyl ether **17** (2.38 g, 9.4 mmol) in dry CH_2Cl_2 (100 mL) was added 1.6 mL of (diethylamino)sulfur trifluoride (DAST) dropwise at 0 °C. After being stirred for 1 h at room temperature, the mixture was diluted with CH_2Cl_2, washed with saturated aqueous $NaHCO_3$, and dried over anhydrous Na_2SO_4. The solvent was removed in vacuo, and the residue was purified by flash chromatography on silica gel (eluent: hexanes:ethyl acetate, 10:1 to 5:1) to give the 4-fluorosugar **18** (0.85 g, 35% yield) as a colorless oil. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.24 (3H, dd, $J = 6.3$, 0.9 Hz, 5-Me), 2.01 (1H, m, 3-H_{ax}), 2.31 (1H, m, 3-H_{eq}), 3.42 (3H, s, OMe), 3.50 (1H, m, 2-H), 3.71 (1H, m, 5-H), 4.02 (1H, dddd, $J = 48.6$, 11.1, 9.3, 4.8 Hz, 4-H), 4.55 and 4.63 (2H, ABq, $J = 12.6$ Hz, benzylic-Hs), 4.60 (1H, d, $J = 3.9$ Hz, 1-H), 7.33 (5H, m, PhHs). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 17.1 (C-6), 30.9 (d, $J = 19.1$ Hz, C-3), 55.0 (OMe), 65.8 (d, $J = 24.4$ Hz, C-5), 71.2 (benzylic-C), 73.4 (d, $J = 10.9$ Hz, C-2), 90.3 (d, $J = 180.2$ Hz, C-4), 96.9 (d, $J = 1.6$ Hz, C-1), 127.8, 127.9, 128.5, 137.9. ^{19}F NMR ($CDCl_3$, 188 MHz) δ −185.3 (ddd, $J = 48.7$, 10.0, 4.5 Hz). HRMS (CI) calcd for $C_{14}H_{23}FNO_3$ ($M + NH_4^+$) 272.1662; found 272.1647.

Methyl 4-Fluoro-3,4,6-trideoxy-α-D-*ribo*-hexopyranoside (19). A solution of compound **18** (0.85 g, 3.3 mmol) in methanol (40 mL) containing 10% Pd/C catalyst (100 mg) was hydrogenated for 2 h under a H_2 atmosphere. The mixture was filtered through Celite, concentrated, and coevaporated with benzene (20 mL) to give the product **19** (0.52 g, 93% yield) as a colorless oil that was used without further purification. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.26 (3H, dd, $J = 6.3$, 1.5 Hz, 5-Me), 1.81 (1H, m, 3-H_{ax}), 1.99 (1H, br s, OH), 2.35 (1H, m, 3-H_{eq}), 3.44 (3H, s, OMe), 3.67 (2H, m, 2-H, 5-H), 4.05 (1H, dddd, $J = 48.9$, 11.1, 9.3, 4.8 Hz, 4-H), 4.58 (1H, dd, $J = 4.2$, 3.9 Hz, 1-H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 17.1 (C-6), 34.5 (d, $J = 18.5$ Hz, C-3), 55.2 (OMe), 65.9 (d, $J = 24.9$ Hz, C-5), 67.3 (d, $J = 11.1$ Hz, C-2), 90.0 (d, $J = 180.4$ Hz, C-4), 98.2 (d, $J = 1.6$ Hz, C-1).^{19}F NMR ($CDCl_3$, 282 MHz) δ −195.3 (m). HRMS (CI) calcd for $C_7H_{17}FNO_3$ ($M + NH_4^+$) 182.1192; found 182.1199.

1,2-Di-*O*-Acetyl-4-fluoro-3,4,6-trideoxy-α-D-*ribo*-hexopyranose (20). To a solution of **19** (0.52 g, 3.1 mmol) in acetic anhydride (5 mL) was added 4 drops of concentrated sulfuric acid at 0 °C. After stirring at room temperature for 12 h, the mixture was diluted with ethyl acetate and H_2O, neutralized with $NaHCO_3$, washed with brine, and dried over anhydrous Na_2SO_4. The solvents were removed by rotary evaporation, and the residue was chromatographed on silica gel (eluent: hexanes: ethyl acetate, 10:1 to 5:1) to afford the diacetyl ester **20** (0.53 g, 72% yield, $\alpha/\beta > 9/1$) as a colorless oil. Analytical data for the major isomer were as follows.^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.25 (3H, d, $J = 6.3$ Hz, 5-Me), 2.00 (3H, s, OAc), 2.02 (1H, m, 3-H_{ax}), 2.13 (3H, s, OAc), 2.36 (1H, m, 3-H_{eq}), 3.82 (1H, m, 5-H), 4.17 (1H, dddd, $J = 48.0$, 11.1, 9.3, 4.8 Hz, 4-H), 4.90 (1H, m, 2-H), 6.10 (1H, dd, $J = 3.9$, 3.6 Hz, 1-H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 17.2 (C-6), 20.7 (OAc), 21.0 (OAc), 30.2 (d, $J = 20.6$ Hz, C-3), 66.8 (d, $J = 12.1$ Hz, C-2), 68.4 (d, $J = 24.9$ Hz, C-5), 88.2 (d, $J = 1.6$ Hz, C-1), 89.1 (d, $J = 180.5$ Hz, C-4), 169.2 (C═O), 169.8 (C═O). ^{19}F NMR ($CDCl_3$, 282 MHz) δ −196.2 (ddd, $J = 48.2$, 9.9, 4.5 Hz). HRMS (CI) calcd for $C_{10}H_{19}FNO_5$ ($M + NH_4^+$) 252.1247; found 252.1241.

