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► **To cite this version:**

Christiane Luley-Goedl, Bernd Nidetzky. Carbohydrate synthesis by disaccharide phosphorylases: reactions, catalytic mechanisms and application in the glycosciences. *Biotechnology Journal*, 2010, 5 (12), pp.1324. 10.1002/biot.201000217. hal-00599450

HAL Id: hal-00599450

<https://hal.science/hal-00599450>

Submitted on 10 Jun 2011

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**Carbohydrate synthesis by disaccharide phosphorylases:
reactions, catalytic mechanisms and application in the
glycosciences**

Journal:	<i>Biotechnology Journal</i>
Manuscript ID:	biot.201000217.R1
Wiley - Manuscript type:	Review
Date Submitted by the Author:	03-Nov-2010
Complete List of Authors:	Luley-Goedl, Christiane; Institute of Biotechnology and Biochemical Engineering, Graz University of Technology Nidetzky, Bernd; Institute of Biotechnology and Biochemical Engineering, Graz University of Technology
Primary Keywords:	Biocatalysis
Secondary Keywords:	Carbohydrates
Keywords:	Glycosyl transfer, Glycobiotechnology, Enzyme engineering

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3 **Review**
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5 **Carbohydrate synthesis by disaccharide phosphorylases:**
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8 **reactions, catalytic mechanisms and application in the**
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10 **glycosciences**
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34 Keywords: glycosyl transfer, inverting and retaining mechanism, enzyme
35 engineering, glycoside synthesis, application
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3 Disaccharide phosphorylases are glycosyltransferases (EC 2.4.1.–) of specialized carbohydrate
4 metabolism in microorganisms. They catalyze glycosyl transfer to phosphate using a disaccharide as
5 donor substrate. Phosphorylases for the conversion of naturally abundant disaccharides including
6 sucrose, maltose, α,α -trehalose, cellobiose, chitobiose, and laminaribiose have been described.
7
8 Structurally, these disaccharide phosphorylases are often closely related to glycoside hydrolases and
9 transglycosidases. Mechanistically, they are categorized according the stereochemical course of the
10 reaction catalyzed, whereby the anomeric configuration of the disaccharide donor substrate may be
11 retained or inverted in the sugar 1-phosphate product. Glycosyl transfer with inversion is thought to
12 occur through a single displacement-like catalytic mechanism, exemplified by the reaction coordinate
13 of cellobiose/chitobiose phosphorylase. Reaction via configurational retention takes place through the
14 double displacement-like mechanism employed by sucrose phosphorylase. Retaining α,α -trehalose
15 phosphorylase (from fungi) utilizes a different catalytic strategy, perhaps best described by a direct
16 displacement mechanism, to achieve stereochemical control in an overall retentive transformation.
17
18 Disaccharide phosphorylases have recently attracted renewed interest as catalysts for synthesis of
19 glycosides to be applied as food additives and cosmetic ingredients. Relevant examples are lacto-*N*-
20 biose and glucosylglycerol whose enzymatic production was achieved on multikilogram scale. Protein
21 engineering of phosphorylases is currently pursued in different laboratories with the aim of broadening
22 the donor and acceptor substrate specificities of naturally existing enzyme forms, to eventually
23 generate a toolbox of new catalysts for glycoside synthesis.
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Introduction

The *O*-glycosidic linkage is a highly stable bond in many natural molecules. Its formation as in disaccharides, polysaccharides, glycoconjugates and numerous other small molecules is usually catalyzed by glycosyltransferases (Scheme 1A). The activated donor substrate required for enzymatic glycosyl transfer is provided from central metabolic pathways of the cell. Glycosyltransferases are often very specific for both the donor and acceptor substrate used in their reactions [1]. The free energy of conversion of the activated sugar donor drives the glycosyltransferase reaction towards the *O*-glycosidic product. Breakdown of the *O*-glycosidic linkage is a task usually fulfilled by glycoside hydrolases (Scheme 1B). In aqueous solution, the thermodynamic equilibrium of the hydrolase reaction lies far on the side of products. The rate acceleration achieved by these hydrolases in comparison to the corresponding uncatalyzed hydrolysis reaction is impressive, sometimes reaching values on the order of 10^{17} [2]. Metabolic incorporation of the hydrolysis products usually requires energy, often in the form of ATP. For example, the glucose produced from sucrose (α -D-glucopyranosyl-1,2- β -D-fructofuranoside) would often be converted to glucose 6-phosphate in a kinase-catalyzed transformation. The expenditure of cellular energy in the phosphorylation of sugars derived from hydrolysis of *O*-glycosidic substrates is a possible disadvantage, especially under conditions in which saccharide reserves are mobilized or in the case of organisms that dwell in a low-oxygen or anoxic environment where ATP production cannot occur via the efficient process of respiratory chain-linked phosphorylation. Phosphorolysis of substrate, that is breakdown of the *O*-glycosidic linkage concomitant with glycosyl transfer to phosphate, yields the corresponding sugar 1-phosphate as a high-energy product [3]. Because no ATP is required in further metabolism of the resulting sugar 1-phosphate, *phosphorolysis* seems to be an energetically more economic way of utilizing saccharide substrates than is *hydrolysis*. In agreement with the notion of the physiological advantages resulting from phosphorolysis, enzymes catalyzing phosphorolytic conversion of *O*-glycosidic substrates have often been identified in microorganism having a facultatively anaerobic lifestyle. Notable examples include *Bifidobacterium* sp., *Leuconostoc* sp., *Lactobacillus* sp., *Cellulomonas* sp., *Cellvibrio* sp., and *Clostridium* sp. Furthermore, intracellular degradation of α -1,4-glucan polymers (glycogen, starch) stockpiled as reserves of carbon and energy occurs in a wide range

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3 of organisms and cell types through the action of a phosphorylase that produces glucose 1-phosphate.
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5 Using the example of phosphorolysis of sucrose, Scheme 1C shows the basic reaction catalyzed by *O*-
6
7 glycoside phosphorylases, henceforth also in brief, phosphorylases.
8

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10 The known phosphorylases utilize disaccharides, oligosaccharides (maltodextrins,
11
12 cellodextrins) or polysaccharides (starch, glycogen) as donor substrates. Herein, we restrict our
13
14 discussion mainly to disaccharide phosphorylases for two reasons. First, the disaccharide
15
16 phosphorylases embrace essentially the complete mechanistic diversity of this group of
17
18 glycosyltransferases. Second, applications of phosphorylase enzymes in glycoside synthesis were
19
20 developed chiefly using disaccharide phosphorylases and considerations of enzymatic synthesis and
21
22 enzyme engineering are a central theme of this article. The family of phosphorylases, which
23
24 irrespective of their divergent substrate preference for maltodextrins, starch or glycogen, are often
25
26 collectively called “glycogen phosphorylases”, is not covered in detail. However, mechanistic and
27
28 synthetic analogies between trehalose, sucrose and glycogen phosphorylase are drawn and will be
29
30 pointed out later.
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34 35 36 **Disaccharide phosphorylases: classification, structure and function**

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38 Phosphorylases bridge in many ways the classes of glycosyltransferases and glycoside hydrolases.
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40 Although categorized by EC number as transferases, phosphorylases have various properties in
41
42 common with glycoside hydrolases. Unlike “true” glycosyltransferases whose physiological function
43
44 is synthesis of *O*-glycosides, phosphorylases are generally believed to fulfil a catabolic role. The
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46 intracellular concentration of phosphate may be in the range <5 mM [4] and is expected to exceed the
47
48 concentration of sugar 1-phosphate (e.g. α -D-glucose 1-phosphate) by up to two orders of magnitude
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50 [5]. The concentration of free acceptor substrate (e.g. D-glucose, D-fructose, *N*-acetyl-D-glucosamine)
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52 in the cell will also be very low [6]. According to the law of mass action, therefore, the direction of the
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54 phosphorylase reaction in which *O*-glycoside is produced (*synthesis*) will be strongly disfavoured as
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56 compared to *phosphorolysis* under conditions prevailing *in vivo*.
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60 According to a widely used sequence-based classification [7], all known disaccharide
phosphorylases except retaining trehalose phosphorylase are placed in one of the families of glycoside

