# Dismutase Activity of ADP-L-*glycero*-D-*manno*-heptose 6-Epimerase: Evidence for a Direct Oxidation/Reduction Mechanism†

James P. Morrison,‡ Jay A. Read,‡ William G. Coleman, Jr.,§ and Martin E. Tanner*,‡

*Department of Chemistry, University of British Columbia, Vancouver, British Columbia, V6T 1Z1, Canada, and Laboratory of Biochemistry and Genetics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20817-0830*

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ABSTRACT: The first positive evidence for the utilization of a direct C-6″ oxidation/reduction mechanism by ADP-L-*glycero*-D-*manno*-heptose 6-epimerase is reported here. The epimerase (HldD or AGME, formerly RfaD) operates in the biosynthetic pathway of L-*glycero*-D-*manno*-heptose, which is a conserved sugar in the core region of lipopolysaccharide (LPS) of Gram-negative bacteria. The stereochemical inversion catalyzed by the epimerase is interesting as it occurs at an "unactivated" stereocenter that lacks an acidic C−H bond, and therefore, a direct deprotonation/reprotonation mechanism cannot be employed. Instead, the epimerase employs a transient oxidation strategy involving a tightly bound $NADP^+$ cofactor. A recent study ruled out mechanisms involving transient oxidation at C-4″ and C-7″ and supported a mechanism that involves an initial oxidation directly at the C-6″ position to generate a 6″-keto intermediate (Read, J. A., Ahmed, R. A., Morrison, J. P., Coleman, W. G., Jr., Tanner, M. E. (2004) *J. Am. Chem. Soc. 126*, 8878−8879). A subsequent nonstereospecific reduction of the ketone intermediate can generate either epimer of the ADP-heptose. In this work, an intermediate analogue containing an aldehyde functionality at C-6″, ADP-$\beta$-D-*manno*-hexodialdose, is prepared in order to probe the ability of the enzyme to catalyze redox chemistry at this position. It is found that incubation of the aldehyde with a catalytic amount of the epimerase leads to a dismutation process in which one-half of the material is oxidized to ADP-$\beta$-D-mannuronic acid and the other half is reduced to ADP-$\beta$-D-mannose. Transient reduction of the enzyme-bound $NADP^+$ was monitored by UV spectroscopy and implicates the cofactor's involvement during catalysis.

Lipopolysaccharide (LPS)[1] is a glycolipid that constitutes the outermost surface of Gram-negative bacteria (*1*, *2*). It is composed of lipid A (endotoxin), which anchors LPS to the outer cellular membrane, a core domain, and the immunogenic O-antigen repeat polymer, which is an oligosaccharide of 1−40 units that varies from strain to strain of bacteria. The core region is a nonrepeating oligosaccharide consisting of 6−10 core sugars (*E. coli* K-12) and contains the unusual higher order sugars 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) and L-*glycero*-D-*manno*-heptose (L,D-Hep). Functionally, the core region is a barrier to antibiotics, as evidenced by studies of mutant strains of *E. coli* K-12 and other Gram-negative mutant bacteria that bear truncated LPS lacking core oligosaccharides. These mutant bacteria with truncated LPS are viable but show increased susceptibility to hydrophobic antibiotics and reduced pathogenicity. Thus, the enzymes involved in the biosynthesis of higher order sugars in the core region of LPS are potential therapeutic targets for the development of agents that could be used in tandem with available antibiotics.

L,D-Hep is incorporated into the LPS via an ADP-linked sugar nucleotide by the action of a transferase enzyme (Figure 1) (*3*). It has recently been determined that the ADP-heptose is formed as the $\beta$-anomer, which is somewhat unusual for sugar nucleotides (*4*, *5*). The biosynthesis of ADP-$\beta$-L-*glycero*-D-*manno*-heptose (ADP-L,D-Hep) involves five steps and four enzymes starting from sedoheptulose 7-phosphate. An isomerase, GmhA, first generates D,D-Hep 7-phosphate, and then the kinase activity of the bifunctional enzyme, HldE,[2] gives $\beta$-D,D-Hep 1,7-bisphosphate. A phosphatase, GmhB, cleaves the phosphate at C-7 to give $\beta$-D,D-Hep 1-phosphate, and the second activity of HldE catalyzes a pyrophosphorylation reaction to give ADP-D,D-Hep. Finally, ADP-L,D-Hep is generated by a reversible epimerization catalyzed by the enzyme ADP-$\beta$-L-*glycero*-D-*manno*-heptose 6-epimerase (HldD or AGME, formerly RfaD).

The mechanism employed by ADP-L,D-Hep 6-epimerase is of interest since the reaction occurs at an "unactivated" stereocenter that lacks an acidic C−H bond ($pK_a < 30$) (*6*,

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* To whom correspondence should be addressed. Phone, (604) 822-9453; fax, (604) 822-2847; e-mail, mtanner@chem.ubc.ca.

‡ University of British Columbia.

§ National Institutes of Health.

[1] Abbreviations: Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; L,D-Hep, L-*glycero*-D-*manno*-heptose; LPS, lipopolysaccharide; SDR, short-chain dehydrogenase/reductase; NOE, nuclear Overhauser enhancement; HMBC, heteronuclear multiple bond connectivity.

[2] The nomenclature HldD and HldE will be employed in this paper as recently suggested in ref 4.

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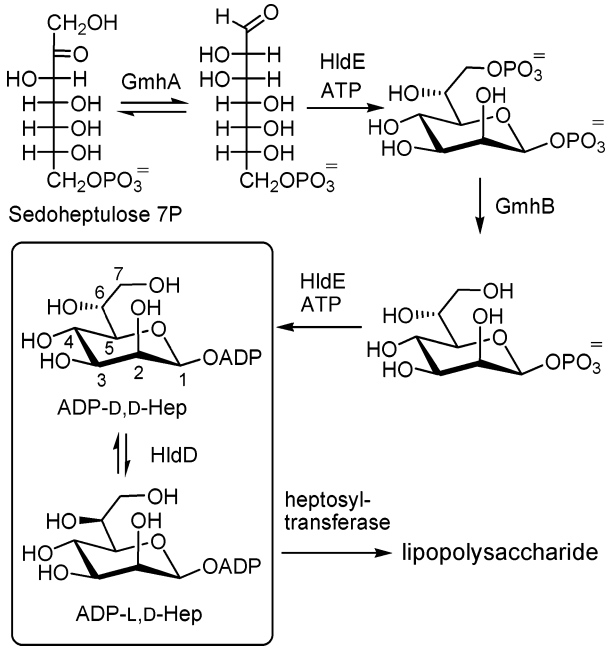


FIGURE 1: The biosynthesis of ADP-$\beta$-L-*glycero*-D-*manno*-heptose and its incorporation into lipopolysaccharide. The reaction catalyzed by ADP-$\beta$-L-*glycero*-D-*manno*-heptose 6-epimerase, HldD, is boxed.

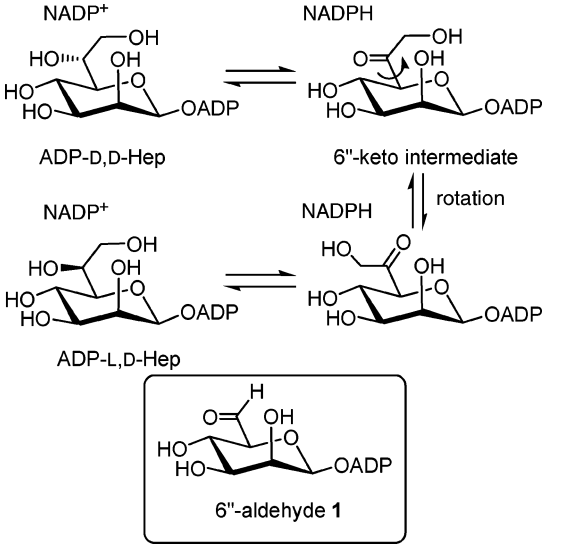


FIGURE 2: The proposed mechanism for the reaction catalyzed by ADP-L,D-Hep 6-epimerase. Inset shows structure of ADP-$\beta$-D-*manno*-hexodialdose **1**.

