Identification and Mechanism of a Bacterial Hydrolyzing UDP-*N*-Acetylglucosamine 2-Epimerase†

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ABSTRACT: This paper reports the first identification of a fully functional hydrolyzing UDP-*N*-acetylglucosamine 2-epimerase from a bacterial source. The epimerase (known as SiaA or NeuC) from *Neisseria meningitidis* MC58 group B is shown to catalyze the conversion of UDP-GlcNAc into ManNAc and UDP in the first step of sialic acid (*N*-acetylneuraminic acid) biosynthesis. The mechanism is proposed to involve an *anti* elimination of UDP to form 2-acetamidoglucal as an intermediate, followed by the *syn* addition of water. The observation that the α-anomer of ManNAc is the true product and that solvent deuterium is incorporated at C-2 is consistent with this mechanism. The use of the ^{18}O-labeled substrate confirms that the overall hydrolysis reaction proceeds via cleavage of the C−O bond. Furthermore, the putative intermediate 2-acetamidoglucal is shown to serve as a catalytically competent substrate and is enzymatically hydrated to give ManNAc exclusively. Isotope effect studies show that cleavage of the C−H bond is not rate limiting during catalysis. Mutagenesis studies show that three active site carboxylate residues are crucial for catalysis. In two of the mutants that were studied (E122Q and D131N), 2-acetamidoglucal was released from the active site during catalysis, providing direct evidence that the enzyme is capable of catalyzing the *anti* elimination of UDP from UDP-GlcNAc.

The display of sialic acids at the termini of oligosaccharide chains of glycoconjugates on the surface of mammalian cells serves a vital role in cellular recognition and adhesion processes (*3*−*5*). Many human bacterial pathogens also present cell surface sialic acids in lipooligosaccharides which mimic human glycolipids (*6*). The neuroinvasive organisms *Neisseria meningitidis* groups B and C and *Escherichia coli* K1 and K92 synthesize a capsule of polysialic acid, consisting of a homopolymer of α-2,8-linked, α-2,9-linked, or mixed α-2,8-α-2,9-linked *N*-acetylneuraminic acid (Neu5Ac)[1] (*7*), which are mimics of the neural cell adhesion molecule (NCAM) (*8*). As a result of its mimicry of the host's sialylated glycoconjugates, these glycolipids and capsules are considered a major virulence factor of these bacterial pathogens which are the cause of meningitis and sepsis (*6*, *9*−*11*).

Whereas our understanding of the biosynthetic pathway of these nine-carbon polyhydroxylated α-keto acid units in bacteria is incomplete, the corresponding mammalian pathway is well-established. In mammals, the route to Neu5Ac begins with the conversion of UDP-*N*-acetylglucosamine (UDP-GlcNAc) to *N*-acetylmannosamine (ManNAc) and UDP (*3*), a process that involves net hydrolysis and inversion of stereochemistry at C-2 (Figure 1a). This reaction is under the direction of a bifunctional UDP-GlcNAc 2-epimerase/ManNAc kinase, which also catalyzes the subsequent phosphorylation of ManNAc to ManNAc 6-phosphate (*12*). ManNAc 6-phosphate is then condensed with phosphoenolpyruvate, followed by dephosphorylation, to give Neu5Ac. Commitment to sialic acid biosynthesis has been found to be dependent on the formation of ManNAc, and thus, the hydrolyzing UDP-GlcNAc 2-epimerase is considered the key regulator in the pathway (*13*).

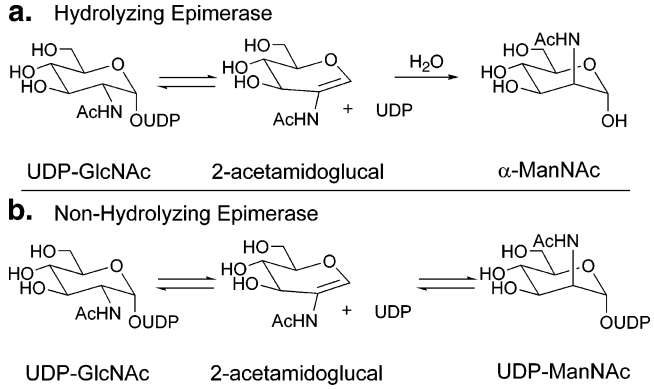

FIGURE 1: Mechanisms of the reactions catalyzed by (a) the mammalian hydrolyzing UDP-GlcNAc 2-epimerase and (b) the bacterial nonhydrolyzing UDP-GlcNAc 2-epimerase.

The bifunctional UDP-GlcNAc 2-epimerase/ManNAc kinase from rat is a 722-amino acid protein and is composed of two discrete domains that catalyze the respective activities (*14*). The N-terminal domain (378 amino acids) catalyzes the epimerase activity, as has been clearly demonstrated by

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[1] Abbreviations: GlcNAc, *N*-acetylglucosamine; ManNAc, *N*-acetylmannosamine; Neu5Ac, *N*-acetylneuraminic acid; PIX, positional isotope exchange; UDP, uridine diphosphate.

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mutagenesis experiments. Several monofunctional homologues of this domain are now known in the literature; however, only the bacterial nonhydrolyzing UDP-GlcNAc 2-epimerase from *E. coli* (sequence 22% identical) has been studied in significant detail. This enzyme is a true epimerase and therefore catalyzes the reversible interconversion of UDP-GlcNAc and UDP-ManNAc (Figure 1b). It provides an activated form of ManNAc for use in a variety of bacterial polysaccharide biosyntheses and has been characterized both mechanistically and structurally (*15*−*17*).

Mechanistic studies have shown that the mammalian hydrolyzing UDP-GlcNAc 2-epimerase catalyzes an *anti* elimination of UDP and a subsequent *syn* hydration of the 2-acetamidoglucal intermediate to yield α-ManNAc (Figure 1a) (*18*, *19*). A similar mechanism is employed by the bacterial nonhydrolyzing UDP-GlcNAc 2-epimerase except that the *syn* addition occurs with UDP instead of water (Figure 1b) (*15*). However, since the product, UDP-ManNAc, is utilized for purposes other than sialic acid synthesis, it is reasonable to expect that a hydrolyzing enzyme similar to the mammalian epimerase is responsible for the production of ManNAc in bacterial strains that display sialic acids. A bacterial version of the hydrolyzing enzyme would be of interest, as it would likely share a similar structure and mechanism with the mammalian enzyme but would not suffer from its known instability (*14*, *18*, *20*, *21*). For instance, numerous attempts to crystallize the mammalian epimerase have failed (*22*), but as is frequently the case, a bacterial enzyme would likely be readily crystallized. Recent attempts to identify such an enzyme have implicated the NeuC protein from *E. coli* K1 as a hydrolyzing UDP-GlcNAc 2-epimerase whose sequence is 53% similar and 23% identical to that of its mammalian counterpart (*1*). NeuC was observed to catalyze the formation of the putative 2-acetamidoglucal intermediate, suggesting that it also operates via an *anti* elimination of UDP, as in the first step in Figure 1a. However, the second step, hydration of the glycal, is apparently hampered *in vitro*, as only trace amounts of ManNAc could be detected under extremely sensitive assay conditions.

