# Eliminations in the Reactions Catalyzed by UDP-*N*-Acetylglucosamine 2-Epimerase

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**Abstract:** Mechanistic studies have been carried out on the bacterial enzyme UDP-*N*-acetylglucosamine 2-epimerase, which catalyzes the interconversion of UDP-*N*-acetylglucosamine (UDP-GlcNAc) and UDP-*N*-acetylmannosamine (UDP-ManNAc). This enzyme is interesting because it epimerizes a stereocenter that does not bear an acidic proton, and therefore it cannot utilize a simple deprotonation/reprotonation mechanism. A coupled enzyme assay employing UDP-ManNAc dehydrogenase has been developed. The epimerization in $D_2O$ is found to be accompanied by the incorporation of deuterium into the C-2″ position of both epimers, supporting a mechanism that ultimately involves a proton transfer at this position. The epimerization of [2″-$^2$H]UDP-GlcNAc is slowed by a primary kinetic isotope effect indicating that C−H bond cleavage is occurring during a rate-determining step of the reaction. A positional isotope exchange (PIX) experiment shows that an $^{18}$O label in the sugar-UDP bridging position will scramble into nonbridging diphosphate positions during enzymatic epimerization. These observations are consistent with a mechanism that proceeds via cleavage of the anomeric C−O bond, with 2-acetamidoglucal and UDP as enzyme-bound intermediates. Additional evidence for this mechanism is found in the unusual observation that during extended incubations, the intermediates are gradually released from the enzyme and accumulate in solution. These intermediates are formed by an *anti* elimination of UDP from UDP-GlcNAc and a *syn* elimination of UDP from UDP-ManNAc. It is likely that E1-like eliminations via oxocarbenium intermediates are involved in the reaction. Further experiments show that 3″-deoxy-UDP-GlcNAc is not a substrate for the enzyme and that the enzyme does not contain a tightly bound $NAD^+$ cofactor.

## Introduction

Most enzymatic racemizations and epimerizations take place at stereogenic carbon centers that are adjacent to electron-withdrawing groups such as carbonyl or carboxylate functionalities.[1,2] These reactions ultimately involve a deprotonation at the stereolabile center followed by a reprotonation in the opposite stereochemical sense. A few epimerases are known that act on "unactivated centers" lacking an acidic proton and must therefore use alternative reaction mechanisms. Perhaps the best-understood example is found in UDP-galactose 4-epimerase, an enzyme that employs hydride transfers instead of proton transfers.[3−8] This enzyme contains a tightly bound $NAD^+$ cofactor and transiently oxidizes the alcohol at the galactose C-4 position to a ketone. Reduction of the ketone in the opposite stereochemical sense produces UDP-glucose and completes the epimerization process. Another example of an epimerase that acts at an "unactivated center" is UDP-*N*-acetylglucosamine 2-epimerase (UDP-GlcNAc 2-epimerase),[9] which catalyzes the interconversion of UDP-*N*-acetylglucosamine (UDP-GlcNAc) and UDP-*N*-acetylmannosamine (UDP-ManNAc) (Scheme 1).[10−12] Very little is known about the mechanism of this process or how the active site is able to accommodate two epimers that differ by the position of a relatively bulky acetamido group. In this paper we report studies that indicate this enzyme uses an unusual $\beta$-elimination/readdition strategy to carry out the inversion process.[13]

The bacterial UDP-GlcNAc 2-epimerase provides bacteria with a source of activated ManNAc residues for use in the biosynthesis of a variety of surface polysaccharides. In Gram-positive bacteria such as *Staphylococcus aureus* H and *Bacillus subtilis*, a ManNAc residue is a component of the "linkage unit" that serves to attach teichoic acids to the peptidoglycan.[14−17] Teichoic acids are polyol phosphate polymers that can account for as much as 60% of the dry weight of the Gram-positive bacterial cell wall and are thought to be important both in

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(9) The epimerase acts at the C-2″ position of UDP-GlcNAc (the C-2 position of the GlcNAc moiety). Throughout the remainder of this text the notation C-*X*″ or H-*X*″ will be employed in reference to atoms in the GlcNAc or ManNAc portions of the sugar nucleotides.
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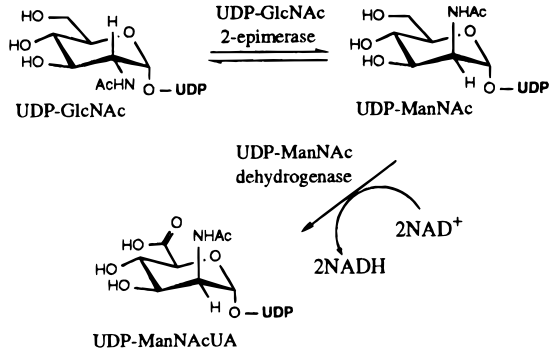
**Scheme 1.** Reactions Catalyzed by UDP-GlcNAc 2-Epimerase and UDP-ManNAc 6-Dehydrogenase

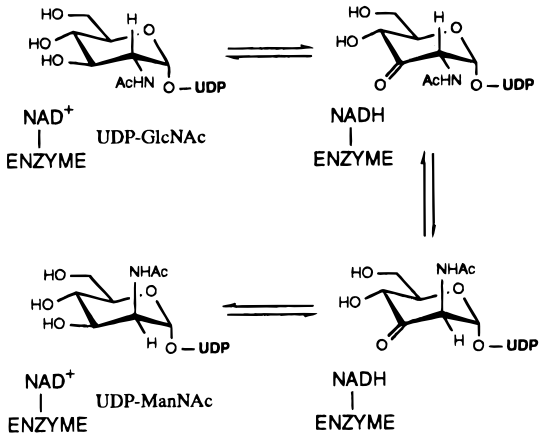



maintaining proper surface cation concentrations and in cell division.[18,19] In Enterobacteriaceae (Gram-negative), such as *Escherichia coli*, *N*-acetylmannosaminuronic acid (ManNAcUA) residues are present in the enterobacterial common surface antigen (ECA).[20] These ManNAcUA residues are derived from a pool of UDP-ManNAcUA that is generated by the sequential action of UDP-GlcNAc 2-epimerase and UDP-ManNAc 6-dehydrogenase on UDP-GlcNAc (Scheme 1).[21−23] *N*-Acetylmannosamine is also found as a component of the polysaccharide capsule in several strains of encapsulated bacteria (both Gram-positive and Gram-negative).[24−26] In the case of *Streptococcus pneumoniae* types 19F and 19A, this capsule allows the bacteria to evade the immune system of the host and is thereby responsible for the virulence of these strains.[24,27] It has recently been shown that the capsular polysaccharide gene cluster of *S. pneumoniae* type 19F contains a gene that encodes for a functional UDP-GlcNAc 2-epimerase.[28]

In mammals an enzyme that is also known as UDP-*N*-acetylglucosamine 2-epimerase has been identified.[29−33] The classification of this enzyme as an epimerase is not entirely rigorous since the mammalian enzyme catalyzes the formation of free ManNAc and UDP from UDP-GlcNAc in an essentially irreversible manner. The ManNAc formed in this reaction is ultimately used in the synthesis of sialic acid. Studies on this enzyme have been somewhat hampered by its instability; however, it appears likely that certain mechanistic elements will be common between the two types of enzymes.[29]

The *E. coli* UDP-GlcNAc 2-epimerase has been purified and was reported to be a dimer with a subunit molecular weight of about 38 000 Da.[10,11] Its activity was unaffected by the addition of either $NAD^+$ or $NADP^+$ indicating that if one of these cofactors were required it must be bound tightly enough to survive the purification procedure. Changes in the rate of epimerization as a function of UDP-GlcNAc concentration showed allosteric behavior with a Hill coefficient of 2.0. In addition, the reaction in the reverse direction (UDP-ManNAc → UDP-GlcNAc) was shown to have an absolute requirement for the presence of UDP-GlcNAc. Together these observations were taken to indicate that the enzyme contains a modulator site which is distinct from the active site and is highly specific for UDP-GlcNAc.