*7*). For this reason, a strategy involving a deprotonation/reprotonation sequence directly at the site of stereoinversion cannot be employed. Clues to the nature of the epimerase mechanism lie in the observations that it tightly binds 1 equiv of NADP(H) and that it belongs to the short-chain dehydrogenase/reductase (SDR) superfamily of enzymes (*8*−*10*). This strongly suggests that ADP-L,D-Hep 6-epimerase uses $NADP^+$ to transiently oxidize its substrate during catalysis. The structure of ADP-L,D-Hep 6-epimerase complexed to ADP-α-glucose is known and clearly supports this notion as the sugar is bound in close proximity to the nicotinamide ring of the cofactor (*9*). It is difficult to deduce the site of oxidation from this structure, however, as the electron density for the glucose is disordered and several bound conformations are observed. This is likely a result of the differing stereochemistry between ADP-α-D-glucose and ADP-$\beta$-L,D-Hep at both C-1″ and C-2″. Reasonable chemical mechanisms can be drawn that involve transient oxidation at C-4″, C-6″, or C-7″, and the distinction between them has been addressed in a recent study (*11*). The observation that neither $^{18}O$ nor $^{2}H$ isotopes were incorporated into product from solvent during catalysis argues against mechanisms that rely on proton transfer or dehydration/rehydration to invert the stereochemistry. In addition, it was observed that C-4″ and C-7″ deoxy substrate analogues also undergo epimerization, which argues strongly against mechanisms that rely on transient oxidation at these positions during catalysis. These observations provided indirect evidence in support of a scenario whereby the epimerase first oxidizes the substrate at C-6″ to generate a 6-keto-intermediate (Figure 2). A reorientation of the ketone and/or cofactor such that the opposite face of the carbonyl is exposed to the cofactor then takes place, and a subsequent reduction generates the product epimer. Precedence for this overall strategy can be found in two other well-studied SDR family members: UDP-galactose 4-epimerase (*6*, *12*−*15*) and CDP-tyvelose 2-epimerase (*16*, *17*). However, both the site of transient oxidation and the required conformational reorientation are quite different in these enzymes.

One strategy that could be used to provide positive evidence for the involvement of oxidation/reduction at C-6″ is to test the catalytic competence of the 6″-keto-intermediate by incubating it with a reduced form of epimerase (NADPH bound) and observing its conversion into a mixture of epimeric ADP-heptoses. This experiment is challenging to perform as it requires a technically demanding synthesis of the 6″-keto-intermediate as well as the preparation of the NADPH form of the enzyme (the cofactor will not exchange with free species under normal incubation conditions) (*10*). Furthermore, product analysis would be hampered by the stoichiometric nature of the reaction since large quantities of enzyme would be required to prepare milligram quantities of product.

In this work, we describe the use of an alternate intermediate analogue, ADP-$\beta$-D-*manno*-hexodialdose **1** (Figure 2, inset) to provide direct evidence for the ability of the epimerase to catalyze redox chemistry at the C-6″ position of a $\beta$-linked ADP-sugar nucleotide. The aldehyde is found to undergo a dismutation into equal amounts of ADP-$\beta$-D-mannuronic acid and ADP-$\beta$-D-mannose when incubated with catalytic amounts of ADP-L,D-Hep 6-epimerase.

## EXPERIMENTAL PROCEDURES

*Materials and General Methods*. All chemicals were purchased from Sigma-Aldrich and used without further refinement unless otherwise noted. Dry solvents were distilled fresh using $CaH_2$ (methylene chloride, pyridine, DMSO) or Na/benzophenone (THF) as drying agent. Inorganic pyrophosphatase (from yeast) was purchased from Roche Diagnostics Corporation. Protein concentrations were determined by the method of Bradford using bovine serum albumin as standard (*18*). $^{1}H$ NMR spectra were obtained on a Bruker AV300 or AV400 spectrometer at a field strength of 300 or 400 MHz, respectively. Proton-decoupled $^{31}P$ NMR spectra were recorded on these spectrometers at 121.5 or 162 MHz, respectively. Mass spectrometry was performed by the Mass Spectrometry Center at UBC by

electrospray ionization (ESIMS) using a Waters Micromass LCT mass spectrometer.

*Cloning of hldD and hldE*. The *hldD* (formerly *rfaD* or *waaD*)[2] and *hldE* (formerly *rfaE*) genes (GenBank accession numbers P67910 and P76658, respectively) (*4*) were amplified by the polymerase chain reaction (PCR) using *Escherichia coli* K-12 W3110 genomic DNA as template. Oligonucleotide primers, synthesized by the Nucleic Acids Protein Services (NAPS) Unit at UBC, included overhangs for ligation-independent cloning: 5′-GGTATTGAGGGTCGCATGATCATCGTTACCGGCGGC-3′ (forward sequence, *hldD*), 5′-AGAGGAGAGTTAGAGCCTTATGCGTCGCGATTCAGCC-3′ (reverse sequence, *hldD*), 5′-GGTATTGAGGGTCGCATGAAAGTAACGCTGCCAGAG-3′ (forward sequence, *hldE*), and 5′-AGAGGAGAGTTAGAGCCATCTGTGAACCGCTTTCC-3′ (reverse sequence, *hldE*).

A general procedure is as follows: 5.0 $\mu$L of 10× PCR buffer (Invitrogen), 1.0 $\mu$L of 10 mM dNTP mix, 1.5 $\mu$L of 50 mM $MgCl_2$, ∼0.1 $\mu$L of *E. coli* K-12 W3110 cell pellet, 25 pmol of each primer, 0.25 $\mu$L of 5 U/$\mu$L *Taq* polymerase, and distilled $H_2O$ to a total volume of 50 $\mu$L were added to a 200 $\mu$L PCR tube. DNA was amplified using an iCycler thermal cycler (Bio-Rad) according to the following cycles: one cycle of 3 min at 94 °C; 30 cycles of 60 s at 94 °C, 60 s at 55 °C, and 90 s at 72 °C followed by cooling to 4 °C. The PCR product was cloned into the pET-30 Xa/ LIC vector (Novagen) using the ligation-independent cloning method according to the manufacturer's directions. The resulting plasmid, that encodes for the protein fused to an N-terminal 43-residue peptide containing a hexahistidine tag, was transformed into NovaBlue Singles chemically competent *E. coli* cells (Novagen). The presence of the gene was confirmed by colony PCR and DNA sequencing.

*Overexpression and Purification of Histidine-Tagged HldD and HldE*. Overexpression of HldD and HldE was performed as described in the following generic procedure. The recombinants were transformed into expression host cells, JM109 chemically competent *E. coli*, which were incubated overnight at 37 °C with shaking at 225 rpm in 10 mL Luria−Bertani (LB) medium containing 30 $\mu$g mL$^{-1}$ kanamycin. The overnight cultures were poured into 500 mL LB medium containing 30 $\mu$g mL$^{-1}$ kanamycin and grown at 37 °C with shaking at 225 rpm until an $OD_{600}$ of 0.6−0.9 was reached. Cells were induced for overexpression by addition of 120 mg L$^{-1}$ (0.5 mM) isopropyl $\beta$-D-galactopyranoside (IPTG), and the cultures were allowed to continue growth until an $OD_{600}$ of 1.6−1.8 was reached (∼6 h). Cells were harvested at 4000 rpm for 30 min and resuspended in lysis buffer. In the case of HldD, TEM lysis buffer was used (10 mM Tris-HCl, 2.5 mM EDTA, pH 8.0, 5 mM $\beta$-mercaptoethanol) with 1 $\mu$g mL$^{-1}$ pepstatin A and 1 $\mu$g mL$^{-1}$ aprotinin added. The buffer used for HldE was 20 mM triethanolamine-HCl (pH 8.0) with 1 mM DTT, 1 $\mu$g mL$^{-1}$ pepstatin A, and 1 $\mu$g mL$^{-1}$ aprotinin added. Cells were lysed at 20 000 psi in an ice-cooled French pressure cell. The cell lysate was clarified by centrifugation at 6000 rpm for 40 min and filtered through a 0.4 $\mu$M membrane prior to affinity chromatography.

