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1,4-Anhydrogalactopyranose is not an intermediate of the mutase catalyzed UDP-galactopyranose/furanose interconversion

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Abstract—UDP-galactopyranose mutase (UGM) catalyzes the isomerization of UDP-galacto*pyranose* (UDP-Gal*p*) into UDP-galacto*furanose* (UDP-Gal*f*), an essential step of the mycobacterial cell wall biosynthesis. The first mechanistic assumption proposed in the literature was the involvement of 1,4-anhydrogalactose **1** as intermediate of this ring contraction. To confirm or rule out this hypothesis, we synthesized **1** and engaged it in reactions with UGM. The expected formations of UDP-Gal*f* and UDP-Gal*p* were never observed, thus showing that **1** is not, in fact, a low energy intermediate of this enzymatic contraction.

Oligo-galacto*furanosides* (Gal*f*) are essential glycoconjugates of all mycobacteria including severe pathogens such as *Mycobacterium tuberculosis*.[1–4] The search for the biosynthetic origin of the Gal*f* residues has led to the discovery of an unusual enzymatic ring contraction: the interconversion of UDP-galactopyranose (UDP-Gal*p*) into UDP-galactofuranose (UDP-Gal*f*), the universal Gal*f* donor for all oligo-galactofuranosides. This ring contraction is catalyzed by a flavoenzyme, UDP-galactopyranose mutase (UGM), whose mechanism has recently attracted much attention. Such an enzymatic isomerization is a key biocatalytic process not only because it is essential for the survival of mycobacteria,[5] but also because its mechanism addresses questions that are at the heart of the enzymology and the chemistry of carbohydrates: (i) what is the transient mode of activation of the anomeric center? (ii) what is the conformational itinerary of the glycoside throughout its transformation? So far, several mechanisms have been proposed: the involvement of 1,4-anhydrogalactopyranose **1** as intermediate,[6,7] a covalent catalysis[8] with the FAD cofactor playing the role of nucleophile[9,10] as well as single electron transfers from the FAD to generate an anomeric radical.[11] From the different mechanistic investigations published to date, several intermediates have been proposed. The inhibition of UGM by fluorinated substrate analogues, under non-reducing conditions, suggested that oxycarbenium **A** and **B** can reasonably be invoked as high-energy intermediates (Fig. 1).[7] To date, the anomeric radicals corresponding to **A** and **B** cannot be ruled out from this mechanistic scheme, given the presence of the FAD cofactor. Moreover, it was recently found that performing this enzymatic reaction in the presence of $NaBH_3CN$ led to the isolation of a covalent adduct between FAD and the galactose residue: this experiment showed that an iminium such as **C** could also be implied as a high-energy intermediate.[10] The constrained acetal **1** has been proposed as a potential low-energy intermediate.[6,7] This hypothesis was strengthened by the fact that protected 1,4-anhydrogalactopyranoses can chemically undergo a regiospecific acetal opening leading to galactofuranosides.[12] In theory, a reversible transformation of intermediate **1** and iminium **C** is also possible (Fig. 1). On the one hand, the hypothesis of **1** as intermediate satisfies the requirement, for this transformation, that oxygens O-4 and O-5 of the galactose residue are in close proximity the anomeric position, thus explaining, conformationally, a pyranose/furanose interconversion. On the other hand, given the presence of the flavin cofactor, direct S_N1-like substitutions involving **1** and UDP seem rather simplistic. In order to confirm or rule

