Enzymatic Formation and Release of a Stable Glycal Intermediate: The Mechanism of the Reaction Catalyzed by UDP-*N*-Acetylglucosamine 2-Epimerase

Rafael F. Sala, Paul M. Morgan, and Martin E. Tanner*

Department of Chemistry
University of British Columbia, Vancouver
British Columbia V6T 1Z1, Canada

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The enzyme UDP-*N*-acetylglucosamine 2-epimerase catalyzes the interconversion of UDP-*N*-acetylglucosamine (UDP-GlcNAc) and UDP-*N*-acetylmannosamine (UDP-ManNAc) in both Gram-positive and Gram-negative bacteria (Figure 1).[1] This provides the bacteria with a source of activated ManNAc residues for use in the biosynthesis of cell wall surface polysaccharides.[2] This epimerase is unlike most known racemases and epimerases in that it must invert a stereogenic center that is not adjacent to an electron-withdrawing carbonyl or carboxylate group and therefore cannot employ a simple deprotonation−reprotonation reaction mechanism.[3] A previous report suggested that the enzyme overcomes this obstacle by transiently oxidizing the C-3 hydroxyl of the GlcNAc residue to a ketone, thus acidifying the proton at C-2.[4,5] Deprotonation at C-2, followed by reprotonation on the opposite face and finally reduction of the ketone, would produce the epimeric sugar nucleotide. In this communication we report evidence in favor of an alternative mechanism that proceeds via cleavage of the anomeric C−O bond, with 2-acetamidoglucal and UDP as enzyme-bound intermediates (Figure 1).[6] We have employed a positional isotope exchange (PIX) experiment[7] in which an ^{18}O label in the sugar−UDP bridging position (darkened atom in Figure 1) has been observed to scramble into nonbridging diphosphate positions during enzymatic epimerization. We have also demonstrated that the enzyme occasionally releases these relatively stable intermediates into solution.

The gene coding for the *Escherichia coli* UDP-GlcNAc 2-epimerase, known as *rff*E, had tentatively been assigned to an open reading frame, *o355*, near min 85 on the *E. coli* chromosome.[8] We have found that *rff*E is actually located 2.4 kb upstream of this sequence and has been designated nfrC in other work.[9] The nfrC gene product has been overexpressed and was reported to be a cytoplasmic protein of unknown activity that is required for bacteriophage N4 adsorption.[10] We have purified this protein to homogeneity and demonstrated that it is UDP-GlcNAc 2-epimerase.[11]

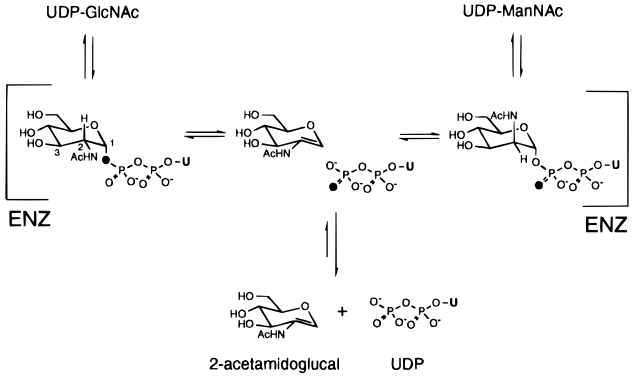

Figure 1. Proposed mechanistic scheme for the reactions catalyzed by UDP-GlcNAc 2-epimerase. Species within brackets are enzyme bound. U = uridine; darkened atoms indicate ^{18}O labels.