The discovery of an "aborted" epimerase activity encoded by the *neuC* gene product prompted the examination of the homologous gene, *siaA*,[2] from *N. meningitidis* MC58 group B. Both genes are located within a cluster that encodes the enzymes responsible for the synthesis and transport of Neu5Ac (*23*−*25*). Intriguingly, Petersen *et al*. (*2*) have reported that the *N. meningitidis* product, SiaA, is a GlcNAc-6-phosphate 2-epimerase; however, their claim was based on the results of a discontinuous coupled assay that did not directly assess ManNAc 6-phosphate formation and that utilized crude cell extracts. In contrast, the 52% similar and 32% identical sequences of NeuC and SiaA suggest that the enzymes share a common catalytic activity.

In this report, we describe the cloning and expression of *siaA* and the characterization of the corresponding enzymatic activity. SiaA is shown to efficiently catalyze the conversion of UDP-GlcNAc to UDP and ManNAc, and therefore, this work is the first demonstration of a fully active hydrolyzing UDP-GlcNAc 2-epimerase from a bacterial source. Through the use of ^{1}H and ^{31}P NMR spectroscopy and mass spectrometry, SiaA is shown to employ a mechanism identical to that of the mammalian enzyme. Additionally, kinetic isotope effect and positional isotope exchange (PIX) experiments are presented to address the nature of the rate-limiting step. Finally, site-directed mutagenesis of three potential active site bases implicates these residues as serving essential catalytic roles and, in two instances, results in mutant enzymes that release the intermediate, 2-acetamidoglucal, during catalysis.

EXPERIMENTAL PROCEDURES

Materials and General Methods. UDP-*N*-Acetylglucosamine, lactate dehydrogenase (type II from rabbit muscle), and pyruvate kinase (type II from rabbit muscle) were purchased from Sigma-Aldrich. ^{18}O-enriched water (95%) was purchased from Icon Isotopes. Protein concentrations were determined by the method of Bradford using bovine serum albumin as the standard (*26*). ^{1}H NMR spectra were obtained on a Bruker AV300 or AV400 spectrometer at a field strength of 300 or 400 MHz, respectively. Proton-decoupled ^{31}P NMR spectra were recorded on one of these spectrometers at 121.5 or 162 MHz, respectively. Mass spectrometry was performed by the Mass Spectrometry Centre at the University of British Columbia (UBC) by electrospray ionization (ESI-MS) using a Waters Micromass LCT mass spectrometer.

Cloning of siaA. The *siaA* gene (GenBank accession number X78068) was amplified by the polymerase chain reaction (PCR) using a *N. meningitidis* B (strain MC58) genomic DNA template. Oligonucleotide primers, synthesized by the Nucleic Acids Protein Services (NAPS) Unit at UBC, included overhangs for ligation-independent cloning: 5′-GGTATTGAGGGTCGCATGAAAAGAATTCTTTGCATTAC-3′ (forward sequence) and 5′-AGAGGAGAGTTAGAGCCTTAAAGATTCAAATCGATAA-3′ (reverse sequence). To a 200 μL PCR tube were added 5.0 μL of 10× PCR buffer, 1.0 μL of 10 mM dNTP mix, 1.5 μL of 50 mM $MgCl_2$, 25 ng of template DNA, 25 pmol of each primer, 0.25 μL of 5 units/μL *Taq* polymerase, and distilled H_2O to a total volume of 50 μL. DNA was amplified using an iCycler Thermal Cycler (Bio-Rad) according to the following cycles: one cycle of 3 min at 94 °C, 30 cycles of 45 s at 94 °C, 30 s at 55 °C, and 90 s at 72 °C, and one cycle of 10 min at 72 °C. The PCR product was cloned into the pET-30 Xa/LIC vector (Novagen) using the ligation-independent cloning method according to the manufacturer's directions. The resulting plasmid, pAM04, which encodes the SiaA protein fused to an N-terminal 43-residue peptide that includes a hexahistidine tag, was transformed into NovaBlue Singles chemically competent *E. coli* cells (Novagen). The presence of the gene in pAM04 was confirmed by colony PCR (*27*) and DNA sequencing.

Overexpression and Purification of Histidine-Tagged SiaA. The recombinant pAM04 plasmid was transformed into the expression host cells, BL21(DE3) chemically competent *E. coli*, which were incubated overnight at 37 °C with shaking

[2] The designations *neuC* and *siaA* have both been used to describe the hydrolyzing UDP-GlcNAc 2-epimerase from bacterial sources. For the purposes of this paper, we will use *neuC* to describe the gene from *E. coli* K1 (*1*) and *siaA* to describe the gene from *N. meningitidis* MC58 group B (*2*).

at 225 rpm in 10 mL of Luria-Bertani (LB) medium containing 30 μg/mL kanamycin. The overnight cultures were poured into 4 × 500 mL of LB medium containing 30 μg/mL kanamycin and grown at 37 °C with shaking at 225 rpm until an OD_{600} of 0.6−0.7 had been reached. Cells were induced for overexpression by addition of 120 mg/L (0.5 mM) isopropyl β-D-galactopyranoside (IPTG), and the cultures were allowed to continue to grow until an OD_{600} of 1.6−1.8 had been reached (approximately 4 h). Cells were harvested at 4000 rpm for 30 min, resuspended in lysis buffer [20 mM sodium phosphate (pH 7.5), 10 mM imidazole, 0.4 M NaCl, 1 μg/mL pepstatin A, and 1 μg/mL aprotinin], and lysed at 20 000 psi in an ice-cooled French pressure cell. The cell lysate was clarified by centrifugation at 6000 rpm for 40 min.

A 9 mL column containing Chelating Sepharose Fast Flow resin (Pharmacia Biotech) was charged with 2 column volumes (CV) of 100 mM $NiSO_4$, followed by washing with 2 CV of distilled H_2O and 3 CV of start buffer (lysis buffer without aprotinin and pepstatin). The clarified cell lysate was loaded at a rate of 2 mL/min, and start buffer (approximately 8 CV) was passed through the column at a rate of 3 mL/min until no more flow-through protein eluted, as determined by monitoring A_{280}. To remove nonspecifically bound proteins, a step gradient was then applied, consisting of 2 CV each of 5, 10, 15, 20, and 50% elution buffer [20 mM sodium phosphate (pH 7.5), 500 mM imidazole, and 0.4 M NaCl]. The histidine-tagged SiaA was finally eluted with 3−4 CV of 100% elution buffer. Fractions containing the enzyme were pooled and dialyzed overnight against a 1:100 volume of dialysis buffer [20 or 50 mM sodium phosphate (pH 7.5)]. The enzyme solution was then divided into 665 μL or 1.33 mL aliquots, flash-frozen in liquid N_2, and lyophilized. The enzyme could be stored in this state at −20 °C for at least 3 months without significant loss in activity. Samples were reconstituted as needed by adding H_2O or D_2O and gently inverting several times.

