Intermediate Release by ADP-L-*glycero*-D-*manno*-heptose 6-Epimerase†

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ABSTRACT: ADP-L-*glycero*-D-*manno*-heptose 6-epimerase (HldD or AGME, formerly RfaD) catalyzes the interconversion of ADP-*β*-D-*glycero*-D-*manno*-heptose (ADP-D,D-Hep) and ADP-*β*-L-*glycero*-D-*manno*-heptose (ADP-L,D-Hep). The latter compound provides the heptose moiety that is used in lipopolysaccharide biosynthesis by Gram-negative bacteria. Several lines of evidence suggest that the enzyme uses a direct oxidation/reduction mechanism involving a tightly bound $NADP^+$ cofactor. An initial oxidation at C-6″ gives a 6″-keto intermediate, and a subsequent reduction on the opposite face of the carbonyl group generates the epimeric product. The reorientation required for the nonstereoselective reduction could take place within a single active site, or it could involve the release of the intermediate and rebinding in an altered conformation. To distinguish between these possibilities, two isotopically labeled substrates (ADP-D,D-Hep) were prepared that contained ^{18}O and ^2H isotopes at C-7″ and C-6″, respectively. A crossover experiment was used to determine whether unlabeled or doubly labeled products were formed upon epimerization of a mixture of the two singly labeled compounds. After an initial epimeric equilibrium was reached, no crossover could be detected, indicating that intermediate release is not intrinsic to the overall mechanism. After extended incubation, however, scrambling of the labels could be detected, indicating that a low background rate of intermediate release does occur. To directly detect the release of the intermediate, the labeled compounds were independently epimerized in the presence of a ketone-trapping reagent, phenylhydrazine. The corresponding phenylhydrazones were identified by mass spectrometry, and the absence of any ^2H isotope in the adduct obtained from the deuterated starting compound confirmed that the oxidation had occurred at C-6″.

ADP-L-*glycero*-D-*manno*-heptose 6-epimerase (HldD or AGME, formerly RfaD) catalyzes the interconversion of ADP-*β*-D-*glycero*-D-*manno*-heptose (ADP-D,D-Hep) and ADP-*β*-L-*glycero*-D-*manno*-heptose (ADP-L,D-Hep) (Figure 1) (*1*−*3*). The latter compound provides the heptose moiety that is a vital component of the inner core region of lipopolysaccharide (LPS)[1] in Gram-negative bacteria (*4*, *5*). Mutant bacteria that lack the ability to carry out the epimerase reaction can only biosynthesize truncated LPS structures and as a result are rendered weakly pathogenic and show an increased susceptibility to hydrophobic antibiotics (*6*−*8*). For this reason the epimerase serves as an attractive target for the development of new antibacterial agents.

ADP-L-*glycero*-D-*manno*-heptose 6-epimerase is a member of the short-chain dehydrogenase reductase (SDR) family and uses a tightly bound $NADP^+$ cofactor during catalysis (*9*, *10*). The mechanism of the epimerization is thought to involve a direct oxidation at C-6″ of the bound substrate to give a 6″-keto intermediate (Figure 1) (*11*−*13*). A subsequent reduction on the opposite face of the carbonyl generates the epimeric product. Studies supporting this mechanism include the observation that the reaction proceeds without the incorporation of solvent-derived deuterium or ^{18}O isotopes and that ADP-[6″-^2H]-D,D-Hep is converted into ADP-[6″-^2H]-L,D-Hep with retention of deuterium at the C-6″ position. Deoxygenated substrate analogues lacking hydroxyl groups at either C-7″ or C-4″ have also been shown to be epimerized by this enzyme, demonstrating that transient oxidation at these positions is not required for catalysis. More direct evidence in favor of a C-6″ oxidation event involved work with ADP-*β*-D-*manno*-hexodialdose (ADP-mannose bearing an aldehydic functionality at C-6″). The epimerase was found to catalyze a dismutation reaction in which this compound was converted into a 1:1 mixture of ADP-mannose and ADP-mannuronic acid. This supports the notion that the enzyme is capable of catalyzing oxidation and reduction at the C-6″ position of a bound ADP-sugar. Most recently, a model

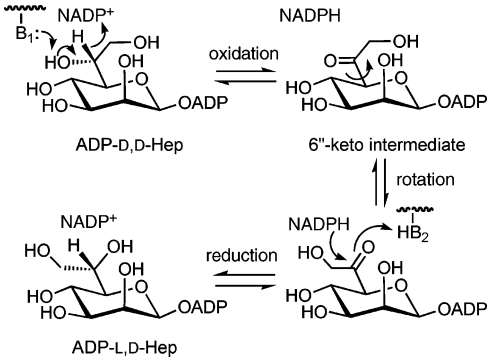

FIGURE 1: Mechanism of the reaction catalyzed by ADP-L,D-Hep 6-epimerase. B_1 and B_2 are active site acid/base residues.