The epimerization of UDP-GlcNAc was monitored by ^1H NMR spectroscopy and ion-pair reversed-phase HPLC.[12] Both techniques showed the appearance of new signals attributable to UDP-ManNAc in the proper equilibrium ratio (10:1 in favor of UDP-GlcNAc).[13] Acidic hydrolysis of the equilibrated mixture produced a 10:1 ratio of GlcNAc:ManNAc identical with authentic samples.[14] The epimerization in D_2O was accompanied by the incorporation of deuterium into the C-2 position of both epimers. This had previously been observed with enzyme obtained from natural sources and supports a mechanism that ultimately involves proton transfer at C-2.[4]

The PIX experiment was performed on UDP-GlcNAc containing an 82% incorporation of ^{18}O label at the GlcNAc anomeric position.[15] This material was enzymatically epimerized in a deuterated phosphate buffer, and the reaction progress was followed by ^1H and ^{31}P NMR spectroscopies. The epimerization was allowed to proceed well past completion as indicated by the complete wash-in of deuterium at C-2. This ensured that each molecule of UDP-GlcNAc had been handled by the enzyme several times. The ^{31}P chemical shift was then

(1) (a) Kawamura, T.; Kimura, M.; Yamamori, S.; Ito, E. *J. Biol. Chem.* **1978**, *253*, 3595. (b) Kawamura, T.; Ishimoto, N.; Ito, E. *J. Biol. Chem.* **1979**, *254*, 8457. (c) Kawamura, T.; Ishimoto, N.; Ito, E. *Methods Enzymol.* **1982**, *83*, 515.

(2) (a) Lew, H. C.; Nikaido, H.; Mäkelä, P. H. *J. Bacteriol.* **1978**, *136*, 227. (b) Yoneyama, T.; Koike, Y.; Arakawa, H.; Yokoyama, K.; Sasaki, Y.; Kawamura, T.; Araki, Y.; Ito, E.; Takao, S. *J. Bacteriol.* **1982**, *149*, 15. (c) Harrington, C. R.; Baddiley, J. *Eur. J. Biochem.* **1985**, *153*, 639. (d) Kuhn, H.-M.; Meier-Dieter, U.; Mayer, H. *FEMS Microbiol. Rev.* **1988**, *54*, 195. (e) Meier-Dieter, U.; Starman, R.; Barr, K.; Mayer, H.; Rick, P. D. *J. Biol. Chem.* **1990**, *265*, 13490.

(3) Adams, E. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1976**, *44*, 69.

(4) Salo, W. L. *Biochim. Biophys. Acta* **1976**, *452*, 625.

(5) The mechanism necessarily invokes a tightly bound NAD^+ cofactor since no requirements for exogenous cofactors have been detected for this enzyme.[1] We have not been able to detect any bound cofactor using standard procedures: Palmer, J. L.; Abeles, R. H. *J. Biol. Chem.* **1979**, *254*, 1217.

(6) A 2-acetamidoglucal intermediate has previously been implicated in the mechanism of the mammalian enzyme that catalyzes both the inversion of configuration at C-2 and the loss of UDP to produce free ManNAc from UDP-GlcNAc: Sommar, K. M.; Ellis, D. B. *Biochim. Biophys. Acta* **1972**, *268*, 590.

(7) Raushel, F. M.; Villafranca, J. J. *CRC Crit. Rev. Biochem.* **1988**, *23*, 1.

(8) Daniels, D. L.; Plunkett, G., III; Burland, V.; Blattner, F. R. *Science* **1992**, *257*, 771. This assignment was based on homology arguments and on previous work that localized the gene onto a 4.9-kb fragment of DNA containing four open reading frames: Meier-Dieter, U.; Barr, K.; Starman, R.; Hatch, L.; Rick, P. D. *J. Biol. Chem.* **1992**, *267*, 746. The *o355* gene has subsequently been assigned to encode for dTDP-glucose-4,6-dehydratase: Macpherson, D. F.; Manning, P. A.; Morona, R. *Mol. Microbiol.* **1993**, *11*, 281. Stevenson, G.; Neal, B.; Liu, D.; Hobbs, M.; Packer, N. H.; Batley, M.; Redmond, J. W.; Lindquist, L.; Reeves, P. *J. Bacteriol.* **1994**, *176*, 4144.

(9) Kiino, D. R.; Rothman-Denes, L. B. *J. Bacteriol.* **1989**, *171*, 4595.

(10) Kiino, D. R.; Licudine, R.; Wilt, K.; Yang, D. H. C.; Rothman-Denes, L. B. *J. Bacteriol.* **1993**, *175*, 7074.

