Unravelling the Specificity of Laminaribiose Phosphorylase from *Paenibacillus* sp. YM-1 towards Donor Substrates Glucose/Mannose 1-Phosphate by Using X-ray Crystallography and Saturation Transfer Difference NMR Spectroscopy

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 Glycoside phosphorylases (GPs) carry out a reversible phosphorolysis of carbohydrates into oligosaccharide acceptors and the corresponding sugar 1-phosphates. The reversibility of the reaction enables the use of GPs as biocatalysts for carbohydrate synthesis. Glycosyl hydrolase family 94 (GH94), which only comprises GPs, is one of the most studied GP families that have been used as biocatalysts for carbohydrate synthesis, in academic research and in industrial production. Understanding the mechanism of GH94 enzymes is a crucial step towards enzyme engineering to improve and expand the applications of these enzymes in synthesis. In this work, with a GH94 laminaribiose phosphorylase from *Paenibacillus* sp. YM-1 (PsLB), we have demonstrated an enzymatic synthesis of disaccharide 1 (β-α-mannopyranosyl-(1→3)-α-glucopyranose) by using a natural acceptor glucose and noncognate donor substrate α-mannose 1-phosphate (Man1P). To investigate how the enzyme recognises different sugar 1-phosphates, the X-ray crystal structures of PsLB in complex with Glc1P and Man1P have been solved, providing the first molecular detail of the recognition of a noncognate donor substrate by GPs, which revealed the importance of hydrogen bonding between the active site residues and hydroxy groups at C2, C4, and C6 of sugar 1-phosphates. Furthermore, we used saturation transfer difference NMR spectroscopy to support crystallographic studies on the sugar 1-phosphates, as well as to provide further insights into the PsLB recognition of the acceptors and disaccharide products.

Introduction

Glycoside phosphorylases (GPs) are a group of carbohydrate-active enzymes that catalyse the reversible cleavage of glycosidic linkages in di- or oligosaccharides by transferring the non-reducing end glycosyl residue to inorganic phosphate. [1–4] The reverse reaction (synthesis reaction) of GPs is of practical importance because it can be used as an alternative method of enzymatic glycosylation by utilising sugar 1-phosphates as donor substrates. GPs have been classified based on their sequence identity into glycosyl hydrolase (GH) and glycosyltransferase (GT) families, or categorised into retaining and inverting phosphorylases, depending on the anomeric configuration in the O-glycoside product, with respect to the sugar 1-phosphate substrates. Substrates for GP-catalysed glycosylations are more readily available, in comparison to those for GT-catalysed reactions; this makes GPs attractive biocatalysts for carbohydrate syntheses. The use of GP biocatalysts has been demonstrated in academic research, such as in the synthesis of homogeneous crystalline cellulose, [5] self-assembled structures of alkylated cellulose, [6] cellulose nanoribbons with primary amino groups, [7] and the formation of oligo(ethylene glycol)-bearing cellulose hydrogels, [8] and more widely at an industrial scale, such as for the synthesis of 2-O-(α-α-d-glucopyranosyl)-sn-glycerol, a cosmetic ingredient, by sucrose phosphorylase; [9] kilogram-scale synthesis of lacto-N-biose, a prebiotic made with lacto-N-biose phosphorylase; [10] and the synthesis of disaccharide sweetener kojibiose, produced with a sucrose phosphorylase variant from *Bifidobacterium adolescentis*. [11]

One of the most studied GP families is found in GH94, which includes GPs acting on β-(1→2)- (sophorose), [12] β-(1→3)- (laminaribiose (LB)), [13] and β-(1→4)-linked glycans (cellobiose, [14, 15] cellooligomers, [16] chitobiose, [17] and cellohexitol) [18]. Several characterised GH94 GPs show broad specificity towards non-physiological acceptor substrates, including cellobextrin phosphorylase (CDP) from *Ruminiclostridium stercorarium*, which

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Cellobiose phosphorylase (CBP) is capable of using xylose as an acceptor, to produce glucopyranosyl xylose, and simple alcohols as acceptors, producing alkyl β-glucosides. Although relaxed acceptor specificity has been demonstrated, the specificity of GPs for sugar 1-phosphate is relatively narrow. The majority of GH94 GPs use glucose 1-phosphate (Glc1P) as a donor substrate, with the exception of chitobiase phosphorylase (CbBP), which uses α-N-acetyl-β-glucosamine 1-phosphate (GlcNAc1P) as its natural donor, although it can also use Glc1P with 20 times reduction in efficiency. Relaxed donor specificity has also been demonstrated for CDP from R. stercorarium, which can use either Glc1P or α-D-galactose 1-phosphate (Gal1P) as its glycosyl donor for glycolipid synthesis, albeit with ten times less efficiency on Gal1P. Both CBP and CDP from Ruminiclostridium thermocellum are capable of using α-D-galactosyl fluoride as a donor for the synthesis of cellobiose and celloctetraose.

Numerous X-ray crystal structures are available for GH94 enzymes, either in the presence of phosphate or sulfate (PDB IDs: 2CQS, 3QDE, 3RSY, and 2CQT), the acceptors (PDB IDs: 3S4B, 1V7X, 5H40, 4ZLG, and 5NZ8), iminosugar inhibitors (PDB IDs: 3QFY, 3QFZ, and 3QG0, 5H41), or disaccharide products (PDB IDs: 354A and 4ZLF). These structures provide valuable resources that can be used to guide the engineering of GPs for noncognate substrates. Structure-guided site-directed mutagenesis has been performed extensively on CBP from Cellvibrio gilvus (CgCBP), including its conversion into a lactose phosphorylase. In addition, a single mutation (E649C) in CgCBP created an enzyme variant capable of using methyl β-glucoside, ethyl β-glucoside, and phenyl β-glucoside as acceptors. Another CgCBP variant was created by mutation of five amino acids within and around the entrance to the enzyme active site, which broadened the acceptor range to include both β- and α-glucosides. In contrast to the situation of acceptor substrate studies, the number of reported GP structures in complex with sugar donors is relatively limited, with only a β-(1→2)-glucan phosphorylase from Lachnoclostridium phytofermentans (LP5OGP) in complex with Glc1P being reported (PDB ID: 5H42), which limits our understanding of the recognition of the sugar 1-phosphate donors by the GH94 family.

