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Research Article

Side Chain Conformation Restriction in the Catalysis of Glycosidic Bond Formation by Leloir Glycosyltransferases, Glycoside Phosphorylases, and Transglycosidases

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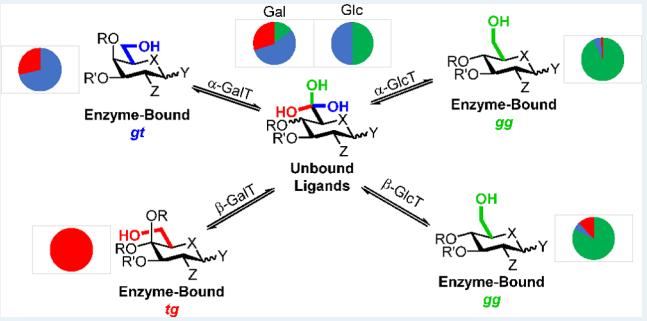
ABSTRACT: Carbohydrate side chain conformation is an important factor in the control of reactivity at the anomeric center, i.e. in the making and breaking of glycosidic bonds, whether by chemical means or, for hydrolysis, by glycoside hydrolases. In nature glycosidic bond formation is catalyzed out by glycosyltransferases (GTs), glycoside phosphorylases, and transglycosidases. By an analysis of 118 crystal structures of sugar nucleotide dependent (Leloir) GTs, 136 crystal structures of glycoside phosphorylases, and 54 crystal structures of transglycosidases bound to hexopyranosides or their analogues at the donor site (−1 site), we determined that most enzymes that catalyze glycoside synthesis, be they GTs, glycoside phosphorylases, or transglycosidases, restrict their substrate side chains to the most reactive *gauche,gauche* (*gg*) conformation to achieve maximum stabilization of the oxocarbenium ion-like transition state for glycosyl transfer. The galactose series deviates from this trend, with α-galactosyltransferases preferentially restricting their substrates to the secondmost reactive *gauche,trans* (*gt*) conformation and β-galactosyltransferases favoring the least reactive *trans,gauche* (*tg*) conformation. This insight will help promote the design and development of improved, conformationally restricted GT inhibitors that take advantage of these inherent side chain preferences.



KEYWORDS: *glycosyltransferase, glycoside hydrolase, glycoside phosphorylase, transglycosidase, inhibitor, oxocarbenium ion, conformational analysis*

INTRODUCTION

Glycosyltransferases (GTs) and transglycosidases are enzymes that catalyze the formation of glycosidic bonds,[1−4] a pivotal transformation in carbohydrate chemistry and the glycosciences in general.[5−8]

To bring about glycosidic bond formation, GTs stabilize oxocarbenium ion-like transition states (TSs) for the displacement of phosphate-based leaving groups from the anomeric position of their substrates (glycosyl donors) by alcohols (glycosyl acceptors) with either inversion or retention of configuration. In this they closely resemble the glycoside hydrolases (GHs), which cleave glycosidic bonds through oxocarbenium ion-like TSs by displacement of an alcohol by water,[9,10] albeit the intimate details the details of GT mechanisms are the subject of ongoing debate.[1,11] As the GTs are prime drug targets given their key involvement in multiple disease states including cancer, diabetes, and autoimmune disorders and in bacterial processes such as cell wall biosynthesis,[12−15] a more complete understanding of the fine details of their mechanisms is necessary to facilitate improved inhibitor design.

Glycoside phosphorylases are enzymes that catalyze the reversible displacement of a glycosidic linkage with a phosphate group. On the basis of their amino acid sequences, these enzymes are categorized into either GT families (such as glycogen and starch phosphorylases) or GH families (sucrose and maltose phosphorylases, among others).[16,17] Transglycosidases are a group of enzymes that, while formally classified as GHs due to their amino acid sequences, catalyze glycoside formation rather than hydrolysis with retention of configuration.[18]

The mechanistic parallels among GTs, glycoside phosphorylases, transglycosidases, and GHs extend to chemical glycosylation, for which the most common approach by far involves displacement of a leaving group from the anomeric center of a glycosyl donor by an acceptor alcohol through oxocarbenium ion-like transition states.[9,19−26] In chemical glycosylation and hydrolysis, the conformation of the donor

