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Carbohydrate Research 343 (2008) 2032–2040
Carbohydrate RESEARCH

Minireview

Mechanistic differences among retaining disaccharide phosphorylases: insights from kinetic analysis of active site mutants of sucrose phosphorylase and α,α-trehalose phosphorylase

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Received 12 October 2007; received in revised form 22 January 2008; accepted 22 January 2008
Available online 2 February 2008

Presented at Eurocarb 14th Lübeck, Germany, September 2007

Abstract—Sucrose phosphorylase utilizes a glycoside hydrolase-like double displacement mechanism to convert its disaccharide substrate and phosphate into α-D-glucose 1-phosphate and fructose. Site-directed mutagenesis was employed to characterize the proposed roles of Asp^{196} and Glu^{237} as catalytic nucleophile and acid–base, respectively, in the reaction of sucrose phosphorylase from *Leuconostoc mesenteroides*. The side chain of Asp^{295} is suggested to facilitate the catalytic steps of glucosylation and deglucosylation of Asp^{196} through a strong hydrogen bond ($\geqslant$23 kJ/mol) with the 2-hydroxyl of the glucosyl oxocarbenium ion-like species believed to be formed in the transition states flanking the β-glucosyl enzyme intermediate. An assortment of biochemical techniques used to examine the mechanism of α-retaining glucosyl transfer by *Schizophyllum commune* α,α-trehalose phosphorylase failed to provide evidence in support of a similar two-step catalytic reaction via a covalent intermediate. Mutagenesis studies suggested a putative active-site structure for this trehalose phosphorylase that is typical of retaining glycosyltransferases of fold family GT-B and markedly different from that of sucrose phosphorylase. While ambiguity remains regarding the chemical mechanism by which the trehalose phosphorylase functions, the two disaccharide phosphorylases have evolved strikingly different reaction coordinates to achieve catalytic efficiency and stereochemical control in their highly analogous substrate transformations.

Keywords: Glycoside hydrolase; Glycosyltransferase; Phosphorylase; Retaining mechanism; Covalent intermediate

Contents

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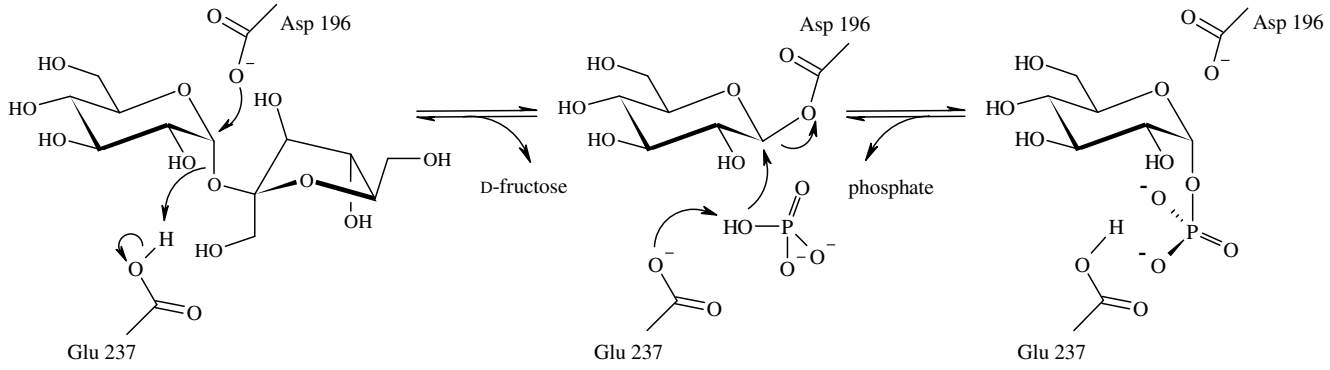
doi:10.1016/j.carres.2008.01.029

1. Introduction

Phosphorylases are carbohydrate processing enzymes that catalyze reversible glycosyl transfer from a saccharide donor substrate to phosphate.[1] Depending on which direction of the reaction, phosphorolysis or synthesis, is preferred under given conditions, they serve to promote breakdown or formation of glycosidic bonds. Structural and mechanistic studies have led to the classification of known phosphorylases into a main group of glycoside hydrolase-like enzymes and a smaller group of enzymes related to glycosyltransferases.[2] Phosphorylases belonging to either one of the two classes are further categorized according to whether they retain or invert the anomeric configuration of the carbohydrate substrate in the sugar 1-phosphate product. Inverting phosphorylases for which the crystal structures have been determined are found in glycoside hydrolase families GH-65 (maltose phosphorylase) and GH-94 (cellobiose and chitobiose phosphorylases).[3–5] Their mechanism is thought to involve a direct S_N2-like displacement reaction where departure of the leaving group, facilitated through partial protonation of the glycosidic oxygen from a general acid catalytic group of the enzyme, and attack of the phosphate nucleophile occur from opposite faces of the glycosyl moiety being transferred.[2] Retaining phosphorylases are found in family GH-13 (sucrose phosphorylase) and glycosyltransferase families GT-4 (trehalose phosphorylase) and GT-35 (glycogen, starch and maltodextrin phosphorylases).[6–8]