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3 hydrolases. Currently, there are 118 glycoside hydrolase families and phosphorylases are found in
4
5 families GH-13, GH-65, GH-94 and GH-112 (Table 1). Families GH-13 and GH-65 contain
6
7 phosphorylases and hydrolases. Families GH-94 and GH-112 so far comprise only phosphorylases.
8
9 Family GH-65 has been categorized into glycoside hydrolase clan GH-L. Member families of clan
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11 GH-L share a common $(\alpha/\alpha)_6$ protein fold. Although, by the criterion of common fold, family GH-94
12
13 would also belong to clan GH-L, family-to-clan assignment has not yet been performed for family
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15 GH-94. The anomeric configuration of the glycosides cleaved by enzymes from family GH-65 (α -D-
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17 glucosides) and family GH-94 (β -D-glycosides) is different, providing a possible reason for not
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19 classifying both families into the same clan. Family GH-13 belongs to clan GH-H, which comprises
20
21 proteins having a common $(\beta/\alpha)_8$ fold. No assignment to a particular GH clan has been made for
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23 family GH-112. However, the catalytic domain of galacto-*N*-biose/lacto-*N*-biose phosphorylase was
24
25 shown to consist of a partially broken TIM (triosephosphate isomerase) barrel fold [8] that is
26
27 structurally similar to a thermophilic β -galactosidase, thus supporting the classification of this and
28
29 related phosphorylase as one of the current GH families. Membership of phosphorylases to glycoside
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31 hydrolase families and clans strongly supports an evolutionary relationship between these two enzyme
32
33 classes at the level of both structure and catalytic function. Interestingly, therefore, the retaining
34
35 trehalose phosphorylases and glycogen phosphorylases are classified in glycosyltransferase family
36
37 GT-4 and GT-35, respectively. It is known from crystal structures (glycogen phosphorylase) and
38
39 suggested by structure modelling (trehalose phosphorylase) that both phosphorylases adopt the so-
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41 called GT-B fold, which comprises two Rossmann fold-like domains. The classification of
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43 glycosyltransferases currently spans 92 families. Membership to GT families for glycogen
44
45 phosphorylase and trehalose phosphorylase would suggest that these two phosphorylases are
46
47 mechanistically distinct from counterpart phosphorylases categorized in GH families. The comparison
48
49 between trehalose phosphorylase (family GT-4) and sucrose phosphorylase (family GH-13) has shown
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51 that the two enzymes utilize strikingly different reaction coordinates to achieve catalytic efficiency and
52
53 stereochemical control in their highly analogous substrate transformations [9], as will be described
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55 later. Family GT-35 comprises no enzyme activity except that of glycogen phosphorylase.
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3 Interestingly, therefore, family GT-4 contains both glycosyltransferase and phosphorylase activities
4 and represents a variety of donor and acceptor substrate specificities.
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8 Like glycoside hydrolases and glycosyltransferases, phosphorylases can be categorized
9 according to whether in the enzymatic phosphorolysis reaction the anomeric configuration of the
10 donor substrate is retained or inverted in the corresponding sugar 1-phosphate product [3]. Reaction
11 via inversion and retention are thought to necessitate different catalytic mechanisms. As shown in
12 Table 1, sucrose phosphorylase and trehalose phosphorylase catalyze an α -retentive reaction,
13 involving an overall axial-to-axial substitution on the anomeric carbon of the glycosyl ring [9].
14 Phosphorylase reaction coordinates featuring the opposite stereochemical course, that is β -retentive
15 conversion via an equatorial-to-equatorial substitution, have not so far been described. Inverting
16 phosphorylases promote conversion of their substrates via either axial-to-equatorial or equatorial-to-
17 axial substitution on the anomeric carbon, as seen in maltose phosphorylase [10] and cellobiose
18 phosphorylase [11], respectively. Chemically, the difference between a phosphorylase reaction and the
19 corresponding hydrolase reaction utilizing the same disaccharide substrate is the choice of nucleophile,
20 phosphate or water. Crystal structures of different phosphorylases reveal how phosphate is
21 accommodated in the enzyme catalytic site and provide suggestions of how phosphorylases might
22 discriminate against reaction with water, preventing “error hydrolysis” of their donor substrate [12,
23 13]. With the exception of sucrose phosphorylase that shows significant inherent hydrolase activity
24 [14], other phosphorylases typically do not show detectable conversion of disaccharide substrate (or an
25 analogue thereof) in a reaction with water as nucleophile [10, 11, 15, 16].
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49 **Phosphorylase mechanisms**

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51 ***Cellobiose and chitobiose phosphorylase.*** The structure and function of these enzymes from family
52 GH-94 have been examined in considerable detail. Crystal structures of cellobiose phosphorylase from
53 *Cellvibrio gilvus* [17] and chitobiose phosphorylase from *Vibrio proteolyticus* [18] have been reported.
54
55 At the level of the three-dimensional structure (overall fold), cellobiose and chitobiose phosphorylase
56 are very similar one to another, and both enzymes are structurally homologous to maltose
57 phosphorylase from *Lactobacillus brevis* (family GH-65) [12] and glucoamylase from
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3 *Thermoanaerobacterium thermosaccharolyticum* (family GH-15, clan GH-L) [19]. Another cellobiose
4 phosphorylase from *Cellulomonas uda* has been successfully crystallized [20].
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8 Structural comparison of chitobiose phosphorylase, maltose phosphorylase and glucoamylase
9 revealed that Asp-492 (amino acid numbering of chitobiose phosphorylase from *V. proteolyticus*)
10 superposed well with the known and putative general catalytic acid of the reaction catalyzed by the
11 glucoamylase (Glu-438; from *T. thermosaccharolyticum*) and maltose phosphorylase (Glu-487; from
12 *L. brevis*), respectively [18]. Asp-492 is part of a highly conserved region in phosphorylases of family
13 GH-94. Substitution of Asp-492 by Asn resulted in complete loss of activity, suggesting that the Asp
14 side of chitobiose phosphorylase serves an analogous Brønsted acid catalytic role as the homologous
15 Glu side chains of glucoamylase and maltose phosphorylase [18]. Interestingly, at the position of the
16 general catalytic base on an inverting hydrolase (Scheme 2A), no candidate residue is found in
17 chitobiose phosphorylase and maltose phosphorylase. However, the position of the base catalyst in
18 glucoamylase (Glu-636) is almost precisely adopted by Gln-690, another highly conserved residue
19 amongst GH-94 phosphorylases (Figure 1), suggesting that activation of the phosphate ion is not
20 necessary in the reaction as it is for the attack of a water molecule by an inverting hydrolase.
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36 The evidence from crystal structure determination together with results of biochemical studies
37 on chitobiose/cellobiose phosphorylase supports the mechanism summarized in Scheme 2B. Note:
38 despite the differences in stereochemical features of the disaccharide substrate, the basic catalytic
39 mechanism of phosphorolysis with inversion of configuration appears to be conserved among
40 cellobiose phosphorylase [13] and maltose phosphorylase [12]. Inverting α,α -trehalose phosphorylase
41 and kojibiose phosphorylase for which no crystal structure has so far been published seem to use a
42 similar direct displacement-like mechanism.
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51 The thorough characterization of chitobiose/cellobiose phosphorylase has provided a wealth of
52 structure-function relationship data that are conducive to the design of novel phosphorylases for
53 carbohydrate synthesis (see later).
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57 **Sucrose phosphorylase.** Crystal structures of sucrose phosphorylase from *Bifidobacterium*
58 *adolescentis* together with evidence from site-directed mutagenesis and other biochemical studies have
59 revealed that this enzyme utilizes a glycoside hydrolase-like double displacement mechanism,
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3 illustrated in Scheme 3, to convert its disaccharide substrate and phosphate into α -D-glucose 1-
4 phosphate and fructose. Three acidic residues (Asp-192, Glu-232, Asp-290) are contributing to the
5 catalytic sub-site, as shown in Figure 2. These residues are highly conserved in members of family
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10 GH-13.