**Scheme 2.** Salo's Proposed Mechanism for the Reaction Catalyzed by UDP-GlcNAc 2-Epimerase[34]




Previous mechanistic studies were limited to the observation that solvent-derived tritium was incorporated into the C-2″ position during epimerization in tritium-enriched water.[34] This supports a mechanism that ultimately involves the removal of a proton at C-2″ and argues against a direct hydride-transfer process such as that employed by UDP-glucose 4-epimerase. An alternative mechanism was proposed by Salo that involves transient oxidation at C-3″ by a tightly bound $NAD^+$ cofactor (Scheme 2).[34] This serves to acidify the proton at C-2″ and permits the stereochemical inversion to occur via a deprotonation/reprotonation process. Reduction of the ketone at C-3″ regenerates the $NAD^+$ cofactor and produces the epimeric sugar nucleotide.

In this paper we report the identification of the gene encoding for *E. coli* UDP-GlcNAc 2-epimerase and the purification of the overexpressed gene product. We present experimental evidence that supports an alternative elimination/readdition mechanism (Scheme 3). In the UDP-GlcNAc-to-UDP-ManNAc direction, an *anti* elimination produces the intermediates 2-acetamidoglucal and UDP. A subsequent *syn* addition generates the epimeric product. The proposed mechanism is further supported by the observation that the intermediates are gradually released into solution and can be isolated and identified.

## Experimental Section

**General Methods.** Uridine 5′-(2″-acetamido-2″-deoxy-α-D-mannopyranosyl diphosphate) was prepared from 2-acetamido-2-deoxy-α-D-mannopyranosyl monophosphate by the method of Salo and Fletcher.[35] 2-Acetamido-2-deoxy-α-D-mannopyranosyl monophosphate was prepared by the method of Yamazaki et al.[36] The synthesis of 3″-deoxy-UDP-GlcNAc was achieved via a slight modification of the route described by Hindsgaul et al.[37,38] Protein concentrations were deter-

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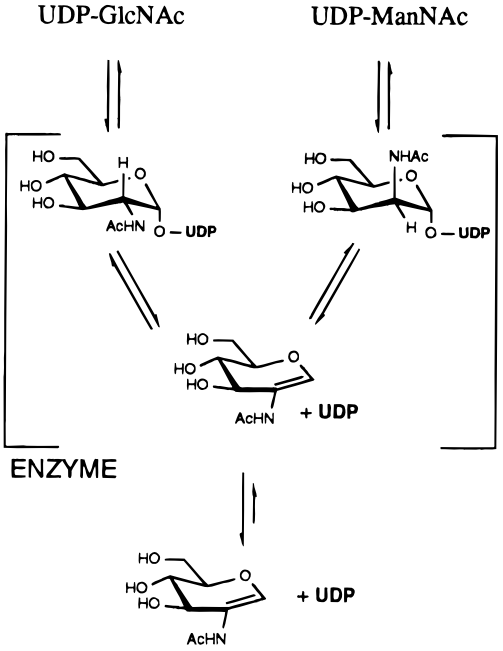
**Scheme 3.** Proposed Glycal Mechanism for the Reactions Catalyzed by UDP-GlcNAc 2-Epimerase[a]




[a] Species within the brackets are enzyme-bound.

mined by the method of Bradford,[39] using bovine serum albumin as the standard. A unit is defined as the amount of epimerase that produces 1 $\mu$mol of UDP-ManNAc/min under standard assay conditions with 4 mM UDP-GlcNAc.

**Purification and Identification of UDP-GlcNAc 2-Epimerase.** The *rffE* (or *nfrC*) gene product was overexpressed in *E. coli* JM109 (DE3, pKI86).[40] The resulting protein was purified to homogeneity (as indicated by a single band on a SDS−PAGE gel) by ion-exchange chromatography over a short plug of DE-52 followed by an HPLC separation using a Waters AP-1 Protein-Pak Q column (50 mM Trien-HCl, pH 8.5, containing 10% glycerol and 2 mM dithiothreitol, 0−0.2 M NaCl gradient). See Supporting Information for full details.

Products of the epimerization reaction were characterized by heating the epimers (3 mM total concentration) in 0.06 N HCl at 100 °C for 15 min.[12] The resulting free sugars were analyzed[41] using a Bio-Rad Aminex HPX-87H column (13 mM $H_2SO_4$ as eluent) and compared with authentic standards. Control reactions confirmed that epimerization did not occur during this analysis.

**Kinetics and Activation of the Epimerase Reaction.** The kinetic parameters of the overexpressed protein were obtained in the UDP-GlcNAc-to-UDP-ManNAc direction with a continuous coupled assay that employs UDP-ManNAc dehydrogenase.[48] Very high concentrations of the dehydrogenase were required in order to ensure complete coupling and to eliminate a "lag phase" that was observed at lower enzyme concentrations. Rates were determined by following the increase in absorbance at 340 nm (NADH formation) in assay mixtures (600-$\mu$L total volume) that contained 50 mM Tris-HCl (pH 8.8), 2 mM dithiothreitol, 4.0 mM $NAD^+$, $5.8 \times 10^{-3}$ units of epimerase, and 60 units of UDP-ManNAc dehydrogenase (5 mg of protein in each cuvette) at 37 °C. A background rate that was due to minor impurities in the dehydrogenase preparation was observed and was found to be independent of substrate concentration. In each kinetic run the rates were determined before and after initiation with UDP-GlcNAc (0.07−3.1 mM). The background rate was then subtracted from the final rate (it typically amounted to 15% of $V_{max}$), and the resulting value was divided by a factor of 2 (to account for the stoichiometry of the dehydrogenase reaction). Kinetic parameters were determined from initial velocities by a direct fit of the data to a Hill equation using the computer program GraFit.[38]

The requirement for UDP-GlcNAc as a specific activator of the epimerase was investigated in the following manner. A solution of UDP-ManNAc (1.1 mM) in potassium phosphate buffer (40 $\mu$L, 50 mM, pH 8.1, containing 2 mM dithiothreitol) was incubated for 10 min at 37 °C with the epimerase ($2.6 \times 10^{-4}$ units). An analogous solution that also contained UDP-GlcNAc (0.35 mM) was treated in the same fashion. The reactions were analyzed by ion-paired reversed-phase HPLC.[42]

**Solvent Deuterium Isotope Incorporation.** A sample of UDP-GlcNAc (15.4 mM) in Trien-HCl buffer (50 mM, pH 8.5) was lyophilized and resuspended in an equal volume of $D_2O$ two times. To 450 $\mu$L of this "deuterated" buffer was added epimerase in storage buffer (10 $\mu$L, 4 mg/mL, 0.27 unit). The sample was kept at 25 °C, and $^1H$ NMR spectra (400 MHz) were collected at timed intervals.