Dibenzyl 2-*O*-Acetyl-4-fluoro-3,4,6-trideoxy-α-D-*ribo*-hexopyranyl Phosphate (21). A mixture of diacetyl ester **20** (0.26 g, 1.1 mmol) and tributyltin methoxide (0.4 mL) in dry CH_2Cl_2 (15 mL) was refluxed for 56 h under N_2. The mixture was concentrated in vacuo and chromatographed on silica gel (eluent: hexanes:ethyl acetate, 5:1 to 2:1) to afford the C-1 deprotected sugar (0.10 g, 86% yield based on converted starting material) along with recovered diacetyl ester **20** (0.12 g). The C-1 deprotected sugar (0.10 g, 0.52 mmol) was dissolved in dry THF (4 mL) at −78 °C. To this solution was added *n*-BuLi in hexanes (1.6 M, 0.4 mL) dropwise. After stirring for 20 min, a solution of dibenzyl phosphorochloridate (prepared from 1 mmol of dibenzyl phosphite and 1 mmol of *N*-chlorosuccinimide in benzene) in dry THF (2 mL) was added. The resulting solution was stirred at −78 °C for 30 min, and then at 0 °C for 2 h. The mixture was concentrated at room temperature, diluted with ether, washed with H_2O, and dried over anhydrous Na_2SO_4. The solvents were removed in vacuo, and the residue was purified by flash chromatography on silica gel (eluent: hexanes:ethyl acetate, 5:1 to 1:1) to give the product **21** (83 mg, 44% yield based on the converted alcohol) as a colorless oil, together with the C-1 deprotected sugar (21 mg). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.20 (3H, dd, $J = 6.3$, 0.9 Hz, 5-Me), 1.92 (3H, s, OAc), 2.01 (1H, m, 3-H_{ax}), 2.34 (1H, m, 3-H_{eq}), 3.85 (1H, m, 5-H), 4.15 (1H, dddd, $J = 48.3$, 11.4, 9.9, 5.1 Hz, 4-H), 4.79−4.87 (1H, m, 2-H), 5.10 (4H, m, 2× PhHs), 5.72 (1H, m, 1-H), 7.36 (10H, m, PhHs). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 16.9 (C-6), 29.6 (d, $J = 20.6$ Hz, C-3), 67.4 (dd, $J = 12.4$, 7.9 Hz, C-2), 68.0 (d, $J = 24.9$ Hz, C-5), 69.4 (dd, $J = 5.5$, 4.2 Hz, benzylic-C), 88.9 (d, $J = 181.0$ Hz, C-4), 93.2 (dd, $J = 5.8$, 1.8 Hz, C-1), 127.8, 127.9, 128.6, 128.7, 135.5, 135.6, 169.9.^{19}F NMR ($CDCl_3$, 282 MHz) δ −186.6 (dd, $J = 48.1$, 4.9 Hz). ^{31}P NMR ($CDCl_3$, 121 MHz) δ −1.5. HRMS (FAB) calcd for $C_{22}H_{27}FNO_7P$ ($M + H^+$) 453.1502; found 453.1490.

CDP-4-deoxy-4-fluoro-D-paratose (14). A suspension of **21** (103 mg) and 10% Pd/C catalyst (40 mg) in a 1:1 MeOH/EtOAc solution (10 mL) was stirred under a H_2 atmosphere at room temperature for 20 min. The mixture was filtered through Celite, and the filtrate was neutralized with 3 drops of triethylamine and dried under reduced pressure to give a colorless syrupy residue. The residue was stirred with 2 N LiOH solution (10 mL) overnight and then with Dowex-50W resin (H^+ form) for 30 min. The resin was removed by filtration, and the aqueous solution was neutralized with triethylamine and lyophilized to give the coupling precursor (112 mg) as a white amorphous solid. A mixture of the coupling precursor (67 mg) and cytidine 5′-monophosphomorpholidate (274 mg) was coevaporated with dry pyridine (3 × 1.5 mL) to form a white powder, to which was added dry pyridine (2.0 mL) and 1*H*-tetrazole (48 mg). After the mixture was stirred for 40 h at room temperature, the solvents were removed under vacuum and the residue was dissolved in H_2O. Purification was accomplished with a Bio-Rad P2 column (2 cm × 1.2 m) using 50 mM NH_4HCO_3 as the mobile phase. The desired fractions were detected by UV absorption at 267 nm and lyophilized to give the coupling product **14** (59 mg, 54% yield) as a white powder.^{1}H NMR (^2H_2O, 300 MHz) δ 1.02 (3H, d, $J = 7.2$ Hz, 5-Me), 1.65 (1H, m, 3-H_{ax}), 2.07 (1H, m, 3-H_{eq}), 3.58 (1H, m, 2-H), 3.74 (1H, m, 5-H), 3.91−4.15 (6H, m, 4-H, 2′-H, 3′-H, 4′-H, 5′-Hs), 5.23 (1H, m, 1-H), 5.72 (1H, d, $J = 3.6$ Hz, 1′-H), 5.90 (1H, d, $J = 7.5$ Hz, 5″-H), 7.77 (1H, d, $J = 7.8$ Hz, 6″-H). ^{13}C NMR (^2H_2O, 75 MHz) δ 16.1, 32.1 (d, $J = 18.8$ Hz), 64.2 (d, $J = 5.4$ Hz), 67.1 (d, $J = 5.4$ Hz), 68.4, 74.2, 82.3 (d, $J = 9.6$ Hz), 89.5, 89.8 (d, $J = 176$ Hz), 94.0 (d, $J = 5.4$ Hz), 96.4, 141.2, 157.5, 160.2, 166.1.^{19}F NMR (^2H_2O, 282 MHz) δ −185.3 (dd, $J = 48.2$, 5.6 Hz). ^{31}P NMR (^2H_2O, 121 MHz) δ −10.5 (d, $J = 21.4$ Hz), −12.1 (d, $J = 21.4$ Hz). HRMS (ESI) calcd for $C_{15}H_{23}FN_3P_2O_{13}$ ($M - H^-$) 534.0690; found 534.0688.

