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Probing UDP-galactopyranose mutase binding pocket: A dramatic effect on substitution of the 6-position of UDP-galactofuranose

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ABSTRACT

UDP-galactopyranose mutase (UGM) catalyzes the isomerization of UDP-galactopyranose (UDP-Gal*p*) into UDP-galactofuranose (UDP-Gal*f*), an essential step of the mycobacterial cell wall biosynthesis. UDP-(6-deoxy-6-fluoro)-D-galactofuranose **1** was tested as substrate of UGM. Turnover could be observed by HPLC. The k_{cat} (7.4 s^{-1}) and the K_m (24 mM) of **1** were thus measured and compared with those of UDP-Gal*f* and other fluorinated analogs. The presence of the fluorine atom at the 6-position had a moderate effect on the rate of the reaction but a huge one on the interactions between the enzyme and its substrate. This result demonstrated that key interactions occur at the vicinity of the 6-position of UDP-galactose in the Michaelis complex.

Oligo-galactofuranosides (Gal*f*) are essential glycoconjugates of all mycobacteria including severe pathogens such as *Mycobacterium tuberculosis*.[1] The search for the biosynthetic origin of the Gal*f* residues has lead 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. In addition, this enzymatic isomerization is a key biocatalytic process because it is essential for the survival of mycobacteria.[2] For instance, lipidic galactofuranosides were recently found to be in vitro inhibitors of mycobacterial growth.[3]

Beyond its medicinal relevance, UGM displays fascinating biocatalytic properties as well. First of all, the role of its FAD cofactor has been the subject of intense investigations mainly designed to elucidate whether redox chemistry[4,5] was occurring during the catalytic cycle and/or to demonstrate its involvement as a catalytic nucleophile.[6] Compared to glycosidases and glycosyl transferases, the two major classes of carbohydrate processing enzymes, the way UGM activates the anomeric position of its substrate is clearly unique and full of surprises.

Several types of molecular probes have been exploited to unveil the intimate mechanism of this ring contraction: transition state (or high energy intermediates) analogs,[7–9] time-dependent inactivators,[10,11] conformationally constrained[9,12,13] and acyclic molecules.[14,15]

Among them, several fluorinated analogs of UDP-galactose have been synthesized both in the pyranose[16,17] and the furanose series.[10] As a matter of fact, fluorinated molecules are valuable tools for elucidating enzyme mechanisms.[18] To date, UDP-2-, 3- and 4-fluoro-galactose have been prepared through chemical[10,16,19] or enzymatic[17,19] procedures and tested with UGM. To complete this 'fluorine scan',[20,†] we decided to synthesize UDP-6-fluoro-galactofuranose **1**[21] (Fig. 1) and measure its binding properties to UGM in order to compare to published data on related UDP-Gal*f* analogs **2**,[10] **3**[10] and **4**.[22] The procedures for the preparation of **1** have been previously described.[21] The *Escherichia coli* enzyme was overexpressed and purified as reported in our preliminary studies.[12]

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† The term fluorine scan has been coined in the recent literature to describe experiments in which systematic exchange of one H (or functional groups) for F atoms in various positions of inhibitors are performed to explore the fluorophilicity/fluorophobicity of the target enzyme active site.

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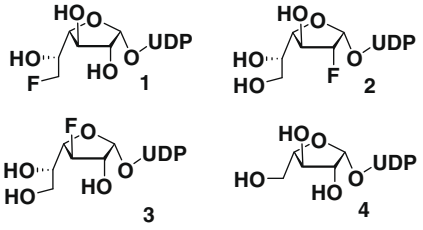


Figure 1. Substrates of UDP-galactopyranose mutase.

