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The Mechanism of the Reaction Catalyzed by ADP-β-L-*glycero*-D-*manno*-heptose 6-Epimerase

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

Department of Chemistry, University of British Columbia, Vancouver, British Columbia, Canada, and the Laboratory of Biochemistry and Genetics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland

Received March 12, 2004; E-mail: mtanner@chem.ubc.ca

ADP-L-*glycero*-D-*manno*-heptose 6-epimerase (AGME, RfaD) is a bacterial enzyme that interconverts the unusual seven-carbon β-linked sugar nucleotides ADP-β-L-*glycero*-D-*manno*-heptose (ADP-L,D-Hep) and ADP-β-D-*glycero*-D-*manno*-heptose (ADP-D,D-Hep) (Figure 1).[1] In Gram-negative bacteria, AGME plays a key role in the biosynthesis of cell surface lipopolysaccharide (LPS) as it provides the L,D-Hep that is a component of the inner core tetrasaccharide.[2] Mutant strains of *Escherichia coli* that lack a functional AGME produce truncated LPS chains, have an increased susceptibility to hydrophobic antibiotics, and show decreased pathogenicity.[3−5]

The mechanism by which AGME catalyzes the inversion of stereochemistry is of interest since the C-6″ stereocenter of the substrate lacks an acidic C−H bond and a direct deprotonation/reprotonation mechanism cannot be employed.[6,7] While few mechanistic studies have been undertaken, it is known that the enzyme bears a tightly bound $NADP^+$ cofactor that is presumably utilized to transiently oxidize the substrate.[8−10] Sequence alignments and X-ray crystallographic studies support this hypothesis by showing that AGME is a member of the short-chain dehydrogenase/reductase (SDR) superfamily.[9,11] Several members of this family are known to catalyze reactions on sugar nucleotides that involve transient oxidations of the sugar moiety (usually at C-4″).[12,13] One potential mechanism for the AGME reaction involves an initial oxidation at C-7″ that serves to acidify the proton at C-6″ (Scheme 1, path A). A subsequent deprotonation at C-6″, followed by reprotonation on the opposite face of the enol(ate) intermediate and reduction of the C-7″ aldehyde, generates the epimeric product. An alternative mechanism involves oxidation directly at C-6″ (Scheme 1, path B). A conformational change would then serve to expose the opposite face of the carbonyl to the cofactor, and reduction would give the epimeric product. Last, the transient oxidation may take place at C-4″ (Scheme 1, path C). A nonstereospecific dehydration/rehydration process could invert the stereochemistry at C-6″, and subsequent reduction of the C-4 carbonyl would generate the product. A nonstereospecific retroaldol/aldol process could also be used to invert the stereochemistry at C-6″ of the 4-keto intermediate (not shown).

In this communication we report evidence in favor of a mechanism involving transient oxidation directly at C-6″ (Scheme 1, path B). Epimerization in either D_2O or $H_2{}^{18}O$ was not accompanied by solvent isotope incorporation, and the 7-deoxy and 4-deoxy analogues of ADP-L,D-Hep (Figure 1, **1a** and **2a**, respectively) were found to be alternate substrates for the enzyme.

ADP-L,D-Hep and ADP-D,D-Hep were obtained via chemical synthesis.[14,15] When either substrate was incubated in buffered D_2O with the recombinant *E. coli* AGME and the reactions were monitored by both ^{1}H- and ^{31}P NMR spectroscopy, an equilibrium mixture of 2.3:1 ADP-L,D-Hep/ADP-D,D-Hep was observed. Samples of the epimeric mixture formed under multiple turnover conditions were analyzed for deuterium content by both ^{1}H NMR spectroscopy and mass spectrometry. No detectable levels of deuterium were observed, indicating that no more than 1% of the enzymatic turnovers results in solvent isotope incorporation. A similar experiment was run using a 50:50 mixture of $H_2{}^{18}O/H_2{}^{16}O$ as solvent, and no ^{18}O-label could be detected in the mass spectrum of the resulting epimeric mixture. The absence of deuterium incorporation argues against a mechanism involving deprotonation/reprotonation at C-6 (Scheme 1, path A). In all known racemases and epimerases that catalyze nonstereospecific proton transfer steps, a two-base mechanism is employed, and solvent deuterium is stoichiometrically incorporated into the product.[6,16,17] Similarly, the absence of ^{18}O-isotope incorporation argues against the dehydration/rehydration mechanism (Scheme 1, path C) since the same molecule of water formed upon dehydration would have to be efficiently redelivered to the opposite face of the enone intermediate.

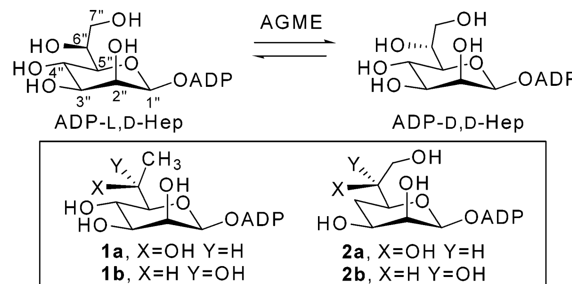

Figure 1. Reaction catalyzed by AGME and structure of deoxy analogues (inset).