Although crystallographic studies provide valuable snapshots of enzyme active sites, they do not capture the dynamics of the enzyme–ligand interaction in solution. Therefore, other techniques to study protein–ligand interactions in solution are needed to complement the crystallographic data. Saturation transfer difference (STD) NMR spectroscopy was developed to study protein–ligand binding interactions in solution, based on the transfer of magnetisation from the protein protons to the protons of the ligand, whilst the ligand is bound. Those ligand protons in close contact with the protein exhibit the strongest STD NMR intensities; thus allowing mapping of the ligand-binding epitope. STD NMR spectroscopy can be used to elucidate the study of protein–glycan interactions, which is often difficult to study in solution due to weak affinity, as well as the complexity and conformational flexibility of the carbohydrate ligands. STD NMR spectroscopy has been used to elucidate the specificity of protein–glycan interactions on different sialyloligosaccharides, and to reveal the importance of glycan polarity, which determines the interaction and subsequent biological activation of its receptor. This technique has also been used to study enzyme–carbohydrate interactions to elucidate recognition features that can be used for inhibitor design, such as the study of ligand recognition by enzymes involved in mycobacterial cell wall biosynthesis, including uridine diphosphate (UDP) galactopyranose mutase and galactofuranosyltransferases. The same technique has been used to elucidate the binding of human blood group glycosyltransferases to their substrates; a process that is crucial to the biosynthesis of human blood group antigen.

Following on from our efforts to understand GP structure–function relationships and their application in carbohydrate syntheses, herein we investigated the GH94 laminariobiose phosphorylase from Paenibacillus sp. YM-1 (PsLBP), which has previously been reported for its specificity towards LB [β-D-glucopyranosyl-(1→3)-α-D-glucopyranosyl; Scheme 1A]. We have evaluated the activity of PsLBP on noncognate donor, α-D-mannose 1-phosphate (Man1P), and cognate acceptor, glucose, in the production of β-D-mannopyranosyl-(1→3)-α-D-glucopyranosyl (disaccharide 1), (Scheme 1B). Furthermore, we used X-ray crystallography, in conjunction with STD NMR spectroscopy, to investigate the interaction between PsLBP and its substrates to understand structural features that contributed to its donor substrate specificity.

**Scheme 1.** Reactions performed by PsLBP. A) Glc1P and Glc as a donor and acceptor, respectively. B) Man1P and Glc as a donor and acceptor, respectively.
Results

Recombinant protein expression and PsLBP activity on the native donor and acceptor

To obtain recombinant PsLBP protein for in vitro characterisation and X-ray crystallography, the gene encoding sequence of PsLBP was obtained from GenBank (accession number AB568298.2), codon-optimised for *Escherichia coli* expression, and synthesised by Gen9. The gene was amplified by PCR and cloned into a PopinF expression vector. The recombinant plasmid containing the *PsLBP* gene was introduced into BL21 (DE3) for protein expression. His<sub>6</sub>-tagged recombinant protein was then produced and purified by immobilised metal affinity chromatography (IMAC), followed by gel filtration. The gel filtration trace showed three different main peaks with different elution volumes (Figure 1A, peaks a, b, and c). To investigate whether the three main peaks have similar activities, the phosphorylase activity of each peak was individually characterised in the synthetic direction (Scheme 1A) by phosphate release assays. No significant difference in activity was observed between these three peaks (data not shown) and the enzyme in peaks a and b was likely to be the higher oligomeric forms of PsLBP. Therefore, only peak c was used for further experiments based on its highest protein yield. SDS-PAGE analysis of peak c showed a major band of protein with an approximate size of 100 kDa, in agreement with the calculated mass of PsLBP monomer (101.6 kDa; Figure 1B). However, gel filtration analysis of peak c against protein standards showed that PsLBP formed a dimer in non-denaturing conditions with an estimated molecular mass of 240 kDa.

To further confirm that the recombinant PsLBP was active, the enzyme was assayed in the synthetic direction (Scheme 1A) in the presence of its natural substrates (Glc and Glc1P) and the reaction mixture was subjected to HPAEC-PAD analysis. The analysis showed that LB was produced (Figure 1C), complemented by the release of inorganic phosphate, which could be detected by a phosphate release assay (Figure S1 in the Supporting Information).

Activity towards noncognate donors

PsLBP activity has previously been screened on several non-cognate acceptors, including mannose, methyl β-glucoside, 2-deoxyglucose, and 6-deoxyglucose, with 50- to 100-fold reduction in activity compared with that of Glc. However, the specificity towards noncognate sugar 1-phosphate donors has not been reported. To assess the donor specificity of PsLBP, the enzyme was assayed in the presence of α-β-galactosamine 1-phosphate (GalN1P), α-β-glucosamine 1-phosphate (GlcN1P), α-galacturonic-acid 1-phosphate (GalA1P), Gal1P, or Man1P as donors and Glc as an acceptor. TLC and HPAEC-PAD analysis of the reactions showed that the enzyme could use Man1P as a donor, as indicated by the presence of an additional spot on TLC, corresponding to the generation of disaccharide 1 (Figure 2A and B). Kinetic parameters for the synthetic reaction with Glc1P or Man1P as donors and Glc as a receptor showed comparable *K₉* values for Glc1P and Man1P, whereas the *k₉* value for Glc1P is more than 100 times higher than that for Man1P (Table 1).

A large-scale (5 mL) enzymatic reaction was performed on 700 μg of the enzyme, 10 mM Glc, and 20 mM Man1P; this was incubated for 15 h to produce milligram quantities of disaccharide 1 (Figure 2C and S2A). Unreacted Man1P and inorganic phosphate by-product were then removed from the reaction mixture by means of anion-exchange chromatography and disaccharide 1 was isolated by means of gel permeation chromatography (GPC; Figure S2A). Isolated disaccharide 1 was analysed by means of TLC, which showed that only one product was obtained with no Glc contamination (Figure S2B). Mass spectrometry analysis of disaccharide 1 on the TLC plate showed a major signal at *m/z* 364.9, corresponding to the mass of a disaccharide with sodium adduct (Figure S2C).

