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Sialic Acid Biosynthesis: Stereochemistry and Mechanism of the Reaction Catalyzed by the Mammalian UDP-*N*-Acetylglucosamine 2-Epimerase

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Abstract: The bifunctional enzyme, UDP-*N*-acetylglucosamine 2-epimerase/ManNAc kinase, catalyzes the first two steps in the biosynthesis of the sialic acids in mammals. The epimerase domain converts UDP-GlcNAc into ManNAc and UDP. This paper demonstrates that α-ManNAc is the first formed anomer and therefore the reaction proceeds with a net retention of configuration at C-1. Studies in deuterated buffer show that solvent-derived deuterium is quantitatively incorporated into the C-2 position of the product during catalysis, but it is not incorporated into the remaining pool of substrate. This indicates that the inversion of stereochemistry is ultimately brought about by the removal and replacement of a proton at C-2 and is consistent with a two-base mechanism. Studies with ^{18}O-labeled UDP-GlcNAc show that the anomeric oxygen of the glycosyl phosphate bond departs with the UDP product and therefore the net hydrolysis reaction involves C−O bond cleavage. Incubation of the putative intermediate, 2-acetamidoglucal, with the enzyme resulted in a slow hydration reaction to give the product, ManNAc. Additional kinetic isotope effect and positional isotope exchange (PIX) experiments address the nature of the rate-determining step of the reaction and show that C−H bond cleavage is not rate limiting. Overall, these results support a reaction mechanism involving an *anti*-elimination of UDP to give 2-acetamidoglucal, followed by a *syn*-addition of water.

Introduction

The sialic acids are nine-carbon polyhydroxylated α-keto acids that are found on the distal end of glycan chains in vertebrate glycoconjugates.[1,2] Most of the sialic acids are derivatives of neuraminic acid (Neu) and contain an amino functionality at the C-5 position. The most common of these is the biosynthetic precursor for the family *N*-acetylneuraminic acid (Neu5Ac). The positioning of the sialic acids at the termini of cell-surface oligosaccharides is responsible for many of their crucial biochemical roles, such as serving as key recognition elements and mediating cellular adhesion processes.[3,4] In addition, sialylation represents an important form of protein glycosylation,[5] and the metastatic potential of tumor cells has been correlated to the extent of cell surface sialylation.[6,7]

The mammalian biosynthesis of the sialic acids begins with conversion of UDP-*N*-acetylglucosamine (UDP-GlcNAc) into *N*-acetylmannosamine (ManNAc) (Figure 1).[1] The ManNAc is then phosphorylated at C-6 and condensed with phosphoenolpyruvate to give Neu5Ac 9-phosphate. Dephosphorylation and coupling with CTP generate the activated form of the sialic acids, CMP-Neu5Ac. The first two steps in this pathway are catalyzed by the bifunctional enzyme, UDP-GlcNAc 2-epimerase/ManNAc kinase.[8−10] This enzyme has been found to

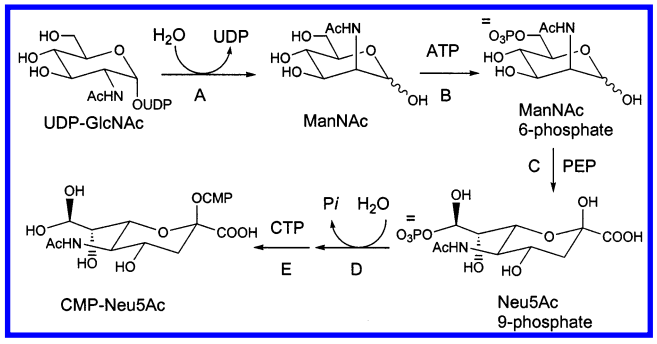

Figure 1. The biosynthetic pathway for CMP-*N*-acetylneuraminic acid in mammals. A = UDP-*N*-acetylglucosamine 2-epimerase, B = *N*-acetylmannosamine kinase, C = *N*-acetylneuraminic acid 9-phosphate synthase, D = *N*-acetylneuraminic acid 9-phosphate phosphatase, E = CMP-*N*-acetylneuraminic acid synthetase.

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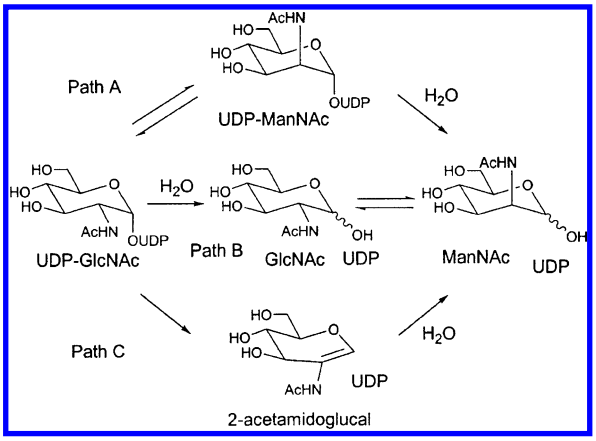

Figure 2. Three potential mechanisms for the reaction catalyzed by the mammalian UDP-*N*-acetylglucosamine 2-epimerase. Path A = epimerization preceding hydrolysis, Path B = hydrolysis preceding epimerization, Path C = elimination preceding hydration.