Identification of the Substrate and Products by NMR. Three 1.15 mL samples of SiaA (0.21 mg each) in 20 mM deuterated sodium phosphate buffer (pD 8.0) were incubated at 37 °C with GlcNAc, GlcNAc6P, or UDP-GlcNAc (5 mM each). ^{1}H NMR spectra were recorded at intervals between 30 min and 2 days.

Enzyme Kinetics As Determined by a Continuous Coupled Assay. Enzyme kinetics were measured using a continuous coupled assay for UDP formation (*28*), under the conditions described by Chou *et al.* (*18*) and using 0.20−10.0 mM UDP-GlcNAc. The UDP-GlcNAc stock concentration was determined by measuring A_{262} using an ϵ_{262} of 9890 M^{-1} cm^{-1}. Reactions were initiated by addition of 10 μL of enzyme solution (2.6 μg), and the decrease in A_{340} was monitored at 37 °C. Kinetic parameters were determined from initial velocities fit to the Hill equation using GraFit (*29*).

Characterization of the Product: Stereochemistry and Solvent Isotope Incorporation. A 665 μL sample of enzyme (35 μg) in 10 mM deuterated sodium phosphate buffer (pD 8.0) was placed in an NMR tube, followed by 35 μL of 100 mM UDP-GlcNAc. ^{1}H NMR spectra were acquired after 1.5 min, 4 min, and 2 days.

Catalytic Competence of 2-Acetamidoglucal. Acetamido-1,2-dideoxy-D-*arabino*-hex-1-enopyranose (2-acetamidoglucal) (*30*) was synthesized by published procedures. To two NMR tubes was added 665 μL of enzyme (170 μg) in 50 mM deuterated sodium phosphate buffer, followed by 17.5 μL of D_2O or 200 mM UDP in D_2O (final concentration of 5 mM). Reactions were initiated by the addition of 17.5 μL of 200 mM 2-acetamidoglucal in D_2O (final concentration of 5 mM), and ^{1}H NMR spectra were acquired after 4.5 h and 2 days. A third tube containing 5 mM 2-acetamidoglucal in 50 mM deuterated phosphate buffer served as a control.

Test for C−O versus P−O Bond Cleavage and Positional Isotope Exchange (PIX) Experiment. [1″-^{18}O]UDP-GlcNAc was prepared according to the method of Morgan *et al.* (*15*) The ^{1}H NMR spectrum was identical to that of the unlabeled compound. The extent of ^{18}O incorporation was determined to be 30% by mass spectrometry: −ESI-MS (H_2O) *m*/*z* 606 ($M - H^+$, ^{16}O, 100), 608 ($M - H^+$, ^{18}O, 40.6). The position of the ^{18}O label was confirmed by ^{31}P NMR spectroscopy: ^{31}P NMR (121.5 MHz, D_2O) δ −10.008 (d, J = 20.5 Hz, P_α), −11.714 (d, J = 20.5 Hz, P_β-^{16}O), −11.726 (d, J = 20.5 Hz, P_β-^{18}O).

A 700 μL solution of 10 mM [1″-^{18}O]UDP-GlcNAc in 20 mM deuterated sodium phosphate buffer (pD 8.0) was placed in an NMR tube, and Chelex 100 resin (∼20 mg, previously rinsed with D_2O) was added. A ^{31}P NMR spectrum was obtained using the following acquisition parameters: spectral frequency of 121.5 MHz, sweep width of 2437 Hz, acquisition time of 13.4 s, pulse delay of 2.0 s, and pulse width of 10 μs. The solution was removed from the tube, mixed with the lyophilized enzyme (340 μg; 20 mM sodium phosphate buffer), and returned to the tube. Reaction progress at 25 °C was monitored by ^{1}H and ^{31}P NMR spectroscopy. After ∼55% completion (30 min), the enzyme was inactivated by addition of 5 μL of 6 M HCl, followed by 30 μL of 1 M NaOH, and a ^{31}P NMR spectrum was acquired: ^{31}P NMR δ −4.559 (d, J = 22 Hz, P_β-^{16}O of UDP), −4.580 (d, J = 22 Hz, P_β-^{18}O of UDP), −9.099 (d, J = 22 Hz, P_α of UDP), −10.049 (d, J = 21 Hz, P_α of UDP-GlcNAc), −11.792 (d, J = 21 Hz, P_β-^{16}O of UDP-GlcNAc), −11.805 (d, J = 21 Hz, P_β-^{18}O of UDP-GlcNAc).

Kinetic Isotope Effect Studies. (i) KIE on k_{cat}. [2″-^{2}H]UDP-GlcNAc was prepared according to the procedure of Morgan *et al.* (*15*). The extent of ^{2}H incorporation was determined to be >97% by ^{1}H NMR spectroscopy and −ESI-MS. Rates of reaction were determined in triplicate for [2″-^{1}H]- and [2″-^{2}H]UDP-GlcNAc (both at 7.5 mM) using the continuous coupled assay described above.

(ii) KIE on k_{cat}/K_m. A 1:1 molar solution (700 μL total, 8.13 mM each) of [2″-^{1}H]- and [2″-^{2}H]UDP-GlcNAc in D_2O was prepared from stock solutions of 17.1 and 15.5 mM, respectively, as determined by A_{262}. Initial ^{1}H NMR (400 MHz) and −ESI-MS spectra were acquired. To a lyophilized sample of the enzyme [340 μg in 40 mM sodium phosphate buffer (pD 8.0)] was added a 665 μL portion of the substrate solution, and reaction progress was monitored by ^{1}H NMR spectroscopy. After 3.5 h (approximately 85% completion), 5 μL of 6 M HCl was added to inactivate the enzyme, followed by neutralization with 30 μL of 1 M NaOH. Final ^{1}H NMR and −ESI-MS spectra were acquired. The KIE on k_{cat}/K_m was determined from the initial and final spectra using the equation $KIE = \ln(1 - F_H)/\ln[(1 - F_H)R/R_0]$, where F_H is the fractional conversion of the protiated species to products and R_0 and R are the initial and final ratios of

protiated to deuterated substrate, respectively (*31*). F_H was calculated from the NMR spectra before and after addition of the enzyme by integration of the substrate H-1″ signals relative to the uracil H-6 signal. R_0 and R were calculated from the mass spectra before and after addition of the epimerase using the relative peak intensities of the protiated substrate [m/z 606 ($M - H^+$)] to the deuterated substrate [m/z 607 ($M - H^+$)], after correction for natural isotope abundance.