Sucrose phosphorylase utilizes a glycoside hydrolase-like double displacement mechanism, illustrated in Scheme 1, to convert its disaccharide substrate and phosphate into α-D-glucose 1-phosphate (α-Glc1*P*) and fructose. The enzymatic reaction was already identified in the 1940s [for a historical review, see Ref. 9] and shown to proceed via a catalytically competent β-glucosyl enzyme intermediate in elegant biochemical studies almost four decades ago.[10] Numerous studies have since reported on the synthesis of α-glucosidic products using transglucosylation catalyzed by sucrose phosphorylases from different bacterial sources.[11–13] Recently, high-resolution structure snapshots along the reaction coordinate of sucrose phosphorylase have been provided by crystallographic studies of the enzyme from *Bifidobacterium adolescentis*. They include the free phosphorylase,[6] enzyme bound with sucrose (Fig. 1A), the β-glucosyl enzyme intermediate (Fig. 1B) and a complex with β-D-glucose resulting from hydrolysis of the glucosylated phosphorylase (Fig. 1C).[14] These X-ray crystal structures have facilitated the examination of the catalytic mechanism of sucrose phosphorylase by site-directed mutagenesis.

While α,α-trehalose phosphorylases from bacteria are inverting enzymes belonging to family GH-65,[15] the counterpart enzymes from *Schizophyllum commune* and several other filamentous fungi utilize a retaining mechanism and have been classified into family GT-4.[16–19] Unlike sucrose phosphorylase whose Ping-Pong kinetic mechanism seems to reflect enzymatic reaction in two catalytic steps, namely glucosylation and deglucosylation,[10,20] trehalose phosphorylase from *S. commune* recruits phosphate to induce α,α-trehalose binding recognition and promotes catalysis from a ternary



Scheme 1. Reaction mechanism proposed for sucrose phosphorylase. The covalent enzyme–sugar intermediate can be intercepted by water or other nucleophiles in the absence of natural acceptors such as phosphate (shown in the scheme) or fructose.[7]

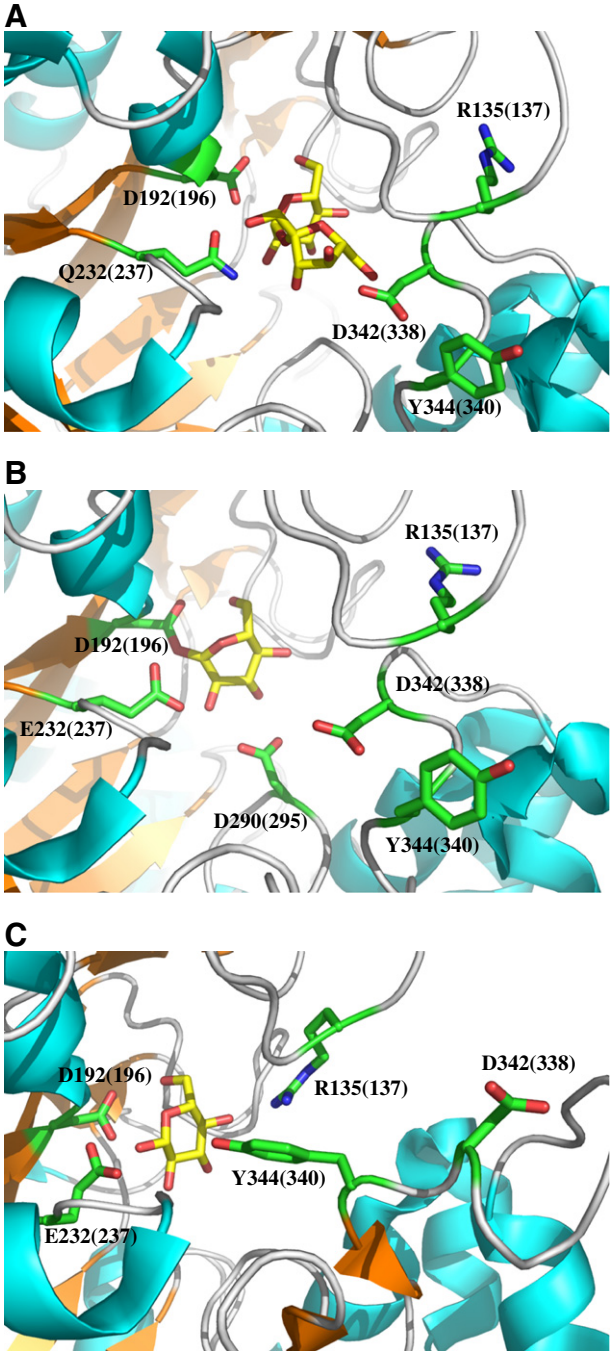


Figure 1. Close-up structures of the active site of sucrose phosphorylase from *Bifidobacterium adolescentis* when bound with sucrose (A) and β-D-glucose (C), and in the β-glucosyl enzyme intermediate (B). Note the conformational change at subsite +1 in panel 1C in comparison to panels 1A and 1B. The picture was generated using coordinates of Protein Data Bank entry 2gdu (A) and 2gdv (B, C) and PyMol v.0.99. Numbering of amino acids is for the *B. adolescentis* enzyme, and the corresponding residues of the *L. mesenteroides* enzyme are given in parentheses.

enzyme–substrate complex (Scheme 2).[21] Attempts to isolate a covalent intermediate of a putative double displacement-like reaction of trehalose phosphorylase have proven not fruitful, suggesting that alternative catalytic mechanisms of the enzyme should also be taken into consideration (see later).[16,21] An internal return-like (S_Ni-like) mechanism was proposed for glycogen phosphorylase and several other α-retaining glycosyltransferases for which exhaustive structural and mutagenesis studies failed to provide support for the double displacement reaction [for reviews, see Refs. 2 and 22]. The mechanism involves a direct front-side displacement of the leaving group by the incoming nucleophile, resulting in retention of the anomeric configuration (Scheme 3). Reaction is suggested to occur via a single transition state featuring a highly developed oxocarbenium-ion character. However, a clear-cut distinction between the catalytic mechanisms of retaining glycoside hydrolases and glycosyltransferases remains uncertain.