A 10 mL column containing Chelating Sepharose Fast Flow resin (Pharmacia Biotech) was charged with 2 column volumes (CV) of 100 mM $NiSO_4$ followed by washing with 2 CV of distilled $H_2O$ and 3 CV of running buffer (lysis buffer minus aprotinin and pepstatin plus 500 mM NaCl) containing 5 mM imidazole. The filtered cell lysate was loaded at 2 mL min$^{-1}$, and start buffer (∼8 CV) was passed through the column at 3 mL min$^{-1}$ until no more flow-through protein eluted, as determined by UV observation at 280 nm. A wash with running buffer containing 125 mM imidazole was used to remove nonspecifically bound proteins (∼4 CV used). Histidine-tagged protein was finally eluted with 3−4 CV of running buffer containing 500 mM imidazole. Fractions containing the enzyme were pooled and dialyzed overnight against a 1:100 volume of dialysis buffer. In the case of HldD, the buffer was 10 mM Tris-HCl, pH 8.0, containing 2.5 mM EDTA, 5 mM $\beta$-mercaptoethanol, and 10% glycerol. In the case of HldE, the buffer was 20 mM triethanolamine-HCl, pH 8.0, containing 1 mM DTT and 10% glycerol. Enzymes were concentrated by ultrafiltration (Amicon Ultra-4, 10 000 MWCO) to 8−20 mg mL$^{-1}$, and 25−500 $\mu$L aliquots were flash-frozen in liquid $N_2$ and stored at −80 °C. Enzymes could be stored at −80 °C for at least 12 months without significant loss in activity. The mass of His-tagged epimerase HldD was 39 860 Da (versus 39 862 Da calculated) as determined by electrospray mass spectrometry.

*Synthesis of 1,2,3,4-Tetra-O-acetyl-6,7-dideoxy-$\beta$-D-manno-hept-6-enopyranose **3***. To a solution of 3.00 g (8.61 mmol) of **2** (*19*) in 45 mL of dry DMSO under argon, 11.0 g (26.0 mmol) of *N*-cyclohexyl-*N*′-(2-morpholinoethyl)carbodiimide methyl-*p*-toluenesulfonate (Fluka) was added. To this solution, 1.1 mL dichloroacetic acid (13.4 mmol) was added via syringe, and the resulting solution was stirred in a cool water bath. After 3 h, the reaction mixture was poured into 250 mL of ice-cold distilled water and extracted with ice-cold ethyl acetate (4 × 100 mL). The pooled organic layers were extracted with ice-cold distilled water (3 × 100 mL), and the pooled aqueous layers were back-extracted with 100 mL of ice-cold ethyl acetate. The pooled organic layers were then washed with ice-cold NaCl brine and dried over sodium sulfate. Evaporation of the solvent gave 2.43 g of a pale yellow residue. The crude aldehyde product was used immediately without further purification in the following methylenation reaction with the Lombardo reagent. The Lombardo reagent was prepared by cooling to −40 °C a solution of 11.5 g (17.6 mmol) of zinc dust in 100 mL of dry THF with 4.05 mL (8.0 mmol) of dibromomethane and adding dropwise 4.6 mL (5.7 mmol) of neat $TiCl_4$. The Lombardo reagent was allowed to stir at −40 °C for 2 h, then at 4 °C for 2 days prior to use. The entire crude aldehyde product was dissolved in 50 mL of dry methylene chloride and cooled on ice. The entire Lombardo reagent was added to the aldehyde solution and stirred for 30 min at 0 °C. The reaction mixture was then poured into 250 mL of saturated sodium bicarbonate solution with 500 mL of ethyl acetate and stirred vigorously until evolution of gas dissipated (∼1 h). The resulting mixture was filtered through Celite, and the two layers were separated. The aqueous layer was extracted with ethyl acetate (2 × 100 mL). The combined organic layers were washed with 200 mL of water and brine (3 × 100 mL) and dried over sodium sulfate. Evaporation of the solvent gave 1.61 g of a yellow residue. Purification by column chromatography (3:1 petroleum ether/ethyl acetate, silica gel) gave 343 mg (0.995 mmol) **3** as a white solid (11.6% yield). $^1$H NMR ($CDCl_3$): $\delta$ 5.89 (d, 1H, $J_{1,2}$

= 1.2 Hz, H1), 5.80 (ddd, 1H, $J_{5,6}$ = 7.2 Hz, $J_{6,7a}$ = 17.2 Hz, $J_{6,7b}$ = 10.4 Hz, H6), 5.49 (dd, 1H, $J_{1,2}$ = 1.2 Hz, $J_{2,3}$ = 3.2 Hz, H2), 5.38 (d, 1H, $J_{6-7a}$ = 17.2 Hz, H7a), 5.30 (d, 1H, $J_{6-7b}$ = 10.4 Hz, H7b), 5.19 (dd, 1H, $J_{3,4}$ = 10.0 Hz, $J_{4,5}$ = 10.0 Hz, H4), 5.13 (dd, 1H, $J_{2,3}$ = 2.8 Hz, $J_{3,4}$ = 10.0 Hz, H3), 3.94 (dd, 1H, $J_{4,5}$ = 8.2 Hz, $J_{5,6}$ = 8.2 Hz, H5), 2.21, 2.10, 2.01, 2.00 (4 s, 12H, 4 × OAc); ESIMS *m*/*z* 367.2 [M + $Na^+$]. Anal. Calcd for $C_{15}H_{20}O_9$: C, 52.32; H, 5.85. Found: C, 52.46; H, 6.24.

*Synthesis of 2,3,4-Tri-O-acetyl-6,7-dideoxy-*D*-manno-hept-6-enopyranose **4***. To a solution of 421 mg (1.22 mmol) of **3** in 5 mL of acetonitrile, 1.1 mL of 5 M dimethylamine (5.5 mmol, in ethanol, Fluka) was added and allowed to stir at room temperature for 1.5 h. Crude product was recovered by rotary evaporation. Purification by column chromatography (5:2 petroleum ether/ethyl acetate, silica gel) yielded 314 mg of **4** (85.2% yield). $^1$H NMR ($CDCl_3$) (∼7:1 ratio of α- to β-anomers, as determined by integration of H5 signals. Assignment of the α- and β-anomeric signals is based upon selective NOE experiments in which H5 the α- and β-anomers were irradiated in individual experiments). α-Anomer: δ 5.78 (ddd, 1H, $J_{5,6}$ = 7.6 Hz, $J_{6,7a}$ = 17.2 Hz, $J_{6,7b}$ = 10.4 Hz, H6), 5.40 (dd, 1H, $J_{2,3}$ = 3.4 Hz, $J_{3,4}$ = 10.2 Hz, H3), 5.33 (d, 1H, $J_{6,7a}$ = 17.2, H7a), 5.26 (d, 1H, $J_{2,3}$ = 3.2 Hz, H2), 5.25 (d, 1H, $J_{6,7b}$ = 10.4 Hz, H7b), 5.19 (s, 1H, H1), 5.15 (dd, 1H, $J_{3,4}$ = 10.2 Hz, $J_{4,5}$ = 10.2 Hz, H4), 4.39 (dd, 1H, $J_{4,5}$ = 9.6 Hz, $J_{5,6}$ = 8.0 Hz, H5), 2.13, 1.98, 1.97 (3 s, 9H, 3 × OAc). β-Anomer: δ 5.83−5.73 (H6, signals overlapped by H6 of the α-anomer), 5.42−5.05 (H2, H3, H4, H7a, H7b, signals overlapped by those of the α-anomer), 4.97 (s, 1H, H1), 3.85 (dd, $J_{4,5}$ = 8.0 Hz, $J_{5,6}$ = 8.0 Hz, H5), 2.19 (s, 3H, OAc), 1.98, 1.97 (2 × OAc, signals overlapped by those of the α-anomer); ESIMS *m*/*z* 325.0 [M + $Na^+$]. Anal. Calcd for $C_{15}H_{18}O_8$: C, 51.65; H, 6.00. Found: C, 52.26; H, 5.87.