(11) The enzyme was purified by ion-exchange chromatography on a Waters Protein-Pak Q column in Tris-HCl buffer at pH 7.0 followed by a second column at pH 8.5. The protein displayed only a single band when analyzed by SDS−PAGE. Electrospray mass spectrometry results were consistent with the reported gene sequence: calcd, 42 246 Da; found, 42 254 Da.

(12) Meynial, I.; Paquet, V.; Combes, D. *Anal. Chem.* **1995**, *67*, 1627.

(13) UDP-ManNAc was eluted from a C-18 reversed-phase silica column with a shorter retention time than UDP-GlcNAc under the conditions described in ref 12. UDP-ManNAc displayed distinctive NMR signals at 5.40 ppm (anomeric proton, 0.06 ppm upfield of that of UDP-GlcNAc) and 1.99 ppm (methyl protons, 0.04 ppm upfield of those of UDP-GlcNAc).

(14) The hydrolysis conditions (0.06 N HCl, 100 °C, 15 min) are known not to cause epimerization.[1a] The sugars were found to have identical retention times to that of authentic standards when analyzed by HPLC using a Bio-Rad Aminex HPX-87H column with 13 mM H_2SO_4 as eluent (see ref 18).

(15) An ^{18}O label was introduced into the anomeric position of 3,4,6-tri-*O*-acetyl-GlcNAc by heating the sugar in 95%-enriched $H_2{}^{18}O/CH_3CN$. The α-dibenzylphosphate was then prepared by phosphitylation and oxidation: Sim, M. M.; Kondo, H.; Wong, C. H. *J. Am. Chem. Soc.* **1993**, *115*, 2260. Complete deprotection of the sugar followed by UMP−morpholidate coupling using standard conditions (Srivastava, G.; Alton, G.; Hindsgaul, O. *Carbohydr. Res.* **1990**, *207*, 259) provided UDP-GlcNAc containing an 82% enrichment of ^{18}O in the anomeric position.

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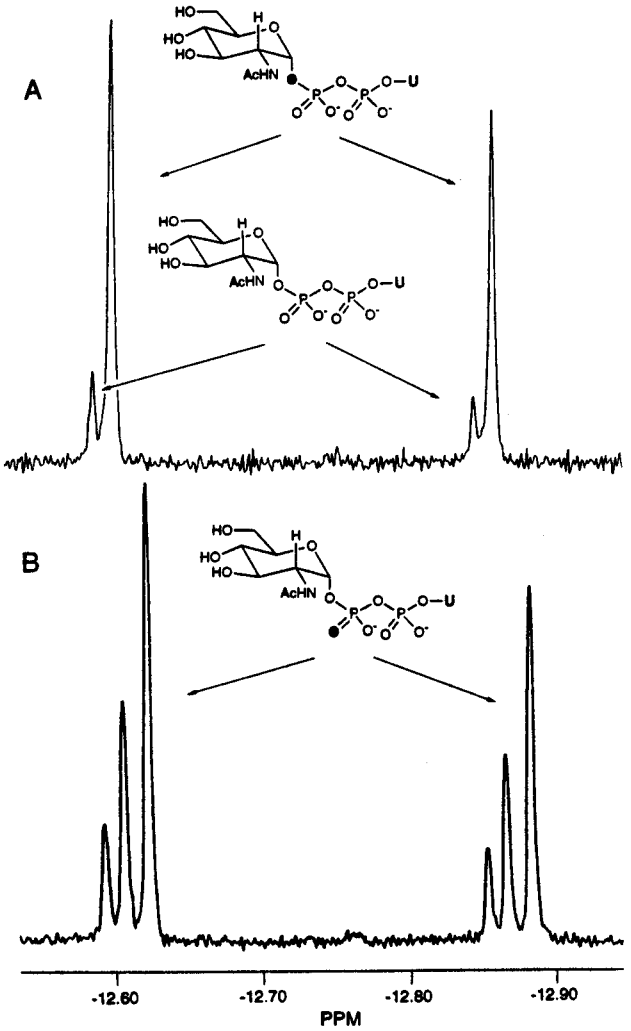