The ability of **1** to be a substrate of UGM was first investigated. Assays were performed under reducing conditions (sodium dithionite, 20 mM) and the enzyme concentration was adjusted at 500 nM to allow significant conversions (20–30%) within 10 min of reaction times. The reaction was monitored by HPLC under standard conditions[12] and we could easily observe the formation of a new peak with a shorter retention time. This trend was expected since all UDP-pyranoses reported to date display a shorter retention time than their corresponding UDP-furanoses.[10,17,22] The product of the reaction could be easily isolated and its analysis by mass spectrometry confirmed the structure.[23]

Table 1
Comparative kinetic parameters of UDP-Gal*f* analogs

	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m
UDP-Gal*f*[5]	27	22	1.23
UDP-6F-Gal*f* **1**	7.4	24,000	3.4×10^{-4}
UDP-2F-Gal*f* **2**[10]	0.033	65	4.1×10^{-4}
UDP-3F-Gal*f* **3**[10]	5.7	861	5.4×10^{-3}
UDP-Ara*f* **4**[22]	12	600	0.02

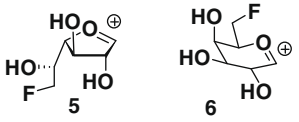


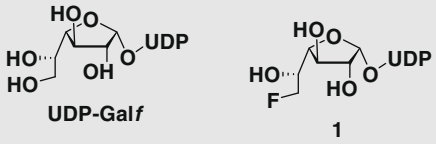
Figure 2. Hypothetical high-energy intermediates of the UGM catalyzed furanose/pyranose interconversion.

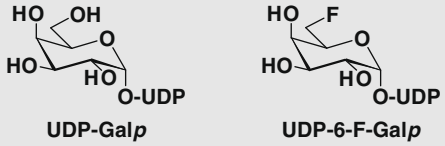
The k_{cat} (7.4 s^{-1}) and the K_m (24 mM) of **1** were thus measured[24] and compared with UDP-Gal*f* and other fluorinated analogs (see Table 1). Thus, a threefold rate reduction compared to the natural substrate was observed. This somewhat expected value was in the same range than the known UDP-3F-Gal*f* **3** and UDP-Ara*f* **4**. The electron-withdrawing character of the fluorine atom likely gives rise to a destabilization of oxacarbenium species such as **5** and/or **6** (Fig. 2) that are likely involved as high energy intermediates of this reaction. However the rate reduction we observed is modest compared to UDP-2F-Gal*f* **2**. As for the glycosidases[25] and the glycosyl transferases[19,26] the kinetic effect of a fluorine substitution is directly related to the distance between the fluorine atom and the anomeric position.

To our surprise, we measured a very large value for the K_m of molecule **1** compared to UDP-Gal*f*. This huge difference of K_m suggests that interactions in the Michaelis complex are dramatically affected when the 6-OH group is substituted for a fluorine atom. Moreover, this effect is much more pronounced than UDP-Ara*f* **4** that lacks the 6-CH_2OH group. This spectacular K_m enhancement may be explained by a repulsive, fluorophobic, effect. The intriguing interactions between organofluorine molecules and proteins have been the subject of discussions and sometimes of controversies.[27] Significant fluorophobic effects have been observed[28] and were indicated to be responsible of the loss of affinity of fluorinated molecules and proteins. This kind of repulsive interaction may be invoked to explain the K_m value of **1**. On the other hand, fluorophilic interactions have also been evidenced,[20] including with fluorinated carbohydrates. For instance, in the family of carbohydrate processing enzymes, we recently described the attractive interaction of the fluorine atom of a 2-fluoro-heptoside in complex with a bacterial heptosyl transferase.[29] If fluorophilic interactions occur between **1** and UGM, the only way to explain the large K_m value would be that the fluorine atom forces **1** to bind UGM in a conformation or a position that is not prone for catalysis.

