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Note

Small-molecule glucosylation by sucrose phosphorylase: structure–activity relationships for acceptor substrates revisited

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ABSTRACT

Sucrose phosphorylase catalyzes the O-glucosylation of a wide range of acceptor substrates. Acceptors presenting a suitable 1,2-diol moiety are glucosylated exclusively at the secondary hydroxyl. Production of the naturally occurring compatible solute, 2-*O*-α-D-glucopyranosyl-*sn*-glycerol, from sucrose and glycerol is a notable industrial realization of the regio- and stereoselective biotransformation promoted by sucrose phosphorylase. The acceptor substrate specificity of sucrose phosphorylase was analyzed on the basis of recent high-resolution crystal structures of the enzyme. Interactions at the acceptor binding site, observed in the crystal (D-fructosyl) and suggested by results of docking experiments (glycerol), are used to rationalize experimentally determined efficiencies and regioselectivities of enzymatic glucosyl transfer.

Starting from the early 1940s, when the enzyme was first discovered, a number of studies have demonstrated the usefulness of sucrose phosphorylase in the synthesis of α-configured *O*-glucosides.[1–3] The natural reaction of the enzyme is conversion of sucrose and phosphate into α-D-glucose 1-phosphate and D-fructose. The catalytic mechanism of sucrose phosphorylase is that of a double displacement-like reaction involving a β-glucosyl enzyme intermediate. In the absence of phosphate, alternative glucosyl acceptors (e.g., monosaccharides, sugar alcohols) can take part in the reaction by intercepting the glucosylated enzyme. This enzymatic transglucosylation therefore leads to the formation of new α-D-glucosides. Scheme 1 shows the kinetic mechanism of sucrose phosphorylase for glucosyl transfer from sucrose to the reactive alcohol group of a suitable acceptor molecule. Application of sucrose phosphorylase as a biocatalyst for small-molecule glucosylation has recently attracted renewed attention as it became clear that two naturally occurring compatible solutes, 2-*O*-α-D-glucopyranosyl-*sn*-glycerol (GG) and 2-*O*-α-D-glucopyranosyl-*R*-glycerate (GGA), can be synthesized very efficiently by the enzyme (from *Leuconostoc mesenteroides*).[4,5] Production of GG has been developed into a commercialized process on industrial scale, and a solution containing 50% GG has been introduced to the market as an active ingredient for cosmetics where it is available under the trade name Glycoin Extremium.[6] Both GG and GGA could find further application as protein stabilizers.[7]

The specificity of sucrose phosphorylase for reaction with glucosyl acceptors is unusually relaxed. Accommodation of phosphate and D-fructose as acceptor substrate for the forward and reverse directions of the natural reaction, respectively, is already remarkable. However, aside from reaction with water (*hydrolysis*), the enzyme additionally promotes glucosyl transfer to hydroxy groups in a diversity of compounds, including polyhydroxylated molecules as well as aromatic alcohols.[8] Under conditions in which a suitable 1,2-diol acceptor group is available for enzymatic reaction (e.g., glycerol, *R*-glycerate), the secondary hydroxyl is glucosylated with essentially complete regioselectivity. However, polyhydroxylated acceptors differ strongly in their reactivity toward glucosylated sucrose phosphorylase. A molecular basis underpinning the observed relationships between acceptor structure and reactivity is currently not available, and the high regioselectivity of sucrose phosphorylase is not well understood. Based on recent high-resolution crystal structures of sucrose phosphorylase from *Bifidobacterium adolescentis*,[9] we compare here (bio)chemical evidence for the enzymatic glucosyl transfer to structural properties of the acceptor binding site of sucrose phosphorylase. Results are used to propose a model of how the enzyme recognizes different acceptor substrates.