catalyze the rate-limiting steps in sialic acid biosynthesis and therefore serves as the key regulator of cell surface sialylation in humans.[11] It has also been shown to be essential in the embryonic development of mice,[12] and point mutations result in the human disease hereditary inclusion body myopathy.[13]

An examination of the UDP-GlcNAc 2-epimerase/ManNAc kinase amino acid sequence suggests that the enzyme is comprised of two domains. The N-terminal domain is responsible for the epimerase activity and catalyzes both the inversion of stereochemistry at C-2 of UDP-GlcNAc as well as hydrolysis to produce free ManNAc and UDP (this reaction is essentially irreversible and technically not a true epimerization). The C-terminal domain is responsible for the kinase activity that generates ManNAc 6-phosphate. These assignments have been supported by both sequence homology studies and mutagenesis studies.[8]

Potential mechanisms for the "epimerization" reaction include the initial epimerization of UDP-GlcNAc to give UDP-ManNAc, followed by a hydrolysis step that releases UDP and ManNAc (Path A, Figure 2). The first step would presumably involve a 2-acetamidoglucal intermediate analogous to the one shown in Path C (vide infra). Alternatively, hydrolysis may occur first, and epimerization of the free sugar would generate ManNAc (Path B, Figure 2). In this case, the epimerization would likely proceed via the open chain form of the sugar and involve a deprotonation/reprotonation of the relatively acidic proton at C-2. Finally, the mechanism may proceed via an initial *anti*-elimination of UDP from UDP-GlcNAc to give the intermediate 2-acetamidoglucal, followed by the hydration of the glycal to give free ManNAc (Path C, Figure 2). Paths A and C are indirectly supported by studies on the bacterial UDP-GlcNAc 2-epimerase that catalyzes the interconversion of UDP-GlcNAc and UDP-ManNAc (and is therefore a true epimerase).[14−19] This

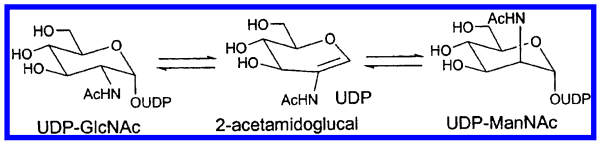

Figure 3. The mechanism of the reaction catalyzed by the bacterial UDP-*N*-acetylglucosamine 2-epimerase.

enzyme is known to catalyze an *anti*-elimination of UDP from UDP-GlcNAc to give 2-acetamidoglucal, followed by the *syn*-addition of UDP to give the product, UDP-ManNAc (Figure 3). The epimerase domain in the rat epimerase/kinase shares a 22% amino acid identity with the bacterial enzyme, suggesting the two have evolved from a common ancestor and likely employ a similar mechanism.[8,15]

Previous isotope incorporation studies have shown that the reaction in tritiated water produces ManNAc containing tritium at the C-2 position.[20] This indicates that the inversion of stereochemistry is ultimately brought about by the removal and replacement of a proton at C-2, and is consistent with all three of the proposed mechanisms. Initial velocity kinetics and product inhibition studies have been performed and were interpreted in favor of an ordered mechanism in which UDP was the first product released, followed by an irreversible formation of ManNAc.[21] This agrees most closely with the mechanism shown in Path C. Further experiments involved incubating the enzyme with the putative reaction intermediates and testing them for catalytic competence. The incubation of the enzyme with UDP-ManNAc did lead to the formation of ManNAc as might be expected if Path A were operative.[22] Further studies in tritiated water, however, showed that the ManNAc produced contained tritium at C-2 and the amount incorporated was about 87% of that incorporated when UDP-GlcNAc was the substrate. In addition, the recovered UDP-ManNAc from an incomplete reaction bore no tritium label, and no trace of UDP-GlcNAc could be detected. These results are inconsistent with either the direct hydrolysis of UDP-ManNAc or the reversible epimerization step shown in Path A. It was suggested that the UDP-ManNAc is simply serving as an alternate substrate and is not an intermediate in the normal reaction mechanism. To test for the possibility that Path B was operative, free GlcNAc was incubated with the enzyme; however, no epimerization was observed.[23] Perhaps the most convincing experiment was performed when 2-acetamidoglucal was incubated with the enzyme.[21] This resulted in the production of a compound that coeluted with ManNAc when analyzed by high voltage paper electrophoresis. This observation supports the reaction mechanism outlined in Path C.

In this work, the epimerase reaction is shown to proceed with a retention of stereochemistry at C-1, and the loss of UDP is found to occur via a C−O bond cleavage process. In addition,

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the product of the incubation with 2-acetamidoglucal is positively identified as ManNAc by ^{1}H NMR spectroscopy. These results support a mechanism involving an *anti*-elimination of UDP followed by the *syn*-hydration of 2-acetamidoglucal. Positional isotopic scrambling experiments and kinetic isotope effect measurements are described that address the nature of the rate-determining transition states in this reaction.

