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COMMUNICATIONS

Published on Web 11/21/2003

# Glycosidase−Substrate Interactions Analysis by STD-NMR Spectroscopy: Study of α-L-Fucosidase

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Saturation transfer difference-nuclear magnetic resonance (STD-NMR) is a rapidly developing method used to analyze the interactions between large proteins and small ligands.[1] This technique is performed in solution and requires only small amounts of protein. Although it has been found useful for the analysis of many kinds of interactions, to date it has mainly been used to study those between oligosaccharides or glycomimetics and various proteins such as lectins[2] and antibodies.[3]

We have used this method to gain new insights into the reaction mechanism of the α-L-fucosidases. α-L-Fucosidases are glycosidases found in all the different kingdoms of living organisms.[4] They are involved in important biological processes including cancer[5] and genetic diseases[6] and have also been implicated in developmental processes.[7] Despite their biological significance and their apparently unique structure (based on their primary amino acid sequence), almost no information is available regarding their catalytic mechanism.

We have used STD-NMR to investigate the interactions between recombinant canine α-L-fucosidase[8] and various inhibitors and oligosaccharides. We demonstrate that STD-NMR can be used to probe the interactions between not only glycosidases and inhibitors but also glycosidases and substrates.

We have used 1-deoxymannojirimycin (DMJ), which has previously been shown to be a good inhibitor of α-L-fucosidase with an inhibition constant in the micromolar range,[9] making it suitable for STD-NMR experiments.

Recently the structural requirements for inhibition of α-L-fucosidase have been determined by analysis of the affinity constants of many different α-L-fucosidase inhibitors.[10] It has been argued from this analysis that the correct stereochemistry of the three hydroxyl groups at the 2-, 3-, and 4-positions of the DMJ ring is critical for binding (Figure 1a). Indeed, L-fucose has the same relative configuration of hydroxyl groups at C-2, C-3, and C-4 (Figure 1a) as DMJ. Furthermore, although DMJ is a good inhibitor of α-L-fucosidase, the imino-derivative of L-fucose, 1-deoxyfuconojirimycin (DFJ) (Figure 1b), is 1000 times more potent as inhibitor, being the strongest α-L-fucosidase inhibitor known.[11] Indeed, its inhibition constant is generally in the nanomolar range. This indicates that the deoxy group of fucose plays a critical role in the recognition of the substrate by the enzyme.

It has also been hypothesized that natural substrates of α-L-fucosidase not only bind in the catalytic site itself but also in an aglycon subsite.[11] We have used STD-NMR to try to obtain direct evidence supporting this hypothesis.

In the first part of our work we have determined that, as for other α-L-fucosidases, DMJ is a potent inhibitor of canine α-L-fucosidase, having a $K_I$ of 23 $\mu$M.

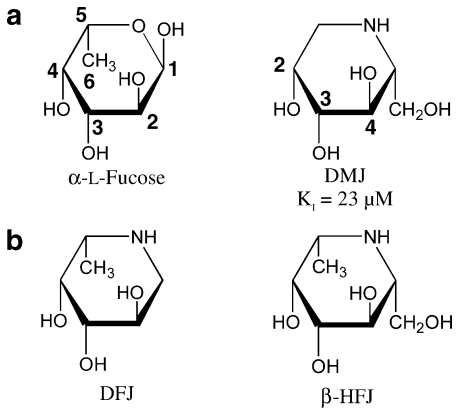


***Figure 1.*** (a) Structure of α-L-fucose and 1-deoxymannojirimycin (DMJ) with, in bold, the relevant numbering of the carbons. (b) Structure of 1-deoxyfuconojirimycin (DFJ) and $\beta$-L-homofuconojirimycin ($\beta$-HFJ).

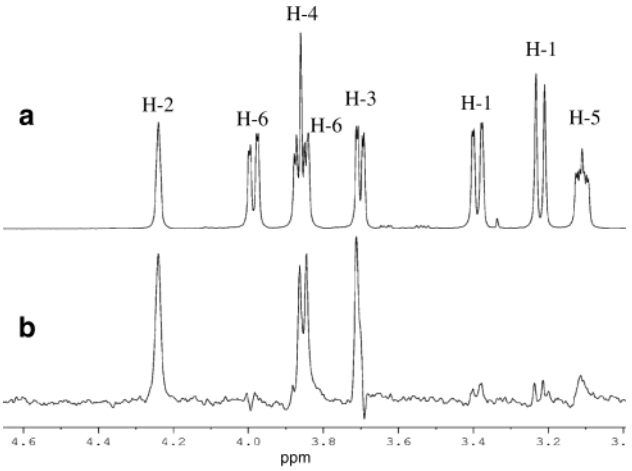


***Figure 2.*** Reference (a) and STD (b) spectra of 5 mM 1-deoxymannojirimycin in 0.1 M deuterated phosphate buffer at 4 °C in the presence of 25 $\mu$M canine α-L-fucosidase. The assignment of the corresponding protons is indicated above.