**Kinetic Isotope Effect Determination.** A 20-mL sample of Tris-HCl buffer (50 mM, pH 8.8, containing 2 mM dithiothreitol) was lyophilized and resuspended in an equal volume of $D_2O$ two times. To this "deuterated" buffer were added UDP-GlcNAc (100 mg, 7.7 mM) and epimerase in the same buffer (400 $\mu$L, 3.5 mg/mL, 9.5 units). The reaction was kept at 37 °C for 2 h, and then it was quenched by heating at 50 °C for 1 h and filtering the mixture through a centricon concentrator (Amicon). To the resulting filtrate were added $NAD^+$ (15 mg) and UDP-ManNAc dehydrogenase (1 mg, 12 units). The solution was incubated at 37 °C for 12 h and analyzed by ion-paired reversed-phase HPLC to ensure that all of the UDP-ManNAc was consumed. The product [$^2$H-2″]UDP-GlcNAc was purified using a column of Dowex AG1 X8 (20 mL, formate form, 100−200 mesh) and eluting with a linear gradient of 0−2 M $NEt_3H_2CO_3$ (400-mL total volume). Fractions containing the product were lyophilized, resuspended in water, and lyophilized again. The product was passed through a column of Amberlite IR-120(plus) resin (20 mL, $Na^+$ form, eluted with water) followed by a column of Bio-Gel P-2 (2.5 × 45 cm, eluted with water). $^1H$ NMR spectral and mass spectral analyses indicated that the extent of deuterium incorporation was >97%: −LSIMS (thioglycerol) *m/z* 629 (M − $H^+$(monosodium salt), $^2$H-2″, 100).

The rates of epimerization were determined in triplicate for the labeled and unlabeled substrate (both at 2.5 mM) using the coupled assay described above. Substrate concentrations were determined using $A_{262}$ with $\epsilon = 8700\ M^{-1}\ cm^{-1}$.

**Positional Isotope-Exchange (PIX) Experiment. (i) Synthesis of Uridine 5′-(2″-Acetamido-2″-deoxy-[1″-$^{18}$O]-α-D-glucopyranosyl diphosphate), $^{18}$O-Labeled UDP-GlcNAc. 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-[1-$^{18}$O]-α-D-glucopyranose.** 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranose[43] (620 mg, 1.8 mmol) was dissolved in 1.0 mL of dry $CH_3CN$ containing 0.4 mL of 95% enriched $H_2{}^{18}O$. The solution was placed in a sealed vial and heated at 90−95 °C for 17 h. The solvent was removed in vacuo, and the resulting brown syrup was chromatographed on silica gel (acetone−hexane, 1:1) to give the labeled compound (445 mg, 71%). $^1H$ and $^{13}C$ NMR spectra were identical to those reported for the unlabeled compound.[43] The extent of $^{18}O$ incorporation was determined to be 89% by mass spectral analysis: +DCIMS ($NH_3$) *m/z* 350 (M + $H^+$,$^{18}O$, 100), 348 (M + $H^+$, $^{16}O$, 11.3), 330 (M + $H^+$ − $H_2{}^{16/18}O$, 71.3).

**Disodium Uridine 5′-(2″-Acetamido-2″-deoxy-[1″-$^{18}$O]-α-D-glucopyranosyl-1″-$^{18}$O diphosphate), $^{18}$O-Labeled UDP-GlcNAc.** 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-[1-$^{18}$O]-α-D-glucopyranose was dibenzylphosphorylated using published procedures.[44] The resulting 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-[1-$^{18}$O]-α-D-glucopyranosyl dibenzylphosphate was fully deprotected and subjected to uridine monophosphomorpholidate coupling using standard conditions.[37] The $^{18}O$ labeled UDP-GlcNAc prepared in this fashion gave $^1H$ and $^{13}C$ NMR spectra that were identical with unlabeled material. The extent of $^{18}O$ incorporation was determined to be 81% by mass spectral analysis:

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+LSIMS (thioglycerol) *m/z* 654 ($M + H^+$,$^{18}O$, 100), 652 ($M + H^+$,$^{16}O$, 23.3). The small decrease in isotopic enrichment observed during this procedure appears to occur during the dibenzylphosphorylation step. The location of the $^{18}O$ label was confirmed by $^{31}P$ NMR spectroscopy: $^{31}P$ NMR ($D_2O$) $\delta$ −12.726 (d, $J_{P-P}$ = 21.0 Hz, 0.2P, $\beta$-P-$^{16}O$), −12.739 (d, $J_{P-P}$ = 21.0 Hz, 0.8P, $\beta$-P-$^{18}O$), −11.014 (d, $J_{P-P}$ = 21.0 Hz, 1P, α-P).

**(ii) Scrambling Experiment.** A solution of $^{18}O$-labeled UDP-GlcNAc (450 $\mu$L, 17 mM) in deuterated phosphate buffer (200 mM, pD 8.0−8.2) was prepared. The sample was placed in an NMR tube, Chelex-100 resin was added (20 mg of 200−400 mesh, $Na^+$ form, previously rinsed with $D_2O$), and both $^1H$ and $^{31}P$ NMR spectra were collected. A solution of UDP-GlcNAc 2-epimerase (1.3 units in 200 $\mu$L of the same buffer containing 20 mM dithiothreitol) was added, and the solution was incubated for 8 h at 25 °C. $^1H$ and $^{31}P$ NMR spectra were collected. The high-resolution, proton-decoupled, $^{31}P$ NMR spectra were obtained on a Bruker AC-200E spectrometer operating at a frequency of 81 MHz. Acquisition parameters were sweep width = 1620 Hz, acquisition time = 10 s, delay between pulses = 0.4 s, and pulse width = 3 $\mu$s.

**Enzymatic Production of 2-Acetamidoglucal and UDP.** Enzyme-produced 2-acetamidoglucal was identified by incubating a solution of UDP-GlcNAc (38 mM) in potassium phosphate buffer (50 mM, 1 mL, pH 8.8) containing 2 mM dithiothreitol and 8.8 units of epimerase at 37 °C for 12 h. The resulting sample was applied to a column of Dowex AG1 X8 (20 mL, formate form, 100−200 mesh), eluted with water (200 mL), and then lyophilized to dryness. The sample was redissolved in $D_2O$, and a $^1H$ NMR spectrum was collected.[38] This was compared to an authentic sample of 2-acetamidoglucal in $D_2O$.