Experimental Section

General Methods. UDP-*N*-acetylglucosamine, lactate dehydrogenase (Type II from rabbit muscle), and pyruvate kinase (Type II from rabbit muscle) were purchased from Sigma Chemical Co. ^{18}O-enriched water (95%) was purchased from Icon Stable Isotopes. Protein concentrations were determined by the method of Bradford using bovine serum albumin as the standard.[24]

Enzyme Expression and Purification. UDP-GlcNAc 2-epimerase/ManNAc kinase from rat was overexpressed in *Spodoptera frugiperda* cells (Sf9, Life Technologies, Inc.) using a baculovirus expression system.[8] Suspension cultures of Sf-9 cells (50 mL) were grown to a density of 2×10^6 cells/mL at 120 rpm and 27 °C, and then infected with the recombinant baculovirus containing the sequence coding for the gene of interest, at a multiplicity of infection of 1. After an optimal infection period of 60 h, the cells were pelleted by centrifugation (5000 rpm, 20 min), and the following purification procedures were carried out at 4 °C.

The resulting cell pellet was lysed in a phosphate buffer (10 mM NaH_2PO_4, pH 7.5, containing 1 mM dithiothreitol, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride) by drawing the cell pellet in and out of a syringe-needle (PrecisionGlide Needle, 26 gauge, 5/8" length, Becton Dickinson & Co.). The crude cell lysate was clarified by centrifugation (5000 rpm, 60 min) and filtration (0.2 μm pore, 25 mm Acrodisc syringe Filter, HT Tuffryn Membrane, Pall Corp.). The filtrate was loaded onto an ion-exchange column (1 mL, HiTrap Q HP, Amersham Pharmacia Biotech) preequilibrated with column buffer (10 mM NaH_2PO_4, pH 7.5, containing 1 mM DTT, and 1 mM EDTA). The column was eluted with a linear gradient of 0−1 M NaCl in column buffer and monitored at 280 nm. Fractions containing the enzyme were used directly in experiments. Enzyme in deuterated buffer was prepared as above, except that both the lysis and the column buffers were prepared with D_2O (pD 7.9).

Stereochemistry and Solvent Deuterium Isotope Incorporation Studies. A sample of UDP-GlcNAc 2-epimerase in deuterated column buffer (950 μL, 950 μg of protein) was prepared, and a ^{1}H NMR spectrum was taken. A sample of UDP-GlcNAc prepared in the same buffer (50 μL, 100 mM) was added, and the resulting solution (5 mM UDP-GlcNAc, 1 mL) was incubated for 10 h at 25 °C. ^{1}H NMR spectra (400 MHz) were taken at timed intervals. A control reaction of UDP-GlcNAc in the same buffer (5 mM, 1 mL) was also monitored under identical conditions.

Tests for C−O versus P−O Bond Cleavage and PIX Experiment. (i) Preparation of ^{18}O-Labeled UDP-GlcNAc. Uridine 5′-(2″-acetamido-2″-deoxy-[1″-^{18}O]-α-D-glucopyranosyl diphosphate), ^{18}O-labeled UDP-GlcNAc, was synthesized according to the procedure by Morgan et al.[16] ^{1}H and ^{13}C NMR spectra were identical to those reported. The extent of ^{18}O incorporation was determined to be 68% by mass spectral analysis: -LSIMS (thioglycerol) *m*/*z* 608 (M − H$^+$, ^{18}O, 100), 606 (M − H$^+$, ^{16}O, 46.5). The location of the ^{18}O-label was confirmed by ^{31}P NMR spectroscopy: ^{31}P NMR (D_2O) δ −11.789 (d, J_{p-p} = 21.0 Hz, 0.32P, β-P-^{16}O), −11.801 (d, J_{p-p} = 21.0 Hz, 0.68P, β-P-^{18}O), −10.095 (d, J_{p-p} = 21.0 Hz, 1P, α-P).

(ii) Positional Isotope Exchange (PIX) Experiment and Test for C−O versus P−O Bond Cleavage. A solution of ^{18}O-labeled UDP-GlcNAc in deuterated buffer (450 μL, 17 mM) was placed in an NMR tube, and Chelex-100 resin was added (20 mg of 200−400 mesh, Na$^+$ form, previously rinsed with D_2O). ^{1}H and proton-decoupled ^{31}P NMR spectra were taken. The scrambling experiment was initiated by the addition of UDP-GlcNAc 2-epimerase in deuterated buffer (200 μL, 200 μg of protein), and the resulting solution was incubated for 24 h at 25 °C. ^{1}H and proton-decoupled ^{31}P NMR spectra were taken at timed intervals. Proton-decoupled ^{31}P NMR spectra were obtained using a Bruker 300 MHz spectrometer operating at a frequency of 121 Hz. Acquisition parameters were sweep width = 2437 Hz, acquisition time = 13.4 s, delay between pulses = 2.0 s, and pulse width = 10 μs.

The final solution was applied to a size exclusion column (Biogel P-2, 200−400 mesh, 2.5 cm × 70 cm) and eluted with distilled water. Fractions exhibiting absorbance at 254 nm (uridine chromophore) were collected and lyophilized to dryness. ^{1}H NMR spectra of relevant fractions revealed that they contained UDP. Mass spectral analysis confirmed the presence of the ^{18}O-label in the UDP produced: -ESIMS *m*/*z* 405 (M − H$^+$, ^{18}O, 100), 403 (M − H$^+$, ^{16}O, 46.5). ^{31}P NMR spectroscopy confirmed that the ^{18}O-label was incorporated into the β-phosphate: ^{31}P NMR (D_2O) δ−8.001 (d, J_{p-p} = 21.0 Hz, 0.32P, β-P-^{16}O), −8.030 (d, J_{p-p} = 21.0 Hz, 0.68P, β-P-^{18}O), −9.645 (d, J_{p-p} = 21.0 Hz, 1P, α-P).