(iii) Solvent Isotope Discrimination during Glycal Hydration. A 2 mL sample of 50% D_2O was prepared by mixing 0.901 g of H_2O (0.500 mol) and 1.000 g of 99.9% D_2O (0.499 mol). This labeled water was used to reconstitute a sample of the lyophilized enzyme (340 μg, 1.33 mL; 20 mM sodium phosphate buffer) and to prepare stock solutions of 2-acetamidoglucal (200 mM), ManNAc (200 mM), and UDP (200 mM). The enzyme solution was divided into two 665 μL portions; to one were added 17.5 μL of UDP and 17.5 μL of ManNAc, and to the other were added 17.5 μL of UDP and 17.5 μL of glycal. The mixtures were incubated at 37 °C for 7.5 h, at which time ~1 mL of AG 1-X8 resin (Bio-Rad) was added to remove anionic species (i.e., UDP and phosphate buffer). The mixtures were filtered through glass wool, frozen, lyophilized, and submitted for mass spectral analysis (+ESI-MS). The ratio of [2-^{1}H]ManNAc [m/z 244 ($M + Na^+$)] to [2-^{2}H]ManNAc [m/z 245 ($M + Na^+$)] was calculated by the relative peak intensities, after correction for natural isotope abundance. The sample containing ManNAc as the starting sugar served as a control and showed no change, indicating the reaction is irreversible. The procedure described above was repeated using UDP-GlcNAc (35 μL of a 100 mM stock in 50% D_2O) as the substrate and with a 40 min period of incubation with the enzyme.

Site-Directed Mutagenesis and Assays of SiaA Mutants. Mutants were prepared according to the protocol of the QuikChange Site-Directed Mutagenesis Kit from Stratagene. Oligonucleotide pairs that were used to introduce mutations in the forward and reverse directions are listed below, with the mutated nucleotides underlined. Primers used for the D100N mutant were 5′-GGTCATGATTCACGGCAACC-GTTTAGAAGCACTAGC-3′ and (antiparallel) 5′-GCGC-CTGCTAGTGCTTCTAAACGGTTGCCGTG-3′. Primers used for the E122Q mutant were 5′-GCAGCCGTTTAG-TTTGCCATATCCAAGGTGGTG-3′ and (antiparallel) 5′-CTGTACCAGATAGTTCACCTTGGATATGGC-3′. Primers used for the D131N mutant were 5′-GGTGGTGAAC-TATCTGGTACAGTAAATGACTCCATTCG-3′ and (antiparallel) 5′-GTTTACTAATAGAATGACGAATGAAGT-CATTTACTGTACCAG-3′. All mutations were confirmed by sequencing the entire gene. Expression of the mutant plasmids and purification of the resulting mutant proteins were conducted using the protocol of the wild-type plasmid expression and protein purification.

Enzyme kinetics of the mutant proteins were determined by the spectrophotometric coupled assay described for the wild-type enzyme using 0.5 mg of protein per reaction. ^{1}H NMR spectroscopy was used to monitor the incubation of 2 mg of each mutant with either UDP-GlcNAc or 2-acetamidoglucal and UDP (all three at 5 mM) at 37 °C for up to 7 days.

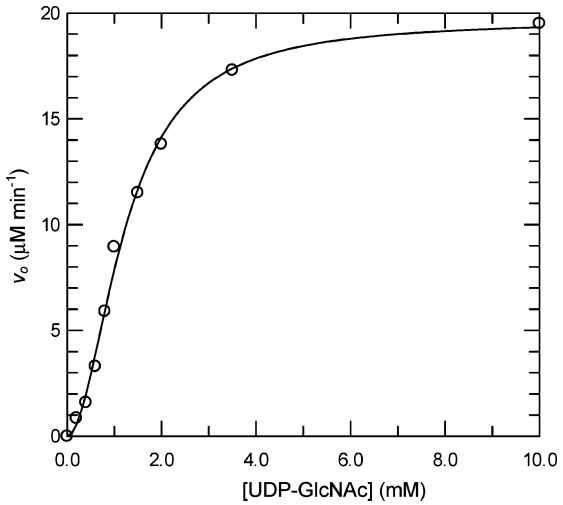

FIGURE 2: Enzyme kinetic plot of initial velocity vs substrate concentration. Kinetic parameters as determined by fitting the data to the Hill equation are as follows: $k_{cat} = 4.7 \pm 0.5$ s^{-1}, $K_{m,app} = 1.49 \pm 0.12$ mM, $k_{cat}/K_{m,app} = (3.2 \pm 0.4) \times 10^3$ M^{-1} s^{-1}, and $n_{app} = 1.94 \pm 0.14$.

RESULTS

Cloning, Overexpression, and Purification of SiaA. The *siaA* gene was cloned from *N. meningitidis* B genomic DNA using a ligation-independent cloning (LIC) method, which also introduced an N-terminal polyhistidine affinity purification tag. After verification of the plasmid insert by colony PCR and DNA sequencing, the recombinant protein was expressed in IPTG-induced *E. coli* BL21(DE3) cells and purified by affinity chromatography. Attempts to buffer-exchange and concentrate the enzyme by ultrafiltration resulted in a substantial loss of protein. Instead, the enzyme sample was dialyzed and lyophilized, at which point it could be stored at −20 °C without a significant loss of activity. SDS−PAGE was consistent with the expected molecular mass (47 kDa) and indicated a purity of >95%.

Identification of the Substrate and Products by NMR. The catalytic function of SiaA was initially determined by ^{1}H NMR assays with UDP-GlcNAc, GlcNAc, or GlcNAc 6-phosphate in a deuterated buffer. It was observed that within 30 min of incubation with the enzyme at 37 °C, only UDP-GlcNAc underwent reaction; in fact, neither GlcNAc nor GlcNAc 6-phosphate showed any degree of reaction after 2 days. The H-1″ signal of UDP-GlcNAc at 5.4 ppm had completely disappeared, while two new signals at 5.02 and 4.92 ppm appeared, consistent with the H-1 signals of the α- and β-anomers of ManNAc, respectively (*18*). No signal that could be attributed to 2-acetamidoglucal was observed during this process. A ^{31}P NMR spectrum also indicated the complete conversion from UDP-GlcNAc to free UDP.

These results established a functional link between SiaA and the mammalian hydrolyzing UDP-GlcNAc 2-epimerase. Accordingly, kinetic and mechanistic characterization of SiaA was undertaken following an approach similar to that with the mammalian enzyme (*18*).