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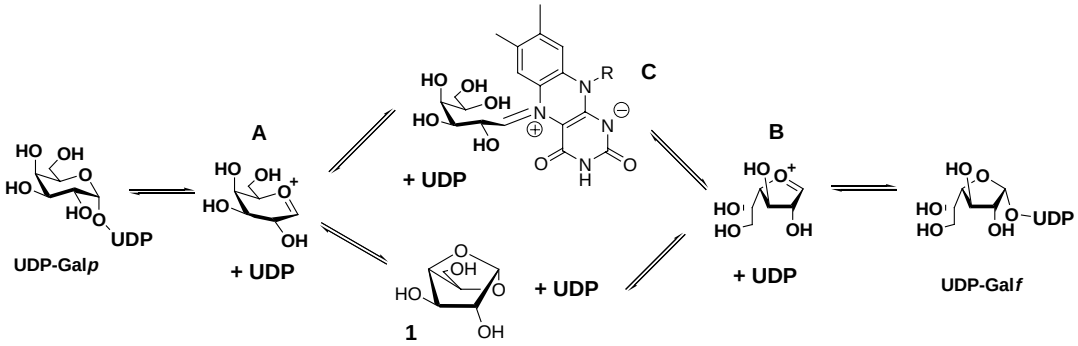
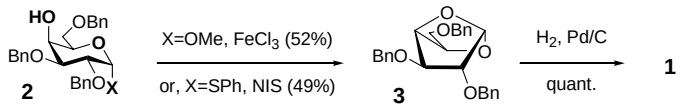


Figure 1. Putative mechanistic pathways for the UGM catalyzed pyranose/furanose interconversion.



Scheme 1. Synthesis of 1,4-anhydrogalactopyranose **1**.

out the hypothesis of **1** as intermediate of this enzymatic process, we synthesized this molecule and engaged it in reactions with both UGM and UDP. The principle of this experiment is straightforward: if UGM is forced to bind to **1** and UDP, then UDP-Gal*f* and UDP-Gal*p* should be produced if the mechanism follows a pathway involving **1** as an intermediate.

Tri-*O*-benzyl-1,4-anhydrogalactopyranose **3** could be synthesized from galactoside **2** either from a known procedure using $FeCl_3$ as Lewis acid[12,13] or by an intramolecular acetalation using NIS as promoter (Scheme 1). Intermediate **3** was then hydrogenolyzed in the presence of Pd/C to give **1** in quantitative yield. Analytical data of molecule **1** were consistent with a $^{1,4}B$ boat-locked galactoside.[†]

None of the reaction conditions depicted in Figure 2 lead to the formation of UDP-Gal*p* and/or UDP-Gal*f*.[‡] These reactions could be followed by analytical HPLC since UDP, UDP-Gal*p*, and UDP-Gal*f* are easily separated on standard C-18 columns.[14,15] For each reaction we systematically controlled that: (i) UGM was still active during all the reaction time (ii) UDP and the constrained acetal **1** were not hydrolyzed or chemically modified. For all reaction conditions, we performed two control experiments: (i) the same experiment, with UDP and UGM, but without **1** (no decomposition of UDP followed by HPLC), and (ii) UGM alone to control its activity (by addition of UDP-Gal*f*). The stability of **1**, at pH 7.3, was assessed by ^{1}H NMR in D_2O: **1** was found absolutely unchanged after several days under these conditions. Moreover, we paid attention to the fact that uncharged monosaccharides usually display low binding affinities to glycosyl processing enzymes. The concentration in constrained acetal **1** was thus comprised between 500 μM and 5 mM. On the other hand, UDP possesses a strong binding affinity to UGM: the concentrations in UDP (from 200 μM to 1 mM) were therefore chosen to insure that **1** was in excess compared to UDP. This trend is very general for proteins binding to nucleoside-diphospho-sugars (NDP-sugars): for instance, glycosyltransferases are usually inhibited, in the micromolar range, by the diphosphonucleoside (NDP) corresponding to their substrates but not by their corresponding free sugar.[16] As expected, UGM is inhibited by UDP in the micromolar range (IC_{50} = 200 μM),[4] thus showing that UGM displays a similar binding behavior than glycosyltransferases: most of the binding strength between the nucleotide-sugar and the protein comes from the charged nucleoside-diphosphate, while the sugar insures binding selectivity. It is now well established that the binding of uncharged monosaccharides to proteins usually requires concentrations of the carbohydrate in the mM range, which is the case for the glycosidases,[17] the lectins,[18] and the glycosyltransferases.[16] Therefore, we believe that in the experiments depicted in Figure 2, concentrations up to 5 mM in **1** were high enough to insure its binding to the galactose binding site of UGM.

