Anomeric Selectivity of Trehalose Transferase with Rare L-Sugars

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ABSTRACT: Retaining LeLoir glycosyltransferases catalyze the formation of glycosidic bonds between nucleotide sugar donors and carbohydrate acceptors. The anomeric selectivity of trehalose transferase from Thermoproteus uzonensis was investigated for both D- and L-glycopyranose acceptors. The enzyme couples a wide range of carbohydrates, yielding trehalose analogues with conversion and enantioselectivity of >98%. The anomeric selectivity inverts from α1- to β1-glycosidic bonds for D-glycopyranose acceptors to α1- to β1-glycosidic bonds for L-glycopyranose acceptors, while (S)-selectivity was retained for both types of sugar acceptors. Comparison of protein crystal structures of trehalose transferase in complex with α,α-trehalose and an unnatural α,β-trehalose analogue highlighted the mechanistic rationale for the observed inversion of anomeric selectivity.

KEYWORDS: glycosyltransferase, glycosidic linkages, trehalose, transferase, Thermoproteus uzonensis

The synthesis of a glycosidic bond is one of the most important reactions within glycochemistry. Enzymes couple sugars to afford oligosaccharides with high selectivity under mild reaction conditions. As enzymes are regarded to have evolved toward the selective conversion of the naturally more abundant D-sugars, their L-sugar enantiomers are often not considered as suitable substrates for enzymatic conversions. For this reason, the coupling of both D- and L-glycopyranose acceptors has rarely been compared for a single enzyme. However, the incorporation of L-sugars offers a broad spectrum of diametrically opposed glycosides or oligosaccharides, which might display new biological activities.

In one example, a retaining non-LeLoir glycosyltransferase (GT) coupled L-glycopyranose acceptors with sucrose as sugar donor in an α,β-(1 → 2)-fashion, while α,α-(1 → 2)-glycosidic bonds were observed with D-glycopyranose acceptors.4 The switch of anomeric selectivity for the sugar acceptor was attributed to the C4 and C4 chair configuration for D- and L-glycopyranoses, which affect the position of the nucleophilic hydroxyl group at the anomeric position. In general, α-D and β-L anomers of the same sugar are structurally more alike (Figure 1a), than the corresponding α-D and α-L anomers (Figure 1b).5 This structural similarity allows the conversion of both α-D and β-L configured substrates by an (S)-selective enzyme.

For retaining glycosyltransferases (GTs) with an internal nucleophilic substitution (Sni) mechanism, the anomeric selectivity can be expected to invert when (S)-selectivity is retained. (Figure 1c-d).3−5 The “same-face” attack of the nucleophile (i.e., sugar acceptor) on the leaving group (i.e., sugar donor) is guided by hydrogen bonding and proceeds with high anomeric selectivity for the sugar donor and acceptor.4 The position of the anomeric hydroxyl of the sugar acceptor might affect the type of glycosidic bond formed.

Here, the anomeric selectivity of the retaining LeLoir GT trehalose transferase (TreT) was investigated. TreT is particularly suitable for the screening of L-glycopyranoses, as it couples nucleotide diphosphate (NDP) sugar donors to a wide spectrum of nonphosphorylated D-sugar acceptors, resulting in an α,α-(1 → 1)-glycosidic linkage.6 We focused on the recently described TreT from Thermoproteus uzonensis (TuTreT) fused to mCherry for the systematic screening of D- and L-glycopyranoses as sugar acceptors.6,7 mCherry TuTreT is an interesting enzyme because of a high thermostability, high activity, the possibility of fluorometric detection that is due to mCherry, and performance as an immobilized catalyst.8

Initially, the reaction conditions were optimized to exclude any possible side reactions or promiscuous activities. TuTreT did not display any phosphorylase or hydrolase activity. The use of glucose-1-phosphate as sugar donor did not result in the formation of trehalose (excluding phosphorylase activity), and
no hydrolase activity was observed when the enzyme was incubated solely with trehalose. However, slow hydrolysis of uridine diphosphate-glucose (UDP-glucose) to UDP and glucose by $Tu$ TreT was observed, resulting in the subsequent formation of trehalose from glucose and UDP-glucose (Figure S3). To minimize the undesired formation of trehalose as a side product via UDP glucose hydrolysis during the screening of other sugar acceptors, the reaction time was limited to 60 min using 1.0 mg mL$^{-1}$ of $Tu$ TreT.$^9,10$