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[1] Abbreviations: LPS, lipopolysaccharide; L,D-Hep, L-*glycero*-D-*manno*-heptose; SDR, short-chain dehydrogenase/reductase.

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explaining how the nonstereospecific reduction of the C-6″ carbonyl can occur has been presented (*14*). The epimerase active site is thought to contain two catalytic "pockets" that can accommodate either the C-6″ hydroxymethylene group or the C-6″ hydroxy group depending on which epimer is bound. These pockets are defined by the position of the key catalytic active site acid/base residues Tyr140 and Lys178 (B_1 or B_2 in Figure 1), each of which is responsible for promoting the hydride transfer to a given face of the carbonyl. Thus, each pocket acts as a stereospecific dehydrogenase, and together they catalyze an overall epimerization.

In order for the bound NADPH to access both faces of the carbonyl of the 6″-keto intermediate, a reorientation event must take place. Perhaps the simplest possibility is that a rotation takes place about the C-5″−C-6″ bond of the intermediate such that the carbonyl moves from one pocket to the other without dissociating from the enzyme (shown in Figure 1). An alternate possibility is that the ketone intermediate is released into solution (or transferred to an adjacent subunit of the pentameric enzyme) and rebound in an altered conformation. In this paper an isotopic crossover study is used to distinguish between these events. Evidence is presented that shows the hydride transfer is intramolecular and that intermediate release is not an integral step in catalysis. On extended incubations, however, a small background rate of intermediate release is observed. Chemical trapping studies are used to provide the first direct evidence that a 6″-keto intermediate is generated in this epimerization reaction.

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 (dichloromethane, pyridine, acetonitrile), Na (toluene), or Na/benzophenone (THF) as drying agent. ^{18}O-Labeled water (97%) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). Inorganic pyrophosphatase (from yeast) was purchased from Roche Diagnostics Corp. (Indianapolis, IN). Protein concentrations were determined by Bradford assay (*15*) using bovine serum albumin as standard. ^{1}H NMR spectra were obtained on Brucker AV300 or AV400 spectrometers 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 spectra were measured on a Bruker Esquire ESI-MS spectrometer.

Preparation of Enzymes. ADP-L-*glycero*-D-*manno*-heptose 6-epimerase (HldD) and the bifunctional D-*glycero*-D-*manno*-heptose 7-phosphate kinase/*β*-D-*glycero*-D-*manno*-heptose 1-phosphate adenylyltransferase (HldE) from *Escherichia coli* K-12 were overexpressed and purified as described previously (*12*). *β*-D-*glycero*-D-*manno*-Heptose 1,7-bisphosphate phosphatase (GmhB) from *E. coli* K-12 was also prepared according to literature procedures (*11*).

*Synthesis of Bis-^{18}O-labeled Phosphoric Acid Dibenzyl Ester **3**.* To a solution of 1.00 g (2.90 mmol) of dibenzyl *N*,*N*-diisopropylphosphoramidite in 5 mL of anhydrous acetonitrile was added 290 *μ*L (14.48 mmol) of water (^{18}O, 97%) followed by 406 mg (5.79 mmol) of 1*H*-tetrazole. After being stirred for 1 h at room temperature, a solution of 1.47 g (5.79 mmol) of iodine in 5 mL of anhydrous pyridine was added dropwise to the reaction mixture. When the addition was complete, the red solution was stirred for 1 h before the volatiles were removed. The residue was dissolved in 50 mL of dichloromethane and washed with saturated aqueous $NaHCO_3$ (5 × 30 mL). The organic layer was discarded, pooled aqueous layers were acidified to pH 1−2 with concentrated HCl, and the product was extracted with dichloromethane (3 × 50 mL). Combined organic layers were treated with 5% aqueous $Na_2S_2O_3$ (50 mL), dried over $MgSO_4$, and evaporated to yield 419 mg (51%) of **3** as a white solid. The product showed spectral properties identical to a sample of commercially available unlabeled material. −ESIMS: *m*/*z* 281.0 $[M - H]^-$.