The rate of UDP/2-acetamidoglucal formation was determined by incubating solutions (1.0 mL) containing 50 mM Tris-HCl buffer, pH 8.8, 2 mM dithiothreitol, 9.2 mM UDP-GlcNAc, and 0.88 unit of epimerase at 37 °C. Aliquots (50 $\mu$L) were removed at timed intervals over the course of 120 min and analyzed by ion-paired reversed-phase HPLC.[42] A linear gradient of $CH_3CN$ (0−10%) in 50 mM potassium phosphate buffer, pH 7.0, containing 2.5 mM tetrabutylammonium hydrogen sulfate was used to separate the components. The concentration of UDP was determined by integration of the corresponding peak and comparison to a previously obtained linear calibration curve. Linear kinetics was obtained during this analysis, and it was found that 10% of the epimeric UDP-sugars had converted to UDP and 2-acetamidoglucal.

An attempt to determine the external equilibrium constant was made by incubating a solution of UDP-GlcNAc (17 mM, 1 mL) in Tris-HCl buffer (50 mM, pH 8.8, containing 2 mM dithiothreitol) with the epimerase (51 units) at 37 °C. Aliquots were removed at timed intervals over the course of 48 h and analyzed by HPLC as described above. The concentration of 2-acetamidoglucal was taken to be equivalent to that of UDP. The equilibrium position was calculated using the ratio of the appropriate peak areas with the assumption that all the nucleotides had an identical $A_{262}$. Control reactions containing heat-killed epimerase were run to ensure that significant amounts of UDP were not spontaneously formed under these conditions.

**Studies with 3″-Deoxy-UDP-GlcNAc.** A solution (0.6 mL) of 3″-deoxy-UDP-GlcNAc (9.6 mM) and the epimerase (20 units) in a deuterated potassium phosphate buffer (200 mM, pD 8.2, containing 2 mM dithiothreitol) was incubated at 37 °C and monitored by $^1H$ NMR spectroscopy over a period of 12 h. Analogous solutions that also contained either 8.0 or 0.5 mM UDP-GlcNAc were analyzed in the same manner.

The requirement for 3″-deoxy-UDP-GlcNAc as a specific activator of the epimerase was investigated in the following manner. A solution of UDP-ManNAc (1.1 mM) and 3″-deoxy-UDP-GlcNAc (0.35 mM) in potassium phosphate buffer (40 $\mu$L, 50 mM, pH 8.1, containing 2 mM dithiothreitol) was incubated for 10 min at 37 °C with the epimerase ($2.6 \times 10^{-4}$ unit). The reaction mixture was analyzed by ion-paired reversed-phase HPLC.[42]

## Results

**Identification of the *rffE* and *rffD* Genes.** In *E. coli* the UDP-GlcNAc 2-epimerase gene, *rffE*, and the UDP-ManNAc dehydrogenase gene, *rffD*, are found in the gene cluster that controls the biosynthesis of the enterobacterial common antigen (ECA).[21,22] The location of the epimerase gene within this cluster had been tentatively assigned[45] based on homology arguments; however, we[13] and others[46] began to suspect that this assignment was incorrect. Ultimately the literature[38] indicated that the epimerase was likely one and the same as a cytosolic protein of unknown function that is required for bacteriophage N4 adsorption.[40,47] The gene encoding for this protein had been given the name *nfrC*, and it had been cloned and overexpressed in *E. coli*. Drs. Diane R. Kiino and Lucia B. Rothman-Denes were kind enough to send us a sample of their pET11a expression vector containing the *nfrC* gene. We have purified the gene product and demonstrated that the NfrC protein is actually an active UDP-GlcNAc 2-epimerase (RffE). The relationship between this protein and the bacteriophage adsorption process is likely due to the requirement of UDP-ManNAc in the ECA biosynthetic pathway. Additional studies in our laboratory have also unambiguously identified the gene that encodes for a functional UDP-ManNAc dehydrogenase (RffD).[48]

**Purification and Identification of UDP-GlcNAc 2-Epimerase.** The epimerase gene was overexpressed in *E. coli* as outlined previously.[40] The resulting protein was purified to homogeneity by ion-exchange chromatography, and its mass was shown (electrospray mass spectrometry) to be consistent with that expected from the gene sequence: calcd, 42 246 Da; found, 42 254 Da. Enzyme prepared in this fashion therefore retains its *N*-terminal methionine residue. In initial studies, ion-paired reversed-phase HPLC was used to detect the activity of the epimerase.[42] Treatment of UDP-GlcNAc with the epimerase led to the generation of a new peak that was assigned to the product UDP-ManNAc (see Figure 3, traces A and B). Integration of the peaks gave a 10:1 ratio of UDP-GlcNAc:UDP-ManNAc, consistent with the reported equilibrium constant.[11] In order to confirm that the product was the expected epimer, the mixture was subjected to acidic hydrolysis under conditions known to cleave the glycosidic bond but not to cause further epimerization.[12] The resulting hydrolysate was shown to contain a 10:1 ratio of GlcNAc:ManNAc by HPLC analysis[41] with comparison to known standards. In subsequent studies authentic UDP-ManNAc was synthesized according to the method of Salo and Fletcher.[35] This allowed for the direct identification of the enzyme product as UDP-ManNAc by comparison of the HPLC retention times. These experiments clearly demonstrated that our preparation contained substantial epimerase activity.

The enzyme UDP-ManNAc dehydrogenase catalyzes the effectively irreversible 2-fold oxidation of UDP-ManNAc to UDP-ManNAcUA with the production of 2 equiv of NADH (Scheme 1).[11] We have overexpressed this enzyme in *E. coli*[48] and used it to develop a continuous spectrophotometric assay of UDP-GlcNAc 2-epimerase activity. This assay allowed us

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(46) Marolda, C. L.; Valvano, M. A. *J. Bacteriol.* **1995**, *177*, 5539−5546.

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(48) We have subcloned the *rffD* gene encoding the dehydrogenase[21] (o379 in ref 45) into a pET11a expression vector giving the plasmid pUS01. High levels of active dehydrogenase are expressed in *E. coli* JM109 (DE3, pUS01) following induction by IPTG. The dehydrogenase used in this study was purified by ion-exchange chromatography and determined to be 90% homogeneous (as analyzed by SDS−PAGE). It should be noted that a sequencing error[45] led to the incorrect prediction that the *rffD* gene contains 379 codons. The correct sequence[40] indicates that it contains 420 codons. Full details will be reported elsewhere (Morgan, P. M., Tanner, M. E., manuscript in preparation).

to determine that the specific activity of our purified enzyme was 6.8 units/mg. This agrees quite well with the specific activity reported for the enzyme isolated from natural sources (7.08 units/mg)[11] and indicates that the previous observations are not simply due to a contamination of endogenous epimerase.