Enzyme Kinetics As Determined by a Continuous Coupled Assay. To quantify the activity of SiaA kinetically, a coupled assay dependent on the production of UDP was employed (*28*). Initial reaction velocities (v) were plotted against UDP-GlcNAc concentration (Figure 2), and the kinetic data were fitted to the Hill equation to give values of 4.7 ± 0.5 s^{-1} for

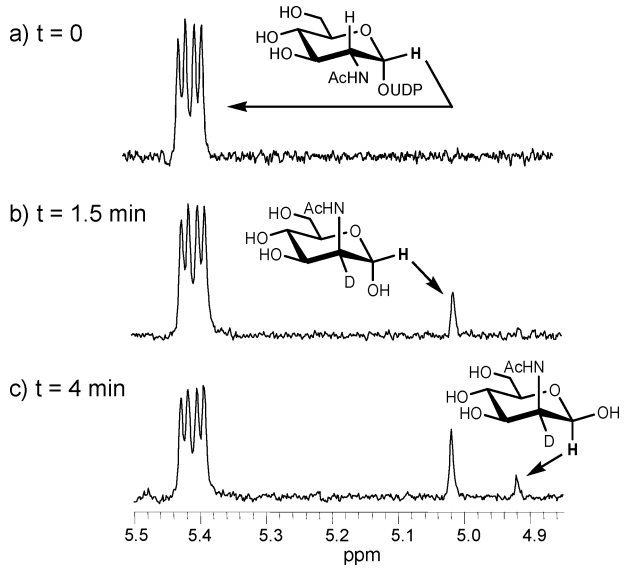

FIGURE 3: Enzymatic formation of α-[2-^{2}H]ManNAc in D_2O by ^{1}H NMR spectroscopy. (a) Before addition of enzyme. (b) Within the first 90 s, only the α-anomer was apparent, but (c) after 4 min, the *β*-anomer had begun to form as a result of nonenzymatic mutarotation. Signals from the anomeric protons (bold) are indicated.

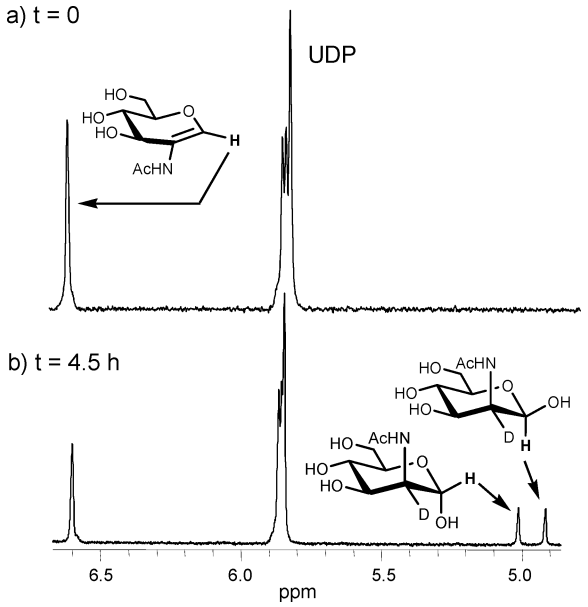

FIGURE 4: Enzymatic conversion of 2-acetamidoglucal to [2-^{2}H]-ManNAc in the presence of UDP. ^{1}H NMR spectra of the incubation of 2-acetamidoglucal and UDP with SiaA at (a) $t = 0$ and (b) $t =$ 4.5 h.

k_{cat}, 1.49 ± 0.12 mM for $K_{m,app}$, and 1.94 ± 0.14 for n_{app}. The curve's sigmoidicity indicates that SiaA is allosterically regulated by UDP-GlcNAc, and the value of the Hill coefficient is consistent with the presence of two substrate binding sites exhibiting strong cooperativity. This value compares to the Hill coefficient of the homologous non-hydrolyzing UDP-GlcNAc 2-epimerase (*15*, *32*). It is likely, therefore, that like that enzyme, SiaA is dimeric, with one substrate binding site per monomer (*16*).

Characterization of the Product: Stereochemistry and Solvent Isotope Incorporation. To establish the stereochemistry of the reaction, it was necessary to determine which anomer of ManNAc is the initially formed product. Complicating the analysis, however, was the fact that the two anomers readily, nonenzymatically interconvert via mutarotation, leading to an approximately equal proportion of each isomer. Because this process was found to be buffer-catalyzed, the buffer had to be diluted to 10 mM to minimize mutarotation. By monitoring the early stages of the epimerase reaction, the ^{1}H NMR spectrum revealed that the first-formed product in the enzymatic reaction is α-ManNAc (Figure 3), whose spectral assignment has been previously reported (*18*). As the reaction continued, mutarotation produced *β*-ManNAc, eventually leading to an equilibrium mixture of the two anomers. Thus, the stereochemistry of the reaction involves a net retention of configuration at C-1.

Furthermore, the NMR experiment also indicated that the reaction proceeds with >97% incorporation of solvent-derived deuterium at C-2. This finding was determined by the absence of H-2 signals from ManNAc (not shown) and by the appearance of the H-1 signals (Figure 3). The anomeric proton signal of UDP-GlcNAc appears as a doublet of doublets due to coupling with H-2″ and the *β*-phosphorus of UDP. When UDP departs and deuterium becomes incorporated at C-2, the anomeric proton signals appear as singlets due to negligible $J_{H1,D2}$ values and to the absence of phosphorus.

Catalytic Competence of 2-Acetamidoglucal. To assess the catalytic competence of the purported glycal intermediate of the hydrolyzing and nonhydrolyzing epimerase reactions, a synthetic sample of 2-acetamidoglucal (*30*) was incubated with SiaA. In the absence of UDP, no turnover was detected after 2 days at 37 °C; however, in the presence of 5 mM UDP, 37% conversion to ManNAc occurred within 4.5 h at 37 °C (Figure 4), as determined by the decrease in the singlet at 6.6 ppm and the concomitant appearance of two singlets at 5.02 and 4.92 ppm. Extended incubation (>2 days) led to the complete disappearance of the H-1 signal of 2-acetamidoglucal. These findings indicate that hydration of the glycal, although slower than the full reaction with the natural substrate, is a catalytically competent process.

Test for C−O versus P−O Bond Cleavage and Positional Isotope Exchange (PIX) Experiment. To determine if the generation of UDP proceeds with C−O or P−O bond cleavage, a sample of UDP-GlcNAc with an ^{18}O label at the "bridging" position between the anomeric carbon and *β*-phosphorus was chemically synthesized (*15*) and treated with the enzyme while the reaction was monitored by ^{31}P NMR spectroscopy (Figure 5a, inset). The presence of a heavier isotope attached to a phosphorus atom is known to cause a small upfield shift relative to that of the unlabeled compound (*33*). After incubation for 30 min at 25 °C, ^{1}H and ^{31}P NMR spectra indicated that the reaction had proceeded to 55% completion (Figure 5b, inset). The UDP product gave a pair of doublets for the *β*-phosphorus with a separation of 0.021 ppm, showing that the ^{18}O label had departed with UDP. Therefore, this confirms that the enzymatic reaction involves C−O bond cleavage.