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12 The proposed mechanism (Scheme 3), which is strongly supported by the crystallographic data shown
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14 in Figure 2, received further support from mutational analyses carried out on the enzyme from
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16 *Leuconostoc mesenteroides*. In this phosphorylase, Asp-196 which is homologous to Asp-192 of the *B.*
17
18 *adolescentis* enzyme was replaced by Ala (D196A) [21]. In agreement with the expected kinetic
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20 consequences resulting from complete removal of catalytic assistance by the nucleophilic group on the
21
22 enzyme, the purified D196A mutant lacked activity ($\leq 0.0001\%$ that of the wild-type enzyme).
23
24 However, when presented with azide as an alternative nucleophile, the conversion of α -D-glucose 1-
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26 phosphate proceeded at a slow rate and resulted in the formation of a new substitution product,
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28 glucose 1-azide with a β -anomeric configuration. Activity enhancement in the D196A mutein was
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30 therefore suggested to result from the direct participation of azide in the now inverting, single
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32 displacement-like mechanism of glucosyl transfer. Results of steady-state kinetic analysis of glucosyl
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34 transfer to phosphate catalyzed by D196A suggested reaction through a ternary enzyme-substrate
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36 complex, in stark contrast to the wild-type enzyme whose reaction in two catalytic steps via a covalent
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38 intermediate appears to dictate Ping-Pong kinetics [21, 22].
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43 Glu-237 (homologous to Glu-232) was replaced by Gln, so the carboxamide group of the Gln
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45 cannot fulfil the acid-base catalytic function proposed for the carboxylic group of the original Glu
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47 (Scheme 3). Detailed kinetic characterization of E237Q showed that catalytic steps involving a poor
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49 leaving group or nucleophile were slowed by five orders of magnitude in the mutein as compared to
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51 the wild-type enzyme whereas other steps expected to proceed without support from an acid-base
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53 catalyst were not strongly affected by the site-directed substitution [23]. Results of pH studies
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55 suggested a role for Glu-237 in which the carboxylic acid/carboxylate side chain of the Glu alternates
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57 between catalytic functions of a general acid and base in glucosylation and deglucosylation half-
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59 reactions of sucrose phosphorylase, respectively. It was proposed that the pK_a of the Glu-237 side
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chain cycles between 7.2 in the free enzyme and 5.8 in the glucosyl-enzyme intermediate [23].

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3 Recently, the key catalytic amino acids in *L. mesenteroides* sucrose phosphorylase (Asp-196,
4 Glu-237) were replaced by the corresponding carboxamide residues, and it was shown that the D196N
5 E237Q double mutein was still capable of promoting slow phosphorolysis [24]. An enzymatic reaction
6 coordinate for α -retaining glucosyl transfer via a covalent intermediate was changed for the first time
7 into one where this intermediate was lacking and configurational retention appeared to be achieved
8 through a direct front-side nucleophilic displacement reaction.
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12 **Retaining α,α -trehalose phosphorylase.** According to Table 1, retaining α,α -trehalose phosphorylase
13 (family GT-4) must be distinguished clearly from “counterpart” inverting trehalose phosphorylase
14 (family GH-65) [25]. No crystal structure of a retaining trehalose phosphorylase has been determined
15 so far. However, the structures of several glycosyltransferases related to the trehalose phosphorylase
16 by common membership to family GT-4 have been solved [26-31]. A three-dimensional model of
17 trehalose phosphorylase from *Schizophyllum commune* has thus been created based on this structural
18 basis. In spite of its possible ambiguity, the model is clear in showing that the active-site of the
19 trehalose phosphorylase (Figure 3A) is very different from the active-site of sucrose phosphorylase
20 (Figure 2). However, active-site similarity between trehalose phosphorylase and glycogen
21 phosphorylase is suggested (Figure 3). Mutagenesis data confirm the importance of Lys-512 and Arg-
22 507 as key phosphate-binding residues for the activity of trehalose phosphorylase. [32]. Both trehalose
23 and glycogen phosphorylase lack a suitable amino acid in their respective active-site whose side chain
24 would be positioned appropriately to be a candidate catalytic nucleophile. A direct front-side
25 nucleophilic displacement mechanism was therefore suggested for both enzymes. In literature, this
26 mechanism is sometimes referred to as “internal return-like” (S_Ni -like) and is considered to be a strong
27 proposal in the current debate about the catalytic mechanism of retaining glycosyltransferases [1].
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51 Biochemical data strongly support the idea that trehalose phosphorylase is mechanistically
52 different from sucrose phosphorylase. Unlike sucrose phosphorylase, which utilizes α -D-glucose 1-
53 fluoride as donor substrate for glucosyl transfer to either phosphate or fructose [22], trehalose
54 phosphorylase recognizes α -D-glucose 1-fluoride exclusively as a slow substrate for the
55 phosphorolysis direction [33]. Trehalose phosphorylase does not promote the conversion of α -D-
56 glucose 1-phosphate into Glc and phosphate when arsenate is present. A two-step reaction via a
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3 glucosyl-enzyme intermediate is a likely mechanistic requirement for the reversible exchange reaction,
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5 α -D-glucose 1-phosphate + arsenate \rightarrow α -D-glucose 1-arsenate + phosphate, which is catalyzed by
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7 sucrose phosphorylase with high specific activity. Note that spontaneous hydrolysis of α -D-glucose 1-
8
9 arsenate makes the overall arsenolysis of α -D-glucose 1-phosphate a completely irreversible process.
10
11 Lacking catalytic assistance to arsenolysis of α -D-glucose 1-phosphate does obviously not prove the
12
13 absence of a double displacement-like intermediate for trehalose phosphorylase. However, the result
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15 supports the notion that in contrast to sucrose phosphorylase, formation and breakdown of any such
16
17 intermediate in trehalose phosphorylase cannot be analyzed as kinetically isolated reaction steps. The
18
19 glucosyl-enzyme intermediate of sucrose phosphorylase can be intercepted by numerous alcohol
20
21 acceptors other than fructose [34-38]. Nucleophilic competition for reaction with a similar
22
23 intermediate was not observed in trehalose phosphorylase [39].
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28 In summary, therefore, although ambiguity remains regarding the chemical mechanism by
29
30 which the trehalose phosphorylase functions, it is clear that trehalose phosphorylase is mechanistically
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32 distinct from sucrose phosphorylase. Scheme 4 is a plausible suggestion for the mechanism utilized by
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34 trehalose phosphorylase. The mechanism employed by glycogen phosphorylase may be similar to that
35
36 of trehalose phosphorylase. It is interesting that glycogen phosphorylases require a pyridoxal 5'-
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38 phosphate cofactor for activity [40] whereas trehalose phosphorylases are active in the absence of a
39
40 cofactor. If the role of pyridoxal 5'-phosphate is that of a catalytic acid that promotes (via the substrate
41
42 phosphate) a partial protonation of the glycosidic oxygen of the donor substrate, there remains the
43
44 question of how trehalose phosphorylase achieves a similar "activation" of the substrate phosphate in
45
46 an S_Ni -like mechanism. Results of pH studies suggested that trehalose phosphorylase recognizes the
47
48 doubly protonated (monoanionic) form of phosphate [41] which may be a sufficiently acidic substrate
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50 to facilitate C–O bond cleavage at the level of the ternary complex in the absence of cofactor (Scheme
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52 4).
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58 **Novel application of disaccharide phosphorylases in synthesis**

59 Although sugar 1-phosphates are valuable compounds for highly specialized applications, there
60
appears to be limited need for them as fine chemicals. α -D-Glucose 1-phosphate is used in medicine as