**Kinetics and Activation of the Epimerase Reaction.** The initial velocity of the UDP-GlcNAc epimerization reaction was measured as a function of UDP-GlcNAc concentration using the coupled assay. The data gave a sigmoidal curve with a Hill coefficient of 2.29, an apparent $K_M$ value of 0.73 mM, and a $k_{cat}$ value of 4.8 $s^{-1}$.[38] Similar kinetic constants and positive cooperativity were also observed with the enzyme obtained from natural sources.[11]

In order to re-examine the report[11] of an absolute requirement for UDP-GlcNAc in the epimerization of UDP-ManNAc, we used synthetic UDP-ManNAc that contained no traces of the epimeric sugar nucleotide. Solutions containing 1.1 mM UDP-ManNAc were incubated in the presence and absence of 0.35 mM UDP-GlcNAc as an activator (0.6 mM UDP-GlcNAc was reported to give half-maximal activation). In the sample lacking UDP-GlcNAc, no evidence of epimerization could be detected, whereas in a comparable sample containing the activator 28% of the UDP-ManNAc had been converted to UDP-GlcNAc. Even when the incubation time of the pure UDP-ManNAc sample was increased 10-fold, less than 1% conversion was observed. This study confirms the previous report that the epimerase is tightly regulated by its substrate. We estimate that the rate of UDP-ManNAc epimerization was reduced by at least 300-fold in the absence of the activator. The specificity of the activation process suggests that the enzyme possesses a modulator site which is distinct from the active site and is specific for UDP-GlcNAc; however, one cannot rule out a model in which one subunit active site must be occupied by UDP-GlcNAc for the other subunit to function.

**Solvent Deuterium Isotope Incorporation.** Salo reported that the enzymatic epimerization of UDP-GlcNAc in tritium-enriched water was accompanied by incorporation of tritium at the C-2″ position.[34] In order to confirm this observation with the recombinant epimerase, we epimerized UDP-GlcNAc in $D_2O$ and followed the process by $^1H$ NMR spectroscopy (Figure 1). The signal corresponding to the proton at the C-2″ position of UDP-GlcNAc is somewhat obscured by signals from the ribose moiety. It is therefore easier to detect the deuterium incorporation process by observing changes in the coupling pattern of the anomeric proton (at C-1″). The anomeric proton appears as a doublet of doublets due to coupling both to the $\beta$-phosphorus of UDP and to the proton at C-2″. Deuterium incorporation at C-2″ will cause this signal to collapse to a doublet. This is clearly seen in the $^1H$ NMR spectra and is accompanied by the appearance of a new doublet due to the minor, C-2″-deuterated epimer, UDP-ManNAc. The observation of deuterium incorporation supports a mechanism involving breakage of the carbon−hydrogen bond at C-2″ via removal of a proton, as opposed to a hydride.

**Primary Kinetic Isotope Effect Measurement.** The solvent isotope incorporation at C-2″ allowed us to prepare [2″-$^2$H]-UDP-GlcNAc and measure the deuterium kinetic isotope effect on the rate of UDP-GlcNAc epimerization. A sample of UDP-GlcNAc was enzymatically epimerized in $D_2O$ to give a mixture of the C-2″-deuterated epimers. The residual [2″-$^2$H]UDP-ManNAc was oxidized by treatment with UDP-ManNAc dehydrogenase and $NAD^+$, and the labeled substrate (>97% isotopic enrichment) was isolated by ion-exchange chromatography. Kinetics for labeled and unlabeled substrates (both at 2.5 mM) were measured using the coupled assay, and the isotope

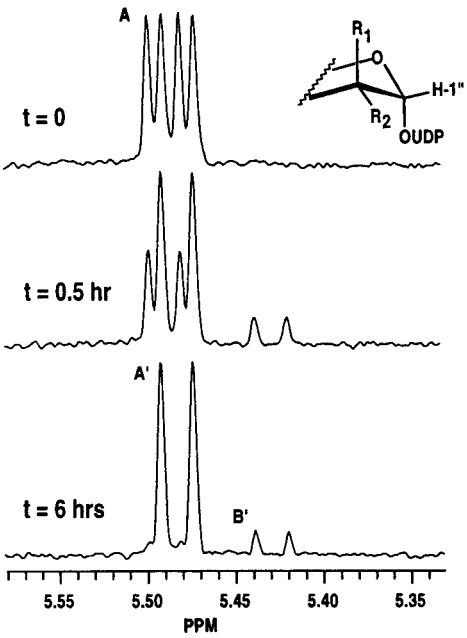


**Figure 1.** $^1H$ NMR spectra obtained during the enzymatic epimerization of UDP-GlcNAc in $D_2O$. Signals shown are from H-1″ of the sugar nucleotides: species A is UDP-GlcNAc ($R_1$ = H, $R_2$ = NHAc); species A′ is deuterated UDP-GlcNAc ($R_1$ = D, $R_2$ = NHAc); species B′ is deuterated UDP-ManNAc ($R_1$ = NHAc, $R_2$ = D).

effect $k_H/k_D$ was found to be 1.8 ± 0.1. There is clearly a primary isotope effect that slows the epimerization of the deuterated substrate, indicating that the C-2″−H bond is broken during a rate-determining step of the reaction.[49]

**Positional Isotope Exchange (PIX) Experiment.** A key difference between Salo's mechanism (Scheme 2) and the glycal mechanism (Scheme 3) is that the bond between the $\beta$-phosphate of the UDP moiety and the C-1 of the hexosamine residue is transiently broken in the latter process only. In order to probe for the intermediacy of enzyme-bound UDP, we have employed a PIX experiment.[50] In this experiment UDP-GlcNAc containing an $^{18}O$ label at the sugar-UDP bridging position is incubated with the epimerase, and the epimeric products are examined for evidence of isotopic scrambling (Scheme 4). If UDP is formed and has a lifetime comparable to, or greater than, that required for bond rotation to occur in the terminal phosphate, then scrambling of the isotope into the nonbridging phosphate positions should be observed in both of the recovered epimers.

The synthesis of the labeled UDP-sugar began with the preparation of 3,4,6-triacetyl-2-acetamido-2-deoxy-D-glucose containing $^{18}O$ at the anomeric position. The $^{18}O$ label was introduced directly into the compound by heating it in $H_2{}^{18}O$/acetonitrile. The labeled material was converted to the α-dibenzylphosphate derivative[44] and then fully deprotected and coupled to UMP under known conditions.[37] The product UDP-GlcNAc was shown to contain an 81% incorporation of $^{18}O$ isotope, and the position of the label was confirmed using the $^{31}P$ NMR signals of the α-phosphorus nuclei (Figure 2A; the signals appear as a doublet due to coupling with the $\beta$-phosphorus). An isotopic substitution of $^{18}O$ for $^{16}O$ is known to cause a small upfield shift in the $^{31}P$ NMR signal of phosphates.[51,52]