Furthermore, this experiment also probes for positional isotope exchange (PIX) in the remaining substrate (*34*). The magnitude of the isotopic shift in a P−^{18}O bond is dependent on the bond order, increasing from ∼0.010 ppm for a bond order of 1 to ∼0.030 ppm for a bond order of 1.5 (*35*). Examination of the *β*-phosphorus signal of the initial UDP-GlcNAc (30% ^{18}O enrichment) revealed a pair of doublets

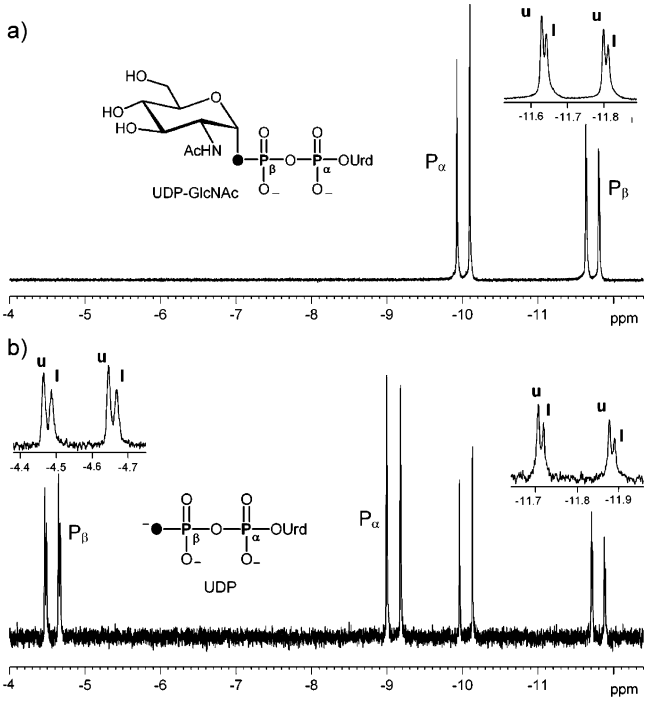

FIGURE 5: ^{31}P NMR spectra showing the conversion of [1″-^{18}O]-UDP-GlcNAc to [β-^{18}O]UDP by SiaA (a) before addition of the enzyme and (b) after 55% reaction completion (30 min). P_α and P_β signals of the pictured compounds are indicated. Signals due to unlabeled (u) and ^{18}O-labeled (l) species can be seen in the selected expansions. Urd is uridine.

separated by 0.012 ppm (Figure 5a), consistent with the label at the "bridging" position. If the label were to shift to the "nonbridging" positions, then a new peak would be expected to appear 0.029 ppm upfield from that of the unlabeled material due to the increased bond order. This had been observed in PIX experiments with the nonhydrolyzing epimerase (*15*). Inspection of the signal of the remaining UDP-GlcNAc after partial conversion (Figure 5b), however, showed no difference, indicating that scrambling had not occurred within the limits of detection (10% scrambling can be observed by this method).

Kinetic Isotope Effect (KIE) Studies. To explore the possibility of a KIE on k_{cat}, the reaction rates of the two isotopologues [2″-^{1}H]UDP-GlcNAc and [2″-^{2}H]UDP-GlcNAc were compared under saturating conditions. [2″-^{2}H]UDP-GlcNAc was prepared as previously reported (*15*), with >97% deuterium content at C-2″ as determined by mass spectrometry and ^{1}H NMR spectroscopy. Both species were independently applied to the kinetic assay described above at 7.5 mM, and from the mean of three measurements, the KIE on k_{cat}, ^DV, was found to be 1.15 ± 0.04. Hence, it is apparent that the C−H bond at the center of inversion is not broken during a rate-limiting step in the reaction.

Whereas the approach described above tests for ^DV by a direct comparison, the KIE on $k_{cat}/K_{m,app}$, $^D(V/K)$, can be determined by an intermolecular competition experiment. In this case, a mixture of two isotopologues of known isotopic composition is treated with the enzyme, and after a certain fractional conversion to products has occurred, the isotopic composition of the recovered starting material is determined. If a KIE exists, the faster reacting species containing the lighter isotope will be depleted to a greater extent than the slower species containing the heavier isotope. Accordingly, a mixture of the two isotopologues (deuterated:protiated ratio of 0.99) was incubated with the epimerase, and after 85% conversion to products, mass spectrometry revealed that the deuterated species had become enriched relative to the protiated species (ratio of 1.36). Following the calculations of Melander and Saunders (*31*), $^D(V/K)$ was determined to be 1.26 ± 0.15. Consistent with the value of ^DV, this indicates that cleavage of the C−H bond is not a rate-limiting step of the reaction.

To probe the nature of the glycal hydration step, a solvent isotope discrimination experiment was performed. Either UDP-GlcNAc or 2-acetamidoglucal/UDP was incubated with the enzyme in a 50:50 mixture of H_2O and D_2O, and the isotopic content at the C-2 position of the resulting ManNAc was analyzed. The irreversible nature of the SiaA-catalyzed formation of ManNAc was first established by the absence of isotope incorporation upon extended incubation of unlabeled ManNAc and UDP in 50% D_2O. Using the same preparation of solvent, UDP-GlcNAc as well as a mixture of UDP and 2-acetamidoglucal was separately incubated with SiaA, and the ManNAc product was analyzed by mass spectrometry. Only a slight preference for protium was determined in both cases (1.26 and 1.24, respectively), indicating that the level of solvent isotope discrimination was lower than what would be expected for a step that reflects a primary kinetic isotope effect disfavoring the transfer of deuterium. One scenario that could explain this result is that the isotope bound to the acidic active site residue does not exchange with solvent while the substrate is bound. Under saturating conditions, the nature of the isotope to be transferred would simply reflect an approximate fractionation factor for the residue [presumably close to unity for an oxygen acid (*36*)].