Investigation of CDP-4-deoxy-4-fluoro-D-paratose (14) as a Substrate. CDP-4-deoxy-4-fluoro-D-paratose (**14**, 7.4 nmol) was mixed with 0.1 mg of CDP-D-tyvelose 2-epimerase in 100 μL of 20 mM Tris-HCl buffer (pH 7.5). After incubation at room temperature for 1 h, the sample was analyzed by HPLC using a SAX column as described under "Enzyme Assay". A large incubation was performed where 37 μmol of **14** and 4.5 mg of CDP-D-tyvelose 2-epimerase were incubated in 1.3 mL of 20 mM Tris-HCl buffer (pH 7.5) for approximately 8 h at room temperature. The product, CDP-4-deoxy-4-fluoro-D-tyvelose, was purified by FPLC as described under "Production and Characterization of CDP-D-tyvelose" and analyzed by ^{1}H NMR. ^{1}H NMR (^2H_2O, 300 MHz) δ 0.94 (3H, d, $J = 6.3$ Hz, 5-Me), 1.71 (1H, dddd, $J = 3.0$, 13.2, 13.2, 13.2 Hz, 3-H_{ax}), 1.87 (1H, m, 3-H_{eq}), 3.73 (2H, m, 2-H, 5-H), 3.84−4.02 (5H, m, 2′-H, 3′-H, 4′-H, 5′-Hs), 4.18 (1H, dddd, $J = 4.8$, 9.3, 9.3, 49 Hz, 4-H), 4.96 (1H, m, 1-H), 5.65 (1H, d, $J = 3.9$ Hz, 1′-H), 5.80 (1H, d, $J = 7.5$ Hz, 5″-H), 7.62 (1H, d, $J = 7.5$ Hz, 6″-H). ^{13}C NMR (^2H_2O, 75 MHz) δ 16.2, 30.3 (d, $J = 20.4$ Hz), 64.5 (d, $J = 5.6$ Hz), 68.2 (d, $J = 25$ Hz), 68.9, 73.9, 82.3 (d, $J = 9.3$ Hz), 89.0 (d, $J = 171$ Hz), 89.2, 94.7 (d, $J = 6.1$ Hz), 96.4, 141.2, 157, 160.4, 165.9. ^{19}F NMR (^2H_2O, 282 MHz) δ −190.4 (dd, $J = 5$, 48 Hz). ^{31}P NMR (^2H_2O, 121 MHz) δ −10.8 (d, $J = 50$ Hz), −13.0 (d, $J = 50$ Hz). HRMS (FAB): calcd for $C_{15}H_{23}FN_3O_{13}P_2$ 534.0690; found 534.0703.

Metal Analysis of CDP-D-tyvelose 2-Epimerase by Inductively Coupled Plasma (ICP) Spectroscopy. CDP-D-tyvelose 2-epimerase

(3 mg) was dialyzed against 1 L of 20 mM Tris-HCl buffer (pH 7.5) containing 10% glycerol overnight. The enzyme (~2 mg) was diluted to 5 mL with 10% HCl. A control containing an equal amount of dialysis buffer was also diluted to 5 mL with 10% HCl. ICP analysis was performed on both samples by the Research Analytical Laboratory in the Department of Soil, Water, and Climate at the University of Minnesota.

Treatment of CDP-D-tyvelose 2-Epimerase with 20 mM EDTA. A solution of 0.3 mg of epimerase in 1 mL of 100 mM potassium phosphate buffer (pH 7.5) containing 20 mM EDTA was left at room temperature for 3 h and then stored at 4 °C. A control without EDTA was also prepared. The activity of the epimerase with and without EDTA was directly assayed after 1 day, 2 days, and 5 days of incubation.

Treatment of CDP-D-tyvelose 2-Epimerase with 1,10-Phenanthroline. CDP-D-tyvelose 2-epimerase (3 mg) was dialyzed for 4 days against 500 mL of 50 mM potassium phosphate buffer (pH 7.5) that contained 5 mM 1,10-phenathroline and 1.5 g of chelex. A control was run in parallel in which CDP-D-tyvelose 2-epimerase (3 mg) was dialyzed against 50 mM potassium phosphate buffer (pH 7.5) with 1.5 g of chelex but without 1,10-phenanthroline. The activity was monitored as described in "Enzyme Assay". At the end of fourth day, the samples were submitted for ICP analysis.

Stopped-Flow Studies. Stopped-flow mixing studies were performed on a Hi-Tech SF-61 DX2 double mixing stopped-flow system configured for fluorescence detection. During fluorescence detection, the excitation wavelength was set at 330 nm, and a cutoff filter of 360 nm was used, while wavelengths from 370 to 700 nm were monitored for emission. All runs were performed at 4 °C unless otherwise indicated. KinetAsyst 2, version 2.1a, software from Hi-Tech was used for fitting the data.

Prolonged Incubations with Deuterated CDP-D-paratose. Two incubations were carried out and each contained 9.3 mg of CDP-D-tyvelose 2-epimerase and either 3.3 μmol of C-2 deuterated CDP-D-paratose or 2.3 μmol of C-4 deuterated CDP-D-paratose. After incubation at room temperature overnight, the bound cofactors were released by boiling for 3 min. The denatured protein was removed by centrifugation, and the resulting supernatant containing the NADH was incubated with 10 units of L-lactate dehydrogenase from rabbit muscle and 1.4 μmol of pyruvate for 20 min at room temperature. The conversion of NADH to NAD^+ was monitored at 340 nm. The NAD^+ was purified by FPLC using a MonoQ HR (10/10) column and a linear gradient from 0 to 40 mM NH_4HCO_3 during the first 12 min and from 40 to 250 mM NH_4HCO_3 during the next 12 min, followed by a 5-min wash with 500 mM NH_4HCO_3. The collected fractions were lyophilized and submitted for electrospray mass spectrometry.

Results

Cloning and Expression of the *tyv* Gene. Production of recombinant CDP-D-tyvelose 2-epimerase was made possible by the cloning and expression of the *tyv* gene (previously known as the *rfbE* gene)[14] from *Y. pseudotuberculosis* IVA.[12b] Initial attempts to amplify the desired gene by PCR using primers that incorporated restriction sites were unsuccessful. Therefore, primers corresponding to the flanking sequences at either end of the *tyv* gene were used to first amplify the desired gene from the genomic DNA of *Y. pseudotuberculosis* IVA by PCR. The resulting PCR product was then used as a template for the second round of PCR with primers that contained the restriction sites needed for cloning. The naturally occurring ribosomal binding site and the AT rich region preceding the start codon were included in the start primer, allowing the PCR product to be cloned directly into a transcription vector, pET-24(+), which carries a T7/*lac* promoter.