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side chain is one of the many factors that influence the reactivity of glycosyl donors, it having been established that donors with the *gauche,gauche* (*gg*) conformation are more reactive than those with the *gauche,trans* (*gt*) conformation, which in turn are more reactive than their counterparts with the *trans,gauche* (*tg*) conformation (Figure 1).[27,28]

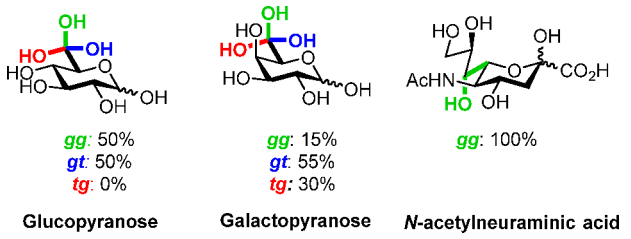

Figure 1. Staggered conformations of pyranoside side chains and their approximate population in free solution in the gluco- and galactopyranosides and in the *N*-acetylneuraminic acids.

The influence of glycosyl donor side chain conformation on reactivity is considered to arise from the interplay of the C−O bond in the side chain with the partial positive charge at the anomeric center in the glycosylation transition state. The *tg* conformation is understood to be electron withdrawing and so to destabilize transition state energies, while the *gg* conformation provides electrostatic stabilization to the partial positive charge at the anomeric center; the *gt* conformation is intermediate between the *tg* and *gg* conformers in its effect.[29−33] The influences of the *gg* and *tg* conformers on the positive charge at the transition state are more easily understood by comparison with the directly analogous axial and equatorial C−O bonds at the 4-position of galactopyranosyl and glucopyranosyl donors, respectively, with the latter being the less reactive of the two (Figure 2).[34,35] Electrostatic

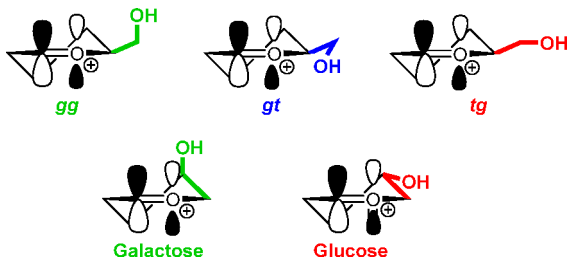

Figure 2. Spatial relationships of side chain and C-4 hydroxyl groups with the oxocarbenium π^* orbital.

stabilization of the positively charged transition state by an ideally located substrate side chain has also been demonstrated for purine nucleoside phosphorylase by Schramm and coworkers.[36]

By an examination of the extensive crystallographic database available in the PDB of GTs bound to hexopyranosides at the donor site we show here that glucosyltransferases typically restrict donor side chains to the reactivity-enhancing *gg* conformation, while galactosyltransferases favor the *gt* and even *tg* conformations. We also examine the glycoside phosphorylases and transglycosidases and find that they too make use of the restriction of side chain conformation to facilitate reactions passing through oxocarbenium ion-like transition states. Side chain restriction by GTs, glycoside phosphorylases, and transglycosidases closely resembles that employed by the GHs[37] and appears to be a broad general phenomenon that can in principle be applied to the design of improved and more selective inhibitors.

RESULTS AND DISCUSSION

Because of the available crystallographic data, we focused on the Leloir GTs, which catalyze displacement of nucleotidyl mono- or diphosphates from the anomeric position of the donor, and on glycoside phosphorylases, as opposed to the non-Leloir GTs employing polyprenyl phosphates and pyrophosphates, for which insufficient crystallographic data are available. Leloir GTs are broadly grouped into two classes, inverting and retaining, according to the stereochemical outcome of the substitution process they catalyze. In the inverting GTs the nucleophilic acceptor alcohol directly displaces the departing phosphate from the anomeric center in an S_N2-like manner (Figure 3), functioning analogously to