*Synthesis of Benzyl 2,3,4-Tri-O-benzyl-7-deoxy-7-diazo-α-D-manno-heptopyran-6-ulose (**2**).* To a solution of 2.25 g (4.05 mmol) of carboxylic acid **1** (*11*) in 50 mL of dry dichloromethane was added 5 drops of dry DMF. The solution was cooled to 0 °C, and 565 *μ*L (6.48 mmol) of oxalyl chloride was added dropwise. The solution was allowed to reach room temperature and stirred for 2 h. Completion of the reaction was confirmed by TLC of aliquots quenched with MeOH. Volatiles were then removed by evaporation, and the syrupy residue was dissolved in 50 mL of anhydrous dichloromethane. The solution was cooled to 0 °C, and 6.1 mL (2.0 M in diethyl ether, 12.15 mmol) of trimethylsilyldiazomethane was added dropwise. The yellow solution was stirred at 0 °C for 2 h and then concentrated to yield a brown syrup. Purification by column chromatography (2:1 to 1:1 petroleum ether/Et_2O, silica gel) afforded 2.05 g (87%) of diazoketone **2**. The spectral properties of the product were identical to data found in the literature (*11*).

*Synthesis of Bis-^{18}O-labeled Benzyl 2,3,4-Tri-O-benzyl-7-dibenzylphosphono-D-glycero-α-D-manno-heptopyranose **4a** and Bis-^{18}O-labeled Benzyl 2,3,4-Tri-O-benzyl-7-dibenzylphosphono-L-glycero-α-D-manno-heptopyranose **4b**.* A 490 mg (0.85 mmol) sample of diazoketone **2** was dissolved in 20 mL of dry toluene, and 382 mg (1.35 mmol) of **3** was added. The solution was stirred at 85 °C for 2 h and cooled to 0 °C, and a solution of 64.1 mg (1.69 mmol) of $NaBH_4$ in 4.5 mL of ethanol was added dropwise. It should be noted that rearrangement products can be obtained if this reduction step is carried out at temperatures above 20 °C. The reaction was stirred for 1 h and then worked up by addition of 60 mL of water and extraction with Et_2O (3 × 60 mL). The combined organic layers were dried over $MgSO_4$ and evaporated. Purification by column chromatography (2:3 to 1:1 ethyl acetate/petroleum ether, silica gel) afforded 371 mg (52%) of a mixture of diastereoisomers **4a**/**4b** in a 1:1.5 ratio. Clean samples were obtained for both products and presented ^{1}H NMR spectra that were indistinguishable from those of the corresponding unlabeled compounds (*11*). +ESIMS: *m*/*z* 857.5 $[M + Na]^+$.

Synthesis of Adenosine 5′-(D-[7″-^{18}O]-glycero-β-D-manno-Heptopyranosyl Diphosphate) Bis(triethylammonium) Salt. The mixture of dibenzyl phosphates **4a** and **4b** was converted to ADP-[7″-^{18}O]-D,D-Hep by following a literature procedure (*11*). The product displayed ^{1}H and ^{31}P NMR spectra that were indistinguishable from those of the reported unlabeled compound. −ESIMS: *m*/*z* 620.2 $[M - 2(Et_3N) - H]^-$.

Isotope Crossover Experiment. A mixture of 3.0 mg (4.8 μmol) of ADP-[6″-^{2}H]-D,D-Hep and 1.9 mg (3.1 μmol) of ADP-[7″-^{18}O]-D,D-Hep was dissolved in phosphate buffer (10 mM NaH_2PO_4, pH 7, containing 10% D_2O) to a final volume of 1.5 mL. Chelex 100 resin beads (30 μL) were added, and the solution was gently shaken for 1 h. Initial mass and ^{31}P NMR spectra were then measured. To 1 mL of this stock solution was added 0.155 mg of epimerase HldD (10 μL of a 15.5 mg/mL solution, 3.9 nmol), and the mixture was incubated at 37 °C. The evolution of the epimerization was followed by ^{31}P NMR spectroscopy. After 1 h of incubation, a 0.5 mL sample was taken, the enzyme was removed by ultrafiltration (Amicon Ultra-4, 10000 MWCO, 5000 rpm for 15 min, 4 °C), and the filtrate was lyophilized. The residue was dissolved in a 1:1 H_2O/MeOH mixture and analyzed by −ESI mass spectrometry. After 18 h of incubation, the rest of the solution was subjected to the same procedure. The enzyme filtered after 18 h of incubation was recovered from the Amicon device and added to a new batch of substrate to test its activity using ^{31}P NMR spectroscopy. It still showed over 90% of activity. The remaining 0.5 mL of the stock solution was incubated with 50 μL of enzyme (15.5 mg/mL, 0.775 mg, 19.4 nmol) at 37 °C. After 18 h, the enzyme was removed, the filtrate was lyophilized and dissolved in a 1:1 mixture of H_2O/MeOH, and a mass spectrum was recorded.