Purification and Characterization of CDP-D-tyvelose 2-Epimerase. SDS-PAGE analysis of the crude cell extract from the induced BL21(DE3) cells containing the pTHEp-10 construct indicated that the target protein was not overexpressed. However, after treatment of the crude cell extract with Ni-NTA resin to isolate the His-tagged protein, a single protein corresponding to the size of CDP-D-tyvelose 2-epimerase was collected (Figure 1). The yield was ~25 mg of purified epimerase from 6 L of culture. Attempts to improve the expression level by varying the cloning vector and expression conditions were unsuccessful. The identity of the purified protein was confirmed by N-terminal sequence analysis, revealing that its first 10 amino acid residue (M-K-L-L-I-T-G-G-C) matched those of the translated *tyv* sequence. The subunit molecular mass of 36 kDa, as approximated by SDS-PAGE, correlates well to the predicted value of 38 987 Da calculated from the translated sequence plus the His-tag. A molecular mass of 147 kDa, estimated by gel filtration, indicated that the native CDP-D-tyvelose 2-epimerase exists as a tetramer.

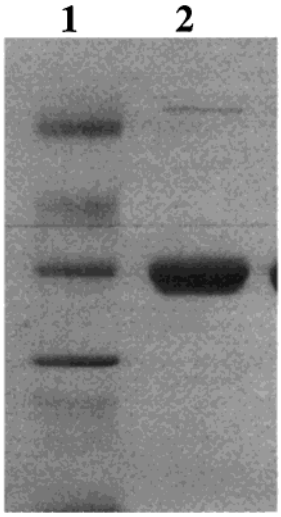


Figure 1. SDS-PAGE of purified CDP-D-tyvelose 2-epimerase. **(1)** Molecular weight standards, (2**)** CDP-D-tyvelose 2-epimerase.

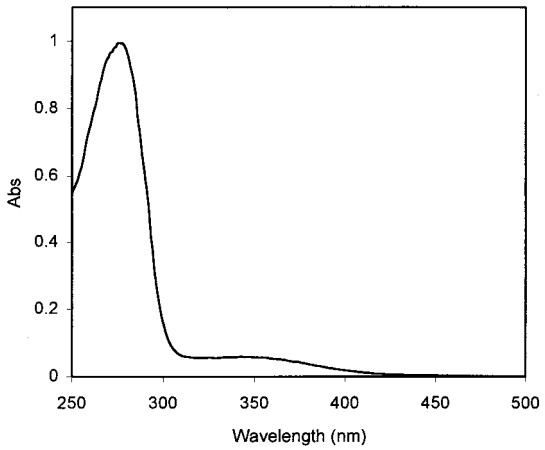


Figure 2. Electronic absorption spectrum of CDP-D-tyvelose 2-epimerase at 0.8 mg/mL in 20 mM Tris-HCl buffer, pH 7.5.

Quantitation of Bound $NAD(P)^+$ and NAD(P)H. Quantitative analysis of NAD^+ and NADH bound to CDP-D-tyvelose 2-epimerase was repeated twice on enzyme obtained from two separate purifications. In the first sample, 64% of the monomers contained bound NAD^+, while 32% had NADH. In the second sample, there were 69% NAD^+ and 22% NADH. Thus, these results clearly indicate one cofactor binding site per monomer, and the majority of coenzymes exist in the oxidized state.

Electronic Absorption Spectrum of CDP-D-tyvelose 2-Epimerase. As shown in Figure 2, the electronic absorption spectrum of CDP-D-tyvelose 2-epimerase displays a λ_{max} at 275

nm. A broad absorption band extending up to 450 nm is likely due to contributions from bound NADH and from a charge-transfer band between the bound NAD^+ and an amino acid residue in the active site. Such charge-transfer bands, known as "Racker Bands",[27] have also been observed in the electronic absorption spectrum of UDP-galactose 4-epimerase.[28]

Equilibrium Constant. The reaction catalyzed by CDP-D-tyvelose 2-epimerase is a reversible process. Separate reactions containing either CDP-D-paratose (**7**) or CDP-D-tyvelose (**8**) led to the same distribution of product and substrate upon reaching equilibrium. The determined K_{eq} of 1.22 at pH 7.5 reflects a nearly equal preference for CDP-D-tyvelose and CDP-D-paratose.

Isolation and Characterization of the CDP-D-tyvelose 2-Epimerase Product. Since the catalysis of CDP-D-tyvelose 2-epimerase has an almost equal preference for substrate and product at equilibrium, an incubation of enzyme and CDP-D-paratose always gave a mixture of CDP-D-tyvelose and CDP-D-paratose. These two compounds could be separated by FPLC, and the identity of purified CDP-D-tyvelose (**8**) was confirmed by ^{1}H NMR.

Kinetic Properties. Due to the highly reversible nature of the reaction, the kinetic parameters were determined in both directions. All reactions were performed in buffer at pH 7.5 since an analysis of activity over a range of pH from 6 to 8.5 revealed a pH optimum between 7.0 and 7.5, with a large decrease near pH 6.5 and a graduate decrease under more alkaline conditions. In the forward direction, the K_m for CDP-D-paratose was determined to be 6.8 ± 0.4 μM with a k_{cat} of 22 ± 1 min^{-1}. In the reverse direction, the K_m for CDP-D-tyvelose and the k_{cat} for the catalysis were determined to be 170 ± 20 μM and 240 ± 10 min^{-1}, respectively. Considering the kinetics were performed with a discontinuous HPLC assay, the actual errors for the K_m and k_{cat} values are likely to be much higher. Thus, such a large intrinsic error may have contributed to the discrepancy in the equilibrium constant ($K_{eq} = 1.22$) determined experimentally from the product and substrate ratio as compared to that ($K_{eq} = 2.3$) calculated from the Haldane relationship of the kinetic constants.

Incubation with CDP-D-[2-^{2}H]paratose. One possible mechanism proposed for C-2 epimerization by CDP-D-tyvelose 2-epimerase involves an intramolecular hydrogen transfer from C-2 to C-3 as shown in Mechanism B, Scheme 2. To test the feasibility of this mechanism, CDP-D-[2-^{2}H]paratose was prepared enzymatically from D-[2-^{2}H]glucose and used in the incubation. This synthetic sample was found to have approximately 50% deuterium at C-2. After incubation with the epimerase, the product and substrate were isolated and analyzed by ^{1}H NMR. Integration of the 2-H and 3-H peaks of the product and substrate revealed that, in both cases, C-2 was still 50% deuterated and C-3 contained no deuterium. Hence, Mechanism B is unlikely a possible mode of action.