![Figure 1. Expression and characterisation of recombinant PsLBP. A) Gel filtration analysis to determine the size of PsLBP. Elution volume of peak c (64.7 mL) was used to estimate the mass of the protein from a calibration curve constructed from protein standards with known molecular mass. B) SDS-PAGE analysis of the recombinant protein after immobilised metal affinity chromatography (IMAC) and gel filtration. C) High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis of the synthetic reaction performed by PsLBP upon incubation with the enzyme with 10 mM Glc and 10 mM Glc1P for 30 min at 45°C. NE = no enzyme control.](image)

| Donor      | *k₉* [s⁻¹] | *K₉* [mM] | *k₉*/*K₉* [s⁻¹ mM⁻¹] |
|------------|------------|-----------|---------------------|
| Glc1P      | 13.0 ± 1.4 | 4.20 ± 1.5 | 3.07                |
| Man1P      | 0.08 ± 0.01| 3.80 ± 1.0 | 0.02                |
| Glc        | 15.4 ± 1.3 | 6.04 ± 1.3 | 2.55                |

Table 1. Kinetic data of PsLBP for Glc1P and Man1P as donors in the presence of 10 mM Glc as an acceptor and for Glc in the presence of 10 mM Glc1P.
data are in line with those reported by Awad et al. for the same disaccharide arising from GH130 β-(1→3)-mannan phosphorylase-mediated synthesis.[49]

Assignment of NMR signals of disaccharide 1 (Figures S3 and S4) was possible with the use of 2D experiments: COSY (Figure S5), HSQC (Figure S6), and 2D non-decoupled HSQC (Figure S7), as well as literature data for model methyl β-mannoside (Table S1).[50] Assignment was also helped by simulated spectra of disaccharide 1 generated by the CASPER program (Table S2).[51] Downfield positions of resonances C-3 of β-Glc (δ = 84.6 ppm) and C-3 of α-Glc (δ = 82.1 ppm) residues, with respect to the corresponding signals in d-glucose (δ = 73.8 and 77.0 for α- and β-anomers, respectively),[52] indicated the presence of the 3-O-glycosylated glucopyranose unit. Coupled HSQC experiments revealed 3J(1H,13C) = 163 Hz for the anomeric signal of the mannosyl residue in the 13C NMR spectrum of 1 split into two very close signals, due to the presence of α/β-anomers of the Glc residue (Figure S3). Anomeric signals of the non-reducing β-Glc residue are expected to appear at δ = 103–104 ppm,[50,53] but there are no signals in that region; therefore, the presence of β-glucoses can be excluded. By comparison with the previously reported Glc-β-(1→3)-Glc 13C NMR spectra,[54] signals at δ = 95.7 and 92.1 ppm can be assigned to C-1 of reducing β-Glc and α-Glc moieties, respectively, whereas signals at δ = 84.6 and 82.1 ppm can be assigned to C-3 β-Glc and C-3 α-Glc, respectively.

Overall crystal structures of PsLBP

Three PsLBP structures were determined and designated according to the ligands found in their active sites: SO₄²⁻, Glc1P, and Man1P complexes. All structures belong to the same space group (P4₁2₁2₁) and contain two subunits per asymmetric unit, which are related by a noncrystallographic twofold axis that superposes them with a root-mean-square deviation (RMSD) of 0.651 Å. The two copies of the molecule in the asymmetric unit formed a biological homodimer with an interfacial area of about 3360 Å², as calculated by jsPISA.[55] The formation of homodimer observed in the crystal structures is in agreement with gel filtration analysis, in which PsLBP is eluted as a dimer. Other GH94 enzymes also form a homodimer with the exception of LpSOGP, which is the only reported monomeric GH94.

Each PsLBP monomer consists of four domains (Figure 3A and B), which are an N-terminal β-sandwich (residues 1–297; yellow), a helical linker region (residues 298–327; lilac), an (α/α)₆ catalytic domain (residues 328–808; green), and a C-terminal domain (residues 809–911; red). The domain organisation in PsLBP is similar to that observed in other GH94 disaccharidase phosphorylases, which include CBPs from Cellulomonas uda (CuCBP),[56] CgCBP,[57] ChBP from Vibrio proteolyticus (VpCBP),[58] and celllobionic acid phosphorylase from Saccharophagus degradans (SdCBAP).[52] PsLBP lacks the extended N-terminal α/β domain (Figure 3B, purple) that is only present in GH94 oligosaccharide phosphorylases, including CDP from R. thermocellum (RtCDP)[50] and LpSOGP.[51] The role of the extended N-terminal domain in RtCDP is proposed to be involved in the interaction of the lower portions of the homodimer, causing the upper portions to move apart, which leads to a widening of the active site to accommodate a larger acceptor (i.e., cello-dextrin) in RtCDP.[50]
Phosphate recognition by PslBP

In the $\text{SO}_4^{2-}$ complex (Figure S9A), $\text{SO}_4^{2-}$, which was derived from the precipitant used for crystallisation, occupied a similar position to those of phosphate and sulfate in other GH94 disaccharide phosphorylases, such as in CgCBP (Figure S9B). The $\text{SO}_4^{2-}$ molecule forms hydrogen bonds with the side chains of R353, T796, and E782. From the amino acid sequence alignment of PslBP, CgCBP, and RtCDP, H739 in PslBP aligned with the conserved histidine residues in CgCBP and RtCDP which formed a hydrogen bond with phosphate in the active site (Figure S9B). However, the distance between the H739 side chain and $\text{SO}_4^{2-}$ in the structure of PslBP is greater than that of a hydrogen-bonding distance; this suggests that H739 may not be essential for phosphate recognition. This is further supported by three pieces of evidence. Firstly, if the corresponding histidine (H666) in CgCBP was mutated to Asn, a phosphate molecule was still bound to the active site of the enzyme (PDB ID: 3ACT). Secondly, the phosphate moiety in Glc1P in complex with LpSOGP does not form a hydrogen bond with the corresponding histidine (H924) (Figure 4B). Lastly, a wild-type CDP from Ruminococcus albus has Gin646 instead of the conserved His residue found in other GH94 phosphorylases.