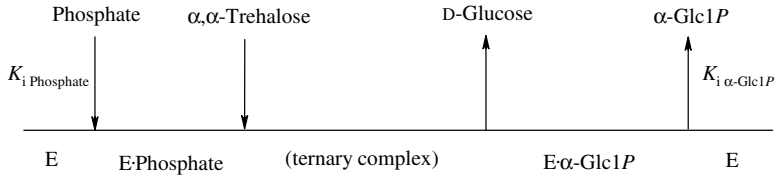
Sucrose phosphorylase and trehalose phosphorylase provide a direct link between the classes of α-retaining glycoside hydrolases and glycosyltransferases. Mechanistic comparisons of the two phosphorylases would therefore seem generally relevant and can be pursued by examining almost the same chemical reaction. In this paper, we review recent mechanistic insights obtained from detailed mutational analysis of sucrose phosphorylase from *Leuconostoc mesenteroides* and trehalose phosphorylase from *S. commune*.

2. Sucrose phosphorylase from *L. mesenteroides*

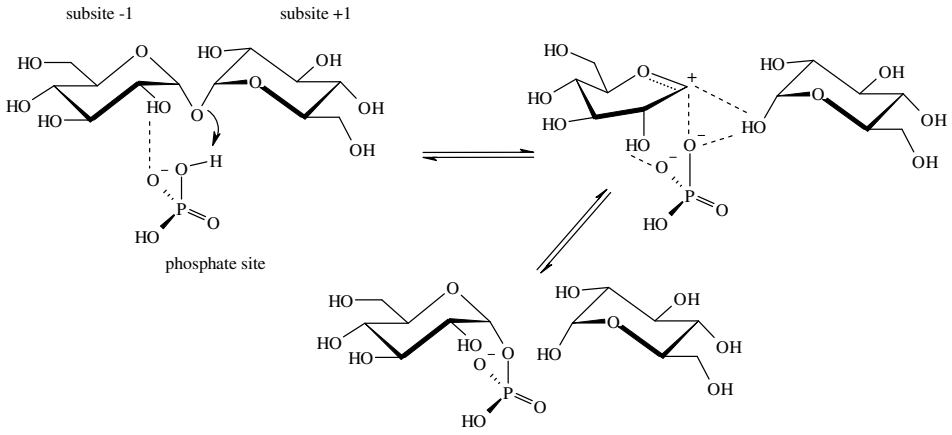
Figure 1B reveals the identity of the catalytic nucleophile of *B. adolescentis* sucrose phosphorylase as Asp^{192}. It also suggests participation of Glu^{232} and Asp^{290} in the catalytic mechanism, as discussed below. Pairwise sequence alignment (not shown) indicates that the three catalytic-site residues of the *Bifidobacterium* enzyme are homologous to Asp^{196}, Glu^{237} and Asp^{295} of sucrose phosphorylase from *L. mesenteroides*.

2.1. Asp^{196}—the catalytic nucleophile

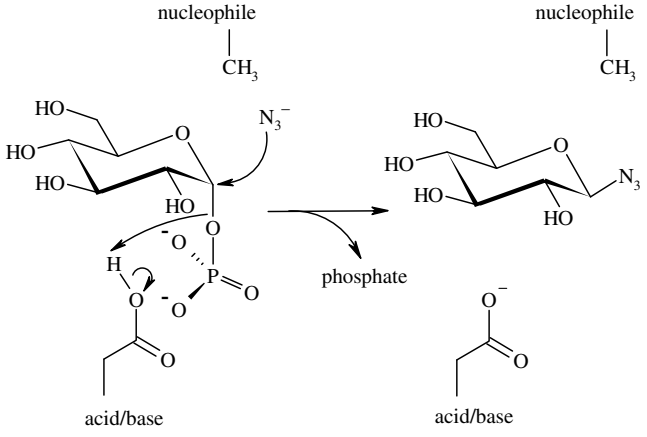
Asp^{196} was replaced by an alanine using site-directed mutagenesis.[20] In agreement with the expected kinetic consequences resulting from complete removal of catalytic assistance by a nucleophilic group on the enzyme, the purified D196A mutant lacked activity (⩽0.0001% that of the wild-type) in assays measuring phosphorolysis and synthesis of sucrose as well as arsenolysis of α-Glc1*P*. However, when presented with azide as an alternative nucleophile, the conversion of α-Glc1*P* proceeded at a slow rate and resulted in the formation of a new substitution product, glucose 1-azide with a β-anomeric configuration. Activity enhancement in the D196A mutant was therefore suggested to result from the direct participation of azide in the now inverting, single displacement-like mechanism of glucosyl transfer (Scheme 4). Consistent with the change of mechanism



Scheme 2. Steady-state kinetic mechanism of *Schizophyllum commune* trehalose phosphorylase. E represents the enzyme and K_i is a dissociation constant of the binary enzyme–substrate complex.