Trapping of the 6″-Keto Intermediate. A solution of ADP-[7″-^{18}O]-D,D-Hep (3.3 mM) and phenylhydrazine (33 mM) was incubated with HldD (130 μM) in sodium phosphate buffer (10 mM, pH 7, total volume of 300 μL) for 18 h at 37 °C. The resulting yellow solution was then subjected to ultrafiltration (Amicon Ultra-4, 10000 MWCO, 5000 rpm for 15 min, 4 °C) to remove the enzyme. The filtrate was analyzed by ^{31}P NMR spectroscopy to confirm that epimerization had occurred and by −ESI mass spectrometry (1:1 H_2O/MeOH) to detect the hydrazone adduct (*m*/*z* 620.2 $[M - H]^-$; *m*/*z* 642.1 $[M + Na - 2H]^-$; *m*/*z* 664.0 $[M + 2Na - 3H]^-$; *m*/*z* 708.1 hydrazone adduct). The experiment was repeated with ADP-[6″-^{2}H]-D,D-Hep using identical conditions (*m*/*z* 619.2 $[M - H]^-$; *m*/*z* 641.1 $[M + Na - 2H]^-$; *m*/*z* 706.1 hydrazone adduct).

RESULTS

Isotope Crossover Experiment. In order to determine whether the hydride transfer to and from the C-6″ position of the epimers is an intramolecular process, an isotopic crossover experiment was devised (Figure 2). This experiment involves incubating a mixture of a C-6″ deuterated substrate (ADP-[6″-^{2}H]-D,D-Hep) and an ^{18}O-labeled substrate (ADP-[7″-^{18}O]-D,D-Hep) with the enzyme and looking for the formation of doubly labeled or unlabeled products. If the C-6″ deuteride is transferred to the $NADP^+$ cofactor and the resulting 6″-keto intermediate dissociates from the active site, an ^{18}O-labeled 6″-keto intermediate may then replace it and a doubly labeled compound would result (active site A in Figure 2). A similar process would also lead to the formation of unlabeled products (active site B in Figure 2). If release of the intermediate occurs upon every turnover, then the rate of isotopic crossover would mirror the rate of epimerization. However, if intermediate release only occurs infrequently, the rate of crossover should be much slower than catalysis.

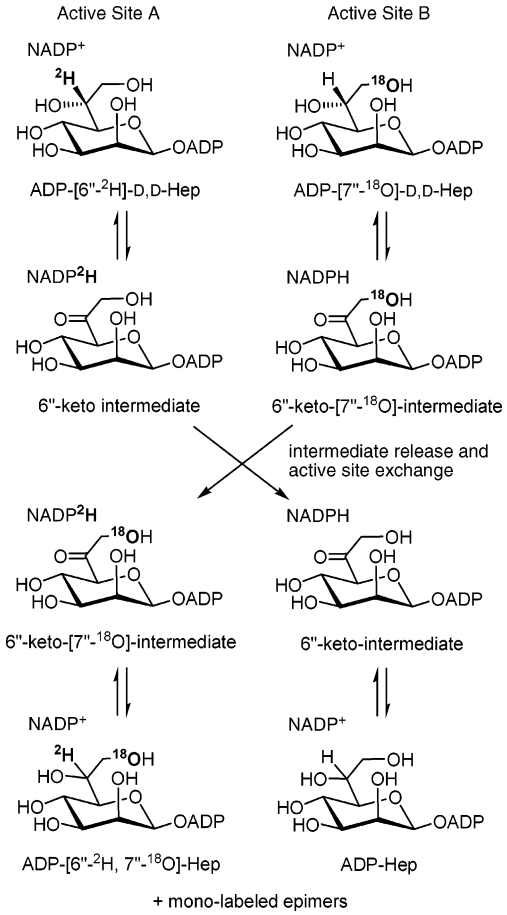

FIGURE 2: Crossover experiment employing ADP-[6″-^{2}H]-D,D-Hep and ADP-[7″-^{18}O]-D,D-Hep.