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2
3 a substitute of inorganic phosphate in parenteral nutrition and as supplement in the case of a phosphate
4 deficiency [42]. Therefore, *phosphorolysis* of a disaccharide substrate is generally of a minor interest
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7 in carbohydrate synthesis, except in situations where this conversion is applied to prepare the sugar 1-
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10 phosphate donor substrate for another phosphorylase-catalyzed transformation, now carried out in
11
12 *synthesis* direction (see later). Small-molecule glycosylations performed by phosphorylases are usually
13
14 highly selective with respect to the site on the acceptor substrate that becomes derivatized. The *O*-
15
16 glycosidic products thus obtained typically have a very high regioisomeric purity. The absence of
17
18 requirement to perform a separation of product isoforms facilitates the downstream processing and
19
20 contributes substantially to a lowering of the overall production costs.
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22
23 Inverting phosphorylases are applied under conditions of equilibrium-controlled synthesis.
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25 Donor and acceptor substrates are usually incubated in the presence of enzyme until the conversion is
26
27 complete. The thermodynamic equilibrium constant (K_{eq}) of the reaction, disaccharide + phosphate \leftrightarrow
28
29 sugar 1-phosphate + monosaccharide, normally favours synthesis of disaccharide from sugar 1-
30
31 phosphate and acceptor. Note: synthesis of sucrose is an exception (see later)! The K_{eq} value may be
32
33 dependent on pH and typically, synthesis becomes more preferred as the pH is decreased. The molar
34
35 ratio of donor and acceptor can be varied to alter the composition of the product mixture at the end of
36
37 the conversion. Considerations of product work-up often dictate the choice of these reaction
38
39 conditions. With some phosphorylases, sugar 1-fluorides are interesting alternate donor substrates that
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41 replace the sugar 1-phosphate in the synthesis reaction. Cellobiose phosphorylase and maltose
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43 phosphorylase [10] are relevant examples. The overall conversion, sugar 1-fluoride + monosaccharide
44
45 \rightarrow disaccharide + fluoride⁻ + H⁺, is essentially irreversible. It was shown for synthesis of cellobiose
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47 from α -D-glucose 1-fluoride and glucose catalyzed by cellobiose phosphorylase that quantitative
48
49 yields of disaccharide product can be obtained [43]. The corresponding yield in the analogous
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51 transformation, in which α -D-glucose 1-phosphate was used instead of α -D-glucose 1-fluoride, was
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53 substantially lower. Fluoride does not serve as nucleophilic acceptor in the conversion of cellobiose by
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55 cellobiose phosphorylase to a measurable extent. Because the phosphorylase cannot hydrolyze the
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57 cellobiose formed, the disaccharide product is stable. By analogy to the “glycosynthase technology”,
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59 which is based on mutated glycoside hydrolases that utilize activated glycosyl donors like sugar 1-
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3 fluorides for glycoside synthesis but are unable to hydrolytically degrade the resulting products [44,
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5 45], these authors previously used the term “glycosynthase-like” to described the synthetic reaction of
6
7 a wild-type phosphorylase with a sugar 1-fluoride as donor substrate [43]. Nakai *et al.* recently
8
9 showed utilization of α -D-glucose 1-fluoride as donor substrate for reaction catalyzed by cellobiose
10
11 and cellodextrin phosphorylases from *Clostridium thermocellum* [46].
12
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14 The donor substrate specificity of the known inverting phosphorylases is narrow. Small
15
16 structural modifications of the substrate like epimerisation or deoxygenation at a single carbon atom
17
18 usually result in large losses of transferase activity. Application of wild-type phosphorylases in
19
20 glycosylation reactions using non-natural glycosyl donors is therefore often not practically useful. The
21
22 replacement of sugar 1-phosphate by the corresponding sugar 1-fluoride may be an exception. The
23
24 scope of acceptor substrates is, however, often broad. Cellobiose phosphorylase has been used for
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26 glycosylation of a variety of acceptors, including disaccharides [47-49] and the same applies to
27
28 maltose phosphorylase [50, 51], inverting trehalose phosphorylase [52] and kojibiose phosphorylase
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30 [53].
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33 Depending on the reaction mechanism utilized, retaining phosphorylases offer the possibility
34
35 to perform glycoside synthesis under thermodynamic or kinetic control. Phosphorylases operating by a
36
37 ternary complex kinetic mechanism in which both substrates must bind to the enzyme before a product
38
39 can be released need to be applied under equilibrium-controlled reaction conditions, as described
40
41 above for the inverting phosphorylases. Trehalose phosphorylase and glycogen phosphorylase belong
42
43 to this group. The scope of donor substrates utilized by these two enzymes is relatively small. While
44
45 there have been reports on transfer of mannosyl [54] and 2-deoxy-glucosyl residues [55] by glycogen
46
47 phosphorylase, the activity of this enzyme for reaction with donor substrates other than glucosyl (α -D-
48
49 glucose 1-phosphate) is extremely small and hardly sufficient for synthesis. α -D-Glucose 1-fluoride is
50
51 a slow substrate of trehalose phosphorylase that is only utilized in phosphorolysis direction [56].
52
53 Conversion of α -D-glucose 1-fluoride by glycogen phosphorylase requires the presence of catalytic
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55 amounts of phosphate [57]. However, as in cellobiose phosphorylase, for example, the acceptor
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57 substrate specificity of trehalose phosphorylase is more relaxed than the corresponding donor substrate
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59 specificity [58].
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3 The covalent β -glucosyl enzyme intermediate formed in the reaction catalyzed by sucrose
4 phosphorylase is point of departure for another route of synthesis, generally called *transglycosylation*,
5 which is distinct from the *direct glycosylation* catalyzed by phosphorylases that operate by a ternary
6 complex mechanism. Glucosylated sucrose phosphorylase (β -Glucosyl-SPase) can be produced using
7 sucrose, α -D-glucose 1-phosphate or α -D-glucose 1-fluoride as donor substrate [59]. Sucrose is often
8 preferred for its high stability in the absence of phosphorylase, the high internal energy that drives the
9 reaction, and the good availability at low costs. In the absence of phosphate that would otherwise react
10 with the intermediate, external acceptors (e.g. glycerol) compete with water for participating as
11 nucleophiles in the overall conversion (Scheme 5). Using sucrose, the synthetic reaction catalyzed by
12 the enzyme is therefore, sucrose + glycerol \leftrightarrow α -D-glucosyl glycerol + D-fructose. Hydrolysis of the
13 β -glucosyl enzyme intermediate occurs as a side reaction in this process and yields D-glucose. An
14 efficient synthesis must therefore strive for preventing loss of donor substrate to the hydrolysis path
15 (see later). Often this can be achieved by using a suitable excess of acceptor substrate [60]. In sucrose
16 phosphorylase, however, use of high sucrose concentration have also been found to be effective in
17 suppressing donor substrate hydrolysis [61, 62]. Because α -D-glucosyl glycerol and other glucosylated
18 acceptors produced by sucrose phosphorylase are by definition substrates for degradation by the
19 enzyme via *secondary hydrolysis* (or *phosphorolysis*), their synthesis takes place under kinetic control.
20 Depending on how fast the respective glucosylated acceptor is broken down, the time of incubation
21 may be a critical parameter in the optimization of the transglycosylation.
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48 ***Multi-step enzymatic synthesis of disaccharides and other glycosides.***

49 Coupling to sucrose conversion. Scheme 6 shows how phosphorolysis of sucrose is utilized to prepare
50 the α -D-glucose 1-phosphate for a coupled enzymatic reaction catalyzed by another phosphorylase.
51 The K_{eq} for the conversion of sucrose and phosphate into α -D-glucose 1-phosphate and D-fructose is
52 around 44 at pH 7.0 and 30 °C [37]. Using the combined action of sucrose and glucan phosphorylase,
53 the synthesis of pure amylose for the use as a functional biomaterial could be implemented in high
54 yield [63, 64]. Additional isomerization of D-fructose into D-glucose provides the acceptor substrate
55 for synthesis of various “glucobioses”, including α,α -trehalose [65, 66] and cellobiose [67]. Other
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3 types of phosphorylase have also been coupled as described in literature [68-72]. However, for
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5 thermodynamic reasons, production of the intermediary α -D-glucose 1-phosphate is much less
6
7 efficient in terms of yield when using cellobiose [73, 74] and even glycogen or starch as donor
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9 substrate [75], as compared to sucrose. This often prevents one-pot synthesis in the presence of two
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11 phosphorylases and requires that α -D-glucose 1-phosphate is prepared first in a separate reaction.
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13 Synthesis of β -D-glucose 1-phosphate from maltose [68, 76] and trehalose [77] is also lacking
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15 efficiency however, because of its relevance as glucosyl donor for both enzymatic and chemical
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17 glucosylation reactions, the concept of a coupled one-pot phosphorolysis/reverse phosphorolysis
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19 reaction using maltose phosphorylase was examined. The costly intermediary β -D-glucose 1-
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21 phosphate, obtained from maltose in the presence of only 2-fold excess of phosphate, is directly used
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23 as substrate for reverse phosphorolysis with different monosaccharides, resulting in >84% yield of
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25 new α -(1 \rightarrow 4)-glucosidic disaccharides [78]. The product spectrum strongly depends on the enzyme's
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27 acceptor substrate specificity.
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34 Lacto-*N*-biose. This disaccharide (Gal β 1 \rightarrow 3GlcNAc) is a key structural component of human milk
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36 oligosaccharides such as lacto-*N*-tetraose (Gal β 1 \rightarrow 3GlcNAc β 1 \rightarrow 3Gal β 1 \rightarrow 4Glc) and lacto-*N*-
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38 fucopentaose (Fuc α 1 \rightarrow 2Gal β 1 \rightarrow 3GlcNAc β 1 \rightarrow 3Gal β 1 \rightarrow 4Glc). It is widely accepted that human milk
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40 oligosaccharides have a "prebiotic effect" in that they can provide selective stimulation to the growth
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42 of microorganisms such as *Bifidobacteria* whose presence in the intestinal flora is considered to be
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44 beneficial for human health and well-being. The physiological efficacy of these human milk
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46 oligosaccharides appears to be linked to the presence of the lacto-*N*-tetraose unit [79]. Nishimoto and
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48 Kitaoka developed an elegant procedure (Scheme 7) in which four enzymes, among them two
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50 phosphorylases, were used in a one-pot transformation of sucrose (660 mM) and GlcNAc (600 mM) to
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52 give lacto-*N*-biose (~500 mM) and fructose in a yield of 83% [80]. Phosphate (P_i) and UDP-glucose
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54 (UDP-Glc) had to be present in catalytic concentrations. The conversion was performed on 10-L scale
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56 and following an easy work-up that included treatment with yeast cells and crystallization, about 1.4
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58 kg of pure product was obtained. Using a similar approach in which GalNAc was used instead of
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60 GlcNAc as galactosyl acceptor substrate, the same authors reported synthesis of galacto-*N*-biose

