Positional Isotope Exchange Catalyzed by UDP-Galactopyranose Mutase

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The unusual sugar galactofuranose (gal*f*) is a component of several pharmacologically interesting macromolecular structures in microorganisms. These include bacterial O-antigens, mycobacterial cell walls, and protozoal cell membrane proteins.[1] The activated precursor of gal*f*, UDP-gal*f*, is synthesized from UDP-galactopyranose (UDP-gal*p*) in an unprecedented reaction catalyzed by the enzyme UDP-gal*p* mutase (Figure 1). Recombinant UDP-gal*p* mutase from several bacterial sources has been purified to homogeneity and biochemically characterized.[2] The structure of the product UDP-gal*f* has been confirmed by NMR spectroscopy.[2a,c] However, little mechanistic information for this reaction has been reported in the literature.[3] Intriguingly, flavin adenine nicotinamide (FAD) is noncovalently bound to UDP-gal*p* mutase. Also, the reduced form of nicotinamide adenine dinucleotide (NAD(P)H) has been reported to be required for UDP-gal*p* turnover. Mechanistic roles for these redox cofactors have yet to be determined. We report here a method to follow the course of positional isotope exchange (PIX),[4] which demonstrates that the anomeric C−O bond is broken during UDP-gal*p*/UDP-gal*f* interconversion, in a kinetically competent step.

Traditionally, PIX experiments have used ^{31}P NMR spectroscopy to detect the shift in ^{31}P resonance that occurs upon positional exchange of bridging and nonbridging ^{18}O atoms.[5] Continuous monitoring of the PIX reaction is difficult because of metal-induced and proton-induced line broadening.[4] Typically, it is necessary to quench, filter, and add EDTA to aliquots of the PIX reaction to observe the isotope shifts. The PIX experiment presented here used ^{13}C NMR to follow PIX continuously. UDP-gal*p* enriched in ^{13}C (99 atom % excess) at the anomeric carbon (C1) and containing ca. 85 atom % excess ^{18}O in the bridge position between C1 and the β-phosphate (**1**) was synthesized.[6] The ^{13}C NMR spectrum of **1** appeared as a doublet ($J_{CP} = 6.7$
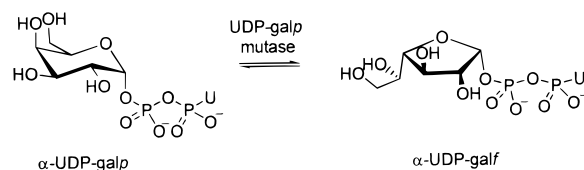
Figure 1. Reaction catalyzed by UDP-galactopyranose mutase. U = uridine
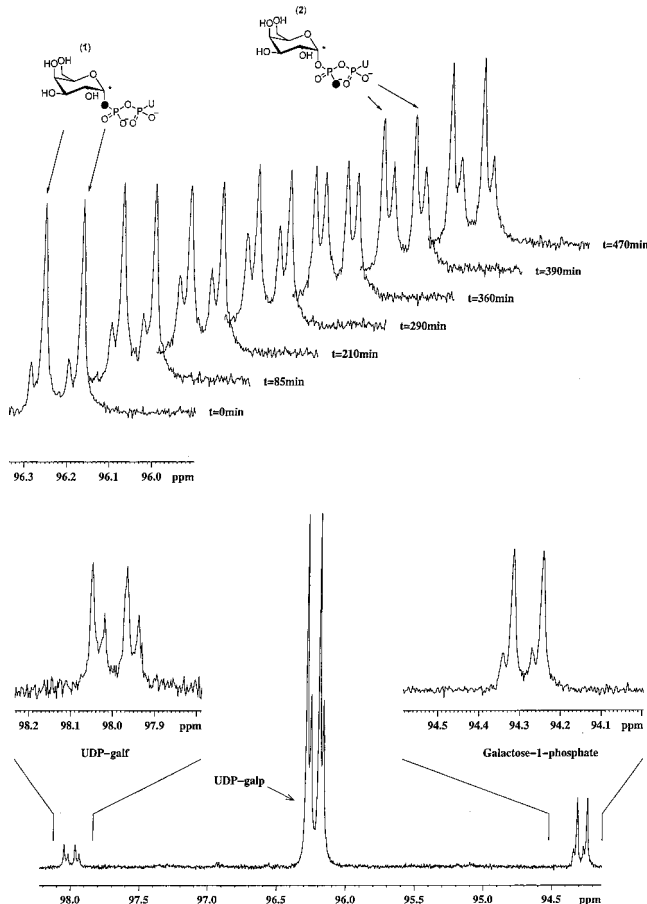
Figure 2. (a)^{13}C NMR time course of the reaction of **1** with UDP-gal*p* mutase. U = uridine; darkened atoms indicate ^{18}O labels. Method: PIX was initiated by the addition of UDP-gal*p* mutase (final concentration = 15 μM) to a solution of **1** (25 mM), DTT (1 mM), FAD (0.1 mM), Tris (20 mM), AMPSO (20 mM), and D$_2$O (10%). The final volume was 350 μL. PIX carried out at pH 7.5 and 25 °C in a 5 mm symmetrical microtube matched with D$_2$O (Shigemi). ^{13}C NMR spectra (proton decoupled) were recorded on a Bruker DRX 300 spectrometer. Acquisition time = 2.75 s; number of scans = 200 (15 min); spectral width = 23.8 kHz. No window function was applied prior to Fourier transformation and phasing. (b) ^{13}C NMR spectrum of PIX reaction at $t = 15$ h.