Using these optimized conditions, the substrate tolerance of $Tu$TreT was probed in a HPLC-based screening of D- and L-sugars (Figure 2). Conversion of L-glycopyranoses resulted in the hypothesized $\beta$-selectivity for $Tu$TreT. Successful enzymatic conversions were repeated on preparative scale and the obtained trehalose analogues were analyzed by NMR and HR-MS (Supporting Information). D-Glucose, D-mannose, D-galactose, and D-xylose exclusively led to the formation of $\alpha\alpha(1\to1)$-linked trehalose derivatives, while L-galactose, L-galactose, and L-gulose led to the formation of $\alpha\beta(1\to1)$-linked trehalose derivatives. The long-range $C−H$ coupling over the glycosidic linkage confirmed the direct coupling of the C1 acceptor with the H1 donor and vice versa in gHMBC experiments. The $4C1$ configuration of $\alpha\alpha\alpha\alpha$-D-glycopyranosides was confirmed by $J_{1,2}$ coupling ($\sim8$ Hz) of the anomeric protons, which are gauche configured. The anomeric protons of $\beta\beta\beta\beta$-glycopyranosides with a $1C4$ chair conformation are anti configured, resulting in larger $J_{1,2}$ coupling constants ($\sim8$ Hz).$^1$

Further analysis of the HPLC screening demonstrates that D- and L-enantiomers of glucose and galactose were accepted, but L-mannose was not. For L-gulose, L-allose, and L-altrose, their D-glycopyranoses enantiomers were not accepted. Anomeric selectivity is dictated by more than the anomeric configuration, and the overall conformation of the sugar acceptor is important as well. The structural variants of L-glycopyranoses with a $4C1$ configuration were readily converted, such as D-glucose, D-mannose, and D-galactose. This is in line with results for other TreTs.$^{11,12}$ C5 sugars were generally less well accepted, with

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**Figure 1.** Position of the anomic hydroxyl of $\alpha\alpha$-glucopyranose ($\delta C_{\alpha}$, cyan) when superimposed to $\beta\beta$-glucopyranose ($\delta C_{\beta}$, green) in panel a is more similar than $\alpha\alpha$-glucopyranose ($\delta C_{\alpha}$, purple) in panel b. The insets in panel a, b show the representative overlay of the Newman projection of the anomeric OH1. The $S_{\alpha\beta}$-like reaction mechanism with an oxocarbenium transition state ($H_2$) that allows the approach of the anomic hydroxyl, $\alpha\alpha$-OH of D-glucose ($\delta C_{\alpha}$) in panel c, or the $\beta\beta$-OH of L-glucose ($\delta C_{\beta}$) in panel d, is guided by hydrogen bonding from the same face as the NDP leaving group. R = NDP.

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**Figure 2.** mCherry $Tu$ TreT catalyzed conversion of D- and L-sugar acceptors with UDP-D-glucose as donor for the screening for the formation of product. The conversion was determined using external calibration curves of sugar acceptor with HPLC. n.d: not detected including a trehalose analogue product. Reaction conditions: substrate (10 mM), UDP-D-glucose (40 mM), HEPES (50 mM), MgCl$_2$ (20 mM), mCherry $Tu$TreT (13.5 nM), pH 7.0, 60 °C, 1 h of reaction time.
the exception of D-xylene, which lacks a CH₂OH group in comparison to D-glucose. The ketohexopyranoses D-fructose and D- and L-tagatose were not converted under the conditions provided, which display dissimilar overall structural conformation of the cyclic ring structure as well as the anomeric configuration. Interactions between active site residues and the carbohydrate substrate were investigated by extending the substrate screening to fluordeoxy-carbohydrates. Unlike hydroxyl groups, fluorine can exclusively function as a hydrogen bond acceptor. All fluoro-deoxy-D-glucopyranoses were quantitatively converted as acceptor substrates, with the exception of 4-deoxy-4-fluoro-D-glucose. The interaction of hydrogen bond donor 4-OH of the sugar acceptor with the deprotonated Asp254 of TuTreT is possibly important for acceptor substrate recognition.

The substrate tolerance toward the glycopyranose moiety of the sugar donor and the sugar acceptor is distinct from one another. For instance, the coupling of UDP-D-glucose with N-acetyl-D-glucosamine (GlcNAc) did not result in any observable conversion. The sugar donor UDP-D-GlcNAc and D-glucose is readily converted by TuTreT with >98% conversion and α,α-(1 → 1)-selectivity. As this is the first report that the α-D-selective retaining glycosyltransferase TreT catalyzes the glycosidic bond formation with β-L-glycopyranosyl acceptors, the protein crystal structure of TuTreT was studied. As the mCherry TuTreT fusion construct did not crystallize satisfactorily, the glycine-rich linker of the fusion protein was cleaved using "in situ" proteolysis with retention of enzyme activity (Figure S1), and the protein was purified (Figure S2). The protein without the mCherry tag subsequently crystallized as apo (PDB: 6ZJ4, 2.1 Å resolution), cocryostallized with magnesium(II) (PDB: 6ZJ7, 2.15 Å resolution), or soaked with D-trehalose (PDB: 6ZJH, 2.1 Å resolution), α-glucopyranosyl-L-galactopyranose (PDB: 6ZN1, 1.75 Å resolution), and UDP-α-D-glucose (PDB: 6ZMZ, 1.9 Å resolution). The latter three are shown in Figure 3a–c.