Incubations Performed in ^2H_2O or $H_2{}^{18}O$. The possibility of hydrogen incorporation from the solvent into CDP-D-tyvelose and CDP-D-paratose during catalysis was investigated. The recovered sugar substrate or product from an incubation performed in deuterated water was analyzed by ^{1}H NMR. The NMR peak integrations in each case showed that no deuterium had been incorporated into the substrate or product. An incubation was also carried out in $H_2{}^{18}O$, and the two sugar derivatives in equilibrium were isolated and analyzed by high-resolution FAB MS. Comparison of the results to a control performed in unlabeled water revealed no significant differences, suggesting that the hydroxyl substituents of **7** and **8** remained intact during the catalysis. These results are inconsistent with Mechanisms B and C.

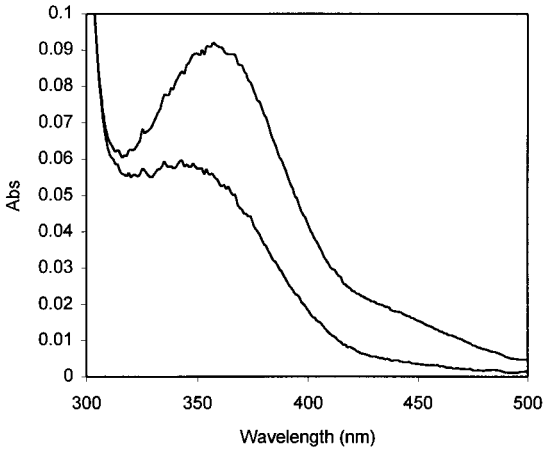


Figure 3. Electronic absorption spectrum of CDP-D-tyvelose 2-epimerase before (bottom line) and after (top line) reduction with $NaBH_4$. The enzyme concentration was 80 μg/mL in 20 mM Tris-HCl buffer, pH 7.5.

KIE of C-2 and C-4 Deuterated Substrate. The kinetic isotope effect (KIE) on *V*/*K* and *V* was calculated according to eq 2. When deuterium was present at C-2 of CDP-D-paratose, a KIE of 1.4 ± 0.2 was found for *V*/*K* and a KIE of 1.3 ± 0.1 was determined for *V*. Substrate containing a deuterium at C-4 displayed a KIE of 1.2 ± 0.1 for *V*/*K* and 0.90 ± 0.02 for *V*. These values are relatively small for a primary KIE, indicating that C−H bond cleavage is not an important rate-determining step. Since these two sets of data are similar, especially considering that the actual error of each value is likely to be much higher due to the intrinsic inaccuracy of the discontinuous assay, they cannot be used as evidence to distinguish which hydrogen is being removed from the sugar substrate during catalysis.

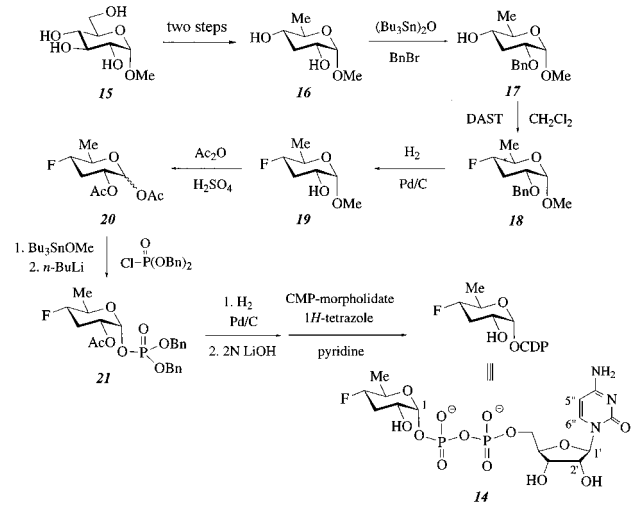
Reduction of Bound NAD^+ with $NaBH_4$. Treatment of CDP-D-tyvelose 2-epimerase with $NaBH_4$ had a pronounced effect on its electronic absorption spectrum. As the NAD^+ is reduced to NADH, a decrease in the charge-transfer band attributed to NAD^+ and an increase in absorbance due to NADH at wavelength longer than 300 nm is expected. As shown in Figure 3, after addition of $NaBH_4$, a large increase in absorbance at 360 nm was indeed observed. Interestingly, when substrate is added to the enzyme before reduction with $NaBH_4$, the large absorption increase occurs at 350 nm. Such a hypsochromic shift may be due to a conformational change of the epimerase in the presence of bound substrate or product.

Incubation of CDP-3,6-dideoxy-D-*glycero*-D-*glycero*-4-hexulose with Reduced Epimerase. All four of the proposed mechanisms for CDP-D-tyvelose 2-epimerase include a redox process in which NAD^+ and the sugar substrate are converted to NADH and a keto sugar intermediate (**9** or **11**), respectively, before being transformed back to NAD^+ and the sugar epimers. The 4-ketohexose intermediate predicted in Mechanisms C and D, CDP-3,6-dideoxy-D-*glycero*-D-*glycero*-4-hexulose (**11**) is also an intermediate (**4**) produced by the E_1/E_3 reaction in the biosynthetic pathway of CDP-D-tyvelose (see Scheme 1). The possibility that this compound **4** could be reduced by epimerase containing NADH was investigated to determine whether **11** is

(27) Rizzo, V.; Pande, A.; Luisi, P. L. In *Pyridine Nucleotide Coenzymes*; Dolphin, D., Poulson, R., Avramovic, O., Eds.; Wiley and Sons: New York, 1987; Vol. IIA, pp 99−161.

(28) Liu, Y.; Vanhooke, J. L.; Frey, P. A. *Biochemistry* **1996**, *35*, 7615−7620.

Scheme 4

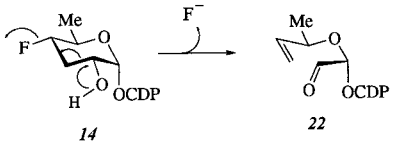


a real intermediate. The desired epimerase with bound NADH was prepared by $NaBH_4$ reduction of the native enzyme. After incubation of **4** with reduced enzyme, the resulting mixture was analyzed by HPLC. Two new peaks with retention times corresponding to those of CDP-D-tyvelose (**8**) and CDP-D-paratose (**7**) were observed in the HPLC chromatogram. These two new peaks were not detected when either enzyme or **4** was omitted from the incubation. Since the 4-keto sugar **4** could clearly be processed by the reduced enzyme, it was concluded that **4** (**11**) is likely an intermediate in this epimerization reaction. This result is most consistent with Mechanisms C and D.