Site-Directed Mutagenesis and Assays of SiaA Mutants. The crystal structure of the homologous nonhydrolyzing epimerase from *E. coli* implicated three carboxylate residues in the active site that could be involved in the transfer of the proton at C-2 or stabilization of an oxocarbenium transition state (*16*). When mutations were introduced at these positions (D95N, E117Q, and E131Q) and the resulting proteins were assayed for activity, it was found that all three exhibited dramatically reduced activity (k_{cat} was >10^4 lower than that of the wild type) (*17*). To determine if the equivalent residues in SiaA are essential, corresponding mutant proteins (D100N, E122Q, and D131N) were also prepared and, using the spectrophotometric coupled assay described above, were found to be significantly catalytically compromised (k_{cat} reduced by >10^3). Instead, to the extent that it was not possible to determine accurate kinetics with the assay at hand, the incubation of UDP-GlcNAc with these mutants was monitored by ^{1}H NMR spectroscopy (Figure 6 for D131N). In all three cases, slow, partial conversion (Table 1) to ManNAc was observed by the appearance of singlets at 5.02 and 4.92 ppm (Figure 6c). Moreover, prior to the appearance of the ManNAc signals, a singlet at 6.59 ppm was detected with the E122Q and D131N mutants (Figure 6b). This signal corresponds to the H-1 proton of the 2-acetamidoglucal that has been prematurely released from the active site during catalysis. This is not seen with wild-type SiaA or the hydrolyzing epimerase from rat and has only been observed with the crippled homologue NeuC (*1*). These assays demonstrate that D100, E122, and D131 are

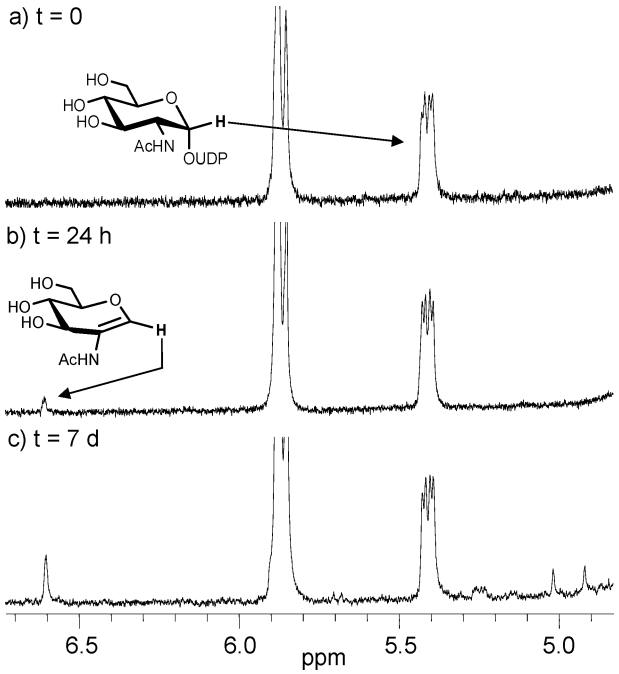

FIGURE 6: Conversion of UDP-GlcNAc to 2-acetamidoglucal and [2-^{2}H]ManNAc catalyzed by the D131N mutant. ^{1}H NMR spectra at (a) $t = 0$, (b) $t = 24$ h, and (c) $t = 7$ days.

Table 1: Extent of Reaction Catalyzed by Wild-Type and Mutant SiaA Proteins, As Determined by ^{1}H NMR Integrations

			% conversion to intermediate or product	
protein	substrate(s)	time	glycal	ManNAc[a]
wild type	UDP-GlcNAc	30 min	0	100
	glycal and UDP	4.5 h	N/A[b]	37
		2 days	N/A[b]	100
D100N	UDP-GlcNAc	24 h	0	0
		7 days	0	3.5
	glycal and UDP	7 days	N/A[b]	0
E122Q	UDP-GlcNAc	24 h	8.7	0
		7 days	14.4	7.2
	glycal and UDP	7 days	N/A[b]	7.4
D131N	UDP-GlcNAc	24 h	2.8	0
		7 days	8.8	11.3
	glycal and UDP	7 days	N/A[b]	9.6

[a] Sum of integrations from α- and β-anomers. [b] Not applicable.

crucial residues for efficient catalysis and provide direct evidence that 2-acetamidoglucal is an intermediate in the reaction.

DISCUSSION

Although ManNAc is known to be a direct precursor to Neu5Ac, which becomes polymerized and displayed as a capsule that surrounds certain strains of pathogenic bacteria, its source in the sialic acid biosynthetic pathway has been uncertain. Recent studies on the NeuC protein of *E. coli* K1 have indicated that ManNAc is generated from UDP-GlcNAc through the action of a hydrolyzing epimerase (*1*); however, since only minor amounts of ManNAc could be detected under the assay conditions, the full activity that is likely expressed *in vivo* remained to be shown. In this paper, we examine the activity and mechanism of the homologous protein, SiaA, isolated from *N. meningitidis* B, and draw comparisons to the mammalian hydrolyzing UDP-GlcNAc 2-epimerase.

Recombinant histidine-tagged SiaA was found to be a robust protein capable of surviving lyophilization without a significant loss of activity. When SiaA was incubated with GlcNAc, GlcNAc 6-phosphate, or UDP-GlcNAc and the reaction monitored by ^{1}H NMR spectroscopy, only the sugar nucleotide served as a substrate, leading to a mixture of ManNAc anomers and UDP. This discovery is at odds with the previous report by Petersen *et al.*, where they claimed SiaA interconverts GlcNAc 6-phosphate and ManNAc 6-phosphate (*2*). Because crude cell extracts were used in their studies, however, it is possible that they had detected background activity from a GlcNAc-6-phosphate 2-epimerase expressed by the host *E. coli* cells, a suggestion that has also been put forth by Ringenberg *et al.* (*37*). In contrast, our approach utilized the purified enzyme and allowed for the direct monitoring of substrate disappearance and product generation. Consistent with the homology of the sequence to that of NeuC, our findings clearly established that SiaA is a hydrolyzing UDP-GlcNAc 2-epimerase. The observation of ManNAc as the sole carbohydrate product, rather than 2-acetamidoglucal, however, marks a distinction from NeuC; thus, this work is the first to demonstrate the uncompromised activity of a bacterial hydrolyzing UDP-GlcNAc 2-epimerase that is functionally identical to the mammalian enzyme.

Kinetic determination of the activity of SiaA revealed that the enzyme is allosterically regulated by its substrate. The corresponding Hill coefficient of 1.9 is consistent with the existence of two UDP-GlcNAc binding sites exhibiting strong cooperativity, like the nonhydrolyzing epimerase from *E. coli* (*15*, *32*). The positive allostery displayed by SiaA is in contrast to that of the mammalian enzyme, which exhibits negative cooperativity ($n_{app} = 0.45$) (*12*). The mammalian epimerase is additionally feedback inhibited by another allosteric modulator, CMP-Neu5Ac, a product further along the sialic acid biosynthetic pathway. It has been suggested that these two sources of negative allostery are important in directing the fate of UDP-GlcNAc by ensuring that the amounts required for oligosaccharide biosynthesis are not diverted to sialic acid biosynthesis (*12*). In contrast, because SiaA displays positive allostery, at higher concentrations of UDP-GlcNAc, the synthesis of sialic acid is promoted. Hence, it is apparent that the sialic acid needs of *N. meningitidis* and other bacteria that synthesize a polysialic acid capsule differ from those of mammals.