*Synthesis of Dibenzyl 2,3,4-Tri-O-acetyl-6,7-dideoxy-β-*D*-manno-hept-6-enopyranosyl Phosphate **5***. To a solution of 291 mg (0.964 mmol) of **4** and 69.0 mg of 1,2,4-triazole (3.86 mmol) in 5.0 mL of dry methylene chloride under an argon atmosphere, 850 μL (2.41 mmol) of 85% dibenzyl *N*,*N*-diethylphosphoramidite was added. After stirring at room temperature for 2 h, 30 mL of diethyl ether was added, and the organic solution was washed with saturated sodium bicarbonate solution (3 × 15 mL) and NaCl brine (3 × 10 mL). The organic layer was dried over sodium sulfate and evaporated to a pale yellow oil. The oil was dissolved in 9 mL of THF and cooled to −78 °C, then 1.9 mL of 30% $H_2O_2$ was added and the solution was allowed to warm to room temperature over 2 h. Diethyl ether (30 mL) was then added, and the organic solution was washed with saturated sodium bicarbonate solution (3 × 15 mL) and NaCl brine (3 × 10 mL). The organic layer was dried over sodium sulfate and evaporated to give a pale yellow oil. Purification by column chromatography (1:1 petroleum ether/diethyl ether to 100% diethyl ether, silica gel) gave 133 mg of **5** as a pale yellow oil (25.3% yield). $^1$H NMR ($CDCl_3$): δ 7.33 (m, 10H, Ph), 5.80 (ddd, 1H, $J_{5-6}$ = 7.2 Hz, $J_{6-7a}$ = 17.2 Hz, $J_{6-7b}$ = 10.4 Hz, H6), 5.48−5.45 (m, 2H, H1 and H2), 5.35 (d, 1H, $J_{6-7a}$ = 16.6 Hz, H7a), 5.28 (d, 1H, $J_{6-7b}$ = 10.4, H7b), 5.16 (dd, 1H, $J_{3,4}$ = 9.8 Hz, $J_{4,5}$ = 9.8 Hz, H4), 5.08−5.02 (m, 5H, PhC$H_2$ and H3), 3.92 (dd, 1H, $J_{4,5}$ = 9.5 Hz, $J_{5,6}$ = 7.4 Hz, H5), 2.15, 2.01, 2.00 (3 s, 9H, 3 × OAc). $^{31}$P NMR ($CDCl_3$): δ −1.65 (s); ESIMS *m*/*z* 585.2 [M + $Na^+$]. Anal. Calcd for $C_{27}H_{31}O_{11}P$: C, 57.65; H, 5.55. Found: C, 58.04; H, 5.76.

*Synthesis of Adenosine Diphospho-β-*D*-manno-hexodialdose **1***. A solution of 19.7 mg of **5** (0.036 mmol) in 10 mL of methylene chloride was cooled to −78 °C in a dry-ice/acetone bath, then ozone was bubbled through until a faint blue color persisted in the solution (∼5 min). Dimethyl sulfide (1 mL) was then added, and the solution was allowed to warm to room temperature. Upon evaporation of the solvent, the subsequent hydrogenolysis was performed without delay. The crude aldehyde was dissolved in 10 mL of 1:1 methanol/ethyl acetate, and 35 mg of 5% Pd/C was added. The solution was vigorously stirred under 1 atm of $H_2$ for 1 h. The reaction mixture was then filtered through Celite, and the solvent was evaporated. The crude product was immediately dissolved in 3 mL of cold 3:4:0.3 methanol/0.1 M triethylammonium bicarbonate buffer/triethylamine (pH 11) and allowed to sit at −20 °C for 4 days. The solution was then diluted 3-fold with water, was frozen, and was lyophilized to a yellow residue. This residue was dissolved in 6 mL of a solution containing 50 mM Tris-HCl buffer, pH 8.0, containing 5 mM $MgCl_2$, 22 mg of ATP (0.040 mmol), 400 μg of HldE, and 20 μg of inorganic pyrophosphatase and was allowed to sit at room temperature for 16 h. Separation by anion exchange column chromatography (DE-52 resin, linear gradient from 0.1 to 0.5 M of triethylammonium bicarbonate buffer, pH 7.5) afforded 0.021 mmol of ADP-sugars (as determined by UV measurement at 259 nm), 58% yield. Compound **1** constitutes ∼75% of the product mixture, as determined by HPLC, $^1$H and $^{31}$P NMR, and mass spectrometry observations. $^1$H NMR ($D_2O$): δ 8.39 (s, 1H, H8), 8.15 (s, 1H, H2), 6.03 (d, 1H, $J_{1',2'}$ = 6.0 Hz, H1′), 5.15 (d, 1H, $J_{1''-P\alpha}$ = 8.8 Hz, H1″), 5.06 (d, 1H, $J_{5''-6''}$ = 2.0 Hz, H6″), 4.60−4.70 (obscured by solvent peak, H2′), 4.41 (dd, 1H, $J_{2'-3'}$ = 5.1 Hz, $J_{3'-4'}$ =3.5 Hz, H3′), 4.28 (dd, 1H, $J_{3'-4'}$ = 2.7 Hz, $J_{4'-5'}$ = 2.7 Hz, H4′), 4.12 (m, 2H, H5′), 3.98 (d, 1H, $J_{2''-3''}$ = 2.6 Hz, H2″), 3.59 (dd, 1H, $J_{3''-4''}$ = 9.3 Hz, $J_{4''-5''}$ = 9.3 Hz, H4″), 3.54 (dd, 1H, $J_{2''-3''}$ = 2.7 Hz, $J_{3''-4''}$ = 9.1 Hz, H3″), 3.23 (dd, 1H, $J_{4''-5''}$ = 9.3 Hz, $J_{5''-6''}$ = 1.8 Hz, H5″), 2.90 (q, 12H, *J* = 7.3 Hz, [HN(C$H_2$-$CH_3$)$_3$]$^+$), 1.09 (t, 18H, *J* = 7.4 Hz, [HN(CH$_2$C$H_3$)$_3$]$^+$). $^{31}$P NMR ($D_2O$): δ −10.24 (d, 1P, $J_{P\alpha-P\beta}$ = 21.0 Hz, Pα), −12.05 (d, 1P, $J_{P\alpha-P\beta}$ = 20.9 Hz, Pβ); ESIMS *m*/*z* 618.1 [M + MeOH − $H^+$] (hemiacetal with methanol solvent).

*NMR Observation of HldD Catalyzed Dismutation of ADP-β-*D*-manno-hexodialdose **1***. The epimerase was exchanged into 10 mM deuterated potassium phosphate buffer (pH 8.0) by ultrafiltration as follows: 70 μL of a 7.9 mg $mL^{-1}$ (0.55 mg) HldD solution that had thawed on ice was added to an ultrafiltration device (Amicon Ultra-4, 10 000 MWCO) with 1.0 mL of deuterated buffer, and the solution was centrifuged at 5000 rpm for 10 min. Addition of 1.0 mL of deuterated buffer followed by centrifugation was repeated twice more, such that the enzyme was finally dissolved in 70 μL of 10 mM deuterated buffer. The entire 70 μL was then added to a solution of 4.94 μmol of ADP-sugars (∼75% aldehyde **1**) and 430 μL of 10 mM deuterated pH 8.0 potassium phosphate buffer to generate a solution containing 9.9 mM ADP-sugar and 28 μM HldD. $^1$H and $^{31}$P NMR spectra were collected immediately and then again after a 12 h incubation at 37 °C.