From the data detailed above and in Table 1 arose a new question: does the K_m value measured for **1** relate to the binding affinity between **1** and UGM? To answer this question, we measured the inhibition of the 'natural' reaction by **1**. To do so, we applied the standard competitive assay against UDP-Gal*f*[8,14,15]: low enzyme concentration (22 nM) and a high inhibitor/substrate ratio ([**1**] = 2 mM, [UDP-Gal*f*] = 200 μM), under reducing conditions (sodium dithionite, 20 mM). The reactions were followed by HPLC. Under these conditions, molecule **1** was not transformed into its

Table 2
Selective relative charges for UDP-Gal*f* and **1**, and their pyranosyl counterparts





	Relative charge	Relative charge	Difference	Relative charge	Relative charge	Difference
C-4	0.004	−0.002	−0.006	0.019	0.016	−0.003
O-4	−0.286	−0.281	0.005	−0.307	−0.303	0.004
H-4	0.092	0.097	0.005	0.101	0.099	−0.002
C-5	0.020	**0.035**	**0.015**	0.038	0.017	**−0.021**
O-5	−0.320	**−0.303**	**0.017**	−0.302	−0.294	0.008
H-5	0.060	0.062	0.002	0.087	0.088	0.001
C-6	0.056	**0.040**	**−0.016**	0.072	0.053	**−0.019**
O-6/F	−0.318	−0.146	na	−0.310	−0.147	na
H-6 (pro *R*)	0.043	0.064	0.021	0.040	0.061	0.021
H-6 (pro *S*)	0.055	0.046	− 0.009	0.031	0.057	0.026

na, non applicable.
Bold values represent significant differences.

pyranose isomer because of the low UGM concentration. As previously described,[14] UDP was also tested in parallel to standardize the inhibition assay. At 30% conversion into UDP-Gal*p*, the inhibition percentages of **1** and UDP were 20% and 22%, respectively. Given that UDP has a K_d of 14 μM,[15] this inhibition assay shows that UDP-6F-Gal*f* has a binding affinity for UGM in the low micromolar range. Therefore, the K_m value of **1** (24 mM) does not refer to the real affinity of **1** for UGM.

In our effort to attempt to rationalize this observation, a computational approach[30] was used to evaluate the relative charges present on all atoms of the hexofuranosyl part of UDP-Gal*f* and the 6-fluorinated derivative **1**. As expected, the substitution of the OH-6 by a fluorine atom has only an impact on short distance and therefore, variations were obtained for atoms on the side arm C-4–C-6 (Table 2). Significant effects were observed on C-6, H-6 (pro *R*), C-5 and more importantly on O-5 since the relative charge increases from −0.320 to −0.303. It results from this less negative charge on O-5 a lower nucleophilicity which might disfavor the biocatalyzed ring expansion of UDP-Gal*f* to UDP-Gal*p*. Finally, considering their pyranosyl counterparts, it is interesting to note that, besides expected effects on C-6, H-6 and C-5, the 6-fluorine atom has only a limited influence on charge, and hence on nucleophilicity of O-4 that is involved in the ring contraction mechanism. Consequently, this underlined the importance of the negative charge held by O-5 in UDP-Gal*f* derivative **1**.

In conclusion, UDP-(6-deoxy-6-fluoro)-D-galactofuranose **1** was tested as substrate and inhibitor of UGM, a key enzyme of the mycobacterial cell wall biosynthesis. Turnover could be observed by HPLC. The k_{cat} (7.4 s^{-1}) and the K_m (24 mM) of **1** were thus measured and compared with those of UDP-Gal*f* and other fluorinated analogs. The presence of the fluorine atom at the 6-position of the galactofuranose moiety had a moderate effect on the rate of the reaction but a huge effect on the interactions of the enzyme and its substrate in the Michaelis complex. These results therefore open the opportunity to design a new family of UGM inhibitors structurally related to UDP-Gal*f*. Such efforts are indeed in progress in our laboratories and will be published in due course.

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30. Hyperchem Molecular Visualization and Simulation software (Hypercube, Waterloo, Ontario, Canada, software HyperChem 7.5) was used to calculate the charges on the interesting atoms using the PM3 force field. The resulting charge values were not considered individually but were rather used to make comparisons between similar species.