In further studies, the 7-deoxy and 4-deoxy analogues of ADP-L,D-Hep (**1a** and **2a**, respectively) were synthesized.[15] These compounds were incubated with AGME, and the resulting reactions were monitored using both ^{1}H- and ^{31}P NMR spectroscopy. In each case, a new set of signals emerged that ultimately equilibrated to an approximately equimolar mixture of two species (Figure 2). The ^{31}P NMR spectra identified the new compounds as sugar nucleotides, and the mass spectra of the isolated mixtures were indistinguishable from those of the starting compounds. These results are consistent with the notion that AGME interconverts both substrate analogues with epimeric sugar nucleotides of similar stability. In the case of compound **1a**, the H-5″ signals for the starting material and product were sufficiently well separated so that a 1D-TOCSY experiment could be used to fully assign the spectrum of the new heptose moiety. Coupling constants were consistent with a mannose configuration for the stereochemistry at

† Department of Chemistry, University of British Columbia.
‡ Laboratory of Biochemistry and Genetics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health.

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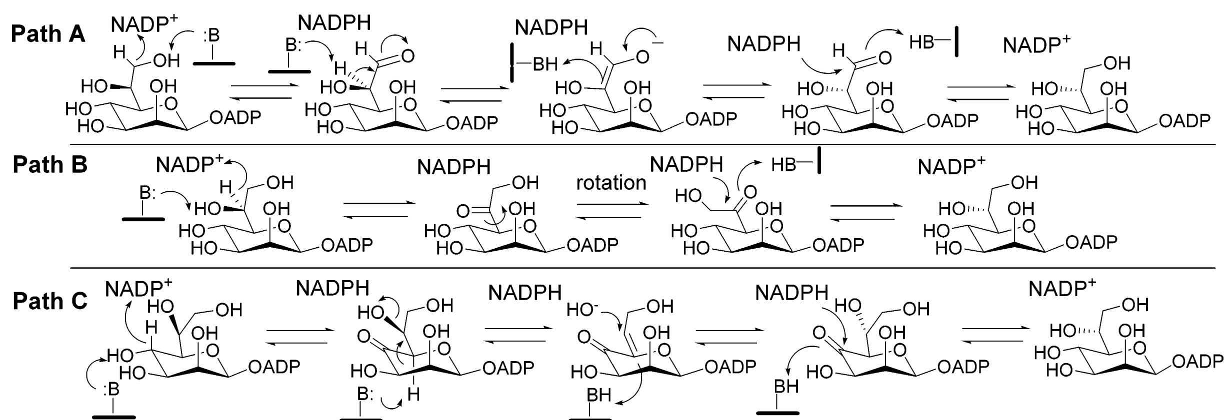
Scheme 1



carbons 2″−5″, indicating that epimerization had occurred at C-6″ and the new compound was **1b**.[15] In the case of compound **2a**, however, the ^{1}H NMR signals of the two species were not sufficiently separated to employ this technique. Instead, a nonstereoselective synthesis was used to generate an authentic 1.8:1 mixture of **2a**/**2b**.[15] A comparison of the ^{1}H- and ^{31}P NMR spectra of the enzymatic mixture to that of the synthetic mixture confirmed that the new product was **2b** (Figure 2). Furthermore, when the synthetic 1.8:1 mixture of **2a**/**2b** was incubated with AGME, it was converted into an equimolar mixture. The ability of **1** and **2** to serve as substrates for AGME rules out all mechanisms that require oxidation at C-4″ or C-7″ (paths A and C).

As AGME is a member of the SDR superfamily, one might expect it would utilize a strategy involving transient oxidation at C-4″.[12,13] In particular, a notable similarity exists between the first two steps in path C and those employed by the sugar nucleotide 4,6-dehydratases.[18−21] However, both the solvent isotope incorporation studies and the deoxy-analogue studies indicate that transient oxidation actually occurs at C-6″. Thus, AGME appears to employ a nonstereospecific oxidation/reduction mechanism (path B). As with other SDR family members, at least one of the required acid/base residues would likely be supplied by the conserved triad, Ser116, Tyr140, Lys144.[9] This strategy is conceptually similar to that of the SDR enzyme UDP-galactose 4-epimerase; however, both the position of oxidation and the required reorientation of the oxidized intermediate are quite different.[6,22−24] This display of flexibility with regard to the position of oxidation is somewhat reminiscent of the reaction catalyzed by CDP-tyvelose 2-epimerase, an SDR family member that is thought to employ transient oxidation at C-2″.[25,26]

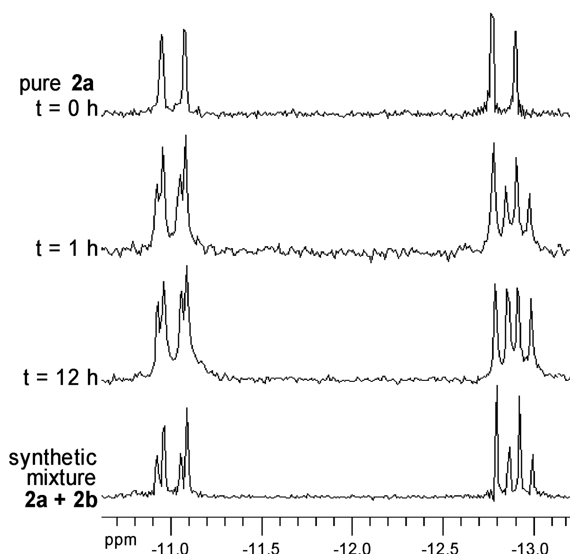


Figure 2. ^{31}P-NMR spectra illustrating the epimerization of **2a**.

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Supporting Information Available: Experimental procedures, ^{1}H NMR spectra of sugar nucleotides, and time courses of enzymatic reactions. This material is available free of charge via the Internet at http://pubs.acs.org.

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