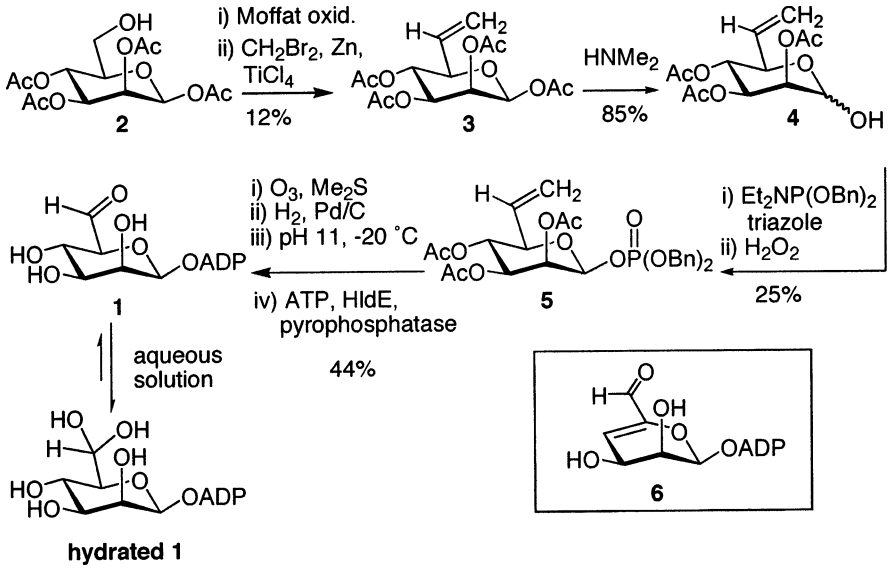


FIGURE 3: Synthesis and buffer-catalyzed hydration of compound **1**. Inset shows putative side-product **6**.

*Isolation of ADP-β-*D*-mannuronate*. Crude dismutation reaction mixtures from several incubation experiments were pooled (total of 0.017 mmol of ADP-sugars) and loaded onto a DE-52 anion exchange column equipped with a UV detector ($\lambda_{obs}$ = 254 nm). A linear gradient from 0.1 to 0.8 M triethylammonium bicarbonate buffer (pH 7.4, total volume 1 L) resulted in elution of two distinct fractions, one which eluted at ~0.25−0.35 M and one which eluted at ~0.4 M buffer concentration. The fractions were lyophilized, and it was determined that the early fraction contained ADP-β-D-mannose (by comparison with the NMR and mass spectrum of a synthetic sample; see Supporting Information) eluting with ADP-sugar impurities, and the last eluting peak is ADP-β-D-mannuronate (3.9 mmol, ~66% of theoretical yield). $^1$H NMR ($D_2O$): δ 8.42 (s, 1H, H8), 8.17 (s, 1H, H2), 6.05 (d, 1H, $J_{1',2'}$ = 6.1 Hz, H1′), 5.16 (d, 1H, $J_{1''-P\alpha}$ = 8.8 Hz, H1″), 4.65−4.75 (obscured by solvent peak, H2′), 4.43 (dd, 1H, $J_{2'-3'}$ = 5.0 Hz, $J_{3'-4'}$ = 3.4 Hz, H3′), 4.30 (dd, 1H, $J_{3'-4'}$ = 2.8 Hz, $J_{4'-5'}$ = 2.8 Hz, H4′), 4.12 (m, 2H, H5′), 3.98 (d, 1H, $J_{2''-3''}$ = 3.0 Hz, H2″), 3.64 (dd, 1H, $J_{3''-4''}$ = 9.5 Hz, $J_{4''-5''}$ = 9.5 Hz, H4″), 3.57 (dd, 1H, $J_{2''-3''}$ = 3.3 Hz, $J_{3''-4''}$ = 9.1 Hz, H3″), 3.55 (d, 1H, $J_{4''-5''}$ = 9.7 Hz, H5″), 2.95 (q, 18H, $J$ = 7.3 Hz, $[HN(CH_2CH_3)_3]^+$), 1.12 (t, 27H, $J$ = 7.3 Hz, $[HN(CH_2CH_3)_3]^+$). $^{31}$P NMR ($D_2O$): δ −10.89 (d, 1P, $J_{P\alpha-P\beta}$ = 20.1 Hz, Pα), −12.96 (d, 1P, $J_{P\alpha-P\beta}$ = 20.6 Hz, Pβ); ESIMS *m*/*z* 646.0 [M − 3H$^+$ + 2Na$^+$].

*UV Observation of Transient HldD-Bound NADPH*. The epimerase was exchanged into 10 mM potassium phosphate buffer (pH 8.0) by ultrafiltration as follows: 250 μL of a 19.0 mg mL$^{-1}$ (4.7 mg) HldD solution was added to an ultrafiltration device (Amicon Ultra-4, 10 000 MWCO) with 2.25 mL of buffer, and the solution was centrifuged at 6000 rpm for 20 min to give a final volume of 250 μL. This was repeated twice more, and buffer was finally added to give 1.5 mL of a final solution in which the protein concentration was measured to be 3.08 mg/mL ([epimerase subunit] = 78 μM). A 500 μL aliquot of the HldD solution was added to a quartz cuvette and was monitored at 354 nm. After 5 min, 10 μL of a 16.9 mM solution of **1** (final concentration of 331 μM) was added with thorough mixing. After 2.5 h, 10 μL of a 1 M $NaBH_4$ solution was added, and the resulting absorbance was immediately measured.

## RESULTS

*Cloning, Overexpression, and Purification of HldD and HldE*. The *hldD* and *hldE* genes were individually cloned from *Escherichia coli* K-12 genomic DNA with the introduction of a coding region for an N-terminal polyhistidine affinity purification tag. The recombinant proteins were expressed in *E. coli* and purified by $Ni^{2+}$ affinity chromatography. An analysis by SDS−PAGE revealed the expected subunit molecular weights (39 and 60 kDa for HldD and HldE, respectively) and indicated purities of >95%. The purified enzymes were stored at −80 °C for a maximum of 12 months without significant loss of activity.

*Synthesis and Characterization of ADP-β-*D*-manno-hexodialdose* **1**. The synthetic strategy used to prepare ADP-β-D-*manno*-hexodialdose **1** (Figure 3) was adapted from a previous report on the synthesis of UDP-α-D-*gluco*-hexodialdose (*20*). From D-mannose, C-6 tritylation, peracetylation, and detritylation gave crystalline **2** (*19*), which was identified as the β-anomer using selective NOE experiments. A mild Moffat oxidation of **2** gave the appropriate aldehyde (*21*); however, this product was found to be unstable and not isolable by conventional silica gel chromatography. This is likely due to the facile β-elimination of the C-4 acetate that is known to cause instability in related compounds (*22*). To protect this sensitive functionality, a mild Lombardo methylenation of the crude aldehyde mixture gave alkene **3**, which proved to be more robust and readily isolable (*23*). The anomeric acetyl group of alkene **3** was then selectively removed with dimethylamine to give **4**, and phosphitylation followed by oxidation yielded a 3:1 mixture of α- and β-glycosyl dibenzyl phosphates, a ratio in agreement with previous phosphorylations of mannopyranosyl sugars (*11*, *24*). The two anomers were separated using conventional silica gel chromatography, and their stereochemistry was assigned based on selective NOE difference spectra of the isolated products. The β-anomer **5** was identified by the observation of an NOE enhancement at H1 upon irradiation

of H5. With the $\beta$-dibenzyl phosphate **5** in hand, it was then necessary to remove the protecting groups and introduce the ADP functionality using the pyrophosphorylase activity of the recombinant HldE (Figure 1). Because of the sensitive nature of the free aldehyde, all four steps were carried out without purification of the intermediate compounds. Ozonolysis was used to unmask the C-6 aldehyde, and hydrogenolysis followed by a mild basic hydrolysis removed both the benzyl and acetyl protecting groups. The resulting crude $\beta$-phosphate was incubated with HldE, ATP, and pyrophosphatase, and the reaction was monitored by $^{31}$P NMR spectroscopy. The appearance of two doublets at $-10.24$ and $-12.05$ ppm confirmed that a sugar nucleotide was being generated and that the HldE enzyme was accepting the 6-aldehydo-mannosyl phosphate as an alternate substrate. Ion-exchange chromatography was used to partially purify the aldehyde **1** which was found to be approximately 75% pure. No further purification was attempted as compound **1** was found to be sensitive to acid, base, and conditions of high ionic strength.