Scheme 3. S_Ni-like reaction mechanism proposed for trehalose phosphorylase and retaining glycosyltransferases. Most probably, the reaction proceeds via the formation of an oxocarbenium ion-like transition state.



Scheme 4. Functional complementation by azide of an otherwise inactive mutant of sucrose phosphorylase in which the catalytic nucleophile Asp^{196} was replaced by the non-nucleophilic Ala.

following the substitution Asp^{196}→Ala, the mutant phosphorylase also promoted phosphorolysis of β-glucose 1-azide to yield α-Glc1*P*. Results of steady-state kinetic analysis of glucosyl transfer to phosphate catalyzed by D196A suggested reaction through a ternary enzyme–substrate complex, in good agreement with expectations from Scheme 4 but in stark contrast to the wild-type enzyme whose reaction in two catalytic steps via a covalent intermediate appears to dictate Ping-Pong kinetics.[10,20]

2.2. Glu^{237}—the catalytic acid–base

Glu^{237} was replaced by Gln, so the carboxamide group of the Gln cannot fulfil the acid–base catalytic function proposed for the carboxylic group of the original Glu. The Ping-Pong kinetic mechanism of the phosphorylase implies that catalytic efficiencies (k_{cat}/K_m) for glucosyl donor and acceptor substrates measure formation and breakdown of the covalent glucosyl-enzyme intermediate as kinetically isolated steps, respectively. Figure 2 summarizes kinetic consequences in the E237Q mutant for reactions that are distinguished according to their requirements for acid–base catalytic assistance during glucosylation and deglucosylation. Catalytic steps involving a poor leaving group or nucleophile were slowed by five orders of magnitude in the E237Q mutant as compared to the wild-type enzyme whereas other steps expected to proceed without support from an acid–base catalyst were not strongly affected by the mutation.[23]

2.2.1. Chemical rescue of the E237Q mutant. Enhancement of activity of the E237Q mutant was observed when the reaction with α-Glc1*P*, a substrate with good leaving group ability, was assayed in the presence of anionic nucleophiles such as azide, acetate and formate. This chemical rescue of catalytic activity showed saturation-like behaviour on the concentration of the nucleophile and in the case of azide, caused up to 330-fold

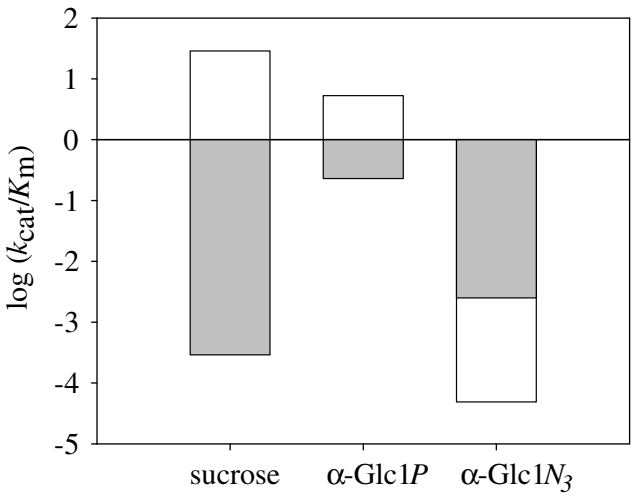


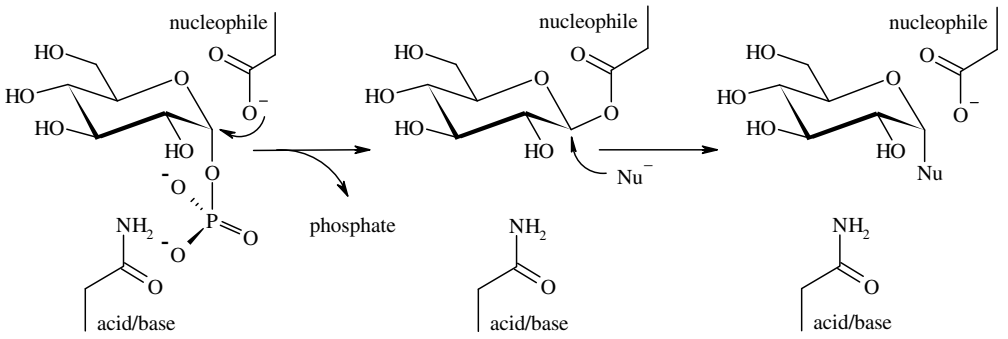
Figure 2. Kinetic consequences of the Glu^{237}→Gln replacement on half-reactions requiring Brønsted catalytic assistance (sucrose) and others that do not (α-Glc1*P*, α-Glc1N_3). Results are expressed as k_{cat}/K_m values for wild-type enzyme (white) and the mutant (grey), and are given in logarithmic form.

increase in the steady-state rate of enzymatic conversion of α-Glc1*P*. The observed kinetic operation of the E237Q mutant under conditions in which deglucosylation was rate-determining is characteristic of a catalytic acid–base function of Glu^{237} in the wild-type enzyme. The proposed mechanism of functional complementation of the E237Q mutant (Scheme 5) whereby external nucleophiles react more rapidly than water with the glucosylated mutant enzyme and thus facilitate catalytic turnover was confirmed by NMR analysis of the respective chemical rescue products, each of which featured the expected α-anomeric configuration. Results of in situ proton NMR studies revealed that unlike α-glucose 1-azide (α-Glc1N_3), which was stable, the α-glucose 1-ester resulting from reaction of the glucosyl-enzyme intermediate with acetate or formate underwent spontaneous transformation due to acyl-group migration, mutarotation and hydrolysis. It should be noted that when incubated under exactly identical conditions as E237Q, the wild-type enzyme hydrolyzed α-Glc1*P* at about 1–2 % of the rate of phosphorolysis of sucrose (≈170 U/mg at pH 7.0 and 30 °C) but did not catalyze glucosyl transfer to azide, acetate or formate.[23]