A chemoenzymatic synthesis of ADP-[6″-^{2}H]-D,D-Hep was previously reported from this laboratory and was used to prepare one of the labeled substrates (*11*). In order to prepare an ^{18}O-labeled substrate, a modified version of this process was employed (Figure 3). The overall strategy involves the chemical synthesis of a mixture of D,D-Hep 7-phosphate and L,D-Hep 7-phosphate followed by an enzymatic conversion of only the former epimer into its corresponding sugar nucleotide. A convenient way to introduce the ^{18}O label into a position other than C-6″ (to avoid a potential washout via the 6″-keto intermediate) was to introduce it at C-7″ during the phosphorylation step. Perbenzylated carboxylic acid **1**, which is available in five steps from D-mannose (*11*), was converted to the α-diazoketone **2** by treatment with oxalyl chloride followed by reaction with trimethylsilyldiazomethane. Doubly ^{18}O-labeled dibenzylphosphoric acid **3** was prepared by treating *N*,*N*-diisopropyldibenzylphosphoramidite with $H_2{}^{18}O$ and 1*H*-tetrazole followed by oxidation with iodine/pyridine/$H_2{}^{18}O$. Mass spectral analysis indicated that >95% of the acid was doubly labeled. Treatment of the α-diazoketone **2** with the acid **3** followed by reduction with $NaBH_4$ gave a mixture of the desired D,D-isomer **4a** and the undesired L,D-isomer **4b** in a 1:1.5 ratio. This epimeric mixture was deprotected by catalytic hydrogenation to give the corresponding mixture of D,D-Hep 7-phosphate and L,D-Hep 7-phosphate, each bearing two ^{18}O labels in the C-7 phosphate group. The former epimer is the natural biosynthetic precursor to the ADP-D,D-Hep, and it has been shown that it can be selectively converted to a β-linked ADP-sugar

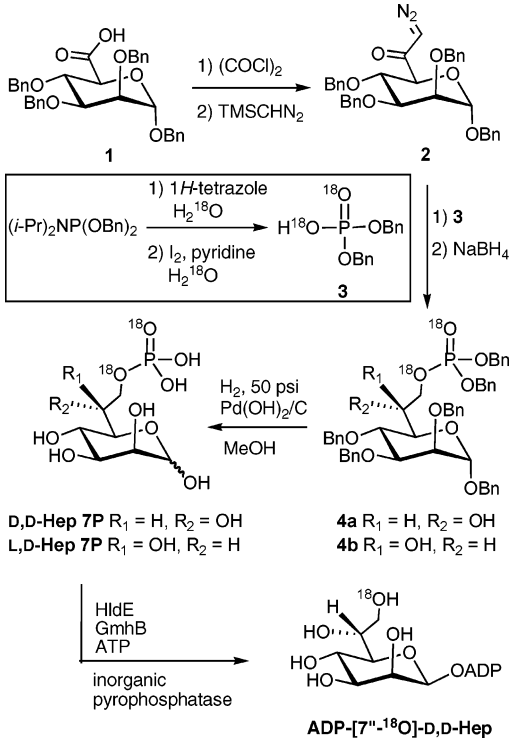

FIGURE 3: Synthesis of ADP-[7″-^{18}O]-D,D-Hep.

upon incubation with the enzymes HldE and GmhB (*11*). D,D-Hep 7-phosphate is first phosphorylated at C-1 by the bifunctional enzyme HldE to give D,D-Hep 1,7-bisphosphate. The phosphatase activity of GmhB then removes the C-7 phosphate to give D,D-Hep 1-phosphate. It was anticipated that this reaction proceeds via a P−O bond cleavage mechanism such that a single ^{18}O label would remain at C-7. Finally, the second activity of HldE installs the ADP functionality to give the product ADP-[7″-^{18}O]-D,D-Hep. Mass spectral analysis of product obtained in this fashion confirmed that >95% of the material bore a single ^{18}O isotope.

The crossover experiment was performed using an approximately 50:50 mixture of ADP-[6″-^{2}H]-D,D-Hep and ADP-[7″-^{18}O]-D,D-Hep and was monitored by both ^{31}P NMR spectroscopy and −ESI mass spectrometry. The mass spectrum taken prior to the addition of enzyme showed peaks of nearly equal intensity at *m*/*z* 619 and 620 that correspond to the two starting materials, respectively (Figure 4a, T = 0). Minor peaks at *m*/*z* 618 and 621 could also be seen and correspond to unlabeled ADP-D,D-Hep formed during the synthesis and ADP-[7″-^{18}O]-D,D-Hep bearing a ^{13}C isotope (natural abundance), respectively. The epimerase was added to the sample, and the progress of the epimerization was monitored by ^{31}P NMR spectroscopy (data not shown). After 10 min under these conditions the epimerization was approximately 50% of the way to equilibrium, and after 30 min the epimerization reaction was completed. After 1 h of incubation, mass spectral analysis showed that no detectable crossover had occurred since the ratio of all four peaks remained unchanged (Figure 4b, T = 1 h). This indicates that intermediate release is not an intrinsic part of the reaction mechanism and that the hydride transfer is an intramolecular process. On average, all of the substrate molecules would have undergone at least one turnover by this point, yet no crossover could be detected. In order to test for a low background rate of intermediate release, the sample was allowed to incubate for an additional 17 h and then analyzed by mass spectrometry (Figure 4c, T = 18 h). A noticeable increase in the relative magnitude of the peaks at *m*/*z* 618 (unlabeled) and 621 (doubly labeled) indicated that crossover was occurring, and integration of these peaks suggested that approximately 10% of the ADP-Hep pool had undergone this process. This observation shows that there is a slow