Compound **1** was characterized by both $^{1}$H and $^{31}$P NMR spectroscopy. A key signal was that of the C-6″ proton which appears at 5.06 ppm and indicates that the aldehyde exists primarily as the hydrate in aqueous solution (Figure 3). This is in excellent agreement with previous reports on UDP-α-D-*gluco*-hexodialdose (*20*). Mass spectral analysis taken from a sample of **1** dissolved in methanol showed a major signal with a mass corresponding to the expected product in a hemiacetal form with a molecule of methanol (ESIMS *m*/*z* 618 [M + MeOH − H$^+$]) and a minor signal for the free aldehyde (ESIMS *m*/*z* 586 [M − H$^+$]) (Figure 4A). The major impurity present (15%) was assigned to be the α,$\beta$-unsaturated aldehyde **6** (Figure 3, inset) based on both mass spectral (ESIMS *m*/*z* 568 [M − H$^+$]) and $^{1}$H NMR data (notably an alkene signal at 5.5 ppm). It is likely that a $\beta$-elimination occurred during the sensitive deacetylation step and the resulting α,$\beta$-unsaturated aldehyde $\beta$-phosphate was also accepted as a substrate by HldE.

*Observation of Dismutase Activity*. When aldehyde **1** was incubated with a catalytic amount of ADP-L,D-Hep 6-epimerase, it was observed to undergo a dismutation reaction in which it was converted into equimolar amounts of the reduced compound, ADP-$\beta$-D-mannose, and the oxidized compound, ADP-$\beta$-D-mannuronic acid (Figure 5). When the reaction was monitored by $^{1}$H NMR spectroscopy, the C-6″ proton of the hydrated aldehyde was observed to completely disappear. When monitored by $^{31}$P NMR spectroscopy, the two doublets of **1** at $-10.24$ (Pα) and $-12.05$ (P$\beta$) ppm were transformed into two new sets of two doublets with similar chemical shifts. Perhaps most tellingly, mass spectral analysis of the reaction showed that the signal corresponding to the methanol adduct of **1** had completely disappeared and had been replaced by signals corresponding to the masses of ADP-$\beta$-D-mannuronic acid (ESIMS *m*/*z* 646 [M − 3H$^+$ + 2Na$^+$]) and ADP-$\beta$-D-mannose (ESIMS *m*/*z* 610 [M − 2H$^+$ + Na$^+$]) in a ∼1:1 ratio (Figure 4B). No reaction was observed when compound **1** was incubated under identical conditions in the absence of the epimerase. No attempts were made to quantify the kinetics of the dismutation process given the presence of other sugar nucleotide impurities; however, a qualitative comparison showed the process took place at a rate 2 orders of magnitude slower than the normal epimerization process, given comparable concentrations of enzyme and substrate.

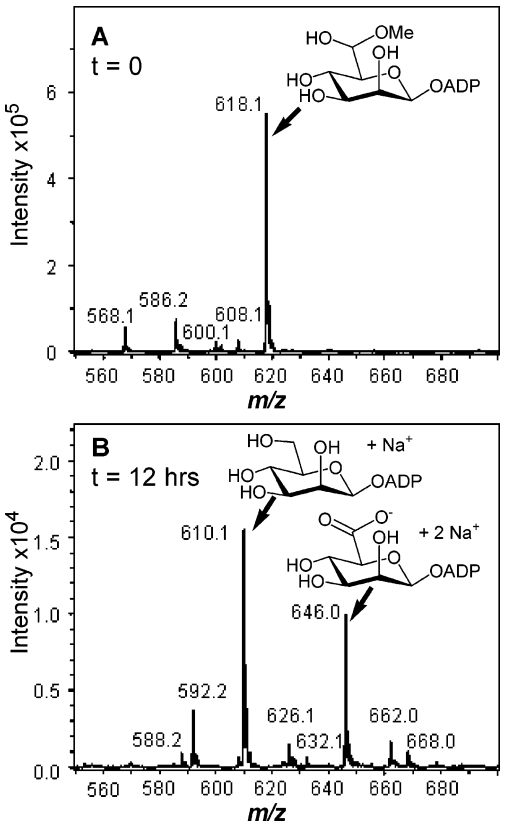


FIGURE 4: ESI mass spectra (MeOH, negative mode): (A) ADP-$\beta$-D-*manno*-hexodialdose **1**; (B) ADP-$\beta$-D-mannuronic acid and ADP-$\beta$-D-mannose, generated by the incubation of **1** with ADP-L,D-Hep 6-epimerase at 37 °C, pH 8, for 12 h.

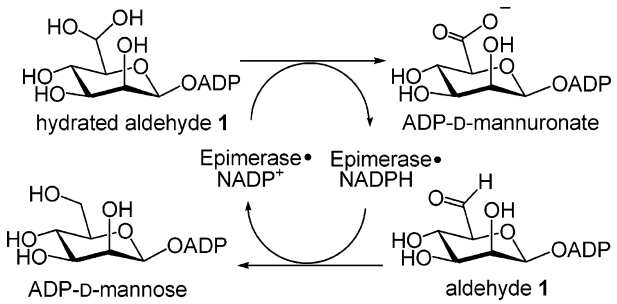


FIGURE 5: The dismutation of ADP-$\beta$-D-*manno*-hexodialdose **1** catalyzed by ADP-L,D-Hep 6-epimerase.

As both ADP-$\beta$-D-mannuronic acid and ADP-$\beta$-D-mannose are previously unknown compounds, their conclusive identification required isolation and characterization of purified samples of these compounds. In the case of ADP-$\beta$-D-mannuronic acid, it was possible to take advantage of the additional negative charge to isolate this product. The acid was separated from the other ADP-sugars present via anion-exchange chromatography and was characterized using $^{1}$H and $^{31}$P NMR spectroscopies and mass spectrometry. The presence of a distinctive C-6″ carboxylate carbon signal at 170 ppm in the $^{13}$C spectrum was demonstrated via its observed coupling with H-5″ using an HMBC NMR experiment.

Isolation of pure ADP-$\beta$-D-mannose from the dismutation reaction via anion-exchange chromatography was less successful. The ADP-sugar impurities, inseparable from ADP-

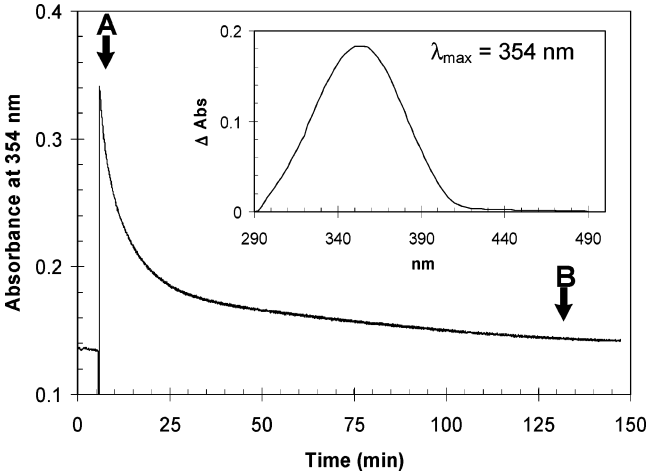


FIGURE 6: UV spectrophotometric detection of enzyme-bound NADPH during dismutation of aldehdye **1**. Trace of absorbance versus time is monitored at 354 nm, and compound **1** was added after 5 min. Inset shows a difference spectrum comparing the sample at time point A versus time point B. Spectra were collected in 10 mM potassium phosphate buffer, pH 8.0, at 22 °C with [HldD] = 78 $\mu$M and initial [**1**] = 331 $\mu$M.

$\beta$-D-*manno*-hexodialdose **1** (vide supra), were also found to coelute with the alcohol. To conclusively assign its structure, ADP-$\beta$-D-mannose was independently synthesized (see Supporting Information for details). A comparison of NMR and mass spectral data obtained with the synthetic sample to that obtained from the dismutation reaction confirmed that ADP-$\beta$-D-mannose was the other product.