Hz) arising from splitting by the β-phosphorus (Figure 2a, $t = 0$ min). A second resonance, shifted 0.03 ppm downfield from that of **1**, corresponded to UDP-gal*p* with ^{16}O in the bridge position. The ratio of (^{16}O)-UDP-gal*p*/(^{18}O)-UDP-gal*p* peak areas was 1/5. Changes in ^{13}C chemical shifts upon isotopic substitution have been well documented.[7] In this experiment, the isotopic shift was used to detect cleavage of the anomeric C−O bond and subsequent scrambling of the ^{18}O from the bridging position into the two

(1) (a) Whitfield, C. *Trends Microbiol.* **1995**, *3*, 178. (b) Brennan, P. J.; Nikaido, H. *Annu. Rev. Biochem.* **1995**, *64*, 29. (c) de Lederkremer, R. M.; Colli, W. *Glycobiology* **1995**, *5*, 547.
(2) (a) Köplin, R.; Brisson, J.-R.; Whitfield, C. *J. Biol. Chem.* **1997**, *272*, 4121. (b) Nassau P. M.; Martin, S. L.; Brown, R. E.; Weston, A.; Monsey, D.; McNeil, M. R.; Duncan, K. *J. Bacteriol.* **1996**, *178*, 1047. (c) Lee, R.; Monsey, D.; Weston, A.; Duncan, K.; Rithner, C.; McNeil, M. *Anal. Biochem.* **1996**, *242*, 1. (d) McMahon, S. A.; Leonard, G. A.; Buchanan, L. V.; Giraud, M.-F.; Naismith, J. H. *Acta Crystallogr.* **1999**, *D55*, 399
(3) Barlow, J. N.; Marcinkeviciene, J.; Blanchard, J. S. In *Enzymatic Mechanisms*; Frey, P. A., Northrop, D. B., Eds.; IOS Press, 1999; p 98.
(4) Raushel, F. M.; Villafranca, J. J. *CRC Crit. Rev. Biochem.* **1988**, *23*, 1.
(5) (a) Villafranca, J. J. *Methods Enzymol.* **1989**, *177*, 390 (b) Sala, R. F.; Morgan, P. M.; Tanner, M. E. *J. Am. Chem. Soc.* **1996**, *118*, 3033.
(6) **1** was synthesised enzymatically from D-galactose-1-(^{13}C) using a protocol similar to those used previously in the synthesis of other UDP-galactose derivatives: (a) Thiem, J.; Wiemann, T. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1163. (b) Hayashi, T.; Murray, B. T.; Wang, R.; Wong, C.-H. *Bioorg. Med. Chem.* **1997**, *5*, 497. ^{18}O was introduced into the anomeric position of D-galactose-1-(^{13}C) by incubation in H$_2$^{18}O for 2 days at 55 °C (Risley, J. M.; Van Etten, R. L. *Biochemistry* **1982**, *21*, 6360). Phosphorylation at the anomeric position by galactokinase and Mg·ATP gave D-α-galactose-1-(^{13}C)-1-(^{18}O)-1-phosphate and uridylation by galactose-1-phosphate-uridyltransferase and UDP-glucose to give **1** was performed in a one-pot reaction. **1** was purified by Mono-Q anion-exchange and P-2 biogel fast performance liquid chromatography (FPLC). Mass analysis by electrospray ionization mass spectrometry (negative mode) gave a mass of 568.0 Da (predicted mass for monoanion = 568.1 Da).
(7) (a) Risley, J. M.; Van Etten, R. L. *Methods Enzymol.* **1989**, *177*, 376. (b) Risley, J. M.; Kuo, F.; Van Etten, R. L. *J. Am. Chem. Soc.* **1983**, *105*, 1647.
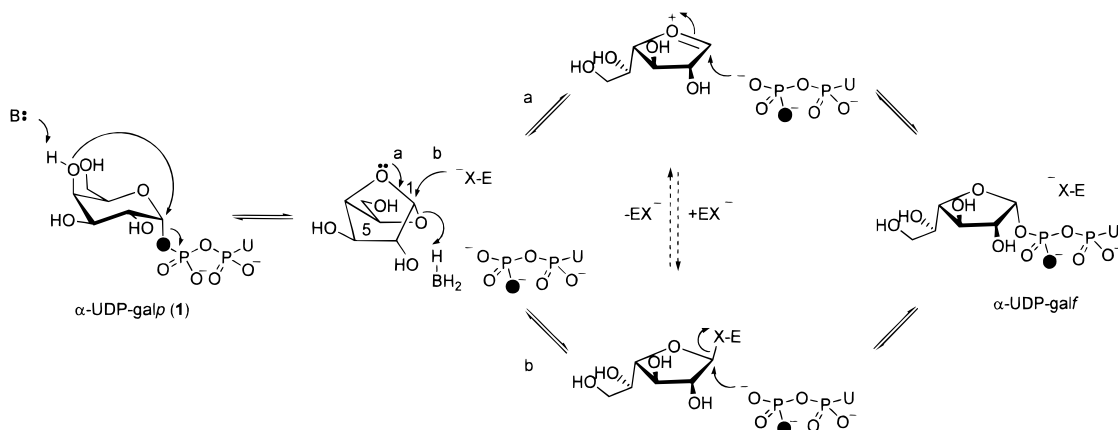
Figure 3. Hypothetical mechanism for UDP-gal*p* mutase based on PIX data. U = uridine; darkened atoms indicate ^{18}O labels; EX = enzyme-based nucleophile.