The stereochemistry of the reaction was determined by monitoring it with ^{1}H NMR spectroscopy. That α-ManNAc was found to be the initially formed anomer indicates that the reaction proceeds with net retention of configuration at C-1 (Figure 1a), a characteristic that is shared with both the mammalian hydrolyzing and bacterial nonhydrolyzing epimerases. Furthermore, this experiment, conducted in D_2O, resulted in the incorporation of deuterium at C-2 in the ManNAc product, indicating that the stereoinversion involves deprotonation at C-2, followed by reprotonation in the opposite configuration with a solvent-derived proton. The lack of an observable internal return of the original protium of UDP-GlcNAc into the product is consistent with a two-base mechanism in which distinct enzymatic residues serve as the catalytic base and acid. Additionally, the H-1″ splitting pattern in the UDP-GlcNAc remaining after 75% conversion appeared to be unchanged from that of the initial material, indicating that no solvent-derived proton is incorporated into

the substrate. This suggests either that the deprotonation of the substrate involves an irreversible step or that the enzymatic residues in the two-base mechanism do not exchange protons with solvent during the lifetime of the intermediates. These findings are in agreement with the observations of Chou *et al*. for the mammalian enzyme (*18*).

Incubations of 2-acetamidoglucal with SiaA in the presence of UDP resulted in the slow generation of ManNAc, indicating that it is an alternative substrate that likely serves as an intermediate in the normal reaction. The decrease in reaction rate relative to that with UDP-GlcNAc as the substrate appears to suggest that the glycal is not kinetically competent to act as an intermediate. However, this may be attributed to slower binding of the glycal to either the free enzyme or the enzyme·UDP complex since these processes are not steps in the normal epimerase reaction. In comparison, the mammalian homologue had also been observed to catalyze the slow, partial turnover of 2-acetamidoglucal to ManNAc (6% in 29 h) and moderately enhanced conversion (12%) by the inclusion of UDP in the reaction mixture. However, the rate and extent of this reaction were much greater for SiaA than for the mammalian enzyme; in fact, extended incubation (>2 days) led to complete conversion to ManNAc. Also differing from the mammalian enzyme is the fact that UDP is absolutely obligatory for catalytic turnover; no reaction was observable in its absence, even after 2 days at 37 °C. This observation suggests that the hydration step may proceed while UDP is still bound in the epimerase's active site. It is even conceivable that UDP may serve as an acid/base catalyst during this step. Taken together with the observed stereochemistry of the reaction, one may conclude that SiaA catalyzes an *anti* elimination of UDP, followed by *syn* addition of water.

Further evidence that the hydrolyzing epimerase reaction proceeds via elimination of UDP was obtained by determining the fate of the ^{18}O label during the incubation of [1″-^{18}O]UDP-GlcNAc with SiaA. As expected, if 2-acetamidoglucal is generated in the process, the label is detected in the UDP product, indicating that the enzymatic reaction involves C−O rather than P−O bond cleavage. Moreover, inspection of the β-phosphorus signals of the remaining UDP-GlcNAc revealed no difference throughout the course of the reaction. If PIX had occurred within the limits of detection, then a new peak should have appeared upfield from the signal corresponding to the bridging position (i.e., approximately 0.029 ppm upfield from that of the unlabeled material). The lack of observed PIX suggests that once formed, UDP does not partition back to the pool of UDP-GlcNAc; in other words, the generation of UDP (or a prior step such as binding or a conformational change) is irreversible. Another possibility, however, is that within the confines of the epimerase's active site, interactions between enzymatic residues and the oxygens of the β-phosphate may impede rotation. Under this restriction, even though the reaction may be reversible, the same oxygen atom will be positioned to re-form the sugar−phosphate linkage. The fact that PIX can be observed with the homologous nonhydrolyzing epimerase (*15*), however, casts some doubt on this possibility.

The lack of a KIE on either k_{cat} or k_{cat}/K_m in the reaction of [2″-^2H]UDP-GlcNAc indicates that breakage of the C-2″−H bond is not a rate-limiting step in the reaction mechanism. Whereas a primary KIE of 1.8 on k_{cat} has been determined for the bacterial nonhydrolyzing epimerase (*15*), the unity value calculated for SiaA is consistent with the mammalian hydrolyzing epimerase (*18*), and therefore, several mechanistic implications are shared between the two enzymes. In light of the absence of PIX, the lack of a KIE on C−H bond cleavage or formation suggests that C−O bond breakage may be rate-limiting, a scenario that requires a stepwise, E1-type mechanism likely involving an oxocarbenium intermediate that is rapidly deprotonated to give the glycal. Alternatively, the lack of PIX may be indicative of restricted rotation of the β-phosphate in the enzyme-bound UDP. In this case, it is possible that a subsequent step such as product release or hydration is rate-limiting. A third possibility that cannot be excluded is the chance substrate binding or a conformational change prior to the first chemical step is rate-limiting.

The crystal structure of the nonhydrolyzing UDP-GlcNAc 2-epimerase shows that three carboxylate residues are located proximal to the hexose-binding region of the active site (*16*). Mutagenesis studies have shown that all three are essential for catalytic activity (*17*). These residues likely play key roles in acting as acid/base residues or in stabilizing transition states with considerable oxocarbenium ion character. In this work, mutation of the corresponding residues in the hydrolyzing enzyme, SiaA, also resulted in >1000-fold drops in k_{cat}, indicating that these three residues play similar roles in catalysis, allosteric regulation, or both. While it was not possible to kinetically characterize these dramatically impaired mutants, extended incubations monitored by NMR spectroscopy revealed important mechanistic information. In the case of both E122Q and D131N, 2-acetamidoglucal was observed as the first formed product and ManNAc appeared after only very long incubation times. The observed release of the proposed reaction intermediates provides direct evidence that the epimerase can catalyze an *anti* elimination reaction from UDP-GlcNAc. This nicely complements the observation that the enzyme can also catalyze the hydration of added 2-acetamidoglucal and suggests that 2-acetamidoglucal was not simply serving as an alternate substrate, as observed with several glycosidases (*38*). Since the wild-type hydrolyzing epimerases do not show any detectable release of intermediates, these mutants must have either a reduced affinity for the intermediates or a more "open" active site that allows them to escape before hydrolysis occurs. Alternatively, the E122Q and D131N mutations may have impaired the second step (hydration) more significantly than the first step, and the normal slow off-rate of the intermediates now competes with forward catalysis. The observation of intermediate release with E122Q mirrors the result obtained when the corresponding mutant (E117Q) of the nonhydrolyzing epimerase was examined (*17*). In that case, a larger ratio of intermediate release to product formation was interpreted as evidence that the mutation was interfering with the second step in catalysis (*syn* addition of UDP). It is also interesting to note that the "crippled" hydrolyzing epimerase, NeuC, formed 2-acetamidoglucal as the only significant product and bears a histidine residue at this normally conserved position (*1*).

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