2.2.2. pH studies. In pH profiles of k_{cat}/K_m, the pH dependences of kinetically isolated glucosylation and deglucosylation steps could be analyzed for wild-type enzyme and the E237Q mutant thereof. Using sucrose or α-Glc1*P* as donor substrate, glucosylation of the wild-type proceeded optimally above and below apparent pK_a values of about 5.6 and 7.2, respectively, whereas deglucosylation was dependent on the apparent single ionization of a group with a pK_a of about 5.8. By contrast, glucosylation of the E237Q mutant was slowed below an apparent pK_a of 6.0 but had lost the high pH dependence of the wild-type enzyme. Deglucosylation of the E237Q mutant was not pH-dependent. These results strongly support an enzymatic mechanism where the side chain of Glu^{237} alternates between catalytic functions of a general acid and base in glucosylation and deglucosylation half-reactions, respectively. It was proposed that its pK_a cycles between 7.2 in the free enzyme and 5.8 in the glucosyl-enzyme intermediate. The low-pH ionization was assigned to Asp^{196}, which must be unprotonated during catalysis for the formation of a covalent intermediate.[23]

2.3. Differential binding of fructose and phosphate as leaving group/nucleophile of the reaction

During analysis of the participation of Glu^{237} in each step of catalysis, the question arose of how glucosylated sucrose phosphorylase manages to accommodate a phosphate ion in the active site while the unprotonated side chain of the Glu remains in place, ready to provide general base catalytic assistance to the attack of the anomeric hydroxyl of β-fructofuranose. An observation oftentimes made with retaining glycoside hydrolases and transglycosidases is that nucleophilic anions are excluded from reactions with glycosyl-enzyme intermediates, probably because of unfavourable electrostatic



Scheme 5. Proposed mechanism for chemical rescue of a mutant sucrose phosphorylase in which the acid–base catalyst Glu^{237} was replaced by Gln. Nu is an external nucleophile such as azide, formate, acetate or a halide.

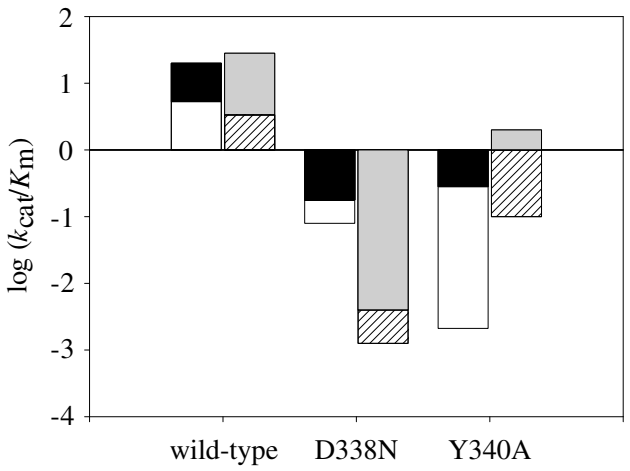


Figure 3. Mutational analysis of subsite +1 of sucrose phosphorylase. Results are expressed as k_{cat}/K_m values for wild-type and mutant enzymes and are given in logarithmic form for phosphate (black), α-Glc1*P* (white), sucrose (grey) and fructose (shaded).

interactions with the negative charge on the Brønsted catalytic group of the enzyme. Recent work has shown that Asp^{338} and Tyr^{340} have key roles in a differential binding mechanism utilized by *L. mesenteroides* sucrose phosphorylase to bring about specificity for, respectively, the fructose leaving group and the phosphate nucleophile during the catalytic steps of phosphorolysis of sucrose (Fig. 3).[24] The results support a suggestion for the reaction cycle of sucrose phosphorylase derived from the crystal structure of the enzyme from *B. adolescentis* where movements of active-site entrance loops cause Asp^{338} and Tyr^{340} to alternate between positions in and out of the catalytic centre, allowing the phosphorylase to switch between the utilization of fructose (Fig. 1A and B) and phosphate (Fig. 1C) upon moving along the reaction coordinate of glucosyl transfer.[14]

2.4. Asp^{295}—transition state stabilization through hydrogen bonding

Results from sequence alignments, X-ray structures and site-directed mutagenesis studies have delineated a common active-site pattern for family GH-13, in which Asp^{295} together with Asp^{196} and Glu^{237} of *L. mesenteroides* sucrose phosphorylase (or the positional homologues thereof) constitutes a conserved triad of catalytic residues.[25,26] A plausible mechanism for Asp^{295} is that bidentate hydrogen bonding of its carboxylate group with C-2 and C-3 hydroxyls of the glucosyl residue undergoing transfer to and from phosphate (Fig. 1B) stabilizes the transition states flanking the β-glucosyl enzyme intermediate. An additional or perhaps alternative role for Asp^{295} is the activation of Glu^{237} for function as catalytic proton donor. We substituted Asp^{295} by Asn and measured kinetic consequences in the purified D295N mutant.