Figure 1A is a close-up view of the substrate binding site in sucrose phosphorylase, revealed by a crystal structure of the *B. adolescentis* enzyme bound with sucrose (PDB-entry 2gdu). The catalytic machinery of this enzyme consists of Asp192 and Glu232. The Asp functions as a nucleophile during formation of the covalent enzyme intermediate[10] while the Glu serves a role as Brønsted acid

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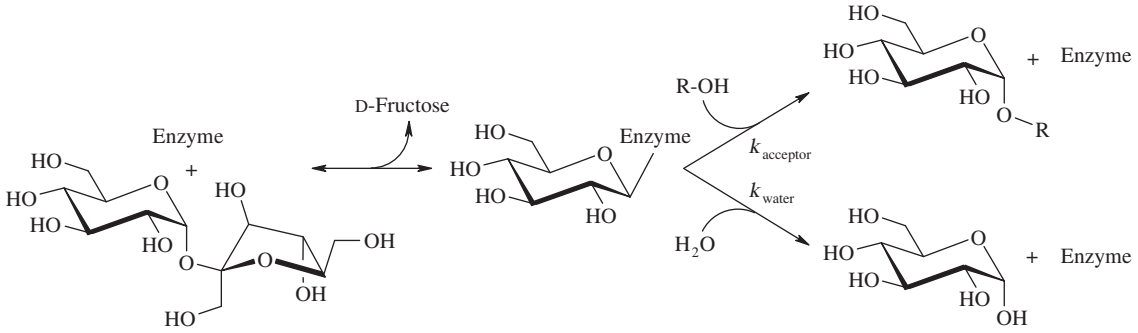

Scheme 1. Kinetic mechanism of sucrose phosphorylase (enzyme) for glucosyl transfer from sucrose to various acceptors. R-OH, monosaccharide, sugar alcohol, acceptor with phenolic, benzylic or other hydroxy groups.

and base during glucosylation and deglucosylation of the enzyme,[11] respectively. Substrate turnover in the crystal was prevented by substitution of Glu232 by Gln, which is not competent as a catalytic Brønsted acid.

The mode of binding of the fructosyl moiety in Figure 1A suggests three types of enzyme–ligand interactions, which are presumably decisive for discrimination between different leaving groups/acceptor substrates. The C1–OH of D-fructosyl appears to assist in bringing the side chain of the native Glu232 into the appropriate place for acid/base catalysis to occur. The C1–OH is therefore expected to provide a major contribution to precise relative positioning of the reactive group of the substrate and the catalytic Glu232 of the enzyme. The distances of the glycosidic oxygen and the fructosyl C1–OH to the N/O atoms of the carboxamide of Gln in the crystal structure are 3.15 and 2.59 Å, respectively. Although the C1–OH does not directly participate in the catalytic event, we propose that its role in the enzymatic reaction can be described as 'substrate-assisted facilitation'.

Figure 1A shows that there are further noncovalent enzyme–substrate interactions that position the D-fructosyl moiety at the binding site for the leaving group/acceptor in sucrose phosphorylase. The C6–OH is accommodated ('sandwiched') between the side chains of Gln345 and Arg399, and the hydrogen bond between the substrate hydroxyl and carboxamide side chain of the Gln is expected to stabilize the bound leaving group/acceptor substrate. Additionally, there are stacking interactions of the fructofuranosyl ring with Phe156 that could provide further stabilization of the leaving group/acceptor molecule in a reactive orientation. We now asked the question: to what extent are noncovalent enzyme–substrate interactions utilized by D-fructosyl (or D-fructose) exploitable by other glucosyl acceptor substrates of the enzyme?