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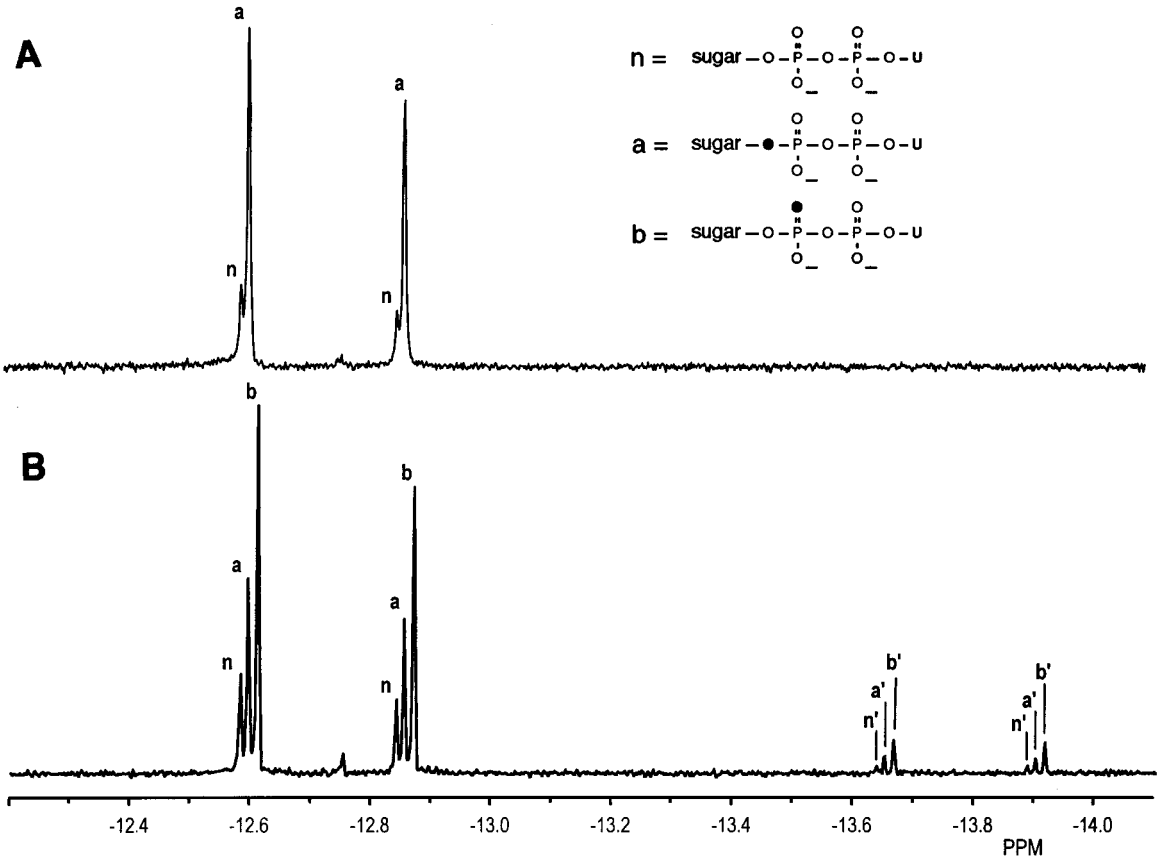
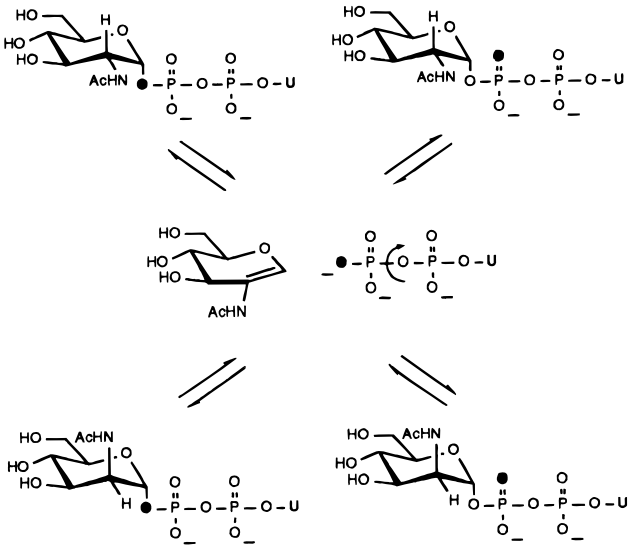



**Figure 2.** $^{31}$P NMR spectra ($D_2O$) of the $\beta$-phosphorus of $^{18}$O-labeled sugar nucleotides: (A) UDP-GlcNAc before treatment with the epimerase and (B) equilibrated mixture of UDP-GlcNAc and UDP-ManNAc after treatment with the epimerase. All peaks appear as doublets due to coupling to the adjacent α-phosphorus. A prime indicates signals due to UDP-ManNAc. U = uridine; darkened atoms indicate $^{18}$O labels.

**Scheme 4.** PIX Experiment That Can Test for C−O Bond Cleavage in the Epimerization Reaction[a]




[a] U = uridine; darkened atoms indicate $^{18}$O labels.

The observed upfield shift of 0.013 ppm in the labeled material is consistent with that expected for a P−$^{18}$O single bond, and the integrals of the signals are consistent with those expected for an 81% incorporation.

The enzymatic epimerization of the labeled substrate was carried out in a deuterated phosphate buffer and monitored using $^1$H NMR spectroscopy. The reaction was allowed to proceed well past completion as indicated by the complete exchange of the protons at C-2″ with solvent deuterium. The isotopic scrambling process was then investigated using the $^{31}$P NMR signals of the α-phosphorus nuclei. The appearance of a new doublet in the spectrum of UDP-GlcNAc confirms that PIX did occur (Figure 2B). This doublet is shifted 0.029 ppm upfield from that of the unlabeled material and can be assigned to a positional isomer with a P−$^{18}$O bond order greater than 1. Integration of the upfield signals shows the expected 2:1 ratio that reflects the statistical distribution of the label between the bridging and nonbridging positions. The analogous $^{31}$P NMR signals of the minor UDP-ManNAc epimer display an identical pattern as expected. The observation of isotopic scrambling supports the notion that the reaction proceeds with C−O bond cleavage and involves the formation of UDP as an intermediate.

**Enzymatic Production of 2-Acetamidoglucal and UDP.** Further insights into the nature of the reaction intermediates could be obtained by monitoring the epimerization during prolonged incubations with high concentrations of enzyme. An HPLC analysis of such a reaction showed a gradual decrease in the peaks due to the epimeric UDP-sugars and a corresponding production of two new peaks that had identical retention times to those of UDP and synthetic 2-acetamidoglucal[42,53] (Figure 3). Control reactions with heat-killed epimerase were used to confirm that this process was enzyme-catalyzed. In order to confirm that 2-acetamidoglucal was produced, the reaction mixture was applied to an anion-exchange column that retained all phosphorylated sugars but allowed neutral species to pass through. Lyophilization of the resulting eluent and analysis by $^1$H NMR spectroscopy clearly showed that 2-acetamidoglucal had been produced.[38] No signals attributable to other neutral carbohydrates such as GlcNAc or ManNAc were observed indicating that the glycal is not significantly hydrated