*UV Spectrum of Transient Enzyme-Bound NADPH*. To provide evidence of redox cofactor involvement in the dismutation reaction, the UV absorption band of bound NADPH was monitored at 354 nm. Previous studies have shown that the recombinant epimerase is isolated with 1 equiv of cofactor tightly bound in each active site; however, a significant fraction is always present as the inactive NADPH form (*8*, *10*). The presence of bound NADPH is evident from an absorbance band at 354 nm that is shifted to slightly higher wavelength than that of free NADPH (340 nm). In this work, the spectrum of the epimerase alone revealed an absorption at 354 nm demonstrating that a significant fraction of the enzyme was in the inactive reduced form. Upon introduction of an approximate 4-fold excess of aldehyde **1**, the absorption at 354 nm immediately increased (within ~20 s), indicating that the bound cofactor had been driven to the NADPH form (Figure 6). Over time, the absorption at 354 nm returned to its previous level, indicating that the transiently formed NADPH had been oxidized back to $NADP^+$ and that no net change in the redox state of the enzyme-bound cofactor had taken place. A difference spectrum, comparing the sample immediately after the addition of aldehyde **1** to the same sample after equilibrium was established, showed that the transient absorbance had a maximum at 354 nm as expected for the bound NADPH cofactor (Figure 6 inset). Once equilibrium was established, an excess of the reducing agent sodium borohydride was added, and an immediate (within ~20 s) increase in the 354 nm absorption was observed, consistent with quantitative reduction of all enzyme-bound $NADP^+$ (not shown). The magnitude of the borohydride-induced absorbance change was comparable to that observed immediately following the injection of aldehyde **1**, indicating that essentially all of the enzyme-bound cofactor had been transiently reduced to NADPH during the dismutation. These observations clearly demonstrate that the dismutation process is mediated by reduction/oxidation of the enzyme bound cofactor.

## DISCUSSION

The observation that ADP-L,D-Hep 6-epimerase catalyzes the dismutation of the aldehyde **1** into a mixture of the reduced compound, ADP-$\beta$-D-mannose, and the oxidized compound, ADP-$\beta$-D-mannuronic acid (Figure 5), provides the first direct evidence that this enzyme employs a catalytic mechanism involving oxidation/reduction at C-6″ of a $\beta$-linked ADP-sugar. Overall, this process is the enzymatic equivalent of the Cannizarro reaction in which two molecules of benzaldehyde disproportionate into an acid and an alcohol in the presence of hydroxide (*25*). The enzymatic version is somewhat different in that the hydride transfer does not occur directly between the two aldehydes but is mediated by an enzyme bound cofactor. Initially, the majority of the enzyme is in an oxidized, $NADP^+$-containing state and readily binds the highly populated hydrated form of aldehyde **1**. Oxidation of the hydrate takes place to generate ADP-$\beta$-D-mannuronate in a thermodynamically favorable process. This rapidly drives all of the enzyme into the reduced, NADPH-containing state as evidenced by the UV spectroscopic analysis. In a much slower process, the reduced enzyme either binds the minor unhydrated form of aldehyde **1** from solution or catalyzes dehydration of the bound hydrate to produce the bound carbonyl version of **1** that may accept a hydride from NADPH and complete the cycle. Precedence for the enzyme-catalyzed dismutation of aldehdyes is well-documented in studies on a variety of alcohol dehydrogenases, most notably horse liver alcohol dehydrogenase (*26*−*33*). For the process to occur, the active site of the dehydrogenase must be able to accommodate the hydrated form of the aldehyde and orient it in a fashion suitable for hydride transfer to the cofactor. In the case of ADP-L,D-Hep 6-epimerase, the normal substrate is a secondary alcohol and the oxidized intermediate is a ketone. This enzyme readily accepts the unnatural hydrated aldehyde **1** since a sterically smaller hydroxyl group occupies the position that is normally taken by the C-7″ hydroxymethylene of the ADP-heptose.

While this study strongly supports a nonstereospecific C-6″ oxidation/reduction mechanism, it does not address the nature of any reorientations that must take place during catalysis. Perhaps the simplest model is to consider an enzyme with two binding sites that may accommodate either a hydroxyl group or a hydroxymethylene group (Figure 7). Each site must also contain a single acid/base residue. One of these bases is almost certainly provided by the conserved triad of Ser116, Tyr140, and Lys144 that act in concert in SDR family members (*9*). The other may be supplied by an active site residue such as Lys178 or Asp210. When one epimer of ADP-heptose binds, the C-6″ hydroxyl occupies one of the sites and the C-7″ hydroxymethylene occupies the other. The C-6″ hydride is oriented toward the $NADP^+$ cofactor, and deprotonation of the C-6″ hydroxyl leads to formation of the oxidized ketone intermediate. When the other epimer binds, the two groups simply occupy the sites in a reversed orientation and the hydride is still positioned appropriately for hydride transfer to occur. In this scenario, the only

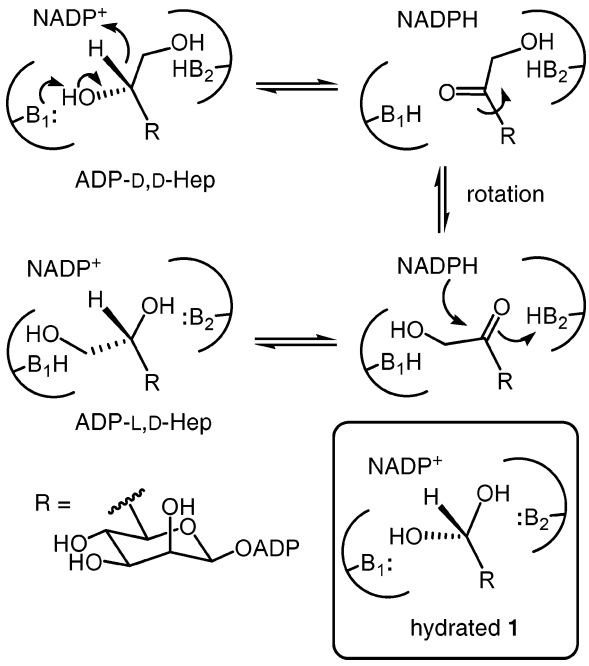


FIGURE 7: Postulated "two-site" mechanism for the reaction catalyzed by ADP-L,D-Hep 6-epimerase. Inset shows conceptual Michaelis complex for hydrated **1**.

reorientation required during catalysis is a rotation about the C5″−C6″ bond of the ketone intermediate and no motion of either the cofactor or the ADP-pyranose moiety is required. This is quite different from the other SDR superfamily members that utilize direct oxidation/reduction strategies to catalyze epimerizations of sugar nucleotides. In UDP-galactose 4-epimerase, the oxidation takes place at the C-4 position of the pyranose ring (*6*, *12*−*15*). In order for the opposite face of the carbonyl group to be exposed to the reduced cofactor, the entire pyranose moiety must flip by 180° within the active site of the enzyme. This occurs via rotations of the bonds linking the pyranose to the UDP group and results in an enzyme that has little recognition for the hexose itself. In CDP-tyvelose 2-epimerase, the oxidation must occur at C-2 of the pyranose ring (*16*, *17*). In this case, much less is understood about the reorientation required during the lifetime of the intermediate, but it is clearly more complex than that required with ADP-L,D-Hep 6-epimerase.

While the full details of the ADP-L,D-Hep 6-epimerase reaction remain to be elucidated, the mechanism proposed above provides guidelines for future experiments. Aldehyde **1** will serve as a useful mechanistic probe as one would expect the hydrated form to bind with one hydroxyl in each of the catalytic sites (Figure 7, inset). Thus, the introduction of key mutations that destroy the ability of a given site to promote hydride transfer should lead to a dramatic loss of epimerase activity yet should leave significant dismutase activity intact.

## SUPPORTING INFORMATION AVAILABLE

$^1$H NMR spectra of adenine diphospho-β-D-*manno*-hexadialdose **1**, ADP-β-D-mannuronate, and ADP-β-D-mannose; synthesis of ADP-β-D-mannose. This material is available free of charge via the Internet at http://pubs.acs.org.