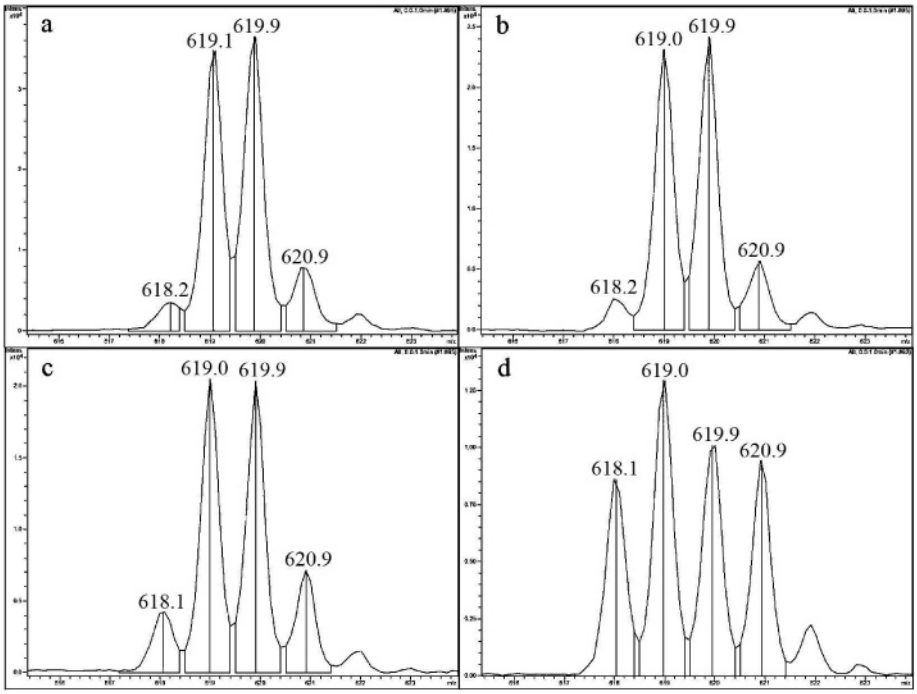

FIGURE 4: ESI mass spectra showing the evolution of the crossover experiment: (a) a mixture of ADP-[6″-^{2}H]-D,D-Hep and ADP-[7″-^{18}O]-D,D-Hep prior to the addition of epimerase; (b) after 1 h of incubation with the epimerase; (c) after 18 h of incubation with the epimerase; (d) after 18 h of incubation with 10× epimerase.

release of the ketone intermediate from the active site of the epimerase, and we estimate that this occurs approximately once every 200 turnovers. Intermediate release followed by substrate binding could lead to the accumulation of an abortive complex (enzyme·NADPH·ADP-Hep) in a process known as substrate-induced inactivation. A qualitative measurement of the residual enzyme activity in the sample that had been incubated for 18 h was therefore made. The enzyme was removed from the equilibrated mixture of ADP-Hep epimers by centrifugal concentration and then added to a fresh sample of ADP-[7″-^{18}O]-D,D-Hep. Monitoring the epimerization reaction by ^{31}P NMR spectroscopy showed that the enzyme had retained nearly all of its activity (>90%) over the course of the incubation (data not shown). This finding is consistent with previous studies showing that the enzyme is surprisingly robust and maintains activity over extended incubation periods (*11*−*13*). In order to demonstrate the occurrence of crossover in a more dramatic fashion, an identical solution of labeled substrates was incubated with a 10-fold greater concentration of epimerase for a period of 18 h. Mass spectral analysis of the resulting sample showed that the crossover process had reached equilibrium as all four mass peaks were found to be of approximately the same intensity (Figure 4d, $T = 18$ h, 10× enzyme).