Figure 2. ^{31}P NMR spectra of the β-phosphorus of ^{18}O-labeled UDP-GlcNAc: (A) before and (B) after treatment with the epimerase. All peaks appear as doublets due to coupling to the adjacent α-phosphorus. U = uridine; darkened atoms indicate ^{18}O labels.

used to deduce the location of the ^{18}O label in the enzyme-treated UDP-GlcNAc. It is well established that substitution of ^{18}O-P for ^{16}O-P causes a small upfield chemical shift in the resulting ^{31}P signal and that the magnitude of this shift will be dependent on the O−P bond order.[16] Figure 2A shows the ^{31}P NMR signals for the β-phosphorus of the 82% ^{18}O-labeled UDP-GlcNAc before epimerization (the signals appear as doublets due to coupling to the adjacent α-phosphorus nucleus). The signal of the labeled material is shifted 0.013 ppm upfield from that of the unlabeled material, indicative of a P−^{18}O single bond.[16] Figure 2B shows the corresponding ^{31}P NMR signals following epimerization. A new signal has appeared 0.029 ppm upfield from that of the unlabeled material. It results from UDP-GlcNAc in which the ^{18}O label has scrambled into a nonbridging position with a P−^{18}O bond order greater than one. The 1:2 ratio of the upfield peaks reflects the statistical distribution expected for bridging versus nonbridging labels. A similar pattern is seen in the β-phosphorus signals of the minor UDP-ManNAc product (not shown in Figure 2).

The observation of deuterium incorporation at C-2 combined with C−O bond cleavage at the anomeric center leads us to suspect that an intermediate glycal is formed during the reaction (Figure 1). Further evidence for this mechanism was obtained upon extended incubation of UDP-GlcNAc with very high concentrations of enzyme.[17] The epimeric mixture of UDP-GlcNAc/UDP-ManNAc initially produced was observed to slowly convert to a 1:1 mixture of free 2-acetamidoglucal and UDP when monitored by HPLC. The glycal was isolated and gave an identical ^{1}H NMR spectrum to that of an independently synthesized sample.[18] No free GlcNAc or ManNAc was detected during this process, indicating that water is not added to the glycal at any significant rate. The external equilibrium constant between the two epimers and the released intermediates was shown to be >600 in favor of UDP and glycal as judged by integration of the HPLC peaks. We estimate that the intermediates are released approximately 1 in every 400 turnovers.[19]

The mechanism we propose involves a *trans*-elimination of monoprotic UDP to form 2-acetamidoglucal, followed by a *syn*-addition (in the UDP-GlcNAc to UDP-ManNAc direction). Occasionally the enzyme releases these relatively stable intermediates which accumulate in solution upon extended incubation. Both steps of the reaction could occur in either a concerted fashion or stepwise via oxonium intermediates. Neighboring group participation by either the acetamido or diphosphate functionalities and the formation of glycosyl-enzyme intermediates could be involved in this process. Further experiments are underway in order to distinguish between these possibilities.

Note Added in Proof: A recent paper that also identifies the correct position of the *rff*E gene has appeared: Marolda, C. L.; Valvano, M. A. *J. Bacteriol.* **1995**, *177*, 5539.

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(16) (a) Cohn, M.; Hu, A. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 200. (b) Singh, A. N.; Hester, L. S.; Raushel, F. M. *J. Biol. Chem.* **1987**, *262*, 2554.

(17) In a typical experiment, 0.08 mg of epimerase was incubated in 0.1 mL of pH 8.5 phosphate buffer containing 0.6 mM UDP-GlcNAc at 37 °C. No glycal formation (or epimerization) was observed using heat-killed enzyme.

(18) Lai, E. C. K.; Withers, S. G. *Biochemistry* **1994**, *33*, 14743.

(19) This was based on the ratio of the reported[1c] specific activity of 7 units/mg for the formation of UDP-ManNAc from UDP-GlcNAc and a measured specific activity of 0.018 units/mg for the formation of UDP and 2-acetamidoglucal from an equilibrating pool of UDP-ManNAc/UDP-GlcNAc (as determined by HPLC analysis of the initial rate of formation of UDP).