Figure 1B shows results of an energy-minimized molecular docking experiment from which interactions of a glycerol acceptor with the β-glucosyl enzyme intermediate of *B. adolescentis* sucrose phosphorylase are predicted. The crystal structure used for ligand docking is that of the wild-type enzyme (PDB-entry 2gdv) having a β-glucosyl residue linked to Asp192. Note the skew boat conformation of the covalently bound sugar. Glycerol is positioned such that the C2–OH is ready for undergoing reaction (distance 3.06 Å) with the anomeric carbon of the β-glucosyl residue. Glu232 has a bidentate hydrogen bonding interaction with C1–OH and C2–OH of glycerol. With a distance of 2.89 Å from one of its oxygens to the oxygen of the glycerol C2–OH, the (ionized) side chain of Glu232 appears to be placed suitably for providing general base catalysis to the nucleophilic attack of the acceptor substrate. The high preference of sucrose phosphorylase for glucosylation of the secondary hydroxyl as compared to the sterically less hindered, hence chemically more active primary hydroxyl of glycerol is thus plausibly explained by the model in Figure 1B. The data also confirm the notion from above that the role of the C1–OH is not just in acceptor substrate recognition. By

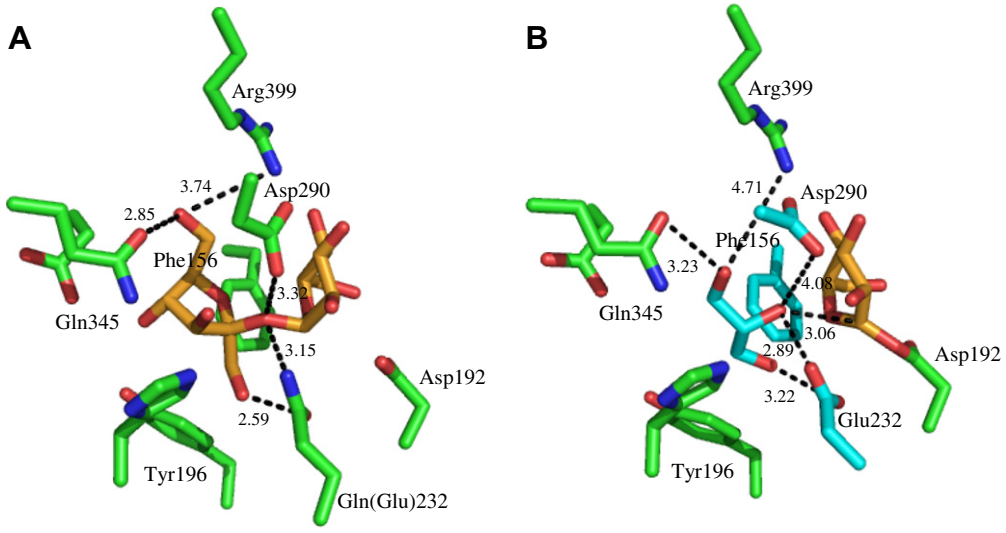

Figure 1. (A) Close-up view of the substrate binding site in *B. adolescentis* sucrose phosphorylase (PDB-entry 2gdu, molecule A). Residues interacting with the fructosyl moiety of sucrose (orange) are displayed. (B) Interactions of glycerol acceptor with the β-glucosyl enzyme intermediate of *B. adolescentis* sucrose phosphorylase (PDB-entry 2gdv, molecule A) derived from an energy-minimized flexible ligand-protein (cyan) docking experiment. Distances are in Å. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

pulling the side chain of Glu232 into the requisite position for 'activation' of the C2–OH, the primary hydroxyl could be directly auxiliary to the catalytic event. The model of bound glycerol shows furthermore that in comparison with the observed binding mode for β-fructosyl in Figure 1A, stacking interactions with Phe156 are not present for obvious reasons. However, the hydrogen bond between the leaving group/acceptor substrate and Gln345 is retained although it is clearly weaker for glycerol than the β-fructosyl moiety. By way of comparison, Arg399 could play a role in accommodating the carboxylate group in a glycerate acceptor substrate.[4,5]