In addition, all reactions were performed under native (oxidized) and reducing conditions (upon sodium dithionite addition) at high enzyme concentration (up to 4 μM). At such concentrations, UGM completes the conversion of UDP-Gal*f* into UDP-Gal*p* within 1 min. In case the reaction between **1** and UDP would be slowed down by the presence of UDP ('substrate' inhibition), the incubation times depicted in Figure 2 were 3 h under reducing conditions and several days under non-reducing conditions. As a matter of fact, both the kinetic (k_{cat}) and the binding properties of UGM (K_d, K_i) strongly depend on the redox state of the flavin

[†] Analytical data of acetal **1**: ^{1}H NMR (400 MHz, D_2O/CD_3OD 6/1) δ 5.42 (br s, 1H, H-1), 4.70 (dd, $J_{4\text{-}5}$ = 1.2 Hz, $J_{4\text{-}3}$ = 3.4 Hz, 1H, H-4), 4.60 (dd, $J_{4\text{-}3}$ = 3.4 Hz, $J_{3\text{-}2}$ = 2.5 Hz, 1H, H-3), 4.15–4.05 (m, 3H, H-5 H-6), 3.84 (d, $J_{3\text{-}2}$ = 2.5 Hz, 1H, H-2); ^{13}C NMR (100 MHz, D_2O/CD_3OD 6/1) δ 107.4 (C-1), 82.1 (d, C-3), 81.3 (C-5), 81.0 (C-2), 79.6 (C-4), 61.7 (C-6); MS (CI^+-NH_3): *m/z* 180 [$M+NH_4^+$]; HRMS for $C_6H_{14}O_5N$: calcd 180.0872, obsd 180.0869.

[‡] The procedures for the expression, purification of UGM, and enzymatic reactions were the same as in our previous studies.[14,15]

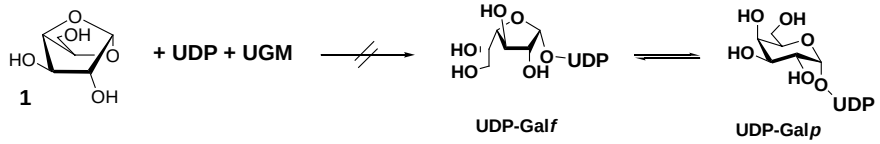


Figure 2. Conditions of the enzymatic assays: pH 7.3, $T = 21$ °C, reducing ($[Na_2SO_4] = 20$ mM, reaction times up to 3 h) and non-reducing conditions (reaction times up to 5 days), [UDP] 200 μM–1 mM, [**1**] 500 μM–5 M, [UGM] 40 nM–4 μM.

cofactor.[19] The K_m values of UDP-Gal*f* are 20 μM and 190 μM under reducing or non-reducing (native) conditions, respectively. UGM is thus much more kinetically competent upon dithionite addition. Moreover, the binding modes of nucleotide-sugars with UGM have also been shown to vary when the flavin is reduced.[14,20] Under both conditions (reducing or not), we never observed the formation of UDP-galactose when UGM was incubated with **1** and UDP.

Very recently, Thomas and co-workers described the preparation of a synthetically challenging analogue of **1** (a methylene moiety replacing O-5).[21] This ether was not found to display competitive inhibition against UGM. However, this inhibition study was performed at low concentrations of this analogue (10–100 μM), a concentration range where uncharged monosaccharides usually do not bind glycosyl-processing enzymes. In a more general perspective, if acetal **1** were a low-energy intermediate of this enzymatic reaction, the UGM catalytic pocket should display some affinity and, at least, a specific reactivity with **1**. Our experiments show the contrary.

In conclusion, the involvement of **1** as a potential intermediate of the enzymatic UDP-galactopyranose/furanose interconversion was the very first mechanistic hypothesis published in the literature. The experiments described here strongly suggest that this assumption is not reasonable. However, these results do not rule out the fact that transient conformations of the galactose moiety, throughout the furanose/pyranose interconversion, are close to a $^{1,4}B$ boat as suggested by our previous experiments.[14] Indeed, the conformations of the cyclic or acyclic high-energy intermediates in complex with UGM are also playing an important role in the catalytic process. Therefore, the conformational itinerary of this carbohydrate during its isomerization has still to be discovered but an important putative intermediate is now excluded from this specific biocatalytic scheme.

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