(iii) Test for Solvent ^{18}O-Isotope Incorporation. A sample of UDP-GlcNAc 2-epimerase in column buffer (300 μL, 300 μg of protein) was mixed with 300 μL of 95% ^{18}O-enriched H_2O. The reaction mixture was incubated at 30 °C for 10 h and then applied to a size exclusion column (Biogel P-2, 200−400 mesh, 2.5 cm × 70 cm) and eluted with distilled water. Fractions exhibiting absorbance at 254 nm were collected and lyophilized to dryness. ^{1}H NMR spectra of relevant fractions revealed that they contained UDP. Mass spectral analysis indicated the absence of the ^{18}O-label in the UDP produced: -LSIMS (thioglycerol) *m*/*z* 425 (M (monosodium salt) − H$^+$, ^{16}O, 100).

Catalytic Competence of 2-Acetamidoglucal. 2-Acetamidoglucal was synthesized by the procedure outlined by Pravdic et al.[25] A solution of UDP-GlcNAc 2-epimerase in deuterated buffer (950 μL, 950 μg of protein) was prepared, and a ^{1}H NMR spectrum was collected. A sample of 2-acetamidoglucal prepared in the same buffer (50 μL, 100 mM) was added, and the resulting solution (5 mM 2-acetamidoglucal, 1 mL) was incubated for 29 h at 25 °C. ^{1}H and ^{31}P NMR spectra were collected at timed intervals. Similar samples, either containing 5 mM UDP or lacking enzyme, were run under identical conditions. The extent of hydration was determined by integration of the H-1 peaks of both the 2-acetamidoglucal and the ManNAc anomers produced.

Kinetic Isotope Effect Studies. [2″-^{2}H]-UDP-GlcNAc was prepared according to the procedure by Morgan et al.[16] ^{1}H NMR and mass spectroscopy confirmed the extent of deuterium incorporation to be >95%: -ESIMS *m*/*z* 607 (M − H$^+$, 2″-^{2}H, 100). Concentrations of stock substrate solutions were calculated from A_{262} measurements with ϵ = 9890 M^{-1} cm^{-1}. Kinetics were measured using a continuous coupled assay for UDP formation.[26] Each assay contained 50 mM NaH_2PO_4 buffer (pH 7.5), 10 mM $MgCl_2$, 2 mM PEP, 0.2 mM NADH, 188 units of pyruvate kinase, 250 units of lactate dehydrogenase, and 5−300 μM UDP-GlcNAc or [2″-^{2}H]-UDP-GlcNAc (800 μL total volume). UDP-GlcNAc 2-epimerase (10 μg) was added to initiate the enzymatic reaction. Reaction rates were measured at 37 °C by monitoring the decrease in absorbance at 340 nm. Kinetic parameters were determined from initial velocities fit to Michaelis−Menten kinetics using the computer program GraFit 4.

Results

Enzyme Purification. The UDP-GlcNAc 2-epimerase/ManNAc kinase from rat was overexpressed in insect cells (Sf-9) using a baculovirus expression system[8] and was purified by a

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single ion exchange chromatographic step. The resulting protein was estimated to be 80% pure as analyzed by SDS−PAGE and was used without further purification because it is unstable to freezing and has limited stability upon storage at 4 °C. The enzymatic activity was also lost upon centrifugal concentration using ultrafiltration membranes, making buffer exchange/concentration difficult. To prepare sufficiently concentrated enzyme in a deuterated buffer for NMR studies, it was necessary to both lyse the insect cells and run the purification column in deuterated buffers, and then use the column fractions directly.

Stereochemistry and Solvent Isotope Incorporation Studies. To elucidate the stereochemistry of the epimerase reaction, it was necessary to determine whether the α-anomer or the β-anomer of ManNAc is the true reaction product. This experiment is complicated by the fact that the anomers interconvert rapidly in the absence enzyme. To determine the rate of this mutarotation process, a sample of commercial ManNAc (predominantly the β-anomer in the solid state, vide infra) was prepared in a deuterated phosphate buffer and immediately monitored using ^{1}H NMR spectroscopy. By integrating the signals corresponding to the anomeric protons, it was determined that the anomers interconvert with a half-life of approximately 10 min at 25 °C. At equilibrium, the ratio of anomers was approximately 1:1. A second problem lay in deducing which of the ^{1}H NMR signals correspond to a given anomer. The values of $J_{H1,H2}$ are very similar for the anomers of mannose derivatives and cannot be used to distinguish between them. Instead, it was necessary to use the technique of Bock and Pedersen that relies on measuring the values of $J_{C1,H1}$.[27] The α-anomers of mannose derivatives are generally found to have $J_{C1,H1}$ values that are 10 Hz greater than those of the β-anomers. An equilibrated sample of the ManNAc anomers in deuterated phosphate buffer was analyzed by 2-D heteronuclear NMR spectroscopy (HMQC), and it was established that the C-1 proton signals at 5.02 and 4.92 ppm corresponded to the C-1 carbon signals at 94.50 and 94.38 ppm, respectively. A proton-decoupled ^{13}C NMR spectrum was then used to determine that the values of $J_{C1,H1}$ were 171 and 161 Hz, respectively. This led to the assignments of the ^{1}H NMR signal at 5.02 ppm to the α-anomer and the signal at 4.92 ppm to the β-anomer.