Based on Figure 1 we can rationalize recognition of different polyhydroxylated acceptor substrates by sucrose phosphorylase and develop structure–activity relationships for these molecules. The compounds shown in Figure 2 were used in the analysis because not only has their reactivity toward enzymatic glucosylation been determined (Table 1) but also the site of chemical transformation has been characterized. We use biochemical data for sucrose phosphorylase from *L. mesenteroides*. Note therefore that the residues contributed to the substrate binding site in the *B. adolescentis* enzyme are fully conserved in the amino acid sequence of the *L. mesenteroides* sucrose phosphorylase. We examine the possible role in enzyme–substrate recognition of the overall conformation of the acceptor (open-chain vs pyranose or furanose ring) as well as the orientation of individual hydroxy groups in it. Energy-minimized conformations of the acceptor substrates in solution were used and from the known binding mode of β-fructosyl (Fig. 1A), their probable orientation in the substrate binding pocket of sucrose phosphorylase could be inferred, as depicted in Figure 3.

The observation that D-fructose behaves as a superior glucosyl acceptor compared to L-sorbose (Table 1) is explained by the predicted loss of interaction between C6–OH and Gln345 in bound L-sorbose. Despite its pyranose conformation, L-arabinose is a better acceptor than L-sorbose. Ability of the C2–OH of L-arabinose to partly reinstall the hydrogen bond with Gln345 that was not available to L-sorbose may be responsible. An alternative possibility is that due to its tilted pyranose ring conformation relative to the furanose ring conformations of D-fructose and L-sorbose (Fig. 3), L-arabinose exploits better than L-sorbose the stacking interactions with Phe156. D-Arabinose is a very poor acceptor substrate for sucrose phosphorylase, and only the β-anomeric conformation is reactive.[12] In an orientation that places the reactive diol moiety into a suitable position (data not shown), there is no hydroxy group of D-arabinose available for bonding with Gln345.

Table 1
Comparison of polyhydroxylated acceptors based on their reactivity to become glucosylated by *L. mesenteroides* sucrose phosphorylase

Acceptor	Analytical synthetic yield[a] (%)	Kinetic partition coefficient (M^{-1})
D-Fructose	23.4	36.4
L-Sorbose	6.8	n.d.
L-Arabinose	12.4	n.d.
D-Arabinose	0	n.d.
D-Glucose	2.4	n.d
L-Arabinitol	24.3	27.8[b]
D-Arabinitol	25.0	8.3[b]
Xylitol	5.1	n.d.
D-Glucitol	2.8	0.2[b]
Glycerol	1.4	2.6

Analytical synthetic yields are taken from Ref. 14.
n.d., not determined.
[a] The analytical synthetic yield is affected not only by the (intrinsic) reactivity of the acceptor in the enzymatic transformation but also by prevalence of the reactive conformation of the acceptor molecule in solution, especially in the case of D-fructose.
[b] Data are taken from Ref. 15.

D-Glucose is an interesting acceptor substrate of sucrose phosphorylase (from *L. mesenteroides*) because it is glucosylated at multiple sites, leading to the formation of two major transfer products, maltose (4-*O*-α-D-glucopyranosyl glucose) and kojibiose (2-*O*-α-D-glucopyranosyl glucose), in about equimolar amounts (Luley-Goedl et al., unpublished results). Other authors have also reported nigerose (3-*O*-α-D-glucopyranosyl glucose) production in significant amounts.[13] No conformation of D-glucose appears to fulfill the requirement of having a suitable diol moiety available for bonding with Glu232. However, a plausible explanation is that recognition of D-glucose does not involve neighboring hydroxyls and exploits both the $^4C_1$ and the energetically less favored $^1C_4$ ring conformation in distinct orientations, as shown in Figure 3. According to the proposed modes of D-glucose binding, glucosylation of C2–OH and C4–OH is rationalized. With D-glucose bound in the $^4C_1$ conformation, the α-anomeric sugar hydroxyl points toward the side chain of Phe156, arguably generating steric conflict.