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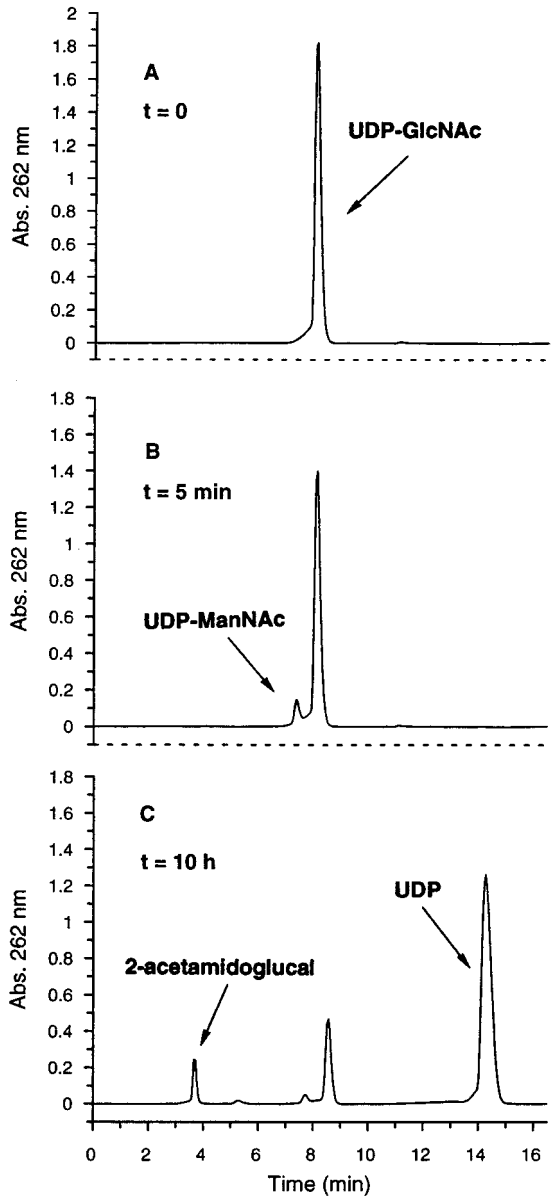



**Figure 3.** Ion-paired reversed-phase HPLC traces obtained during an extended incubation of UDP-GlcNAc with UDP-GlcNAc 2-epimerase.

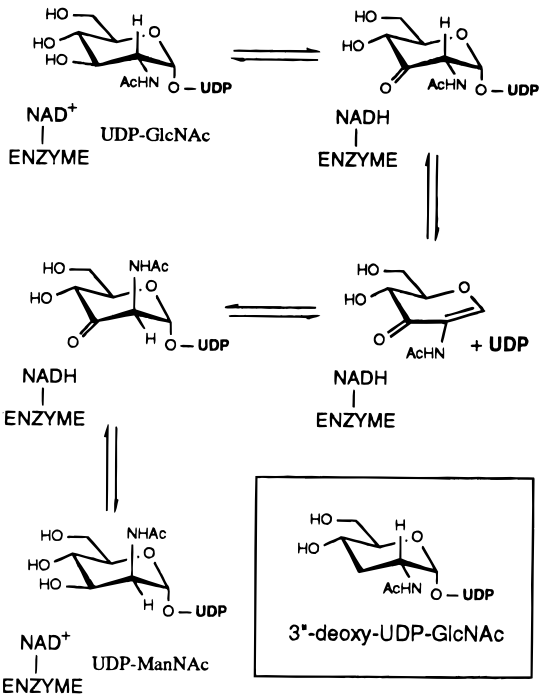
under these reaction conditions. The formation of UDP during extended incubations was also confirmed using $^{31}$P NMR spectroscopy.

The observation that the elimination products accumulate in solution indicates that they are thermodynamically more stable than the equilibrating mixture of UDP-sugar epimers. We previously reported[13] that the equilibrium constant had a value of greater than 600 in favor of 2-acetamidoglucal and UDP. We have made further attempts to quantitate this number using an HPLC analysis of an equilibrated reaction mixture and have found that the equilibrium constant actually has a value that is in excess of 25 000 favoring the intermediates. An inability to accurately integrate peaks of widely differing areas prevented us from obtaining the true value in this fashion.

The kinetics of UDP formation was also followed by HPLC, and the enzyme was found to have a specific activity of 0.017 units/mg in the presence of a saturating equilibrium mixture of the epimers. This compares to the specific activity for UDP-GlcNAc epimerization of 6.8 units/mg in the presence of saturating UDP-GlcNAc.

One explanation for the appearance of 2-acetamidoglucal and UDP during extended incubations is that these are the true

**Scheme 5.** Potential Hybrid Mechanism for the Reaction Catalyzed by UDP-GlcNAc 2-Epimerase[a]




[a] The structure of 3″-deoxy-UDP-GlcNAc is shown in the inset.

reaction intermediates and that the enzyme is simply "sloppy" and occasionally releases them into solution (Scheme 3). An alternative explanation invokes a hybrid mechanism that combines elements of both previously discussed possibilities. The hybrid mechanism also begins with a transient oxidation at C-3″ by a tightly bound $NAD^+$ cofactor (Scheme 5). The enzyme then promotes the elimination of UDP to produce an enone intermediate. A conjugate addition of UDP combined with protonation in the opposite stereochemical sense inverts the stereochemistry at C-2″, and reduction of the ketone at C-3″ completes the reaction. The postulate of a transient oxidation process in combination with an elimination event has ample precedence in the reactions catalyzed by dehydroquinate synthase[54] and *S*-adenosylhomocysteinase.[55] Furthermore, the hybrid mechanism is consistent with all of the isotopic studies. The release of UDP and 2-acetamidoglucal could be explained by a premature reduction of the ketone during the lifetime of the enone intermediate. This would produce 2-acetamidoglucal that could not serve as a conjugate-addition acceptor and would simply be released into solution along with UDP. In order to distinguish between these two possible mechanisms, we investigated the reactivity of 3″-deoxy-UDP-GlcNAc (inset of Scheme 5) and tested for the presence of a bound $NAD^+$ cofactor.

**Studies with 3″-Deoxy-UDP-GlcNAc.** The possibility that a transient oxidation of the hexosamine residue at C-3 is involved in the epimerization reaction led us to investigate whether the deoxygenated analog could serve as an alternate substrate. If 3″-deoxy-UDP-GlcNAc were enzymatically epimerized, one could rule out mechanisms involving such an oxidation step.

When 3″-deoxy-UDP-GlcNAc was incubated with the epimerase in $D_2O$, no epimerization or deuterium incorporation was detected.[38] The lack of epimerization could possibly be

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attributed to the inability of the deoxygenated compound to act as an activator of the enzyme. Indeed when 3″-deoxy-UDP-GlcNac was incubated with UDP-ManNAc and the epimerase, no detectable traces of UDP-GlcNAc were produced. These experiments showed that the removal of the hydroxyl group at C-3″ destroys the ability of the sugar−nucleotide to act as an activator of the enzyme.

The incubation in $D_2O$ was therefore repeated in the presence of UDP-GlcNAc (both at saturating and subsaturating concentrations). Again no signs of epimerization of, or deuterium incorporation into, the 3-deoxy-GlcNAc were detected upon extended incubation. The enzyme was clearly active, however, since the UDP-GlcNAc was rapidly epimerized/deuterated and then gradually converted to UDP and 2-acetamidoglucal. These experiments showed that the 3″-deoxy compound was not a substrate for the epimerase; however, they do not allow for the distinction between the glycal mechanism (Scheme 3) and the hybrid mechanism (Scheme 5). The lack of reactivity could be due to either the inability of the enzyme to oxidize the 3″-deoxy compound at C-3″ or the removal of a group that affects the conformation, electronic properties, and crucial binding interactions of the modified nucleotide. The compound did, however, appear to bind to the enzyme since it was found to act as an inhibitor of the epimerase at millimolar concentrations.[56]