Samples of UDP-GlcNAc in a deuterated phosphate buffer (pD 7.9) were treated with the epimerase, and spectra were immediately collected (Figure 4). New peaks that were identical to those of UDP and free ManNAc gradually replaced those of UDP-GlcNAc, as expected. Inspection of the spectra at the earliest time points (5 min) showed that the α-anomer was the predominant product by a factor of at least 3:1. At later time points, rapid nonenzymatic mutarotation allowed the anomers to interconvert and resulted in a 1:1 equilibrium mixture. This result shows that the reaction of the mammalian enzyme proceeds with a net retention of configuration at C-1 and produces the α-anomer of ManNAc.

The experiment described above also served to demonstrate that the reaction proceeds with the incorporation of solvent-derived deuterium at C-2 and that the level of incorporation was >95%. Signals for the C-2 protons of ManNAc were absent in the spectra (not shown in Figure 4), and the signals for the C-1 protons appeared as singlets (Figure 4). In addition, the experiment confirmed the report that solvent-derived isotope is not incorporated into recovered starting material to any significant extent.[20] Inspection of the signal corresponding to the anomeric proton of UDP-GlcNAc showed no change in the coupling pattern even after 80% of the material had been converted to product.

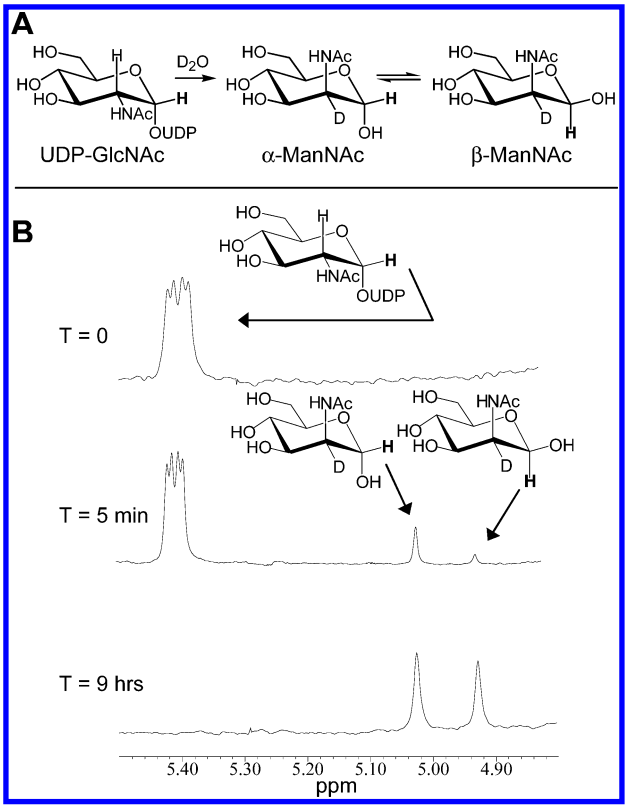

Figure 4. (A) The enzymatic formation of α-[2-^{2}H]-ManNAc in D_2O followed by nonenzymatic mutarotation. (B) ^{1}H NMR spectra following the time course of the reaction.

Test for C−O versus P−O Bond Cleavage and PIX Experiment. To test whether the loss of UDP proceeds via C−O bond cleavage or P−O bond cleavage, a sample of UDP-GlcNAc in a phosphate buffer (pH 7.5) containing 50% $H_2{}^{18}O$ was treated with the epimerase. The reaction was allowed to proceed to completion, and the resulting UDP was isolated. Mass spectral analysis confirmed the absence of any ^{18}O-isotope in the UDP. This observation suggests that the hydrolysis proceeds via C−O bond cleavage and that the solvent-derived oxygen resides in the ManNAc. Because this relies on a negative result, and because the detection of the ^{18}O-label in the anomeric position of ManNAc may be complicated by nonenzymatic wash in/out, the experiment was repeated with ^{18}O-labeled UDP-GlcNAc.

A sample of UDP-GlcNAc bearing an ^{18}O-label at the anomeric or "bridging" position was chemically synthesized as described previously.[16] ^{31}P NMR spectroscopy and mass spectral analysis confirmed the location of the label and indicated that the extent of label incorporation was 68%. By treating this compound with the epimerase in a deuterated phosphate buffer and monitoring the reaction using ^{31}P NMR spectroscopy, it was possible to track the fate of the ^{18}O-label in the UDP produced (Figure 5a). In addition, this experiment tests for the possibility of positional isotope exchange (PIX) in the remaining starting material.[28] A spectrum of the starting material before the addition of enzyme shows that the two signals corresponding