When assayed in the direction of phosphorolysis of sucrose at pH 7.0 and 30 °C, the D295N mutant retained only about 0.01% of the specific activity of the wild-type enzyme. Heat treatment of D295N (100 °C, 10 min) caused significant re-gain of specific activity in the mutant, most likely resulting from partial deamidation of Asn^{295} that was clearly traceable with LC–ESIMS/MS analysis of tryptic peptides derived from a boiled sample of the D295N mutant.[27] The catalytic steps of glucosylation and deglucosylation of the enzyme were affected uniformly, about 20,000-fold, in the D295N mutant as compared to the wild-type enzyme. The catalytic defect resulting from the substitution of Asp^{295} was independent of the leaving group ability and nucleophilic reactivity of the substrate. The pH dependences of k_{cat}/K_m for glucosyl donor and acceptor substrates in the directions of phosphorolysis and synthesis of sucrose were not significantly altered in the D295N mutant in comparison with the wild-type enzyme. The 10^5-fold preference of wild-type phosphorylase for *glucosyl* transfer compared with *mannosyl* transfer from phosphate to fructose was completely lost in the D295N mutant. These results support an auxiliary catalytic function for Asp^{295} in which the carboxylate side chain facilitates the steps of glucosylation and deglucosylation of Asp^{196} through a strong hydrogen bond ($\geqslant$23 kJ/mol) with the equatorial 2-hydroxyl of the glucosyl oxocarbenium ion-like species believed to be formed in the transition states flanking the β-glucosyl enzyme intermediate.[27] Crystal structures of *B. adolescentis* sucrose phosphorylase portray a partial conformational itinerary for the glucosyl moiety along the reaction pathway, revealing relaxed $^4C_1$ chair conformations in enzyme-bound sucrose (substrate) and glucose (hydrolysis product) as well as a $^{1,4}B$ boat conformation for the covalent intermediate.[14] The immediate vicinity of $^4C_1$ and $^{1,4}B$ conformations to a $^4H_3$ conformation in a pseudo-rotational cycle of glucopyranose strongly suggests that the half-chair conformation ($^4H_3$) is used for the oxocarbenium ion-like transition state of sucrose phosphorylase.[28,29] A tentative explanation for why the stabilization provided by Asp^{295} is apparently selective for the transition state of the reaction is that the conformational change $^4C_1 \rightarrow {}^4H_3$ in the glucosylation step and $^{1,4}B \rightarrow {}^4H_3$ (probably via a $^1S_3$ skew boat) in the deglucosylation step contributes to significant shortening, hence strengthening of the proposed hydrogen bond between the side chain of Asp^{295} and the sugar 2-hydroxyl. Electronic effects such as gradual depression of the pK_a of the 2-hydroxyl in response to progressive generation of partial positive charge at the anomeric carbon as heterolysis of the exocyclic C-1–O bond takes place could also be responsible for the optimization of this hydrogen bond in the transition state.[30] Partial ionization of the 2-OH was proposed to provide electrostatic stabilization of the oxocarbenium ion-like

transition state of β-galactoside cleavage by *Escherichia coli* β-galactosidase (lac *Z*).[31]

3. Trehalose phosphorylase from *S. commune*

Trehalose phosphorylase differs from sucrose phosphorylase in many respects, structurally as well as mechanistically. Recent crystal structures of three representatives of family GT-4 [see Refs. 32 and 33], revealed a common protein structural organization typical of glycosyltransferases of fold family GT-B,[34] where the catalytic centre, which features a highly conserved architecture [see Ref. 7] is situated in a deep cleft formed by two Rossmann-fold domains. Sucrose phosphorylase, by contrast, adopts the characteristic $(\beta/\alpha)_8$ fold of members of family GH-13. Trehalose phosphorylase was isolated from *S. commune* as a truncated 61-kDa protein that contained one Mg^{2+} and was enzymatically active as a monomer.[16,21] Heterologous expression of the coding gene in *E. coli* yielded the full-length phosphorylase with a molecular mass of about 80 kDa. The recombinant enzyme lacked bound Mg^{2+}.[7] Intact and truncated forms of the phosphorylase showed the same specific activity of about 6 U/mg.[7] MS analysis revealed that proteolytic processing had occurred at the N-terminus of the enzyme, indicating that critical elements of catalysis and specificity of trehalose phosphorylase reside in the C-terminal part of the protein.

Unlike sucrose phosphorylase, which utilizes α-D-glucose 1-fluoride (α-Glc1*F*) as an alternate donor substrate for glucosyl transfer to either phosphate or fructose,[10] trehalose phosphorylase recognizes α-Glc1*F* exclusively as a slow substrate for the phosphorolysis direction.[35] Trehalose phosphorylase does not promote the conversion of α-Glc1*P* into Glc and phosphate when arsenate is present. A two-step reaction via a glucosyl-enzyme intermediate is a likely mechanistic requirement for the reversible exchange reaction, α-Glc1*P* + arsenate → α-D-glucose 1-arsenate (α-Glc1*As*) + phosphate, which is catalyzed by sucrose phosphorylase with high specific activity (200 U/mg). Note that the spontaneous hydrolysis of α-Glc1*As* makes the overall arsenolysis of α-Glc1*P* a completely irreversible process. Lacking catalytic assistance to arsenolysis of α-Glc1*P* does obviously not prove the absence of a double displacement intermediate for trehalose phosphorylase. However, the result supports the notion that in contrast to sucrose phosphorylase, formation and breakdown of any such intermediate in trehalose phosphorylase cannot be analyzed as kinetically isolated reaction steps.