Intermediate Trapping Experiment. The observation of crossover during extended incubations of the isotopically labeled substrates indicates that the oxidized intermediate is occasionally released from the active site during catalysis. Thus, attempts were made to detect the released intermediate directly. Since the intermediate release did not result in the inactivation of the enzyme sample, the intermediate must be able to effectively compete with the pool of substrate in rebinding to the NADPH-containing form of the enzyme, thereby re-entering the catalytic cycle. For this reason, the intermediate would not be expected to accumulate in solution to a significant extent. To overcome this potential pitfall, a chemical trapping agent, phenylhydrazine, was added to the incubation mixture. Phenylhydrazine is expected to react with the ketone functionality of the intermediate to give a stable phenylhydrazone derivative. Furthermore, phenylhydrazine is sterically bulky and is unlikely to enter the active site and react with the intermediate prior to release. In this type of trapping experiment, only one equivalent of phenylhydrazone derivative could be generated per enzyme active site since the act of trapping leaves the enzyme in a reduced, NADPH-containing state. A 3.3 mM sample of ADP-[7″-^{18}O]-D,D-Hep was therefore incubated with 130 μM epimerase in the presence of excess phenylhydrazine. The enzyme was removed by centrifugal ultrafiltration, and mass spectral analysis of the resulting solution showed a peak at *m*/*z* 708 (Figure 5a). This mass corresponds to that of the expected phenylhydrazone adduct of the ^{18}O-labeled ketone intermediate. A second sample containing ADP-[6″-^{2}H]-D,D-Hep was incubated and analyzed in an identical fashion. In this case a peak at *m*/*z* 706 was observed that corresponds to the phenylhydrazone adduct of the unlabeled ketone intermediate (Figure 5b). The fact that two different masses were observed with the two isotopically labeled substrates confirms that the adducts were substrate-derived and not simply impurities in the sample. Furthermore, the two observed adducts differed by two mass units (as opposed to a difference of one mass unit in the starting materials), indicating that the ADP-[6″-^{2}H]-D,D-Hep has lost its isotopic label as predicted for a C-6″ oxidation process. Overall, this experiment provides the first direct evidence for the formation of a 6″-keto intermediate in the reaction catalyzed by ADP-L-*glycero*-D-*manno*-heptose 6-epimerase.

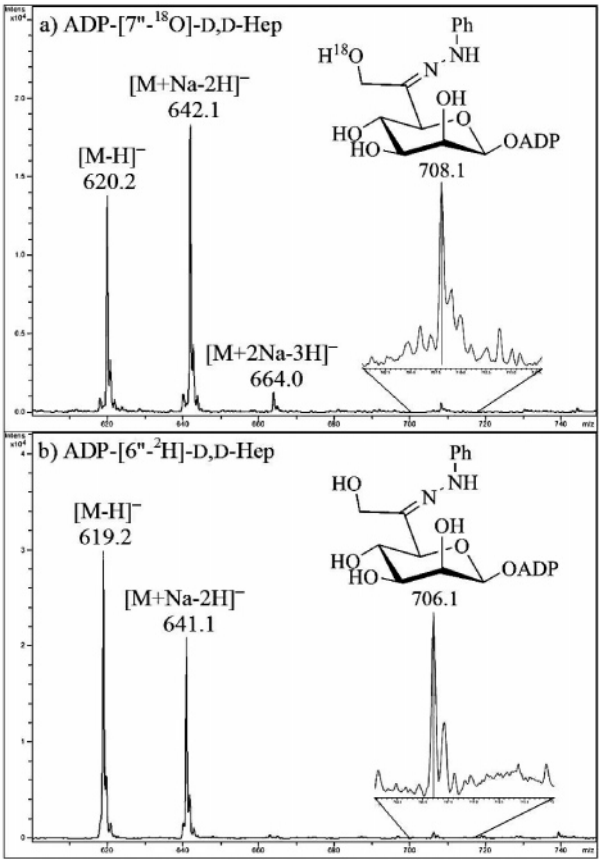

FIGURE 5: ESI mass spectra showing the detection of the hydrazone adduct formed upon incubation of the substrate with the epimerase and phenylhydrazine: (a) adduct formed from incubation with ADP-[7″-^{18}O]-D,D-Hep; (b) adduct formed from incubation with ADP-[6″-^{2}H]-D,D-Hep.