We obtained optimal STD signals with a 200-fold excess of ligand over protein. One- and two-dimensional STD-NMR analyses were performed using previously described methods[1,12] (see Supporting Information for experimental conditions). Our results unambiguously show that H-2, H-3, and H-4 of DMJ are in close contact with α-L-fucosidase, as they each give strong, equally intense signals from STD-NMR (Figure 2). This is the first direct proof of the involvement of the C-2, C-3, and C-4 groups in the inhibitory properties of DMJ. No signals from H-5, H-6, and H-1 were recorded, indicating that they are not directly involved in the interaction with the enzyme. This result is supported by the observation that inhibitors substituted at C-1, such as $\beta$-L-homo-

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10.1021/ja038562g CCC: $25.00 

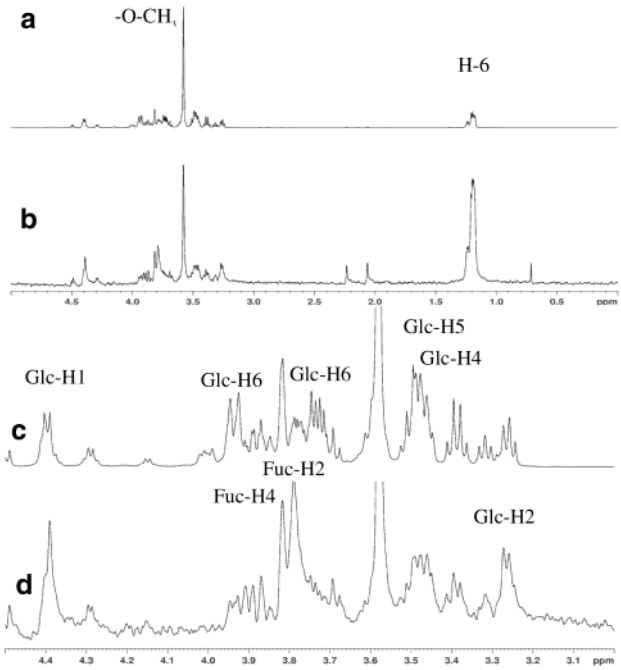


***Figure 3.*** Reference (a and c) and STD (b and d) spectra of a mixture of Fuc$\alpha$-*O*-Glc$\beta$*O*Me (chemical shifts reported[4]) in 0.1 M deuterated phosphate buffer at 4 °C in the presence of 25 $\mu$M canine $\alpha$-L-fucosidase. On the expansion of the STD spectrum (d) the protons with increased signal intensities are given. Assignments of these protons were obtained by 2D STD-TOCSY.

fuconojirimycin (Figure 1b), have the same inhibitory activity as the unsubstituted compound DFJ.[13]

Inhibitors are invaluable compounds for probing the mechanism of enzymes but ultimately only provide models, or mimics, of the transition state of the substrate. Therefore, we determined if it was possible to monitor the interaction between $\alpha$-L-fucosidase and various oligosaccharides including fucosylated milk oligosaccharides (see Supporting Information) and a mixture of four disaccharides ($\alpha$-L-Fuc linked to the 2-, 3-, 4-, and 6-positions of $\beta$-D-Glc-*O*Me) obtained by transglycoslation using this enzyme.[4] To slow the hydrolysis reaction, we performed the reaction at 4 °C.

With each of the oligosaccharides used we always observed a large signal in the STD spectra, corresponding to the deoxy group of fucose (C-6 methyl group) (Figure 3). This is direct proof of a strong interaction of this group with the enzyme and explains why any modification of the corresponding group in inhibitors decreases their inhibitory properties. However, the relative high intensity of the signal from the C-6 methyl group is probably not proportional to the strength of the interaction, as it has recently been demonstrated that STD-NMR does not give accurate quantitative results, especially with strong signals from methyl groups.[14]

Nevertheless, in this example of the interaction between a synthetic mixture of Fuc$\alpha$-O-Glc$\beta$*O*Me disaccharides[4] and $\alpha$-L-fucosidase, it was obvious that the C-6 methyl group of fucose gave rise to a stronger STD signal than the $\alpha$-*O*-methyl (increased by a factor of 9 compared to the control experiment), confirming that the major part of the signal is due to specific interaction with the protein (Figure 3, a and b).

It is also clear that the intensity of several signals coming from the glucose moieties of these compounds varied, providing direct proof of a subsite responsible for the recognition of the aglycon part of the ligand. We obtained similar results with various other oligosaccharides and always observed strong signals from the $\alpha$-L-fucose unit. We were able, as with DMJ, to clearly identify signals arising from H-2 and H-4, thus confirming their involvement in the interaction (Figure 3, c and d). Our results also demonstrate the importance of the deoxy group of L-fucose in the substrate−enzyme interaction.

In conclusion, we have demonstrated that STD-NMR is suitable for analysis of the interaction of glycosidases not only with their inhibitors but also with their substrates. Only one previous report makes similar use of STD-NMR,[15] where it was used to analyze the interaction between a galactosyltransferase and one of its substrates (UDP-Gal). However, in this case catalysis was blocked by the omission of an acceptor molecule.

Furthermore, our data for the first time provides direct evidence of the structural requirements for the interaction of compounds with an $\alpha$-L-fucosidase and hence allows the rational design of more specific and stronger inhibitors.

**Acknowledgment.** The European Network Glycotrain (HPRN-CT2000-00001) and an NH&MRC Australia program grant supported this work.

**Supporting Information Available:** Experimental procedures, 1D- and 2D STD-NMR spectra of DMJ and Fuc$\alpha$-*O*-Glc$\beta$*O*Me in the presence of $\alpha$-L-fucosidase. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA038562G