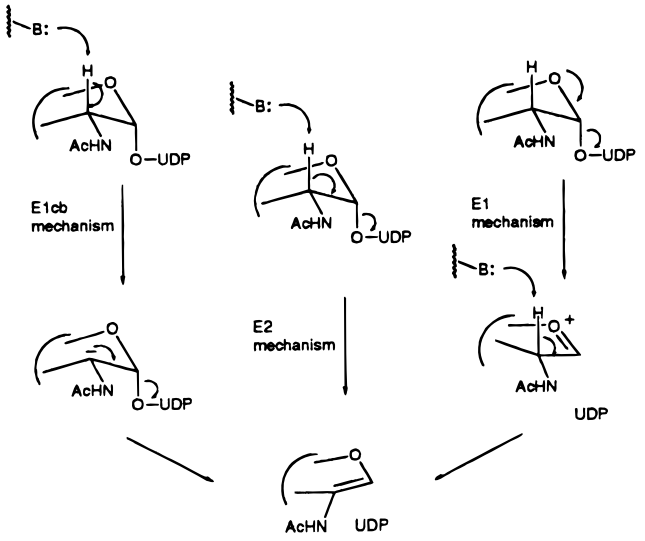
**Tests for Bound NAD$^+$.** Our inability to rule out an NAD$^+$-dependent hybrid mechanism (Scheme 5) led us to specifically test for the presence of the bound cofactor. A direct UV spectroscopic examination of the purified epimerase failed to show any significant absorbances past 310 nm that might be attributed to a chromophoric cofactor.[38] A spectrum taken in the presence of saturating levels of substrate gave similar results.

In a subsequent experiment, a relatively large sample of the epimerase was proteolytically digested and then assayed[55] for the presence of released NAD$^+$.[38] It was found that less than 5% of the epimerase could have contained the bound cofactor. This experiment, taken with the observation that the addition of NAD$^+$ to the purified epimerase does not affect its activity, indicates that the enzyme does not utilize this cofactor during epimerization. This conclusion is consistent with an analysis of the amino acid sequence which fails to show any of the common NAD$^+$ binding site motifs.[57]

## Discussion

The observation of solvent deuterium isotope incorporation and of a primary kinetic isotope effect supports a mechanism involving removal of the C-2″ proton during a rate-determining step of the epimerization reaction. The observation of isotopic scrambling in the UDP-α-phosphate supports a mechanism involving cleavage of the anomeric C−O bond during the reaction. Together these results suggest that an *anti* elimination occurs with UDP-GlcNAc as the substrate and a *syn* elimination occurs with UDP-ManNAc as the substrate. The absence of any cofactor requirements or any tightly bound NAD$^+$ indicates that an oxidation at C-3″ is not involved in the reaction. This leaves the glycal mechanism (Scheme 3) as a reasonable minimal description of the overall reaction pathway. This mechanism is entirely consistent with the observed production

**Scheme 6.** Three Mechanisms for the $\beta$-Elimination of UDP from UDP-GlcNAc




of free UDP and 2-acetamidoglucal since these species are the proposed reaction intermediates. It is of further interest to note that these reaction intermediates are more thermodynamically stable than the equilibrating substrates (when free in solution). Precedence for the eliminations can be found in the enzymatic hydrations/dehydrations of glycals by glycosidases[58] and nucleoside 2-deoxyribosyltransferase.[59] *Anti* additions normally occur in the hydration of glycals by retaining $\beta$-glucosidases,[58] and a *syn* addition is observed in the hydration of 2-acetamidoglucal by $\beta$-*N*-acetylhexosaminidase.[42] In nonenzymatic acid-catalyzed additions of alcohols to glycals, *syn* products are found to predominate; however, *trans* addition products are also observed.[60]

Three nonradical mechanisms are possible for $\beta$-elimination reactions as shown in Scheme 6 for the *anti* elimination of UDP from UDP-GlcNAc. The first is an E1cb mechanism in which an initial deprotonation event generates a carbanionic intermediate prior to expulsion of the leaving group. There is ample precedent for both *syn* and *anti* E1cb mechanisms in enzymatic reactions; however, in all cases an electron-withdrawing functionality such as a carbonyl or carboxyl group serves to stabilize the anionic intermediate.[61,62] It is highly unlikely that this mechanism is operative in the epimerase reaction due to the high p$K_a$ value of the C-2″ proton in the UDP-sugar epimers. The second possibility is a concerted E2 mechanism that proceeds without any intermediate. In enzymatic reactions, convincing evidence for an E2 elimination reaction is limited to the example of crotonase that has been reported to catalyze a concerted *syn* elimination of water.[63] In this light, it is of interest to point out that certain acyl-CoA $\beta$-epimerases are thought to be unique crotonases that catalyze sequential hydrations/dehydrations of opposite stereochemistry.[64−66] Perhaps the

(56) Insufficient amounts of the 3″-deoxy-UDP-GlcNAc were available to complete a thorough analysis of the mode of inhibition. The inhibition was studied under the standard assay conditions at a fixed concentration of UDP-GlcNAc (0.73 mM = $K_M$), and the concentration of 3″-deoxy-GlcNAc was varied from 0.6 to 6.0 mM. An assumption of competitive inhibition led to the calculation of $K_I$ = 1 mM.

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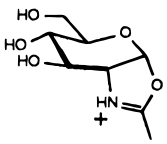
most likely possibility is that the UDP-GlcNAc 2-epimerase eliminations are E1-like processes. The combination of a good leaving group and a relatively stable oxocarbenium intermediate should permit this type of mechanism to take place. Precedence for an E1 elimination mechanism can be found in the reaction catalyzed by imidazoleglycerol-phosphate dehydratase where the cationic intermediate is thought to be stabilized by conjugation to an imidazole ring.[67]

The observation of a primary kinetic isotope effect indicates that C−H bond cleavage occurs in a rate-determining step of the reaction. This is consistent with either an E2 mechanism or an E1 mechanism in which deprotonation of the oxocarbenium intermediate is partially rate-determining.

The simple mechanisms outlined above may be more complicated by the involvement of covalent catalysis with enzyme-bound intermediates (as in the glycosidase reactions described above) or by the possibility of neighboring group participation by the acetamido functionality. In particular, the oxazolines **1** and **2** could be intermediates in the *anti* and *syn* eliminations, respectively. Nonenzymatic oxazoline formation in both GlcNac and ManNac systems often occurs under reaction conditions that promote oxocarbenium formation.[68] In addition, a study has recently been published that strongly implicates the involvement of **1** as an intermediate in the reaction catalyzed by *N*-acetyl-$\beta$-hexosaminidase.[69]



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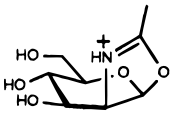

**2**

**Acknowledgment.** We are grateful to Drs. Lucia B. Rothman-Denes and Diane R. Kiino for supplying the plasmid containing *nfrC* and to Dr. P. D. Rick for supplying a plasmid containing *rffD*. We thank Drs. S. G. Withers and E. C. K. Lai for supplying samples of synthetic 2-acetamidoglucal. We also thank the University of British Columbia and the Natural Sciences and Engineering Research Council of Canada (NSERC) for financial support.

**Supporting Information Available:** Further experimental details on purification and identification of UDP-GlcNAc 2-epimerase and selected results (9 pages). See any current masthead page for ordering and Internet access instructions.

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