A number of studies report on glucosylation of sugar alcohols by sucrose phosphorylase.[12,14,15] There is clear evidence supporting the notion that glucosyl transfer is regioselective and orientation of the individual hydroxyls affects the acceptor reactivity. Analytical syn-

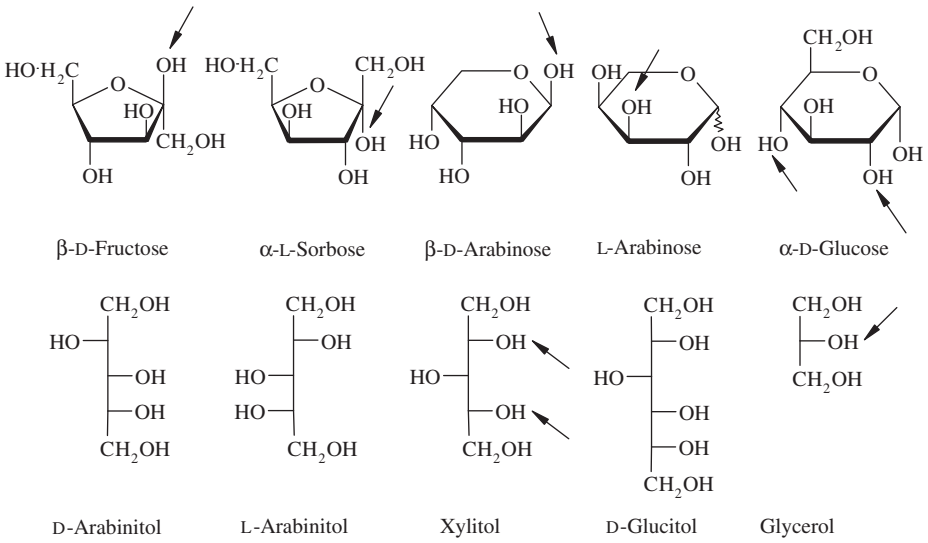

Figure 2. Chemical structures of different polyhydroxylated acceptors for glucosyl transfer by sucrose phosphorylase. The site(s) of glucosylation, identified by X-ray diffraction and NMR analysis,[8] are indicated by an arrow.

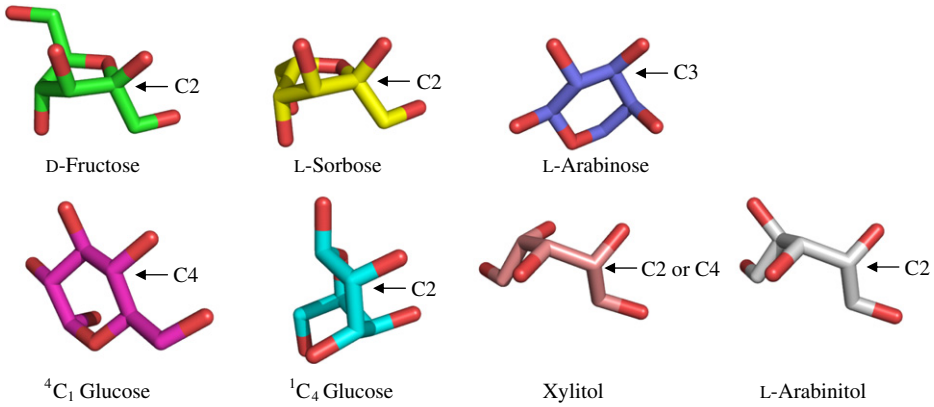

Figure 3. Proposed mode of recognition of polyhydroxylated acceptors by sucrose phosphorylase compared to the experimentally determined (PDB-entry 2gdu, molecule A) orientation of the D-fructosyl moiety in the acceptor/leaving group binding site of the enzyme (from *B. adolescentis*).