Glc1P and Man1P recognition by PslBP

The overall structure of the $\text{SO}_4^{2-}$ and Glc1P complexes are very similar (RMSD of 0.23 Å for a dimer on dimer superposition). Glc1P was bound with the pyranose ring in the $\alpha$-conformation and the $\alpha$-anomeric configuration of phosphate at the C1 position; this supports the enzyme specificity for sugar 1-phosphate in an $\alpha$-anomeric configuration. Glc1P is completely buried within a donor subsite ($-1$ subsite), which is formed entirely within a single subunit of PslBP. A comparison between the Glc1P complex of PslBP and that of LpSOGP (Figure 4B, PDB ID: 5H42) showed that Glc1P recognition by the two proteins was different. In PslBP, the hydroxy group on C3 forms a hydrogen bond with the R374 side chain (Figure 4A), whereas, in LpSOGP, the same hydroxy group forms hydrogen bonds with R630 and D631. In the LpSOGP structure, D631 is also involved in the recognition of the hydroxy group on C2 through hydrogen bonding, whereas, in PslBP, the hydroxy group forms a hydrogen bond with R353. The equivalence of D631 in PslBP (D375) forms a hydrogen bond with neither of the hydroxy groups on C2 or C3 because the distance between the hydroxy groups on C2 and C3 and D375 is greater than that of the hydrogen-bonding distance. The phosphate moiety

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**Figure 3.** Structural comparison of CgCBP, RtCDP, and PslBP. A) A comparison of the GH94 structures. All proteins are in dimeric form. The domains are coloured in one subunit only; the adjacent subunit is coloured in grey. Green spheres represent ligands bound in the active sites. B) Sequences of RtCDP, PslBP, and CgCBP coloured according to the domains. C) Summary of conserved residues found in the amino acid sequence alignment of CgCBP, PslBP, and RtCDP. The conserved residues are involved in Glc1P binding (blue), phosphate binding (magenta), or the predicted catalytic aspartate (red). Details of the alignment can be found in Figure S8.
Catalytic loop in GH94 enzymes

The catalytic loop (purple; Figure 4) is a highly conserved feature among GH94 structures. In PsLB, this loop consists of a WND motif (WS24, N525, and D526), with D526 as a predicted catalytic residue. The tryptophan residue (WS24) is structurally conserved among GH94 members and provides a hydrophobic platform for the binding of the donor. A comparison of the CgCBP and PsLB structures showed that the PsLB active site was slightly more closed than that of CgCBP because of several different structural features. Firstly, the length of the PsLB catalytic loop is ten amino acid residues shorter than that of CBP, and only partially occludes the active site. In contrast, the catalytic loop in CBP forms a lid-like structure that extends over the active site (Figure S10A and B, purple). Secondly, the position of the "adjacent loop" (Figure S10A and B, brown) runs parallel to a significant proportion of the catalytic loop in CgCBP, forming a zipper-like interface that further encloses the CgCBP active site. The absence of the extended catalytic loop in PsLB may be compensated for by the presence of a loop in the β-sandwich domain of the opposing subunit, which is six residues longer than that of the equivalent loop in CgCBP. This forms a β-hairpin "gate," which overlaps with the extended catalytic loop in CgCBP (Figure S10B–D, cyan). Moreover, the opposing loop in CgCBP projects into the active site more than that of PsLB (Figure S10, red). In PsLB, the active site is relatively open because the opposing and adjacent loops are located further away from the active site, with respect to those in the CgCBP structure. The relatively "open" state of the PsLB active site may be representative of the initial binding of the sugar 1-phosphate donor before the synthetic reaction occurs or the final stage of phosphorolysis during which the sugar 1-phosphate is about to be released. On the other hand, the relatively "closed" state observed in the CgCBP structure is likely to represent the intermediate state of catalysis at which the glycosidic bond is either being broken during phosphorolysis or being formed during the synthetic reaction.

STD NMR spectroscopy and CORCEMA-ST suggest different binding affinities of Glc1P and Man1P to PsLB

To study to the interaction between PsLB and Glc1P or Man1P, binding epitopes for Glc1P and Man1P were constructed (Figure 5A and B) by using the initial rates approach (SI 1 in the Supporting Information) to avoid overestimation of slow relaxing protons at large saturation times and to eliminate any effect of ligand rebinding. The epitope maps provide a qualitative measure of proximity of the protons of the ligands to the protein surface; larger values indicate more intimate contact. For Glc1P (Figure 5A), all ligand protons received strong saturation from the protein, thus indicating that the sugar was intimately recognised and made contacts all along the ring. Nevertheless, the exocyclic H6 protons exhibit the strongest STD

Figure 4. A comparison between A) PsLB and B) LpSOGP in complex with Glc1P. C) The active site of PsLB in complex with Man1P. The C2 position on the pyranose ring is indicated with a black arrow head. The protein backbone is shown in cartoon representation and the neighbouring side chains in stick representation. The conserved catalytic loop is presented in purple. The catalytic residue (D526) is underlined.
can be used to predict STD intensities based on STD NMR spectroscopy experiments on Glc1P and Man1P. A) Binding epitope of Glc1P in the presence of PsLBP. B) Binding epitope of Man1P in the presence of PsLBP. Colours represent normalised values of STD, at each position depicted in the structure. For each ligand, the values of STD are normalised against the largest value (see Table S3 for the normalised STD). C) Experimentally determined STD build-up curves for Glc1P binding to PsLBP (top) and complete relaxation and conformational exchange matrix analysis of saturation transfer (CORCEMA-ST)-calculated STD intensities (bottom). The NOE factor (RNOE) between the experimental and calculated data is 0.09. D) Experimentally determined STD build-up curves for Man1P binding to PsLBP (top) and CORCEMA-ST-calculated STD intensities (bottom). The RNOE between the experimental and calculated data is 0.23. For experimental data, circles show observed STD intensities, whereas curves are determined from least squares fitting to the equation in SI 1.