Synthesis of CDP-4-fluoro-4-deoxyparatose (14). Synthesis of CDP-4-deoxy-4-fluoroparatose **14** was achieved as shown in Scheme 4. The precursor, methyl 3,6-dideoxy-α-D-*ribo*-hexopyranoside **16**, was prepared from methyl α-D-glucopyranoside **15** in two steps according to literature procedures.[26] Regioselective protection of the C-2 hydroxyl of **16** was effected by a tributyltin-activated benzylation procedure.[29] Directed fluorination at C-4 of **17** with diethylaminosulfurtrifluoride (DAST) afforded product **18** with retention of configuration in 35% yield. The corresponding 4-F epimer also formed, but at a lower yield. Although fluorination of secondary alcohols using DAST usually leads to inversion of stereochemistry, examples of retention of configuration are also known.[30] An alternative approach using the C-4 epimer of **17** as the reactant was also attempted. Preparation of this 4-epimer was accomplished by standard Mitsunobu conditions; however, no fluorinated product was discernible when it was subjected to the DAST reagent. The final steps of the synthesis shown in Scheme 4 were straightforward. Hydrogenation of **18**, followed by acetylation, afforded ester **20**. Selective deacetylation at C-1 with tributyltin methoxide, followed by phosphorylation and then coupling with cytidine 5′-monophosphomorpholidate, gave the desired sugar dinucleotide **14**.

Alternate Substrates for CDP-D-tyvelose 2-Epimerase. Although incubation of CDP-D-glucose (**2**) and epimerase failed to generate any products, incubation with CDP-6-deoxy-D-glucose did produce a new compound. NMR analysis of this new product from a large-scale incubation revealed that it is CDP-6-deoxy-D-mannose. These results clearly show that CDP-6-deoxy-D-glucose is a substrate and that a hydroxyl at C-6 of the hexose interferes with binding and thus prevents epimerization. However, the presence of a hydroxyl at C-3 of the substrate is apparently tolerated by the enzyme.

Scheme 5



Another compound that proved to be a substrate for CDP-D-tyvelose 2-epimerase is the 4-fluoro analogue of CDP-D-paratose (**14**), exhibiting a K_m of 42 ± 2 μM and a k_{cat} of 0.7 ± 0.1 min^{-1}. This molecule was originally designed as a probe for Mechanism D. If the retro-aldol mechanism (Mechanism D) is operative, incubation of **14** with reduced epimerase may lead to ring opening by releasing a fluoride ion to give **22** (Scheme 5). Should oxidation takes place at C-2 as in Mechanism A, this 4-fluoro analogue is expected to act as a substrate and be converted to the corresponding C-2 epimer. The latter was found to be the case.

Inhibitors of CDP-D-tyvelose 2-Epimerase. A comparison of CMP and CDP as inhibitors of CDP-D-tyvelose 2-epimerase demonstrated that a 23-fold excess of CMP was necessary to achieve a 50% decrease in activity, whereas a 12-fold excess of CDP was enough to completely inhibit this enzyme. The 4-F analogue of CDP-D-paratose (**14**) is also an inhibitor of CDP-D-tyvelose 2-epimerase, albeit less effective than CDP. A 23-fold excess of **14** displayed a 90% inhibition of activity.

Metal Content of CDP-D-tyvelose 2-Epimerase. Divalent metal ions were detected in the purified enzyme by ICP analysis. Interestingly, two nickel ions per tetramer were found in one case, and two zinc ions per tetramer but no nickel were found in a different batch. In an effort to determine if metal ions are necessary for activity, epimerase was treated with metal chelators. A comparison of epimerase treated with 20 mM EDTA to a control sample showed that the control lost 40% of its activity over 5 days at 4 °C, while the epimerase in EDTA maintained its activity. Interestingly, epimerase dialyzed against 5 mM 1,10-phenathroline displayed a similar rate of lossing activity to a control. By day 4, both the 1,10-phenanthroline sample and the control had lost 30% of their activity. ICP analysis revealed that the zinc had been removed from the epimerase dialyzed against 1,10-phenanthroline, but not the control, yet both exhibited the same activity. Thus, neither zinc nor nickel is necessary for activity.

Stopped-Flow Results. The possible accumulation of transiently formed NADH was investigated by stopped-flow spectrophotometry with fluorescence detection. When 75 μM of epimerase (3 mg/mL) and 50 μM of CDP-D-paratose were mixed, a very sharp increase in fluorescence (10%) was observed between 0.001 and 0.01 s. This experiment was repeated with either C-4 or C-2 deuterated substrate, and the rate of the increase was compared to that with unlabeled substrate to determine whether there is any KIE associated with hydride transfer to NAD^+. However, no significant rate change was found for either of the labeled substrates. These results suggest that either the increase in fluorescence is not due to NADH formation or a KIE cannot be detected under these conditions.

Accumulation of NADH Upon Prolonged Incubation with Excess Substrate. When an excess (10×) of CDP-D-paratose was incubated with CDP-D-tyvelose 2-epimerase over a long period of time (4−18 h), an increase in absorbance at 345 nm was observed. This increase is most likely due to the formation

(29) Dasgupta, F.; Garegg, P. J. *Synthesis* **1994**, 1121−1123.
(30) Wolba, D. M.; Razavi, H. A.; Clark, N. A.; Parmar, D. S. *J. Am. Chem. Soc.* **1988**, *110*, 8686−8691.

of an enzyme−NADH−CDP-D-paratose ternary complex resulting from the replacement of the keto sugar intermediate (**9** or **11**) with the excess substrate. Activity assays of the epimerase after such an incubation revealed a 30−50% loss of activity, since the NADH-containing enzyme is catalytically inactive. A similar accumulation of NADH was also observed with UDP-galactose 4-epimerase but on a much faster time scale.[31]