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3 (Gal β 1 \rightarrow 3GalNAc), which is an important core structure in functional sugar chains such as T-antigen
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5 disaccharide and the core 1 sugar chain in mucin glycoproteins [81]. Synthesis of lacto-*N*-biose as well
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7 as that of galacto-*N*-biose are beautiful examples of the large potential of multi-step enzymatic
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9 transformations in making complex carbohydrates available as fine chemicals. For example, the
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11 possible application of lacto-*N*-biose as functional food additive appears to have been opened up by
12
13 the biocatalytic process indicated in Scheme 7.
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19 Glucosylglycerol. Glycosylglycerols are powerful osmolytes that are produced by various plants,
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21 algae, and bacteria in adaptation to salt stress and drought [82]. Among them, 2-*O*-(α -D-
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23 glucopyranosyl)-*sn*-glycerol (GG, Figure 4) has attracted special attention for its promising application
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25 as moisturizing agent in cosmetics [83-85] but also for use as low-caloric sweetener that does not
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27 cause tooth decay [86] and may have a prebiotic effect. Possible therapeutic applications of GG that
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29 are inferred from its ability to stabilize proteins and cells are currently evaluated [87, 88]. However,
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31 the development of industrial applications for GG (and related carbohydrate-based compatible solutes)
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33 has been severely restricted by compound availability. A new biocatalytic process using sucrose
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35 phosphorylase according to Scheme 5 has been described which overcomes the chemical and
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37 technological challenges of production of stereochemically pure GG as an industrial chemical [60].
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41 Under optimized reaction conditions using excess of glycerol (2.0 mol/L) over sucrose (0.8
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43 mol/L), about 90% of the applied donor substrate was converted into GG. Enzymatic production of
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45 GG [89] was implemented on industrial scale by the German company bitop AG, and GG was
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47 commercialized under the tradename Glycoin®. A product termed Glycoin Extremium is available on
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49 the market and will be used as active ingredient for cosmetic formulations [90]. Technical features
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51 important for successful development of the enzyme-catalyzed process included the use of cheap raw
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53 materials, ability of the donor substrate sucrose to drive the reaction to near completion, and the high
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55 efficiency and regioselectivity of sucrose phosphorylase in the glucosylation of glycerol.
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Engineering of phosphorylases for altered substrate specificity.

Cellobiose phosphorylase. Lactose would be an interesting donor substrate for phosphorylase-catalyzed transformations but natural enzymes capable of transferring the galactosyl moiety of lactose efficiently appear to be lacking. Cellobiose phosphorylase (from *C. uda*), which displays a low level of activity towards lactose in the wild-type form, was used as point of departure for the development of engineered lactose phosphorylases [91]. Directed evolution was combined with site saturation mutagenesis to obtain a doubly mutated cellobiose phosphorylase (Thr-508→Ile and Asn-667→Ala) in which a 7.5-fold increase in specific activity on lactose was observed. With Asn-667 being situated adjacent to the donor binding site and Thr-508 being located on a loop at the active-site entrance these mutations mainly affected steps of catalysis or product release rather than substrate binding. Using permeabilized *E. coli* cells, the engineered “lactose phosphorylase” was employed in the synthesis of expensive α -D-galactose 1-phosphate from low-cost substrates such as lactose and phosphate, lactose + phosphate \rightarrow α -D-galactose 1-phosphate + glucose. Highly pure α -D-galactose 1-phosphate (9.5 g) was obtained from a 1-L reaction mixture (500 mM lactose, 50 mM phosphate at 50°C for 48h) after anion exchange chromatography and ethanol precipitation [92].

To assist in the engineering of phosphorylases, De Groeve *et al.* (2010) developed a novel high-throughput screening assay [93]. This assay is based on colorimetric determination of inorganic phosphate released by phosphorylase-catalyzed glycosyl transfer. Application of the assay was demonstrated in the screening of phosphorylase libraries for both novel donor and acceptor specificities. A Glu-649→Cys variant of cellobiose phosphorylase from *C. uda* was identified that showed activity towards alkyl- β -glucoside and phenyl- β -glucoside acceptors. A robust high-throughput screening platform is a prerequisite for targeted development of glycoside phosphorylases with altered substrate specificities to be employed in the synthesis of new glycosides. A triple mutant, containing both donor (T508I/N667A) and acceptor (E649C) mutations, was used as starting point for creation of more promiscuous enzymes using semi-rational design and site-saturation mutagenesis. Two residues near the active-site entrance, Asn-156 and Asn-163 were identified to define specificity towards anomeric substituted acceptors. While the wild-type can not glycosylate these acceptors at all, N156D/N163D/T508I/E649C/N667A showed activity towards alkyl- and aryl- β -glucosides and β -

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3 linked disaccharides including β -cellobiosides whose glycosylated derivatives are of interest as novel
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5 surfactants, cellulose inhibitors or prebiotic sugars [94].
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10 Maltose phosphorylase. By using a multiple sequence alignment (Figure 5) and by careful inspection
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12 of the crystal structure of maltose phosphorylase from *L. brevis* [12], Svensson and co-workers
13
14 identified loop 3 of the $(\alpha/\alpha)_6$ -barrel catalytic domain as key determinant for regioselectivity in this
15
16 group of phosphorylases. Relevant regions of loop 3 in maltose phosphorylase from *L. acidophilus*
17
18 (His-413 to Glu-421) were substituted by the corresponding segments from bacterial inverting
19
20 trehalose and kojibiose phosphorylases that are related to maltose phosphorylase by common
21
22 membership to family GH-65. Phosphorylase variants with superior activity for formation of trehalose
23
24 (α -1,1) and kojibiose (α -1,2) than formation of maltose (α -1,4) were obtained [95].
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30 Sucrose phosphorylase. Using random mutagenesis, eight amino acid substitutions, all located
31
32 between β -strand 2, 3, and 4 (numbering refers to the structure of *B. adolescentis* sucrose
33
34 phosphorylase) were identified to contribute to enhancement of high-temperature stability of sucrose
35
36 phosphorylase from *Streptococcus mutans* [96]. The wild-type enzyme was almost completely
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38 inactivated when incubated at 60°C for 20 min whereas a combination of those eight mutations gave
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40 an enzyme retaining 60% of its initial activity under these conditions (Figure 6). Interestingly, the
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42 temperature and pH optimum were still the same as reported for the wild-type enzyme while covalent
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44 immobilization of sucrose phosphorylase from *B. adolescentis* on Sepabeads EC-HFA resulted in a
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46 shift of the temperature optimum from 58 to 65°C [97]. The same phosphorylase was stabilized by
47
48 formation of cross-linked enzyme aggregates [98]. Immobilized preparations of sucrose phosphorylase
49
50 (from *L. mesenteroides*) are common in the literature [37, 99]. Noteworthy, variants of *S. mutans*
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52 sucrose phosphorylase engineered for enhanced thermostability are employed in a state-of-the-art
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54 process for the enzymatic production of amylose from sucrose [100].
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Conclusions

Disaccharide phosphorylases represent a relatively small group of carbohydrate-active enzymes. Notwithstanding, they embrace a marked diversity of catalytic mechanisms utilized in breakdown or formation of *O*-glycosidic bonds that spans the classes of glycoside hydrolases and glycosyltransferases. Phosphorylases, in native or engineered form, are useful catalysts for the synthesis of disaccharides and other *O*-glycosides. An industrial process for synthesis of GG has been developed using sucrose phosphorylase. The use of phosphorylases as a modular assembly system for generation of a whole new glycosidic assortment might be not that far off in the future.

Financial support from the Austrian Science Funds FWF (project L-586-B03 to B.N.; DK Molecular Enzymology W901-B05) is gratefully acknowledged.

The authors have declared no conflict of interest.

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Figure and Scheme legends

Figure 1. Close-up structure of the active-site of chitobiose phosphorylase from *Vibrio proteolyticus*, showing bound *N*-acetyl-D-glucosamine and sulfate. PDB code 1V7X.