The glucosyl-enzyme intermediate of sucrose phosphorylase can be intercepted by numerous alcohol acceptors other than fructose.[11,13,36,37] Its ability to promote transglucosylation reactions was utilized to synthesize a range of α-glucosidic products, glucosyl-xylitol, for example.[11] Nucleophilic competition for reaction with a double displacement intermediate was not observed in trehalose phosphorylase.[16]

3.1. Biochemical probes of catalytic mechanism

3.1.1. Attempts to form and trap a covalent intermediate. Results of experiments reviewed above suggest that, if a glucosylated enzyme is formed as intermediate of the reaction catalyzed by trehalose phosphorylase, it is not synthesized before all substrates have bound to the active site. In an effort to form and possibly trap a glucosyl-enzyme intermediate, 1,5-anhydro-glucitol ($K_i = 0.72$ mM) and D-glucal ($K_i = 0.31$ mM) were used as incompetent analogues of the α-D-glucose acceptor substrate. The idea was that binding of these inhibitors to the complex of trehalose phosphorylase and α-Glc1*P* could induce the reactive conformational state of the enzyme, thus allowing C-1–O bond cleavage to take place and promoting formation of the glucosylated enzyme with bound phosphate. We expected that, if this complex occurred, it would be stable in the absence of the reacting 1-OH group of α-D-glucose, and the enzyme would be inactive when assayed in the direction of trehalose phosphorolysis, as follows. Since α,α-trehalose cannot bind the putative glucosyl-enzyme intermediate, it will not serve as glucosyl acceptor in place of α-D-glucose or, alternatively, induce the reverse glucosyl transfer to phosphate bound to the enzyme. However, inactivation of trehalose phosphorylase did not occur upon incubation of the enzyme in the presence of α-Glc1*P* (40 mM) and 1,5-anhydro-glucitol (40 mM) or D-glucal (20 mM).[21]

In an alternative approach, similar to that successfully used for the characterization of retaining glycoside hydrolases,[38,39] 2-deoxy-2-fluoro glucosyl donors were examined as substrate analogues of sucrose phosphorylase (unpublished results) and trehalose phosphorylase.[40] The electronegative fluorine at position C-2 is expected to inductively destabilize the oxocarbenium ion-like transition states through which the S_Ni-like mechanism and both steps of a double displacement mechanism proceed. In the case of sucrose phosphorylase, the important hydrogen bond with Asp^{295} will also be removed. Under conditions in which introduction of the fluorine affects deglucosylation much more severely than glucosylation, a 2-deoxy-2-fluoro-glucosyl-enzyme intermediate with a significant lifetime is expected to accumulate, arguably leading to inactivation of the phosphorylase. Unfortunately, the fluoro-sugar approach was not effective with both disaccharide phosphorylases, which do not display detectable reaction with either one of the donor substrate analogues. It would seem that in sucrose phosphorylase, the catalytic step of glucosylation has been slowed dramatically by the substitution OH→F at C-2.

3.1.2. Transition state-like inhibition by vanadate. Orthovanadate is a competitive inhibitor against phosphate and α-Glc1*P* and binds to free trehalose phosphorylase with a K_i of 1 μM about four orders of magnitude more tightly than phosphate.[7,41] We found that the K_i for vanadate, determined in assays for the synthesis direction, varied strongly in response to structural alterations of the α-D-glucose acceptor substrate at C-2 and O-5 that are tolerated in enzymatic catalysis. A linear free-energy relationship was derived between the reciprocal K_i and the catalytic efficiency for reaction with the glucosyl acceptor, as shown in Figure 4.[41] We proposed that a noncovalent adduct of vanadate and the nucleophile leaving group bound at subsite +1 is the true inhibitor and by mimicking the effective leaving group of the reaction (Scheme 6), it displays partial transition state analogy. A hydrogen bond between the phosphate nucleophile and the 1-OH of the α-D-glucose leaving group (see Schemes 3 and 6) would seem to be an essential component of S_Ni-like mechanism of trehalose phosphorylase. However, although perhaps unusual, it is certainly also compatible with a double displacement mechanism where it could take place in each of the two catalytic steps.

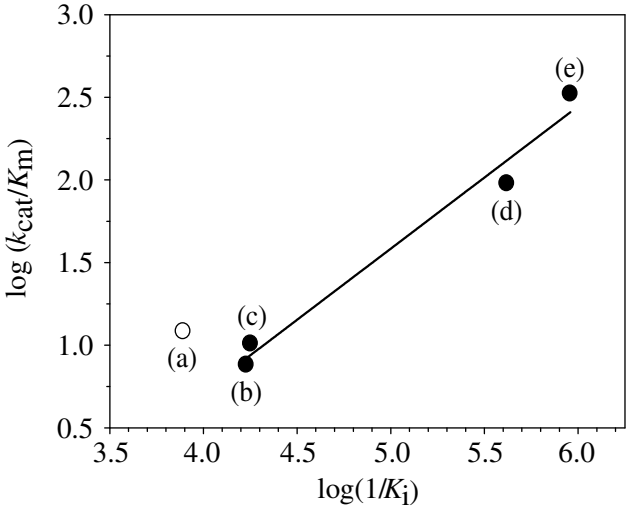
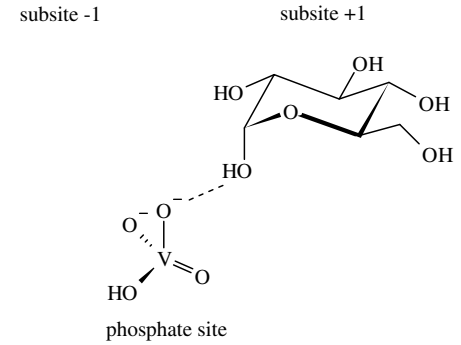