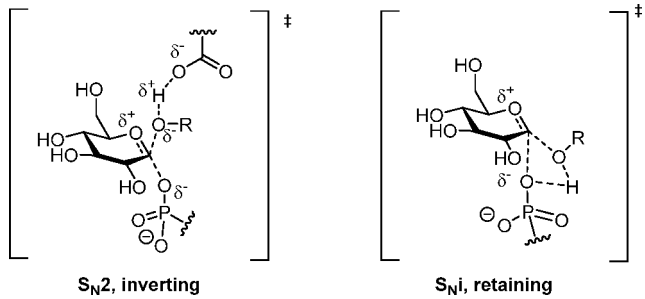

Figure 3. Approximate transition states for inverting and retaining GTs illustrated for D-glucose with the *gg* side chain conformation.

the inverting GHs. The retaining GTs are considered to achieve substitution by an S_Ni mechanism (Figure 3), as for the most part they lack a suitably placed internal nucleophile for the type of double inversion with the intermediacy of a covalent glycosyl enzyme adduct that is operative in the retaining GHs. Computational and mass spectrometric studies nevertheless support the formation of a glycosyl−enzyme adduct in a limited number of cases.[38,39] The transglycosidases, on the other hand, employ a mechanism comparable to that of retaining GHs, forming an enzyme-bound covalent intermediate in a classic Koshland double displacement.[18] Despite these differences, glycosidic bond formations by each of the inverting and retaining GTs, the glycoside phosphorylases, and the transglycosidases pass through transition states with significant oxocarbenium ion character and thus a positive charge at the anomeric center.

Applying the same strategy as for the GHs, we used the Carbohydrate Active Enzymes database (CAZy, http://www.cazy.org)[40] to search the PDB for crystal structures of glycosyltransferases, glycoside phosphorylases, and transglycosidases containing hexopyranosides or corresponding analogues bound at the donor subsite of the enzyme, manually inspecting each individual structure to avoid errors such as incorrect binding site identification. The Privateer software[41] was used to validate each of the glycoside-based structures, with a minimum allowable real space correlation coefficient (RSCC) of 0.8.[42] To minimize crystallographic errors in the data set,[43] we limited the resolution of the included crystal structures to ≤2.50 Å. This cutoff is more relaxed than the ≤2.00 Å cutoff previously applied for the GHs but is necessary in order to obtain a sufficiently large data set: a comparison

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Table 1. Side- Chain Conformations of Ligands Bound to Leloir Glycosyltransferases[a]

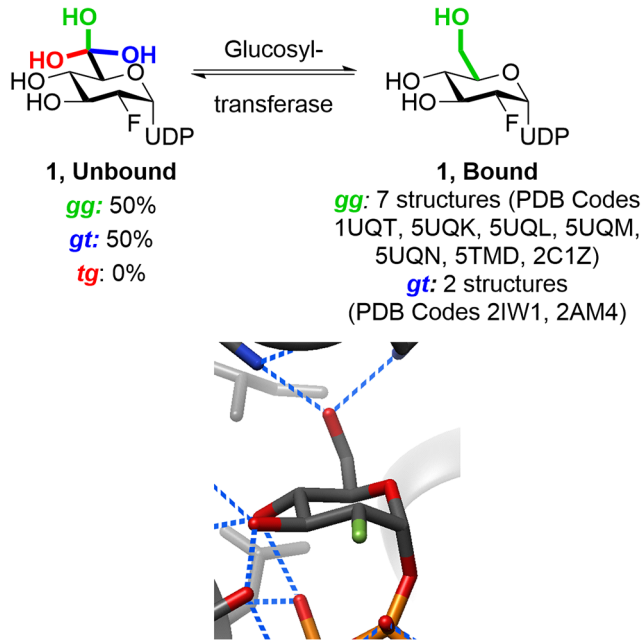
enzyme function	*gg*	*gt*	*tg*	eclipsed	total	H bonding to O6
α-galactosyltransferase		6 (5)	2 (2)		8 (7)	8 (7)
β-galactosyltransferase			8 (3)		8 (3)	8 (3)
α-*N*-acetyl galactosaminyltransferase	1 (−)	2 (2)	5 (2)		8 (4)	8 (4)
α-glucosyltransferase	30 (15)	3 (3)	1 (-)	3 (1)	39 (19)[b]	37 (18)
β-glucosyltransferase	10 (6)	1 (1)	1 (1)		12 (8)	12 (8)
α-*N*-acetyl glucosaminyltransferase	4 (2)	1 (−)	1 (−)	1 (−)	7 (2)	6 (2)
β-*N*-acetyl glucosaminyltransferase	10 (6)	4 (3)		2 (−)	16 (9)	14 (7)
α-*N*-acetyl hexosaminyltransferase[c]	1 (−)	1 (−)			2 (−)	2 (−)
α-mannosyltransferase			1 (−)		1 (−)	1 (−)
β-mannosyltransferase	2 (2)				2 (2)	2 (2)
α-valeniolyltransferase	1 (−)	1 (1)			2 (1)	2 (1)
heptosyltransferase	2 (−)				2 (−)	2 (−)
α-sialyltransferase	9 (7)		1 (−)	1 (−)	11 (7)	7 (6)
total	70 (38)	19 (15)	20 (8)	2 (−)	118 (62)	109 (58)