Figure 5. STD NMR spectroscopy experiments on Glc1P and Man1P. A) Binding epitope of Glc1P in the presence of PsLBP. B) Binding epitope of Man1P in the presence of PsLBP. Colours represent normalised values of STD, at each position depicted in the structure. For each ligand, the values of STD are normalised against the largest value (see Table S3 for the normalised STD). C) Experimentally determined STD build-up curves for Glc1P binding to PsLBP (top) and complete relaxation and conformational exchange matrix analysis of saturation transfer (CORCEMA-ST)-calculated STD intensities (bottom). The NOE factor (RNOE) between the experimental and calculated data is 0.09. D) Experimentally determined STD build-up curves for Man1P binding to PsLBP (top) and CORCEMA-ST-calculated STD intensities (bottom). The RNOE between the experimental and calculated data is 0.23. For experimental data, circles show observed STD intensities, whereas curves are determined from least squares fitting to the equation in SI 1.

STD NMR spectroscopic identification of the binding subsite for Glc and the directional binding of LB and disaccharide 1 to PsLBP

In the study of Glc binding, exchange between its α- and β-anomers precluded such a detailed study, due to the combination of different concentrations of species and the significant signal overlap observed from both anomers. However, it is clear from the spectra that, although similar concentrations of each anomer are present, the STD intensities from the α-anomer are very much weaker than those from the β-anomer (Figures 6A, C and S11). This indicates that the β-anomer is preferentially recognised by the enzyme, which suggests several things. Firstly, the subsite occupied by Glc1P and Man1P must require a sugar 1-phosphate, otherwise it would be expected that α-Glc would bind well. This then means that Glc must bind to a separate subsite, in agreement with its role as the acceptor substrate. Finally, the structure of this subsite must be such that α-Glc, with its axial C1-hydroxy group, is unable to bind, perhaps due to steric interactions.

As in Glc, the reducing end of LB exists as an equilibrium between both of its α- and β-anomers. Again, it is observed that the β-anomer is preferentially recognised by the enzyme (Figures 6B and S12). Given the proposed reaction mechanism and polarity of the disaccharide, it appears that the non-reducing ring of LB (Glc1) binds to the same subsite as Glc1P/Man1P, whereas the corresponding reducing sugar (Glc2β) binds to the same subsite as Glc. Saturation transferred to the reducing end of LB (Figure 6D, bottom) is much stronger than that received by the nonreducing ring (Figure 6D, top); this suggests that the key interaction between the ligand and enzyme is formed with the reducing sugar hexopyranose moiety. This agrees with the previous observation that the −1 subsite can only recognise sugar 1-phosphates effectively.

STD NMR spectroscopy was also performed on disaccharide 1 and PsLBP to determine the interaction between the non-
cognate product and the protein. Overall, interaction between PsLBP and disaccharide 1 is similar to that described in LB, with the main contacts appearing to be with Glc2, in particular, with H6, as previously mentioned for LB (Figure 7A and B). The magnitude of the STD intensities is comparable to those of LB, thus suggesting that the affinity is similar; this would make sense, given that Glc2, common to both, seems to be most important for recognition. Moreover, only the β-anomer binds strongly to PsLBP; the STD intensities for the α-anomer are very much weaker (Figure 7C). However, the STDs for the H6 of Man1 are much stronger than that of Glc1 in LB. This may be because Man binds in a slightly different orientation, which is probably to be expected given the different stereochemistry of C2.

Discussion

GPs are attractive biocatalysts for the synthesis of oligo- and polysaccharides due to their broad specificity towards acceptor substrates and relatively low cost of donors, compared with other enzymes used for glycan syntheses. Understanding the mechanism of GP action on both natural and noncognate substrates would therefore provide background knowledge that could underpin applications of GPs in carbohydrate synthesis, in both academic and industrial settings. Unlike other conventional substrate screening experiments, which have been conducted by various groups on GPs,[13–15, 26, 30, 31, 44, 61, 62] we aimed to pinpoint the mechanism by which PsLBP recognised and utilised Man1P as its noncognate donor, through X-ray crystallography and STD NMR spectroscopy.

The use of GPs for β-(1→3)-mannosylation has been previously conducted by using a GH130 β-(1→3)-mannan mannoside phosphorylase (Zg0232) from Zobellia galactanivorans DSM 12802, which transfers mannose from Man1P to a variety of sugar acceptors, including a noncognate acceptor, Glc.[49] In contrast, our study demonstrates the relaxed specificity of PsLBP towards the sugar 1-phosphate donor, Man1P, from which mannose was transferred onto a Glc acceptor, resulting in the production of Man-(1→3)-Glc disaccharide 1.

Significant interactions between Glc1P and PsLBP, as indicated by the crystal structure and STD NMR spectroscopic analysis, suggests that the specificity of this enzyme towards the donor substrate is more restricted than that of the acceptor site. Therefore, any manipulation to broaden the donor specificity may be challenging. Nevertheless, our work demonstrated a relaxed specificity of PsLBP towards Man1P, which indicated

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Figure 6. STD NMR spectroscopy experiments on Glc and LB. A) Binding epitope of Glc(β) in the presence of PsLBP. B) Binding epitope of LB(β) in the presence of PsLBP. Colours represent normalised values of STD, at each position depicted in the structure. For each ligand, the values of STD, are normalised against the largest value (see Table S3 for the normalised STD). C) Experimentally determined STD build-up curves for Glc(β) binding to PsLBP. D) Experimentally determined STD build-up curves for LB(β) binding to PsLBP. Circles show observed STD intensities, whereas curves are determined from least squares fitting to the equation in SI 1.