Figure 2. Close-up structure of the active-site of sucrose phosphorylase from *Bifidobacterium adolescentis*, showing the covalent β -glucosyl enzyme intermediate. PDB code 2GDV, chain A.

Figure 3. Close-up structures of the active-sites of GT-4 trehalose phosphorylase from *Schizophyllum commune* as modeled (A) and GT-35 glycogen (starch) phosphorylase from *Corynebacterium callunae*. PDB code 2C4M.

Figure 4. Appearance and chemical structure of 2-*O*-(α -D-glucopyranosyl)-*sn*-glycerol (GG).

Figure 5. Multiple sequence alignment of loop 3, connecting α 3 and α 4 of the (α/α)₆-barrel catalytic domain in GH-65 disaccharide phosphorylases. Maltose phosphorylase: *LaMPase*, *Lactobacillus acidophilus*; *LbMPase*, *Lactobacillus brevis*; inverting trehalose phosphorylase: *GsTPase*, *Geobacillus stearothermophilus*; *TbTPase*, *Thermoanaerobacter brockii*; kojibiose phosphorylase: *TbKPase*, *Thermoanaerobacter brockii*. Arrows (\downarrow) indicate region of substitution.

Figure 6. Thermal stability of wild-type and engineered *Streptococcus mutans* sucrose phosphorylase. Residual activity was determined after incubation at 55°C (black), 57°C (light grey) and 60°C (gray) for 20 min.

Scheme 1. Reactions catalyzed by glycosyltransferases (A), glycoside hydrolases (B) and phosphorylases (C). Synthesis, hydrolysis and phosphorolysis of sucrose are used as examples. Glucosyl acceptors are labelled in red.

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3 **Scheme 2.** Proposed catalytic mechanism of inverting glycoside hydrolase (A) and cellobiose
4 phosphorylase (B). Upper residue, general catalytic acid; lower residue, general catalytic base. The
5 general catalytic acid from *Cellvibrio gilvus* cellobiose phosphorylase (Asp-490) is indicated.
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11 **Scheme 3.** Proposed catalytic mechanism of sucrose phosphorylase. Catalytically important residues
12 from *Bifidobacterium adolescentis* sucrose phosphorylase are indicated. Asp-192, catalytic
13 nucleophile; Glu-232, catalytic acid/base.
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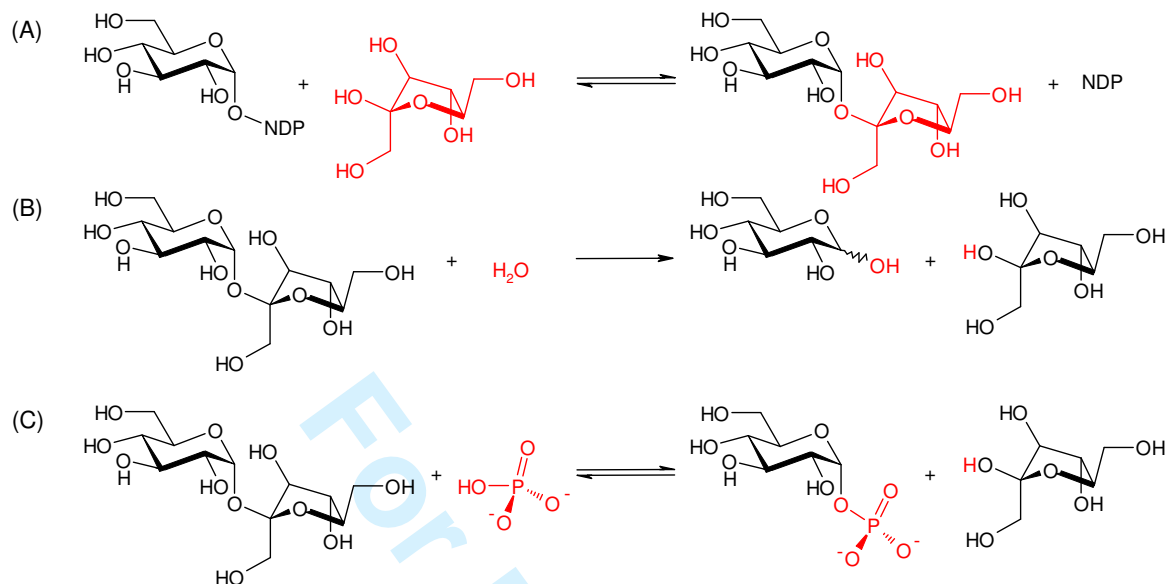
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20 **Scheme 4.** Proposed “internal return-like” catalytic mechanism of retaining trehalose phosphorylase
21 from *Schizophyllum commune*. Residues, important for phosphate binding, are indicated.
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27 **Scheme 5.** Transglucosylation catalyzed by sucrose phosphorylase. SPase, sucrose phosphorylase.
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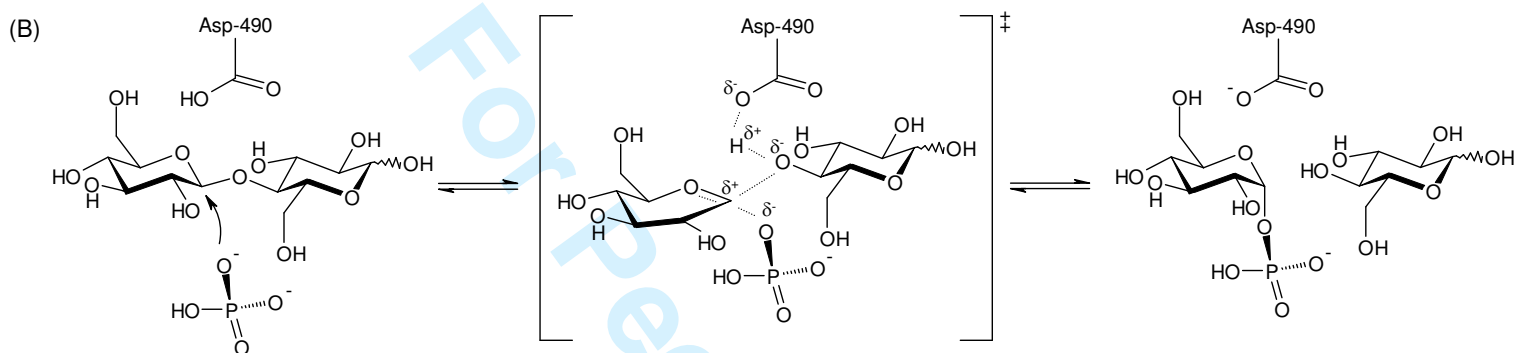
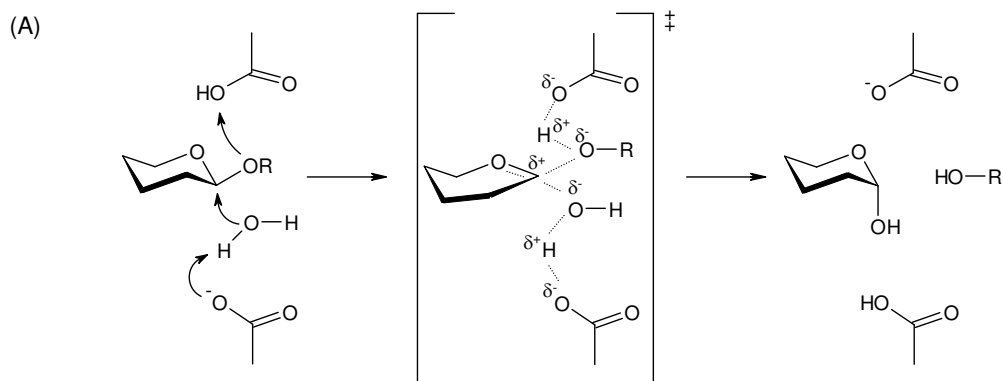
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31 **Scheme 6.** Coupled enzyme system for disaccharide synthesis from sucrose. SPase, sucrose
32 phosphorylase; Glc-I, glucose isomerase; TPase, retaining trehalose phosphorylase; CBPase,
33 cellobiose phosphorylase; α Glc1P, α -D-glucose 1-phosphate.
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40 **Scheme 7.** Multi-step enzymatic synthesis of lacto-*N*-biose. Enzymes employed: SPase, sucrose
41 phosphorylase; Gal-T, UDP-hexose 1-phosphate uridylyltransferase; Gal-E, UDP-glucose 4-epimerase;
42 LNB Pase, lacto-*N*-biose phosphorylase.
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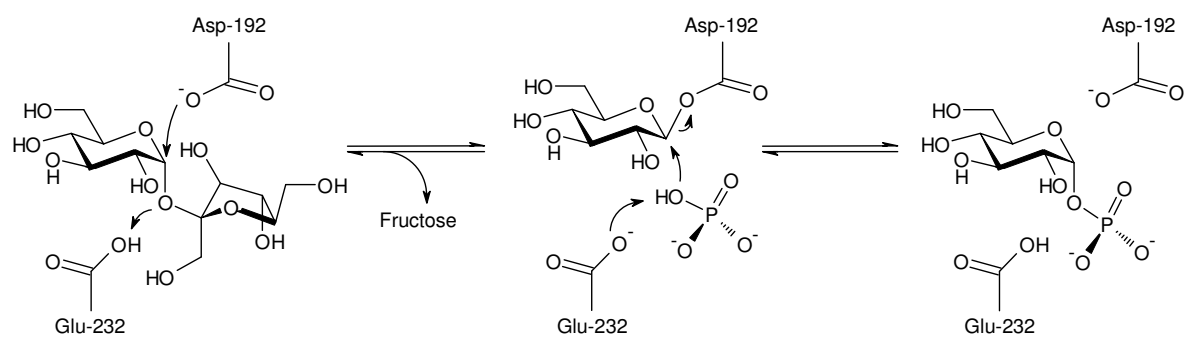
Scheme 1 ((Please keep schemes in file))