Prolonged Incubation with Deuterated CDP-D-paratose. The accumulation of NADH after prolonged incubation with excess substrate provided a convenient means to pinpoint which hydrogen, 2-H or 4-H, is transferred to the bound NAD^+ during catalysis. If the oxidation occurs at C-2, incubation with C-2 deuterated substrate is expected to give deuterated NADH, and conversely, if C-4 oxidation is operative, a reaction with C-4 deuterated substrate should produce deuterated NADH. After the two separate overnight incubations of CDP-D-tyvelose 2-epimerase and either excess C-2 or C-4 deuterated CDP-D-paratose were performed, the accumulated NADH in each sample was released by heat denaturation. However, purification of the resulting NADH proved to be difficult in the presence of the excess substrate. Therefore, the NADH was converted to NAD^+ by the pro-*R* stereospecific L-lactate dehydrogenase from rabbit muscle, which uses NADH to reduce pyruvate to L-lactate. The NAD^+ was then purified by FPLC and analyzed for deuterium content by positive ion electrospray mass spectrometry. NAD^+ from the incubation with C-4 deuterated substrate displayed a $(M + H + 1)^+/(M + H)^+$ of 0% after correction for natural isotope abundance. Interestingly, NAD^+ from the incubation with C-2-labeled substrate had a $(M + H + 1)^+/(M + H)^+$ of 10% after the same correction, indicating that the hydrogen is transferred from C-2 during the epimerization reaction.

Discussion

Inversion of stereocenters is a common transformation found in sugar biosynthesis and appears to be a convenient strategy used by nature to generate structural diversity of carbohydrates. Unlike most epimerases that catalyze stereochemical inversions adjacent to a carbonyl or a carboxylate, CDP-D-tyvelose 2-epimerase is an unusual enzyme because it epimerizes a stereocenter that bears no acidic proton and, thus, cannot utilize a deprotonation−reprotonation mechanism. Only a few enzymes are known that are capable of inverting such unactivated stereocenters, among which, the best-studied example is UDP-galactose 4-epimerase. This enzyme contains a tightly bound NAD^+ and catalyzes the interconversion between UDP-D-galactose and UDP-D-glucose.[6,28] Results from many studies support a mechanism in which the epimerase binds one of the substrate epimers and undergoes a conformational change to activate the cofactor toward hydride transfer. The nucleotide unit of the substrate serves as an anchor in the active site, but the 4-keto-hexopyranosyl moiety is allowed to rotate about the P_β of UDP and the glycosyl oxygen bond. Hydride transfer from C-4 of the hexose to the oxidized coenzyme produces NADH and a UDP-4-ketohexose intermediate. Rotation and exposure of the opposite face of the 4-ketohexose to the reduced cofactor permits hydride transfer to either side of the planar C-4, resulting in the formation of the epimeric products. Other nucleotide sugar 4-epimerases, such as UDP-*N*-acetylglucosamine 4-epimerase and UDP-glucuronate 4-epimerase, have been postulated to follow mechanisms analogous to that of UDP-galactose 4-epimerase.[6a]

Interestingly, the other two sugar epimerases that act on an unactivated stereocenter of their substrates are NAD^+ independent. One of these enzymes is UDP-*N*-acetylglucosamine 2-epimerase, which catalyzes the interconversion of UDP-*N*-acetylglucosamine and UDP-*N*-acetylmannosamine and is devoid of any redox cofactor.[32,33] On the basis of a recent positional isotope exchange (PIX) experiment, its catalysis has been shown to involve a C−O bond cleavage at C-1 to form a 2-acetamidoglucal intermediate.[8] The release and the re-ligation of the UDP moiety at C-1 is a reversible process. What ultimately leads to the observed epimerization is the deprotonation and reprotonation at C-2 taking place at opposite sides of the glucal intermediate.

The enzyme L-ribulose-5-phosphate 4-epimerase is another example of a carbohydrate epimerase that is able to invert an unactivated stereogenic center without using the redox chemistry of NAD^+. The interconversion of L-ribulose-5-phosphate and D-xylulose-5-phosphate by this epimerase requires a divalent metal cation per subunit for activity.[34] Since the N-terminal portion of L-ribulose-5-phosphate 4-epimerase is homologous to a class II L-fuculose-1-phosphate aldolase from *E. coli*, this epimerase was thus investigated whether it makes use of an aldol chemistry.[35,7] Results from these and other studies[34,36−38] suggest that the epimerase is capable of promoting carbon−carbon bond cleavage and support a mechanism that follows a retroaldol cleavage process, generating glycolaldehyde phosphate and the metal-bound enolate of dihydroxyacetone. Rotation of the aldehyde carbonyl exposes the opposite face, and upon aldol addition of the enolate, the epimeric sugar is formed.

As a new member of sugar epimerase that acts on an unactivated stereocenter, CDP-D-tyvelose 2-epimerase resembles UDP-D-galactose 4-epimerase more than other epimerases. Preliminary examination of the translated sequence of CDP-D-tyvelose 2-epimerase from *Y. pseudotuberculosis* IVA revealed a possible NAD^+ binding motif at its N terminus. Further analysis by BLAST search found that this epimerase shows strong homology (29% identity and 47% similarity) to TDP-glucose 4,6-dehydratase of *Salmonella typhimurium* (accession no. P26391) and UDP-galactose 4-epimerase of *E. coli* K12 (26% identity and 41% similarity, accession no. P09147). Since both of these enzymes operate on nucleotide sugars and use NAD^+ as a redox cofactor, the above sequence analysis provided the initial clue for a catalytic role of NAD^+ in the mechanism of CDP-D-tyvelose 2-epimerase.

Many experiments designed to differentiate the possible mechanisms proposed in Schemes 2 and 3 were carried out. As indicated earlier,[9] neither oxygen nor hydrogen from the solvent was found to be incorporated into the sugar products during catalysis as expected in Mechanisms B and C. In addition, labeling studies revealed that the hydrogen at C-2 was retained in both the product and the recovered substrate, negating a hydride transfer from C-2 to C-3 as predicted in Mechanism B. Thus, both Mechanisms B and C are disfavored based on these observations.

(31) Nelsestuen, G. L.; Kirkwood, S. *J. Biol. Chem.* **1971**, *246*, 7533−7543.

(32) Kawamura, T.; Kimura, M.; Yamamori, S.; Ito, E. *J. Biol. Chem.* **1978**, *253*, 3595−3601.

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(36) Deupree, J. D.; Wood, W. A. *J. Biol. Chem.* **1970**, *245*, 3988−3995.

(37) Davis, L.; Lee, N.; Glaser, L. *J. Biol. Chem.* **1972**, *247*, 5862−5866.