Figure 7. STD NMR spectroscopy experiments on 1. A) Binding epitope of disaccharide 1(β) in the presence of PsLBP. Colours represent normalised values of STD, at each position depicted in the structure. For each ligand, the values of STD, are normalised against the largest value (see Table S3 for the normalised STD). B) Experimentally determined STD build-up curves for Man1 binding to PsLBP. C) Experimentally determined STD build-up curves for Glc2(β) and Glc2α in 1 binding to PsLBP. Circles show observed STD intensities, whereas curves are determined from least squares fitting to the equation in SI 1.
that the alternative configuration of the hydroxy group at C2 on the pyranose ring is tolerated. A strengthening of the interaction of Man1P with the active site could be the strategy to make the production of disaccharide 1 more efficient. However, the enzyme crystal structure showed that the axial configuration of C2–OH of Man1P pointed into empty space (at the dimer interface), thus suggesting that a simple mutation approach was unlikely to restore a hydrogen bond with C2–OH on Man1P. On the other hand, Glc1N1P was not a substrate for PsLBP, despite having the same configuration as that of Glc1P at C2. In this case, the C2–OH group is substituted by NH2, which is likely to cause steric and/or electrostatic clashes with R353; thus, disfavouring the binding of Glc1N1P to the active site. Size restriction of the substituent at C2 has been reported in CgCBP, which cannot accommodate GlcNaC1P (OH is replaced by CH2CONH at C2), whereas VpChBP can accommodate both GlcNaC1P and Glc1P, despite having the same amino acid for interaction with the hydroxy group at C2.[17] The difference is only in the placement of the Arg side chain that interacts with the C2 group, which is more distant in VpChBP to accommodate a larger substitution.[27]

Crystallographic structures and STD NMR spectroscopy data generated in this study enabled us to explain the PsLBP preference for other sugar 1-phosphates (see Figure S13 for structures of all sugar 1-phosphate mentioned in the discussion below). For instance, a hydrogen bond formed between C4–OH and the side chains of R374 and the strong saturation transfer signal from the protein to the hydrogen on C4 in the STD NMR spectroscopy experiment indicate that C4–OH and its configuration might be crucial for the recognition of Glc1P by PsLBP, and therefore, any modification at this position may compromise the enzyme activity on the donor. This hypothesis is supported by the fact that PsLBP could not use Gal1P as a sugar donor (Figure 2A, TLC enzyme screening), which has C4–OH in an axial rather than in an equatorial position. The same explanation can be used to explain the lack of PsLBP activity towards GalN1P and GalA1P, both of which are derivatives of Gal1P. We can also predict if PsLBP can work on donors that have not been screened here. For instance, GlcA1P, which has carboxyl group substitution at C6, would likely cause steric and electrostatic clashes with E732, and therefore, may not permit binding of GlcA1P to PsLBP. The importance of C6 for binding to PsLBP was evident from the strong STD intensities at the geminal protons at this position in both Glc1P and Man1P.

Both PsLBP and CgCBP work on disaccharides, but with different linkage specificity (i.e., β-(1→3) vs. β-(1→4)). However, each enzyme may employ a different mechanism to restrict the length of the substrate/product. The extended catalytic loop is a unique characteristic to CgCBP and is not observed in our PsLBP structure, nor in any other characterised disaccharide phosphorolysins in the GH94 family. It is likely to be involved in the substrate specificity of CgCBP with regards to the degree of polymerisation of the product.[27] In contrast, PsLBP contains a unique β-hairpin “gate”, which, if superposed with the CgCBP structure, overlaps with the position of the extended catalytic loop of the latter. Therefore, the β-hairpin gate in PsLBP may perform a similar role to that of the CgCBP extended catalytic loop in restricting the degree of polymerisation.

Conclusions

In summary, we have demonstrated the simple enzymatic synthesis of an unnatural disaccharide by utilising the promiscuity toward noncognate sugar 1-phosphates of PsLBP and provided structural insight into the mechanisms, whereby the enzyme distinguishes sugar 1-phosphate donors. Our work provides a stepping stone towards the design and engineering of GPs for tolerance towards other noncognate sugar donors, which will help to expand the range of GP applications in carbohydrate synthesis.

Experimental Section

Expression and purification of PsLBP: The PsLBP cDNA sequence was synthesised and optimised for E. coli expression (custom DNA synthesis by Gen9, Inc.). The sequence was amplified by PCR and cloned into the PopInF plasmid vector[46] by using In-Fusion (TakaraBio, Mountain View, CA, USA) following the manufacturer’s protocol. The recombinant PopInF-PsLBP was transformed into E. coli (BL21(DE3)) and a 1 L culture of the transformant was grown at 37 °C in lysogeny broth with agitation (180 rpm) until OD600 = 0.7. Heterologous protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM and incubated overnight at 18 °C. The cells were harvested (6721 g, 10 min) and lysed by sonication in buffer A (20 mM HEPES pH 7.0, 250 mM NaCl) supplemented with DNase (1 mg mL⁻¹, Sigma). Supernatant containing the recombinant proteins was separated from cell debris by centrifugation (3291 g, 30 min), then purified with an AKTA pure FPLC system (GE Healthcare) at 4 °C. The supernatant containing His₆-tagged PsLBP was loaded onto a 1 mL HisTrap HP column (GE Healthcare) pre-equilibrated with buffer A (10 mM HEPES pH 7.5, 250 mM NaCl, and 500 mM imidazole. The sample was further purified by gel filtration on a Superdex 200 16/600 column (GE Healthcare) eluted with 20 mM HEPES at pH 7.5, 150 mM NaCl, at 1 mL min⁻¹. Fractions containing the proteins were pooled and concentrated to 10 mg mL⁻¹ by using an Amicon Ultra-15 30 kDa MW cut-off concentrator. The protein was stored in 30 μL aliquots at −80 °C until required. To estimate the assembly of PsLBP in solution, a calibration curve was generated by using standard proteins from Gel Filtration Calibration Kit HMW (GE Healthcare). Approximately 2 mg mL⁻¹ of the standard proteins were analysed individually by using the same method as that described for PsLBP to determine the elution volumes. These values were then used to construct a calibration curve, following the manufacturer’s protocol.