The overall three-dimensional fold observed in all determined crystal structures are similar to the one found in trehalose phosphate synthase (OtsA, PDB: 1GZS, RMSD of 2.0 Å for 304 Cα atoms) from E. coli and in trehalose transferase from Pyrococcus horikoshii (PDB: 2X6Q, RMSD of 2.5 Å for 363 Cα atoms), showing in each domain a characteristic Rossmann fold (Figure S4). Furthermore, all TuTreT structures show a monomer in the asymmetric unit, and this state was confirmed by size-exclusion small-angle X-ray scattering (SEC-SAXS) measurements in aqueous solution (Figure S9–S10). The overall protein conformation remained unchanged when bound to ligands in aqua, according to SEC-SAXS (Figure S9–S10). The protein crystal structures also show high structural similarity (Table S3); however, a minor conformational change was observed for the sugar donor binding site of TuTreT when it was soaked with UDP-D-glucose. Hydrogen bond interactions between the

![Figure 3. Protein crystal structure of TuTreT containing UDP-D-glucose in panel a (PDB: 6ZMZ, inset in panel d), TuTreT bound with α,α-trehalose in panel b (PDB: 6ZJH, inset shown in panel e), and TuTreT in complex with enzymatically synthesized α-glucopyranosyl-β-L-galactopyranoside in panel c (PDB: 6ZN1, inset shown in panel f). The nature of the glycosidic bond is demonstrated showing a high overlap for the sugar donor and the glycosidic linkages between panels d and e, or panels d and f, while the orientation of the sugar acceptor changes slightly between panels e and f.](https://dx.doi.org/10.1021/acscatal.0c02117)
uracil moiety and a disordered loop region of the protein (Figure S6, res. 250–262), were inducing a shift of an α-helix by 2.0 Å (Figure S5). This finding is not in agreement for what has been found for PfTreT, where a larger conformational change was observed for the whole domain after soaking the crystals with trehalose.

The active site of TuTreT is located between the N- or C-terminal domains of the acceptor and donor binding sites (Figure S4). Substrate-bound structures demonstrated clear electron densities at the active site (Figure S6). The active site residues for the sugar donor and acceptor binding sites are conserved for TuTreT, PhTreT,15 and OtsA (Figure S7).14 In TuTreT, the pyrophosphate moiety of the nucleotide sugar donor interacts with Arg221 and Lys226, and the active site can accommodate pyrimidine or purine nucleobases (Figure 3a,d). This leads to the ability of the enzyme to convert nucleotide sugar donors with different nucleotides, which holds for TreTs in general17,18 as was observed with UDP- and ADP-d-glucose with TuTreT previously.6,7

The natural product α,α-d-trehalose shows an α,α-(1 → 1)-glycosidic bond when bound to TuTreT (Figure 3b,c). In trehalose, the α-d-glucopyranosyl donor moiety binds at the same sugar binding site as the UDP-α-d-glucopyranosyl donor (Figure S8a–c). The sugar donor binding site of UDP-d-glucose of TuTreT (shown in Figure S8d) is similar to what has been reported for TreT from Pyrococcus hortikisi.15 Interestingly, the α- and β-trehalose derivatives the α-d-glucopyranosylacetor interacts with TuTreT in a different binding mode than the β-L-galactopyranosylacetor (Figure S8e,f). Arg221 might bind with the 2OH and 3OH of the β-L-galactopyranosylacetor (Figure S8e), while the 3OH and 4OH of the α-d-glucopyranosylacetor is at closer distance to Arg221 (Figure S8f). The movement of the highly conserved Arg221 when UDP-d-glucose is bound was notable (Figure S5f), which has been postulated to play a role in substrate recognition in PhTreT.15 Also, Asp256 is at a hydrogen bonding distance to sugars, leading to the formation of distinctive α-d-β- or α-β-1,6-glycosidic linkages. This paves the way for further studies of utilizing rare 1-glycopyranosides with retaining LeLoir glycosyltransferases, which are especially interesting for the formation of oligosaccharides and glycoses with unnatural glycosidic linkages.

ASSOCIATED CONTENT
■ Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscatal.0c02117.

Full experimental detail including fermentation, production and purification of the enzyme, details of the enzyme catalyzed conversions, HPLC and HRMS and NMR data for the complete characterization of the products, SAXS data and X-ray crystal data of the enzyme and DNA and protein sequence (PDF)

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Notes
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