Scheme 2

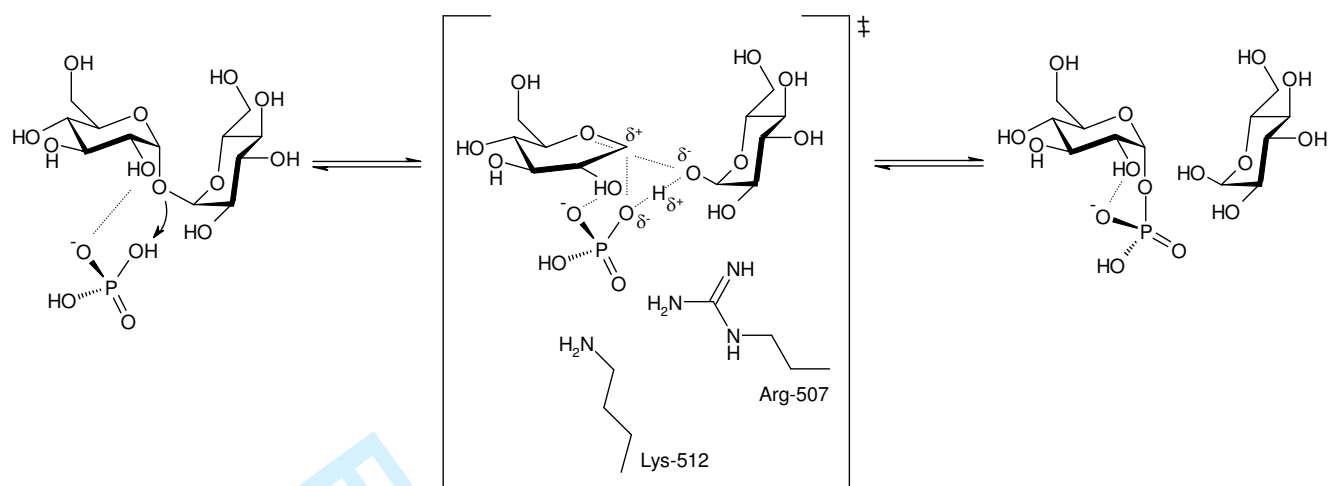


Scheme 3



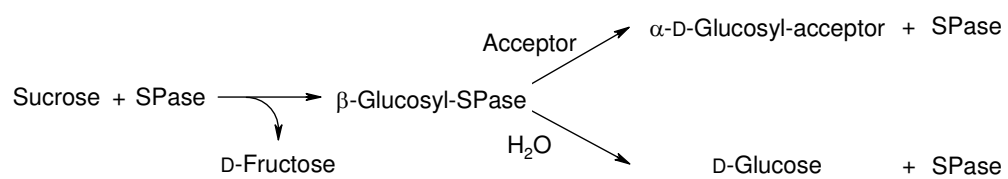
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Scheme 4



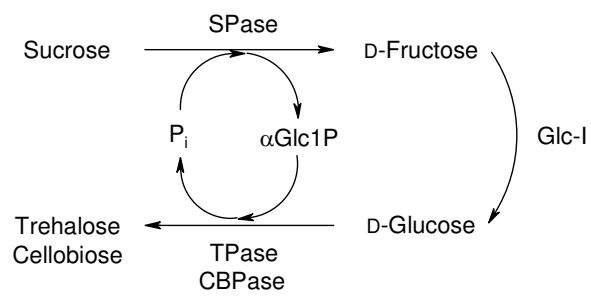
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Scheme 5



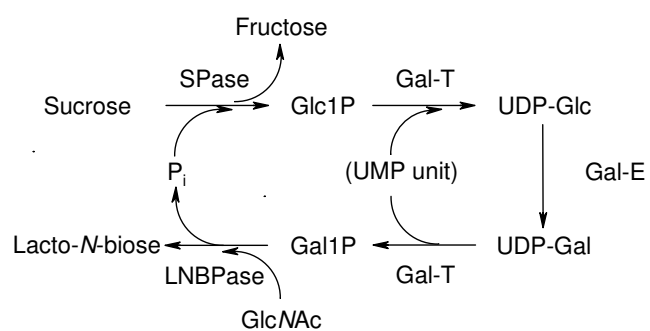
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Scheme 6



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Scheme 7



For Peer Review

Table 1. Disaccharide phosphorylases.

Name	EC number	Family	Clan	Fold	Stereochemistry	Product	Catalytic residues
Sucrose phosphorylase	2.4.1.7	GH-13	GH-H	(β/α) ₈	retaining	α -D-Glc-1-phosphate	Asp, Glu
Maltose phosphorylase	2.4.1.8	GH-65	GH-L	(α/α) ₆	invertig	β -D-Glc-1-phosphate	Glu
Trehalose phosphorylase (invertig)	2.4.1.64					β -D-Glc-1-phosphate	
Kojibiose phosphorylase	2.4.1.230					β -D-Glc-1-phosphate	
Cellobiose phosphorylase	2.4.1.20	GH-94	-	(α/α) ₆	invertig	α -D-Glc-1-phosphate	Asp
Chitobiose phosphorylase	2.4.1.-					α -D-GlcNAc-1-phosphate	
Laminaribiose phosphorylase	2.4.1.31					α -D-Glc-1-phosphate	
Lacto- <i>N</i> -biose phosphorylase	2.4.1.211	GH-112	-	TIM barrel	invertig	α -D-Gal-1-phosphate	Asp
D-Galactosyl-1,4-L-rhamnose phosphorylase	2.4.1.247					α -D-Gal-1-phosphate	
Trehalose phosphorylase (retaining)	2.4.1.231	GT-4	-	GT-B	retaining	α -D-Glc-1-phosphate	unknown

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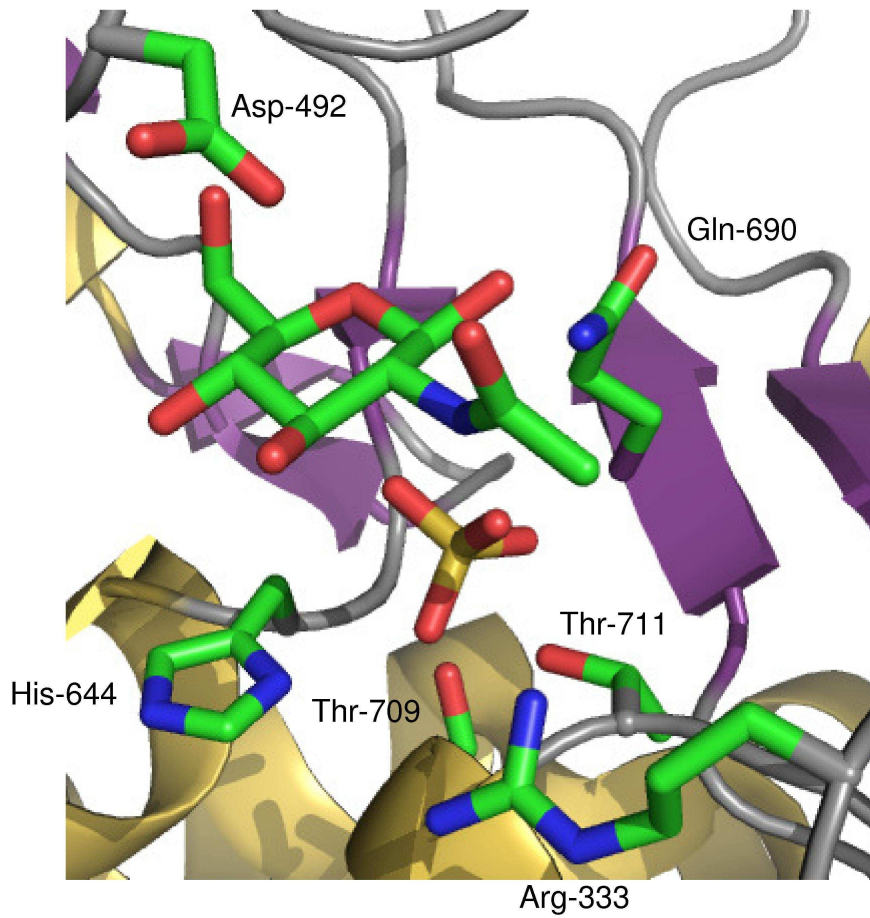