DISCUSSION

This report describes an isotopic crossover experiment that probes the intramolecularity of the hydride transfer steps in the reaction catalyzed by the enzyme ADP-L-*glycero*-D-*manno*-heptose 6-epimerase. The lack of any observed isotopic crossover during the initial approach to equilibrium indicates that the reaction predominantly takes place within a single active site and the hydride transfer is intramolecular in nature. This rules out mechanisms involving multisite hydrogen relay schemes and indicates that a simple conformational reorientation of the ketone intermediate is responsible for the nonstereospecific hydride transfer process. The most likely scenario is that a rotation about the C-5″−C-6″ bond exposes alternate faces of the carbonyl to an immobilized NADPH cofactor. The observance of crossover products during extended incubations indicates that a low level of intermediate release does occur. This is likely due to a "sloppiness" in the enzyme and is not an intrinsic part of the catalytic process. It was possible to take advantage of this intermediate release and trap the ketone functionality with phenylhydrazine. Mass spectral analysis of the resulting adducts confirmed that the intermediate is formed and has

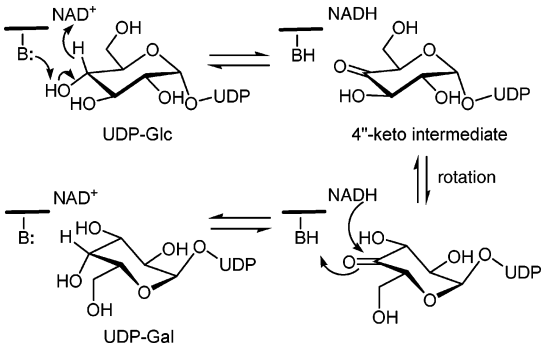

FIGURE 6: Mechanism of the reaction catalyzed by UDP-galactose 4-epimerase.

been oxidized at C-6″. This is the first direct evidence for the 6″-keto intermediate in the reaction catalyzed by this epimerase and strongly supports the proposed direct oxidation/reduction mechanism (Figure 1).

UDP-galactose 4-epimerase is perhaps the most extensively studied epimerase to date, and it is informative to compare its properties with those of the ADP-heptose 6-epimerase (*16*−*18*). UDP-Gal 4-epimerase is also a member of the SDR family and shares considerable structural and sequence homology with the ADP-Hep 6-epimerase. It also contains a tightly bound NAD^+ cofactor and uses a direct oxidation/reduction strategy during catalysis (Figure 6). An initial oxidation at C-4″ generates the 4″-keto intermediate. Extensive evidence supports the notion that the entire pyranose ring rotates by 180° in the active site such that the opposite face of the carbonyl is exposed to the reduced cofactor (*19*). A subsequent reduction gives the epimeric product.

Early studies on UDP-Gal 4-epimerase employed a mixture of UDP-glucose-d_7 and UDP-glucose to show that there were no crossover products (d_1 and d_6 species) formed upon limited incubation with the enzyme (*20*). This established the intramolecularity of the hydride transfer process. Early studies also confirmed that release of the 4″-keto intermediate occurred and it could be trapped by borotritide to give a mixture of the labeled epimeric substrates (*21*−*24*). This process also leads to the observation of substrate-induced inactivation that occurs upon extended incubations (*21*). Release of the 4″-keto intermediate followed by binding of a substrate molecule leads to the formation of an inactive abortive complex (enzyme·NADH·UDP-Gal/Glc). It was found that, after a 2 h equilibration of the epimerase in the presence of 3.5 mM substrate, 67% of the activity is lost due to the reversible accumulation of this abortive complex. The observation of intermediate release also suggests that crossover products should build up after extended incubations. This was verified by using a mixture of UDP-[4″-^{3}H]-glucose and UDP-xylose and showing that tritiated UDP-xylose and UDP-arabinose were generated after prolonged incubation (*21*).

One notable difference between ADP-L,D-Hep 6-epimerase and UDP-Gal 4-epimerase is that the former enzyme does not suffer from substrate-induced inactivation despite the fact that it occasionally releases the oxidized intermediate into solution. Ultimately, the extent of this inactivation will be dictated by the ability of the keto intermediate to compete with the pool of epimeric substrates for binding to the NAD(P)H form of the enzyme. If the two species are bound with similar affinity, the extent of inactivation will be large since the substrates are present in large excess. If the intermediate is bound more tightly than the substrate, the extent of inactivation will be lessened. In the case of UDP-Gal 4-epimerase, it has been well established that the hexose portion of substrate contributes little to the overall binding affinity and most of the recognition comes from the UDP moiety (*25*). This is likely due to the requirement of a dramatic reorientation of the pyranose ring that is intrinsic to the mechanism of catalysis. In the case of ADP-L,D-Hep 6-epimerase, however, a much more subtle reorientation is required for catalysis (rotation about the C-5″−C-6″ bond), and the enzyme may have evolved to bind the pyranose portion of the molecule with greater affinity. Similar reasoning may explain how this enzyme discriminates between the substrate and intermediate more effectively and thereby diminishes the extent of substrate-induced inactivation. Further studies will be required to analyze the affinity of the reduced enzyme for the 6″-keto intermediate in a quantitative fashion.

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