Enzymatic assays: To screen for the PsLBP phosphorylase activity in peaks a, b and c, phosphate release assays[30] were performed in an assay buffer (20 μL, 100 mM HEPES pH 7.0, 20 mM Glc1P, 10 mM Glc, 200 mM sodium molybdate) in the presence of purified protein from peak a, b or c (1 μL). The reactions were incubated at 45 °C for 30 min. A coloured solution (90 μL, 0.1 M HCl, 13.6 mM sodium ascorbate) was added to the boiled reaction mixture and incubated for 30 min at room temperature to allow colour development. A stop solution (90 μL, 68 mM sodium citrate tribasic dihydrate, 2% acetic acid) was added to the mixture to stop colour development.
The absorbance of final solution was measured at λ = 620 nm on a 96-well plate reader. The amount of phosphate release was calculated from the absorbance by comparison with a phosphate standard curve ranging from 0 to 10 mM. All assays were performed in triplicate. To screen for glycan synthetic activity on various sugar 1-phosphate donors, recombinant PsLB P (8 µg) was assayed in the presence of sugar 1-phosphates (Gal1P, Gal1N1P, Gal1P, Glc1P or Man1P; 10 mM) and Glc (10 mM) in a buffer solution (20 µL, 100 mM HEPES pH 7.0). The reactions were incubated at 45°C for 30 min. Kinetic parameters of PsLB P were determined by using the phosphate release assay (20 µL) with the enzymes (25 µg mL−1) in the presence of 0.2–10 mM Glc and 10 mM Glc1P or Man1P. The amount of phosphate released from the assays was measured and the values were fitted through non-linear regression with a Michaelis–Menten model by using GraphPad Prism to determine Vmax and Km.

Oligosaccharide analysis: TLC was performed by spotting the recovered reaction mixture (0.5 µL) onto a silica gel plate (10 cm x 5 cm), then eluted with a mobile phase containing NH4OH/H2O/isopropanol (3:1:4) in a sealed glass container for 2 h to allow oligosaccharide separation. The plate was air-dried and stained with orcinol, which was prepared by adding concentrated sulfuric acid (20 mL) to an ice-cold solution of 3,5-dihydroxytoluene (360 mg) in ethanol which was prepared by adding concentrated sulfuric acid (20 mL) to an ice-cold solution of 3,5-dihydroxytoluene (360 mg) in ethanol until oligosaccharide spots were visible.

HPLC-PAD analyses were performed by diluting the reaction mixtures in MilliQ water (500 µL) followed by desalted on mixed-bed ion-exchange resin (Sigma). The desalted mixtures were filtered through a disposable polytetrafluoroethylene (PTFE) 0.45 µm filter disc (Merck Millipore), and subjected to HPLC-PAD analysis by using a Dionex ICS3000 chromatography system equipped with PAD and controlled by Chromeleon software. A PA100 CarboPac column (analytical: 4 x 250 mm, guard: 4 x 50 mm) was used for all analyses. The solutions for elution of the oligosaccharides were as follows: solution A: 100 mM sodium hydroxide; and solution B: 100 mM sodium hydroxide + 400 mM sodium acetate. The separation was achieved by gradient elution (0–100% solution B) from 1 to 30 min, followed by 30–50 min of 100% B, then 50–60 min re-equilibration of the column with solution A. The solutions were delivered to the column at a rate of 0.25 mL min−1.

NMR spectroscopy: 1H NMR spectra were recorded at 298 K on a Bruker Avance III 800 spectrometer at 800 MHz and 13C NMR spectra were recorded at 298 K on a Bruker Avance III 400 spectrometer at 100 MHz. Chemical shifts (δ) are reported with respect to the residual HOD signal in D2O (δH = 4.79 ppm). Coupling constants (J) are reported in Hz. NMR signal assignments were made with the aid of COSY and HSQC experiments.

Mass spectrometry: Disaccharide 1 was analysed by spotting a 2 mg mL−1 solution (1 µL) in water onto a silica gel plate. The spot was analysed by TLC/MS (Plate Express, Advion Biosciences, Ithaca, NY, USA), which subjected the compound to electrospray ionization by using a spray voltage and sample delivery pressure of 3.5 kV and 3000 psi, respectively, for positive-ion mode, at a flow rate of 0.3 mL min−1. The sample was analysed at a capillary temperature of 250°C, collision energy, and scan time of 1799 ms.

Optical rotation analysis: Disaccharide 1 was dissolved in water to a final concentration of 2.2 mg mL−1. The specific rotation of disaccharide 1 was recorded on a model 341 polarimeter (PerkinElmer) at 20°C, λ = 589 nm.

Physical data for β-d-mannopyranosyl-(1→3)-d-glucopyranose (1): [α]D20 +7 (c = 0.2, H2O); 1H NMR (800 MHz, D2O); δ = 5.172 (d, J = 3.7 Hz, 1H; H-1 α-Glc), 4.839 (d, J = 1.1 Hz, 1H; H-1 β-Man), 4.823 (d, J = 1.0 Hz, 1H; H-1 β-Man), 4.611 (d, J = 8.1 Hz, 1H; H-1 β-Glc), 4.081–4.049 (m, 2H; H-2 β-Man), 3.897–3.851 (m, 2H; H-2 β-Man), 3.865–3.808 (2H; H-3 α-Glc, H-6 β-Glc), 3.801 (d, J = 10.0, 5.0, 2.3, 0.6 Hz, 1H; H-5 α-Glc), 3.772 (dd, J = 12.3, 2.3 Hz, 1H; H-6 α-Glc), 3.718 (dd, J = 12.3, 5.0 Hz, 1H; H-6 α-Glc), 3.694–3.636 (4m, 4H; H-6 β-Man, H-3 β-Glc, H-6′ β-Glc, 3.633–3.579 (m, 3H; H-3 Man, H-2 β-Glc), 3.527 (t, J = 9.7 Hz, 1H; H-4 β-Man), 3.491–3.454 (m, 2H; H-4 α-Glc, H-4 β-Glc), 3.429 (d, J = 10.0, 5.5, 2.2 Hz, 1H; H-5 β-Glc), 3.374–3.334 (m, 2H; H-5 β-Man, 3.314 ppm (dd, J = 9.3, 8.1 Hz, 1H; H-2 β-Glc); 13C NMR (101 MHz, D2O): δ = 100.56 and 100.51 (C-1 Man), 95.7 (C-1 β-Glc), 92.1 (C-1 α-Glc), 84.6 (C-3 β-Glc), 82.1 (C-3 α-Glc), 76.35 and 76.31 (C-5 Man), 75.4 (C-5 β-Glc), 73.7 (C-2 β-Glc), 72.9 and 72.8 (C-3 Man), 71.1 (C-5 α-Glc), 70.9 (C-2 α-Glc, 70.5 (C-2 Man), 68.2 and 68.4 (C-4 α-Glc and C-4 β-Glc), 66.7 (C-4 Man), 61.0 (C-6 Man), 60.7, 60.53 ppm (C-6 Glc); HRMS (ESI): m/z calculated for C36H34NaO12+: 365.1054; found: 365.1062.