## REFERENCES

1. Raetz, C. R. H., and Whitfield, C. (2002) Lipopolysaccharide endotoxins, *Annu. Rev. Biochem. 71*, 635−700.
2. Raetz, C. R. H. (1996) in *Escherichia coli and Salmonella* (Neidhardt, F. C., Ed.) pp 1035−1063, American Society for Microbiology, Washington, DC.
3. Zamyatina, A., Gronow, S., Oertelt, C., Puchberger, M., Brade, H., and Kosma, P. (2000) Efficient chemical synthesis of the two anomers of ADP-L-*glycero*- and D-*glycero*-D-*manno*-heptopyranose allows the determination of the substrate specificities of bacterial heptosyltransferases, *Angew. Chem., Int. Ed. 39*, 4150−4153.
4. Kneidinger, B., Marolda, C., Graninger, M., Zamyatina, A., McArthur, F., Kosma, P., Valvano, M. A., and Messner, P. (2002) Biosynthesis pathway of ADP-L-*glycero*-β-D-*manno*-heptose in *Escherichia coli*, *J. Bacteriol. 184*, 363−369.
5. Valvano, M. A., Messner, P., and Kosma, P. (2002) Novel pathways for biosynthesis of nucleotide-activated *glycero*-*manno*-heptose precursors of bacterial glycoproteins and cell surface polysaccharides, *Microbiology 148*, 1979−1989.
6. Samuel, J., and Tanner, M. E. (2002) Mechanistic aspects of enzymatic carbohydrate epimerization, *Nat. Prod. Rep. 19*, 261−277.
7. Tanner, M. E. (2002) Understanding nature's strategies for enzyme-catalyzed racemization and epimerization, *Acc. Chem. Res. 35*, 237−246.
8. Ni, Y., McPhie, P., Deacon, A., Ealick, S., and Coleman, W. G., Jr. (2001) Evidence that NADP$^+$ is the physiological cofactor of ADP-L-*glycero*-D-*manno*-heptose 6-epimerase, *J. Biol. Chem. 276*, 27329−27334.
9. Deacon, A. M., Ni, Y. S., Coleman, W. G., Jr., and Ealick, S. E. (2000) The crystal structure of ADP-L-*glycero*-D-*manno*-heptose 6-epimerase: catalysis with a twist, *Structure 8*, 453−461.
10. Ding, L., Seto, B. L., Ahmed, S. A., and Coleman, W. G., Jr. (1994) Purification and properties of the *Escherichia coli* K-12 NAD-dependent nucleotide diphosphosugar epimerase, ADP-L-*glycero*-D-*manno*-heptose 6-epimerase, *J. Biol. Chem. 269*, 24384−24390.
11. Read, J. A., Ahmed, R. A., Morrison, J. P., Coleman, W. G., Jr., and Tanner, M. E. (2004) The mechanism of the reaction catalyzed by ADP-β-L-*glycero*-D-*manno*-heptose 6-epimerase, *J. Am. Chem. Soc. 126*, 8878−8879.
12. Wei, Y., Lin, J., and Frey, P. A. (2001) $^{13}$C NMR analysis of electrostatic interactions between NAD$^+$ and active site residues of UDP-galactose 4-epimerase: Implications for the activation induced by uridine nucleotides, *Biochemistry 40*, 11279−11287.
13. Berger, E., Arabshahi, A., Wei, Y., Schilling, J. F., and Frey, P. A. (2001) Acid−base catalysis by UDP-galactose 4-epimerase: Correlations of kinetically measured acid dissociation constants with thermodynamic values for tyrosine 149, *Biochemistry 40*, 6699−6705.
14. Thoden, J. B., and Holden, H. M. (1998) Dramatic differences in the binding of UDP-galactose and UDP-glucose to UDP-galactose 4-epimerase from *E. coli*, *Biochemistry 37*, 11469−11477.
15. Frey, P. A. (1996) The Leloir pathway, *FASEB J. 10*, 461−470.
16. Koropatkin, N. M., Liu, H.-W., and Holden, H. M. (2003) High resolution X-ray structure of tyvelose epimerase from *Salmonella typhi*, *J. Biol. Chem. 278*, 20847−20881.
17. Hallis, T. M., Zhao, Z., and Liu, H.-W. (2000) New insights into the mechanism of CDP-tyvelose 2-epimerase: an enzyme catalyzing epimerization at an unactivated stereocenter, *J. Am. Chem. Soc. 122*, 10493−10503.
18. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem. 72*, 248−254.
19. Helferich, B., and Leete, J. F. (1929) Trityläther der mannose. Eine neue tetracetyl-mannose, *Ber. Dtsch. Chem. Ges. 62*, 1549−1554.
20. Campbell, R. E., and Tanner, M. E. (1997) Uridine diphospho-α-D-*gluco*-hexodialdose: Synthesis and kinetic competence in the reaction catalyzed by UDP-glucose dehydrogenase, *Angew. Chem., Intl. Ed. Engl. 36*, 1520−1522.
21. Nicoll-Griffith, D., and Weiler, L. (1984) Stereospecific introduction of an asymmetric centre at C-6 in a glycoside, *J. Chem. Soc., Chem. Commun*, 659−661.
22. Binkley, R. W. (1977) A mild process for the oxidation of partially protected carbohydrates, *J. Org. Chem. 42*, 1216−1221.

23. Lombardo, L. (1982) Methylation of carbonyl compounds with $Zn-CH_2Br_2-TiCl_4$. Applications to gibberellins, *Tetrahedron Lett. 23*, 4293−4296.
24. Sim, M. M., Kondo, H., and Wong, C.-H. (1993) Synthesis and use of glycosyl phosphites: An effective route to glycosyl phosphates, sugar nucleotides and glycosides, *J. Am. Chem. Soc. 115*, 2260−2267.
25. Gardner Swain, C., Powell, A. L., Sheppard, W. A., and Morgan, C. R. (1979) Mechanism of the Cannizzaro reaction, *J. Am. Chem. Soc. 101*, 3576−3583.
26. Ge, X., Penney, L. C., van de Rijn, I., and Tanner, M. E. (2004) Active site residues and mechanism of UDP-glucose dehydrogenase, *Eur. J. Biochem. 271*, 14−22.
27. Henehan, G. T. M., Chang, S. H., and Oppenheimer, N. J. (1995) Aldehyde dehydrogenase activity of *Drosophila melanogaster* alcohol dehydrogenase: Burst kinetics at high pH and aldehyde dismutase activity at physiological pH, *Biochemistry 34*, 12294−12301.
28. Henehan, G. T. M., and Oppenheimer, N. J. (1993) Horse liver alcohol dehydrogenase-catalyzed oxidation of aldehydes: Dismutation precedes net production of reduced nicotiamide adenine dinucleotide, *Biochemistry 32*, 735−738.
29. Hara, A., Yamamoto, H., Deyashiki, Y., Nakayama, T., Oritani, H., and Sawada, H. (1991) Aldehdye dismutation catalyzed by pulmonary carbonyl reductase: Kinetic studies of chloral hydrate metabolism to trichloroacetic acid and trichloroethanol, *Biochim. Biophys. Acta 1075*, 61−67.
30. Eisses, K. T. (1989) On the oxidation of aldehdyes by alcohol dehydrogenase of *Drosophila melanogaster*: Evidence for the *gem*-diol as the reacting substrate, *Bioorg. Chem. 17*, 268−274.
31. Moxon, L. N., Holmes, R. S., Parsons, P. A., Irving, M. G., and Doddrell, D. M. (1985) Purification and molecular properties of alcohol dehydrogenase from *Drosophila melanogaster*: Evidence from NMR and kinetic studies for function as an aldehdye dehydrogenase, *Comp. Biochem. Physiol. 80B*, 525−535.
32. Hinson, J. A., and Neal, R. A. (1975) An examination of octanol and octanol metabolism to octanoic acid by horse liver alcohol dehydrogenase, *Biochim. Biophys. Acta 384*, 1−11.
33. Abeles, R. H., and Lee, H. A., Jr. (1960) The dismutation of formaldehyde by liver alcohol dehydrogenase, *J. Biol. Chem. 235*, 1499−1503.

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