Figure 4. Linear free energy relationship analysis for the inhibition by vanadate and the catalytic efficiency for synthetic glucosyl transfer by trehalose phosphorylase. The data are taken from Nidetzky and Eis.[41] (a) 2-Deoxy-2-fluoro-D-glucose, (b) 2-keto-D-glucose, (c) 2-deoxy-D-glucose, (d) 5-thio-D-glucose and (e) D-glucose. The line represents the best fit of the data excluding the value shown by an open circle.



Scheme 6. Transition state-like inhibition of trehalose phosphorylase by vanadate in combination with α-D-glucose. Vanadate, a dead-end inhibitor, is competitive against phosphate and α-Glc1*P*.

3.2. Proposed catalytic centre of trehalose phosphorylase

Results of structure-based sequence comparisons with retaining glycosyltransferases of fold family GT-B revealed a high level of residue conservation for the two

Enzyme	GT family	Phosphate site	Sugar subsite +1	Sugar subsite -1	Phosphate site	GPGT motif
*At*GS	5	-IKTGG[18]LADVV	MVHAHD[138]WQAAM	SLLIIH[163]NIAFQ	ISR[299]TTWQK[304]GLD	FE[376]PCGLTQLY[384]A
OtsA	20	AASAGG[23]LAVGI	IIWIHD[131]YHL-L	IGGFLH[155]IP---	VER[263]LLYSK[268]GLP	RD[362]GMNLVAKE[370]Y
*Ec*MalP	35	ALGNGG[115]LGRLA	VLQLND[308]THP-T	FAYTNH[346]TL---	IKR[535]THEYK[540]RQH	KE[638]ASGTGNMK[646]L
PimA	4	FDVPGG[16]VQSHV	VLHIHE[95]PNA-P	IVATFH[118]TS---	GR[196]YDEPRK[202]CMA	GE[274]SFGIVLVE[282]A
*Sc*TPase	4	-PQGGG[292]VALMR	IAFIDD[379]PQM-P	IIYRSH[403]IE---	IAR[507]FDPAK[512]GIP	RE[606]GFEVKVSE[614]A

*Ec*MalP
*Sc*TPase
α6(11) β12(11) β13(12) β19(15) α18(20) α21(23)

D379N $10^{5.3}$ H403A $10^{3.2}$ R507A $10^{5.6}$ K512A $10^{5.1}$

Figure 5. Conservation of active-site residues among glycosyltransferases of fold family GT-B, and kinetic consequences of their replacement in trehalose phosphorylase. Partial multiple sequence alignment of *At*GS (*Agrobacterium tumefaciens* glycogen synthase, acc. no. AAL44876), OtsA (*E. coli* trehalose 6-phosphate synthase, acc. no. AAS99849), *Ec*MalP (*E. coli* maltodextrin phosphorylase, acc. no. AAC76442), PimA (*Mycobacterium smegmatis* phosphatidylinositol-mannosyltransferase, acc. no. ABK72422) and *Sc*TPase (*S. commune* trehalose phosphorylase, acc. no. ABC84380). The Figure also shows secondary structural elements (black cylinders for α-helices and arrows for β-sheets) for *Ec*MalP, whereby the numbering corresponds to that of Watson et al.,[8] aligned to the predicted secondary structural elements for *Sc*TPase, with numbers indicated in parentheses. Secondary structure prediction was done with the public PSIPRED server (http://bioinf.cs.ucl.ac.uk/psipred/) using mGenTHREADER method. The degree of reduction of catalytic activity for the indicated site-directed mutants of *Sc*TPase (arrows) is given as well.[7]

sugar-binding subsites and the phosphate-binding subsite of trehalose phosphorylase, as shown in Figure 5. The putative active centre of trehalose phosphorylase was examined with site-directed mutagenesis. Large between $10^{3.2}$ and $10^{5.6}$-fold catalytic defects in Asp^{379}→Asn, His^{403}→Ala, Arg^{507}→Ala and Lys^{512}→Ala mutants confirmed the crucial importance of the respective residue for the activity of the enzyme.[7]

4. Conclusions

Results of studies by X-ray crystallography and site-directed mutagenesis provide a detailed portrait of the reaction mechanism of sucrose phosphorylase. While great ambiguity remains regarding the chemical mechanism by which the trehalose phosphorylase functions, it is, however, clear that the two disaccharide phosphorylases have evolved strikingly different reaction coordinates to achieve catalytic efficiency and stereochemical control in their highly analogous substrate transformations. The model of the active site of trehalose phosphorylase serves as point of departure for analyzing the glycosyltransferase-like catalytic mechanism of this enzyme.

Acknowledgements

Financial support from the Austrian Science Funds (FWF P15208-B09, P18038-B09, P15118, P18138-B09 and the DK 'Molecular Enzymology' W901-B05) is gratefully acknowledged.

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