Figure 1
124x132mm (600 x 600 DPI)

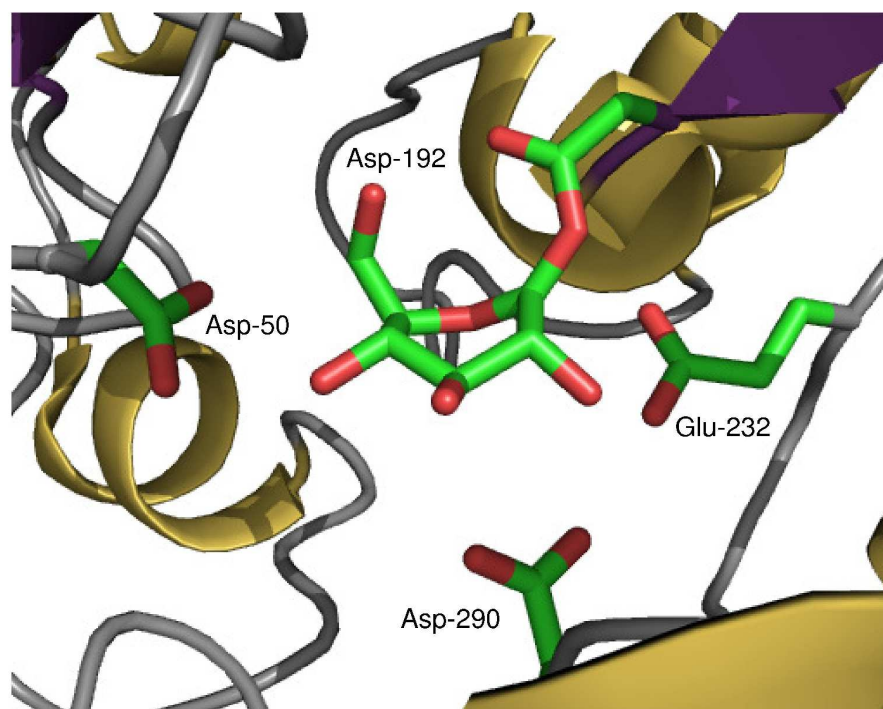


Figure 2
144x127mm (600 x 600 DPI)

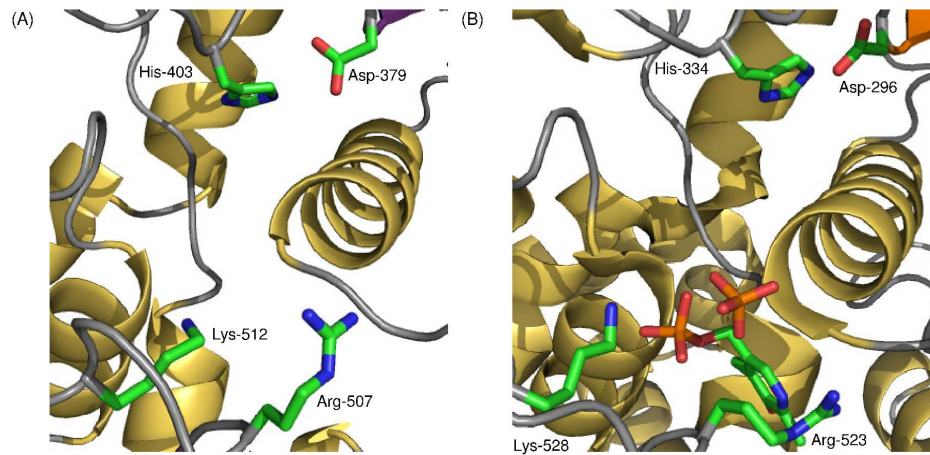


Figure 3
247x129mm (600 x 600 DPI)

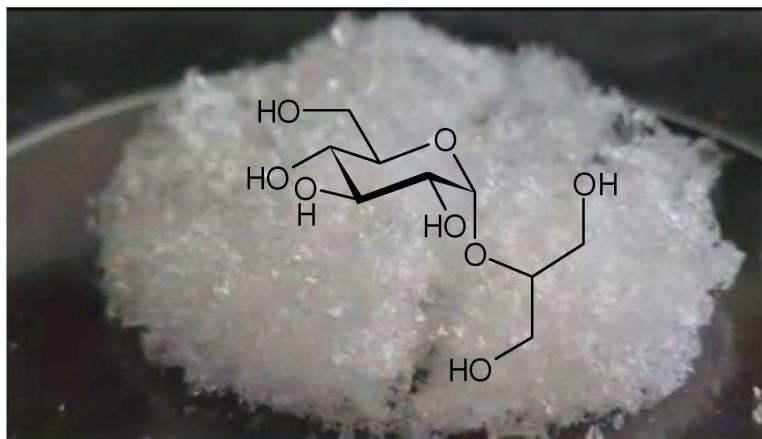


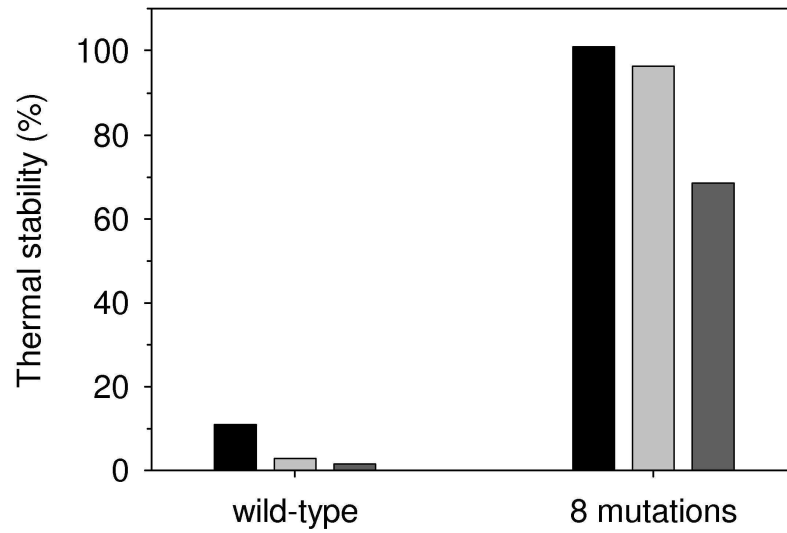
Figure 4
84x52mm (600 x 600 DPI)

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                                α3                                His-413                                α4  
                                ←----->                                ↓                                ↓----->  
LaMPase : 386-PCAYHNAKEQGL-PCALFPMVTFN-GTECHNEWEITFE-----EIHRNADIPH-431  
LbMPase : 384-DGAYINAEEQCL-KGALFPMVTFD-GTECHNEWEITFE-----EIHRNGDIAP-428  
GsTPase : 383-DKARERAREMCHRKGALFPWRTIS-GGECSSYFPAGTA-----QYHISADIAY-428  
TbTPase : 400-DAARSRAKELCH-KGALYPRTID-GPECSAYFPAGTA-----QYHINADIVY-444  
TbKPase : 392-DARKNAALNCY-KCAQYWESADTCETPKWGFDYMGNPVRIWTGDLEHHITADIAF-448
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177x44mm (600 x 600 DPI)

For Peer Review



114x77mm (600 x 600 DPI)