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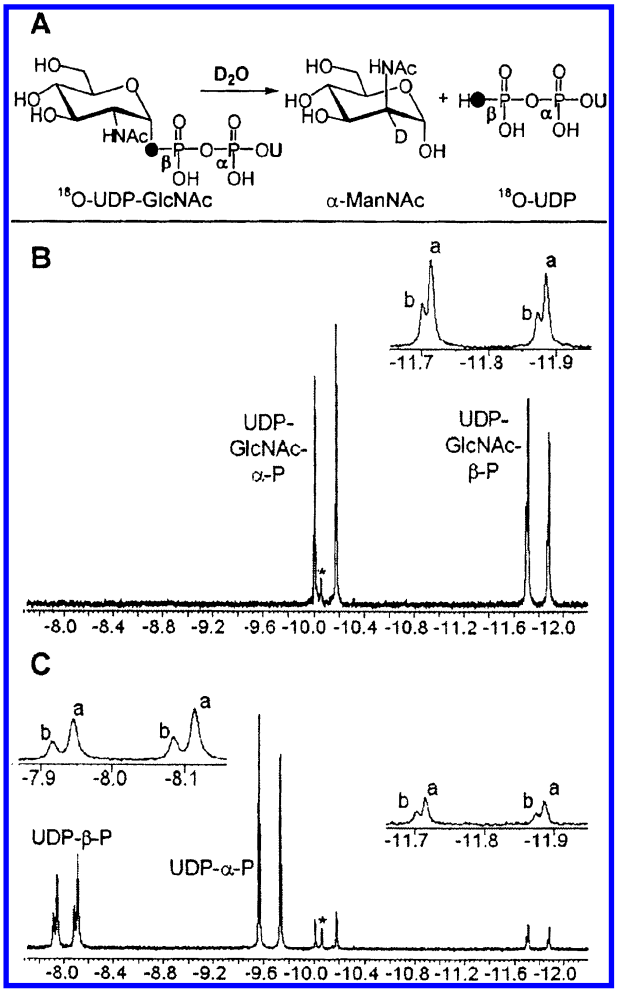

Figure 5. (A) The production of ^{18}O-labeled UDP from ^{18}O-labeled UDP-GlcNAc. (B) ^{31}P NMR spectrum of ^{18}O-labeled UDP-GlcNAc before the addition of enzyme. (C) ^{31}P NMR spectrum after the reaction proceeded to 80% completion (a = ^{18}O-labeled material, b = unlabeled material, * stands for 3% impurity of UMP dimer formed during the morpholidate coupling step of the synthesis).

to the ^{18}O-labeled and unlabeled β-phosphorus nuclei appeared as two doublets separated by 0.012 ppm (Figure 5b, the signals appear as doublets due to coupling with the adjacent phosphorus).[16] The isotopic substitution of ^{18}O for ^{16}O is known to cause an upfield shift of this magnitude,[29,30] and the 1:2 integration of these signals confirms that the upfield doublet corresponds to the labeled material. This material was incubated with the epimerase, and a spectrum was taken after the reaction had proceeded to approximately 80% completion (Figure 5c). An examination of the signals due to the β-phosphorus of the UDP product clearly shows two doublets present in a 1:2 ratio with a separation of 0.029 ppm. Thus, the spectrum indicates that the ^{18}O-label resides in the UDP, a fact that was further established using mass spectrometry. This proves that the enzymatic reaction proceeds via a C−O bond cleavage process. An examination of the signals due to the residual UDP-GlcNAc indicates that no detectable scrambling of the ^{18}O-isotopic label has occurred (it would be possible to detect the presence of 10% of the scrambled product). Material in which the label has scrambled into a "nonbridging" position would show a doublet

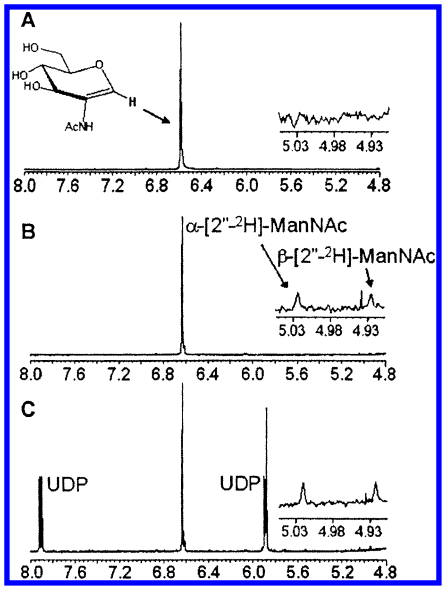

Figure 6. ^{1}H NMR spectra following the incubation of 2-acetamidoglucal (A) without enzyme, (B) with epimerase, and (C) with both epimerase and UDP.

that is shifted upfield by 0.029 ppm from the unlabeled material due to the higher P−O bond order. This was seen in PIX experiments with the bacterial UDP-GlcNAc 2-epimerase.[16] Instead, only the 1:2 ratio of signals present in the starting mixture was observed. To ensure that it would be possible to detect this signal, the experiment was repeated with the bacterial enzyme, and the previously reported scrambling was clearly observed.

Catalytic Competence of 2-Acetamidoglucal. To test whether 2-acetamidoglucal is catalytically competent to serve as a substrate for the second half of the reaction, samples were incubated with the enzyme in the presence and the absence of added UDP. 2-Acetamidoglucal was synthesized using previously reported methods.[25,31] When this material alone was incubated in deuterated phosphate buffer (pD 7.9), and monitored by ^{1}H NMR spectroscopy, no nonenzymatic hydration was observed (Figure 6a). In the presence of added epimerase, however, the formation of [2-^{2}H]-ManNAc was confirmed by the appearance of two singlets at 5.02 and 4.92 ppm in a 1:1 ratio (Figure 6b). No signals corresponding to GlcNAc were detected. The formation of ManNAc was quite slow, and after 29 h only 6% conversion was observed. Under identical concentrations of UDP-GlcNAc and enzyme, the normal reaction proceeded to completion within 2 h under these conditions. When the incubation was repeated in the presence of 5 mM UDP, a 12% production of ManNAc was observed (Figure 6c). The increase in the amount of ManNAc produced was reproducible and grew with increasing levels of added UDP. These experiments demonstrate that 2-acetamidoglucal is a catalytically competent substrate for the mammalian epimerase.