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The two remaining mechanisms, A and D, differ in the position where oxidation is initiated during the catalysis. Attempts to distinguish between these two mechanisms by determining the KIE of substrate labeled with deuterium at C-2 or C-4 led to inconclusive results. Since all of the proposed mechanisms in Schemes 2 and 3 involve the transient formation of NADH, stopped-flow spectrophotometry was used to determine the rate of NADH accumulation with the expectation that substrate labeled at C-2 or C-4 may display different rates for the reduction of NAD^+ to NADH. Unfortunately, the observed isotope effects were again ambiguous.

Interestingly, HPLC analysis of an incubation mixture containing reduced enzyme with bound NADH and the 4-ketohexose **11** revealed the conversion of **11** to CDP-D-tyvelose and CDP-D-paratose. This result is most consistent with the retroaldol mechanism (Mechanism D) that involves the formation of **11** as the intermediate. To further test the implicated retroaldol mechanism, a 4-fluoro analogue of CDP-D-paratose (**14**) was synthesized as a mechanistic probe. It was anticipated that **14**, lacking the 4-OH group required for oxidation to initiate the retro-aldol reaction, may simply act as a competitive inhibitor. It was also considered that **14**, upon incubation with reduced enzyme, could undergo ring opening and elimination of the 4-F to give an acyclic product, **22**, as shown in Scheme 5. Since the reactive aldehyde moiety in **22** may trap an active-site nucleophile, **14** may act as an inactivator for the epimerase. However, to our surprise, **14** was not processed by the reduced enzyme (E·NADH), but instead was recognized as a substrate for the native enzyme containing NAD^+ (E·NAD^+). This observation is clearly incompatible with Mechanism D in which 4-oxidation is a necessary step. It is possible that processing of **11** by the reduced enzyme, which was cited earlier as evidence supporting Mechanism D, may simply be a result of an aberrant reduction by NADH to generate CDP-D-paratose and the NAD^+-containing enzyme. Beyond the initial reduction, the reaction proceeds just like a normal incubation to give a mixture of CDP-D-paratose and CDP-D-tyvelose. Structural studies of CDP-D-tyvelose 2-epimerase will undoubtedly be helpful in revealing the basis for this anomalous reduction at C-4.

As Mechanism D can be ruled out as a viable pathway, Mechanism A, the only scenario that has not been eliminated, must be considered. The key difference between Mechanisms A and D is the position of the oxidation/reduction. Therefore, the best evidence to distinguish between these two possibilities would be a conclusive proof as to the regiochemistry of the initial oxidation. An opportune discovery led to a solution that allowed us to address this problem directly. Since prolonged incubation of CDP-D-tyvelose 2-epimerase with a 10-fold excess of CDP-D-paratose resulted in NADH accumulation, analysis of deuterium incorporation into the NADH after incubation with either C-2 or C-4 deuterated substrate should reveal the origin of the hydride that is transferred from CDP-D-paratose to NAD^+. A similar phenomenon had also been observed with UDP-galactose 4-epimerase, where a dead-end complex formed between the enzyme, NADH, and substrate after prolonged incubation with excess substrate.[31] In this experiment, the accumulated NADH from CDP-D-tyvelose 2-epimerase was converted to NAD^+ to facilitate purification, since NAD^+, not NADH, showed baseline separation from the substrate. To ensure that the newly acquired deuterium on NADH was retained on NAD^+, the L-lactate dehydrogenase from rabbit muscle was used in this transformation. The rationale to use this pro-*R* stereospecific dehydrogenase is based on the fact that CDP-D-tyvelose 2-epimerase is a member of the short-chain dehydrogenase family, and the stereospecificity of a NAD^+-dependent dehydrogenase can be predicted based on its designation as either a long-chain or short-chain dehydrogenase as first defined by Jörnvall et al.[39,40]

The group I long-chain dehydrogenases are characteristic for a polypeptide chain of greater than 350 amino acids, having the NAD(P) binding motif, known as the Rossmann fold, located at the *C*-terminus, requiring a zinc ion for the catalytic activity, and a pro-*R* stereospecificity with regard to the transfer of hydride to and from NAD^+/NADH. In contrast, the short-chain dehydrogenases are composed of approximately 250 amino acids and are zinc ion-independent. The location of their NAD(P) binding site is at the N terminus, and a YXXXK motif is conserved in most short-chain dehydrogenases. Most importantly, the hydride transfer step in short-chain dehydrogenases is *pro*-S stereospecific. Although CDP-D-tyvelose epimerase is composed of 338 amino acids, it does contain a NAD(P) binding sequence, ^{7}**G**GC**G**FL^{13}**G**, at its N terminus, it does not require zinc, and it does contain the conserved motif, ^{164}**Y**GCS^{168}**K**. According to these characteristics, CDP-D-tyvelose epimerase is clearly a member of the short-chain dehydrogenase family, and thus, should also be *pro*-S stereospecific.

On the basis of this information, the conversion of the NADH from CDP-D-tyvelose 2-epimerase to NAD^+ was performed with *pro*-R specific L-lactate dehydrogenase. Upon purification by FPLC, the NAD^+ was analyzed for deuterium incorporation by mass spectrometry. Only NAD^+ from the incubation with C-2-labeled substrate was determined to contain deuterium. The deuterium incorporation of 10% is significant considering that the purified CDP-D-tyvelose 2-epimerase already contained a large quantity of NADH (~25%) prior to incubation and less than 30−50% of the NAD^+ is converted to NADH during turnover. In addition, the C-2-labeled substrate was only 50% deuterated, and a preference for C−H bond cleavage over C-^{2}H bond cleavage is expected for the hydride transfer even though the C-2 deuterated substrate did not display a significant KIE under steady-state conditions.

In summary, these results provide compelling evidence that the reaction catalyzed by CDP-D-tyvelose 2-epimerase follows Mechanism A. Although this epimerization is reminiscent of that of UDP-galactose 4-epimerase, the motion required in this case to allow hydride transfer from NADH to either side of the 2-ketohexose intermediate is expected to be different. It is conceivable that the hexulose and the nicotinamde ring are arranged in an edge-to-edge configuration and a similar rotation about the P_β of CDP and the glycosyl oxygen bond may be sufficient to expose both faces of the 2-ketohexose to the pyridine cofactor. However, an understanding of the actual substrate motion required in the active site to achieve epimerization will have to await further studies of this intriguing enzyme.

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