nonbridging phosphate positions during enzymatic turnover of **1**. The low extent of UDP-gal*p* conversion into UDP-gal*f* by UDP-gal*p* mutase (ca. 7% at equilibrium) precluded the continuous monitoring of the formation of UDP-gal*f* by ^{13}C NMR. Instead, UDP-gal*p* (**2**) formation was monitored in the thermodynamically favorable back reaction during approach to equilibrium. A small amount of contaminating galactose-1-phosphate, an intermediate in the synthesis of **1**, served as an internal control for nonspecific scrambling. This signal also consisted of a pair of doublets, corresponding to ^{18}O-bridged and ^{16}O-bridged galactose-1-phosphate. The isotopic ratio of ^{16}O:^{18}O in the galactose-1-phosphate was also 1/5.

Upon addition of UDP-gal*p* mutase[8] to **1**, the ratio of ^{16}O:^{18}O bridged UDP-gal*p* increased, reaching a maximum of 2:1 after 7 h (Figure 2a). This ratio is that expected statistically from scrambling of the ^{18}O between the single bridging and two nonbridging diphosphate positions of **1**. No change in the ^{16}O/^{18}O isotopic ratio of galactose-1-phosphate was observed (Figure 2b). UDP-gal*f* formation was simultaneously monitored by withdrawing aliquots from the reaction at regular intervals for analysis by ion-exchange HPLC.[9] Attainment of equilibrium between UDP-gal*p* and UDP-gal*f* was coincident with the PIX equilibrium. The ^{13}C NMR spectrum of UDP-gal*f*, taken at equilibrium, displayed the same 2:1 ratio of ^{16}O:^{18}O peaks as that displayed by UDP-gal*p* (Figure 2b), suggesting that cleavage of

the anomeric C−O bond during UDP-gal*p*/UDP-gal*f* turnover is required for product formation.

In view of the results presented in this study, mechanistic possibilities for the unprecedented UDP-gal*p* mutase reaction can be restricted to those that involve the cleavage of the anomeric C1−O bond of the nucleotide sugar. A hypothetical mechanism is shown in Figure 3. In the first step, the anomeric C1−O bond is broken in a reaction drawn here to involve the direct nucleophilic attack of the axial 4′-hydroxyl group on C1, displacing UDP and generating a bicyclo acetal. The β-phosphorus atom of enzyme-bound UDP is torsionally unrestricted, and the oxygen atoms are torsiosymmetric and scramble. In the second step, bond cleavage between the ring oxygen O-5 and C1 must take place since the α-anomer is formed as the sole product,[2a,c] and direct nucleophilic attack by UDP on the anomeric center of the bicyclo acetal would generate β-UDPgal*f*. This step may proceed either with anchimeric assistance (pathway a) or via an S$_N$2 displacement involving an enzyme-based nucleophile (pathway b). In the third step, nucleophilic attack by UDP on the anomeric C1 position generates the product α-UDP-gal*f*. This simple mechanistic proposal does not invoke redox transformations involving the enzyme-bound flavin, or the effects of exogenously added reduced pyridine nucleotides.

(8) Recombinant UDP-gal*p* mutase from *Klebsiella pneumoniae* was purified from *E. coli*. Details of the cloning, expression, and purification of the enzyme will be published separately.

(9) The method from ref 2c was used.