Kinetic Isotope Effect Study. The reaction rates of [2″-^{2}H]-UDP-GlcNAc and unlabeled UDP-GlcNAc were compared to determine if cleavage of the C−H bond is rate determining during catalysis. The deuterium label was introduced into the C-2″ position of UDP-GlcNAc using the bacterial UDP-GlcNAc 2-epimerase in a deuterated buffer, as described previously.[16]

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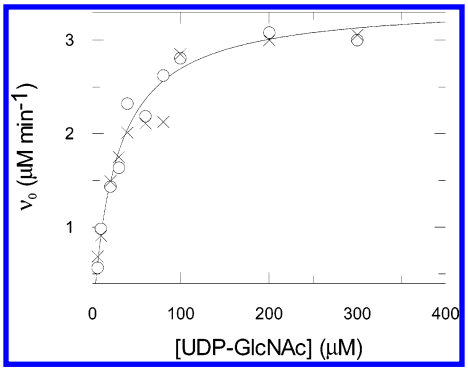

Figure 7. Plots of initial velocity versus substrate concentration for unlabeled UDP-GlcNAc (○) and for [2″-^{2}H]-UDP-GlcNAc (×). The line was fitted to the data obtained with the unlabeled substrate.

The isotopic enrichment in the resulting [2″-^{2}H]-UDP-GlcNAc was found to be >95% as judged by ^{1}H NMR and mass spectral analysis. A continuous spectrophotometric assay employing pyruvate kinase and lactate dehydrogenase gave values of 0.33 ± 0.01 s^{-1} for k_{cat} and 26 ± 4 μM for K_M with unlabeled UDP-GlcNAc (Figure 7). The corresponding values obtained with [2″-^{2}H]-UDP-GlcNAc were 0.32 ± 0.01 s^{-1} for k_{cat} and 27 ± 4 μM for K_M. These values give a k_{cat} isotope effect of 1.0 ± 0.1 and a k_{cat}/K_M isotope effect of 1.0 ± 0.4 and demonstrate that cleavage of the C−H bond is not a rate-determining step in catalysis.

Discussion

Most known racemases and epimerases operate at chiral centers that are adjacent to carbonyl functionalities and bear a relatively acidic proton.[32−34] In these cases, the enzymes employ nonstereospecific deprotonation/reprotonation mechanisms to catalyze an inversion of stereochemistry. The UDP-GlcNAc 2-epimerases belong to the small group of enzymes that operate at chiral centers lacking an acidic proton and must therefore use alternate catalytic strategies. Most of the work to date has focused on the bacterial UDP-GlcNAc 2-epimerase that has been shown to catalyze an initial *anti*-elimination of UDP to give 2-acetamidoglucal, followed by a *syn*-addition of UDP to give UDP-ManNAc (Figure 3).[14−19] These elimination reactions presumably employ E2- or E1-like mechanisms and thus avoid the generation of any carbanionic intermediates. The homologous mammalian UDP-GlcNAc 2-epimerase catalyzes the irreversible formation of UDP and ManNAc and has only recently become available in a recombinant form.[8,9] In this paper, we probe the stereochemistry and mechanism of the mammalian enzyme and compare the results to those obtained with the bacterial enzyme.

By monitoring the reaction with ^{1}H NMR spectroscopy, it was possible to determine that the ManNAc was initially formed as the α-anomer and therefore the reaction proceeds with a net retention of stereochemistry at C-1. This is also the case with the bacterial enzyme. This experiment also showed that the reaction in D_2O generates [2-^{2}H]-ManNAc, implying that the inversion of stereochemistry is ultimately brought about by the removal of a proton from C-2, followed by the replacement of a solvent-derived proton in the opposite stereochemical sense. The previous work with tritiated water also demonstrated that incorporation occurred,[20] but because it is a tracer labeling experiment and subject to discrimination from isotope effects, the observed extent of incorporation is difficult to interpret. This work showed that the extent of incorporation was essentially quantitative and that there was no significant internal return of the original C-2 proton of UDP-GlcNAc into the product. Internal return could only be seen with a one-base mechanism in which the same residue is responsible for both the deprotonation and the reprotonation events.[34,35] Internal return will not be seen with a two-base mechanism in which two different residues play the roles of catalytic base and acid. The fact that no solvent-derived isotope is observed in the remaining pool of starting material after 80% conversion can be explained if one assumes the deprotonation step is irreversible or is preceded by an irreversible step. Alternatively, it can be explained by a "two-base" mechanism involving monoprotic residues that do not exchange protons with bulk solvent during the lifetime of the intermediates. This is the case with the reversible reactions catalyzed by glutamate and proline racemase in which two cysteine thiol(ate)s act as the catalytic acid/base residues and solvent isotope incorporation is observed in the product but not the starting material.[36−38] These observations are consistent with all three mechanisms shown in Figure 2.