Crystallographic methods: Crystallisation trials were set up for purified PsLB P (∼10 mg mL−1) in 20 mM HEPES pH 7.0, 150 mM NaCl with a range of commercial crystallisation screens (Molecular Dimensions) in MRC2 96-well sitting-drop vapour diffusion crystallisation plates (Swissc) with a mixture of 0.3 µL well solution and 0.3 µL protein solution by using an OrxNano robot (Douglas Instruments). After optimisation, the best crystals were obtained from drops containing 0.4 µL of protein and 0.2 µL of a crystallisation solution comprised of 18% (w/v) polyethylene glycol 3350, 0.1 M Tris-citrate buffer, pH 6.0, 0.3 M ammonium sulfate. Crystals were cryo-protected with well solution containing 20% (v/v) ethylene glycol and flash-cooled in liquid nitrogen. For phasing, crystals were soaked in 30 min for a saturated solution of mercury(II) chloride made up in the cryoprotectant solution; the ligand bond complexes were obtained by soaking crystals for 5 min in cryoprotectant containing 20 mM of the compound (Glc1P or Man1P).

The pre-cooled crystals were transferred robotically to the goniostat on either beamline I03 or I04 at the Diamond Light Source (Oxfordshire, UK) and maintained at −173°C with a Cryojet cryocooler (Oxford Instruments). XRD data were recorded by using a Pi-latus 6M hybrid photon-counting detector (Dectris), then integrated and scaled by using XDS[68] through the XIA2 expert system[44] and merged by using AIMLESS[69] All crystals belonged to space group P42,2 with approximate cell parameters of a = b = 147 Å, c = 222 Å (see Table 54 for a cell data collection statistics).

Analysis of the likely composition of the asymmetric unit (ASU) suggested that it would contain two copies of the 102 kDa protein chain, giving an estimated solvent content of 58%. The structure was solved at 2.9 Å resolution by SAD phasing with the CRANK2 pipeline[66] by combining data collected from two mercury-soaked crystals at the Lm X-ray absorption edge of mercury (wavelength = 1.0052 Å); SHELX[70] located 11 sites in the ASU with occupancies of >0.25, and Buccaneer[71] went on to build a model in which 59% of the sequence was fitted with Rwork and Rfree values of 0.342 and 0.400, respectively. This was then edited in COOT[69] before refining in REFMACS[72] against native data processed to 1.95 Å resolution. Phases calculated from this model were used as input for a second Buccaneer task, which produced a model with 97% of the sequence fitted and Rwork and Rfree values of 0.282 and 0.323, respectively. The model was finalised by further iterations of manual rebuilding in COOT and restrained refinement in REFMAC5 by using isotropic thermal parameters and TLS group definitions obtained from the TLSMD server (http://skuld.bmsc.washington.edu/~tlsmd/).[73] In each of the expected active sites, residual density
consistent with an oxyanion was present. This was interpreted as sulfate derived from the precipitant solution. This sulfate-bound structure was used as the starting model for the Glc1P and Man1P complexes, which were built and refined as outlined above.

The geometries of the final models were validated with MOLPRO-BITV[28] before submission to the Protein Data Bank (see Table S4 for a summary of model statistics). Omit mFobs−dFcalc difference electron density maps were generated for the bound ligands by using phases from the final model without the ligands after the application of small random shifts to the atomic coordinates, re-setting temperature factors, and re-refining to convergence (Figure S1A). All structural figures were prepared by using CCP4i4.[29]

STD NMR spectroscopy: All samples were prepared in D2O with [D6]Trit (25 mM, pH 7.4) and contained final protein and ligand concentrations of 50 μM and 6 mM, respectively. All experiments were performed at 278 K on a Bruker Avance III 800 MHz spectrometer equipped with a 5 mm TXI 800 MHz H- C/N- D-05 Z BTO probe. STD NMR spectroscopy experiments were performed by using a train of 50 ms Gaussian pulses applied on the $\phi$ channel at either 0.8 (on-resonance) or 40 ppm (off-resonance). A spoil sequence was used to destroy unwanted magnetisation and a spin lock was used to suppress protein signals (stddiff.3). The recycle delay (d1) was set to 5 s. The total saturation time and number of scans were selected according to Table 2.

| Total saturation time [s] | No. of scans | Total saturation time [s] | No. of scans |
|---------------------------|-------------|---------------------------|-------------|
| 0.5                       | 512         | 2                         | 128         |
| 0.75                      | 512         | 3                         | 128         |
| 1                         | 256         | 5                         | 128         |
| 1.5                       | 256         |                            |             |

Preparation of models: Crystal structures were imported into Schrödinger Maestro[30] and prepared with the Protein Preparation Wizard. All non-protein or non-ligand atoms were removed. Protons were then added to the model, by using PROPKA to predict the protonation state of polar side chains at pH 7.[31] The hydrogen-bonding network was automatically optimised by allowing asparagine, glutamine, and histidine side chains to be flipped. The model was then minimised by using the OPLS[32] force field and a heavy-atom convergence threshold of 0.3 Å. Because STD NMR spectroscopy experiments were performed in D2O, polar protons were removed from the ligand prior to CORCEMA-ST analysis.[33]

CORCEMA-ST calculations: Protein chemical shifts were calculated by using the SHIFTX2[34] webserver, according to experimental conditions. All protein protons or non-ligand atoms were removed. Protons were then added to the model, by using PROPKA to predict the protonation state of polar side chains at pH 7.[31] The hydrogen-bonding network was automatically optimised by allowing asparagine, glutamine, and histidine side chains to be flipped. The model was then minimised by using the OPLS[32] force field and a heavy-atom convergence threshold of 0.3 Å. Because STD NMR spectroscopy experiments were performed in D2O, polar protons were removed from the ligand prior to CORCEMA-ST analysis.[33]

Conflict of Interest

The authors declare no conflict of interest.

Keywords: biocatalysis · enzymes · glycosylation · NMR spectroscopy · structural biology

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