thetic yields are useful, however not always directly revealing (Table 1). Kinetic partition coefficients, obtained in assays in which breakdown of the glucosylated enzyme via reaction with acceptor and reaction with water (*hydrolysis*) is compared, are direct measures of reactivity. According to Table 1, the glucosyl transfer efficiency decreases in the order D-fructose > L-arabinitol > D-arabinitol > D-glucitol. Using data from synthesis, L-arabinitol is preferred about fivefold over xylitol. The product of enzymatic glucosylation was determined as 2-*O*-α-D-glucopyranosyl xylitol.[14] However, the absolute configuration of the compound, in which two diastereomers (3*R* and 3*S*) are possible, was not determined. Figure 3 depicts how the energy-minimized solution structure of xylitol might be accommodated in the acceptor binding site of sucrose phosphorylase. In the shown orientation, xylitol directs its C4–OH toward Phe156 and would therefore be unable to form a hydrogen bond with Gln345. In the case of L-arabinitol for which unfortunately the site of glucosylation is not known, we would predict a hydrogen bond between C4–OH and Gln345, implying reaction at C2–OH. Furthermore, glucosyl transfer to C4–OH is predicted for D-arabinitol whereby the C2–OH would interact with Gln345. The relatively poor reactivity of D-glucitol could be explained by structural similarity to xylitol, that is, the absence of bonding with Gln345 due to 'wrong' orientation of C4–OH.

In conclusion, this communication brings structural information on sucrose phosphorylase and biochemical data on the acceptor specificity of the enzyme into a coherent whole. We hope that it will be considered useful in the selection and perhaps development of novel acceptor substrates to become glucosylated by the phosphorylase. The proposed model of enzyme–substrate recognition is expected to support molecular design approaches in which phosphorylase mutants having altered acceptor specificities will be generated.

1. Experimental

1.1. Energy-minimized molecular docking

Spartan'02 (http://wavefun.com), AutoDockToolsv4,[16] and PyMOL (http://pymol.sourceforge.net) were used for the determination of energy-minimized solution structures, enzyme–ligand docking, and visualization, respectively. The X-ray crystal structure of *B. adolescentis* sucrose phosphorylase having a β-glucosyl residue covalently bound to Asp192 (PDB-entry: 2gdv, molecule A) was used as macromolecule in a molecular docking experiment that employed glycerol as the ligand. One twistable bond was allowed in the side chain between C_β–C_γ in Asp290 and Phe156 and between C_γ–C_δ in Glu232. Glycerol was set flexible with two torsions (C1–C2, C2–C3). Three clusters were obtained using a genetic algorithm with 10 runs and a root-mean-square-deviation tolerance of 2.0 Å for clustering. The interactions of the enzyme with bound glycerol in the obtained models were compared with those of the bound fructosyl moiety in an experimental structure of sucrose phosphorylase in complex with sucrose (PDB-entry 2gdu; molecule A). The model giving the best correspondence to the experimental data was chosen for structural interpretation.

1.2. Transglucosylation—determination of kinetic partition coefficients

Purified sucrose phosphorylase from *L. mesenteroides* was prepared by reported methods.[10] The reaction mixture for kinetic studies of transglucosylation contained 0.1 M α-D-glucose 1-phosphate, 0–0.2 M acceptor, and 3 U ml^{-1} (determined as described in Ref. 15) of sucrose phosphorylase dissolved in 20 mM MES buffer, pH 7.0. It was incubated at 30 °C using an agitation rate of 550 rpm (Thermomixer comfort; Eppendorf). Reactions were stopped after 10 min by heating (99 °C, 5 min), and sample work-up included centrifugation at 10,000 rpm for 10 min to remove precipitated protein. The amount of released phosphate was determined colorimetrically at 850 nm.[17] Glucose was measured using a coupled enzymatic assay in which hexokinase and glucose 6-phosphate dehydrogenase were employed.[18] Data analysis was performed according to Scheme 1. Kinetic partition coefficients ($k_{acceptor}/k_{water}$) were determined from plots of the ratio of phosphate and glucose release rates ($v_{phosphate}/v_{glucose}$) versus the acceptor concentration using following equation: $v_{phosphate}/v_{glucose} = 1 + (k_{acceptor}/k_{water}) \times [acceptor]$. Note: $v_{phosphate}$ is the total reaction rate whereas $v_{glucose}$ measures the hydrolysis rate.

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