The experiment with ^{18}O-labeled UDP-GlcNAc showed that the label was retained in the released UDP and confirms that the reaction proceeds with C−O bond cleavage. In Paths A and B (Figure 2), the hydrolysis step could occur via either C−O or P−O bond cleavage depending on whether the water attacks at carbon or at phosphorus, respectively. This finding rules out the latter possibilities and is also consistent with Path C that follows a mandatory C−O bond cleavage process. The same experiment showed that there was no isotopic scrambling (PIX) in the pool of starting material even after 80% of the material was converted to products. The absence of PIX suggests that the C−O bond cleavage is irreversible (or a step prior to it is irreversible) and that once UDP is formed it does not partition back into the pool of free starting material. An alternate explanation is that the terminal phosphate in the bound UDP is prevented from rotation by interactions with active site residues (no divalent metal cations have been implicated in catalysis[39]). Thus, even if the bond cleavage were reversible, the same oxygen would add back to the anomeric position of the GlcNAc residue. While this is certainly a possibility, it is not the case in the bacterial enzyme where PIX is clearly observed.[16] Even when the bacterial reaction is run under irreversibly coupled conditions using UDP-ManNAc dehydrogenase, complete scrambling of the isotope is observed upon each turnover.[40]

The experiments described thus far are consistent with all of the mechanisms shown in Figure 2, but do not distinguish between them. A previous report briefly mentioned that 2-acetamidoglucal was hydrated to give ManNAc, providing evidence in support of Path C.[21] Because the product had only

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been identified by paper chromatography, this experiment was repeated and monitored by ^{1}H NMR spectroscopy. The experiment clearly showed that ManNAc was produced; however, it was formed at a rate much slower than the normal reaction. Thus, 2-acetamidoglucal is catalytically competent, but not kinetically competent, to serve as an intermediate in this reaction. It is likely that the slow rate of reaction is due to the fact that the free enzyme does not normally bind 2-acetamidoglucal from solution (or release it) and only a small fraction of the enzyme is in the correct conformation and protonation state to do so.[41] The reaction was also run in the presence of UDP, and a modest, but reproducible, increase in the amount of produced ManNAc was observed. This demonstrates that UDP is not an inhibitor of the hydration step and suggests that the hydration may occur while UDP is still bound in the active site of the enzyme. Alternatively, the increase may be simply due to a stabilizing effect that keeps a greater proportion of the enzyme active during the course of the experiment. As we do not have a sensitive assay for the formation of 2-acetamidoglucal in this very slow reaction, we were not able to measure initial rates and test this possibility. The possibility that the hydration was catalyzed by impurities of glycosidases can be ruled out by the observations that only ManNAc was formed (as opposed to GlcNAc)[31,42] and that the rate was increased by the presence of UDP.

The hydration of the glycal strongly supports the mechanism shown in Path C (Figure 2). This is also consistent with kinetic studies that were interpreted in favor of an ordered mechanism in which UDP was the first product released, followed by an irreversible formation of ManNAc.[21] Path B seems quite unlikely as GlcNAc is not epimerized by the enzyme,[23] and 2-acetamidoglucal would not be expected to serve as an alternate substrate. Path A also seems unlikely because the 2-acetamidoglucal (a putative intermediate in the first step) should not be hydrated in the absence of UDP. In addition, the earlier reports of solvent isotope incorporation studies during the hydrolysis of UDP-ManNAc argue against Path A.[22] It is likely that UDP-ManNAc simply serves as an alternate substrate and that the net hydrolysis occurs via an elimination of UDP to give 2-acetamidoglucal, followed by the normal hydration of this intermediate. Thus, Path C remains the most reasonable description of the minimal catalytic mechanism for the mammalian UDP-GlcNAc 2-epimerase. When one considers the overall stereochemistry of the reaction, the enzyme catalyzes an *anti*-elimination of UDP followed by the *syn*-addition of water. This ability to catalyze two reactions of opposite stereospecificity is similar to the bacterial enzyme that catalyzes the nonstereospecific addition of UDP to a 2-acetamidoglucal intermediate.

The absence of any kinetic isotope effect in the reaction of [2″-^{2}H]-UDP-GlcNAc shows that cleavage of the C−H bond is not a rate-determining step in the reaction mechanism. This is somewhat different than in the case of the bacterial enzyme that shows a primary KIE of 1.8 on the value of k_{cat} for the epimerization of UDP-GlcNAc.[16] When this is considered together with the observation that PIX does not occur, several possibilities emerge. One possibility is that the absence of PIX indicates cleavage of the C−O bond is the rate-determining step of catalysis. This would imply that the elimination of UDP proceeds in a stepwise E1 mechanism with deprotonation of the oxocarbenium intermediate occurring in a rapid second step that forms 2-acetamidoglucal. The intermediate in this scenario could also be a species that is covalently bound to an enzyme nucleophile, or an oxazoline, as has been implicated in certain glycosidase mechanisms.[43,44] Another scenario is that binding, or a conformational change preceding the first chemical step, is rate determining and there is a large commitment to catalysis. Finally, it is possible that PIX was not observed due to restricted rotation of the terminal phosphate in the bound UDP. In this case, the rate-determining steps may be hydration of the glycal intermediate or product release. Further studies must be performed to distinguish between these possibilities.

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