[a]Data are listed at the 2.50 Å cutoff, with those for the 2.00 Å cutoff given in parentheses. [b]Two structures bear ambiguous side chain conformations. [c]These enzymes act on both glucosides and galactosides.

between data limited by a 2.00 Å cutoff and those limited by the 2.50 Å cutoff reveals no significant difference and justifies the use of the more relaxed limit. Accordingly, all subsequent discussions refer to the larger data set. By these criteria, a total of 118 crystal structures of Leloir GTs with carbohydrate-based ligands bound in the donor site were located and are summarized in Table 1, with the raw data compiled in Table S1. For each category of enzyme, Table 1 shows the distribution of bound staggered and eclipsed side chain conformations, along with the number of crystal structures showing hydrogen bonding between the side chain hydroxyl group and the enzyme. Structures with multiple chains in the unit cell were listed as one entry if the side chain conformation of the ligand in each active site was the same; when this was not the case, the entries were classed as ambiguous and were not considered further. As, unlike the GHs,[44] actual transition-state analogues of GTs have proven to be elusive,[15,45,46] the vast majority of ligands are simple glycosides.

α- and *β*-Glucosyltransferases and *β*-*N*-acetyl glucosaminyltransferases show moderate to strong preference for binding their ligands at the donor site in the *gg* conformation (77%, 83%, and 63% *gg*, respectively; Scheme 1). Here, it is informative to consider the different CAZy families encompassed by the *α*-glucosyltransferases and *β*-*N*-acetyl glucosaminyltransferases (Table S4). Thus, in the latter case, while the overall side chain population distribution is an ∼2:1 mix of *gg* and *gt* conformers, the crystal structures featuring the *gg* conformation all belong to GT families 2 and 41, while those favoring the *gt* conformation are predominantly members of GT family 13, indicating a family-specific influence on side-chain binding. Interestingly, all three GT13 structures hold the catalytic aspartic acid directly above the ring instead of in the typical *syn* or *anti* positions, sterically disfavoring the *gg* conformation;[47,48] a similar observation was made for the GH116 glucoside hydrolases, whose members also favor the *gt* conformation.[37] These trends are nicely illustrated by the natural substrate UDP-*α*-*N*-acetylglucosamine **2**, as shown in Scheme 2. For the *α*-glucosyltransferases, GT families 20, 44, and 88 show high selectivity for the *gg* conformation, while families 4 and 24 bind their substrates in the *gt* conformation, though the limited sample size restricts the conclusions that can be drawn. Notwithstanding the small sample size for the mannosyltransferases, the population trends closely resemble

Scheme 1. Bound and Unbound Side Chain Populations of UDP-2-deoxy-2-fluoro-*α*-glucose (1), as Exemplified by *E. coli* Trehalose-6-phosphate Synthase (PDB ID 1UQT)



those found in the corresponding GHs and stand in stark contrast with the roughly 1:1 *gg*:*gt* ratio observed both in solution and in the carbohydrate binding domains of lectins. Collectively, the data suggest that glucosyl- and likely *β*-mannosyltransferases exploit the same phenomenon as the corresponding GHs to gain additional transition-state stabilization.

As with the GHs, the *α*-galactosyltransferases follow a different pattern (Table 1), which must stem in part from the higher energy of the *gg* conformation in free galactose, as is evident from its relatively minimal population in solution. Thus, 75% of *α*-galactosyltransferases bind their ligand side chains in the *gt* conformation, which parallels the high *gt* preference of *α*-galactosidases. The *α*-*N*-acetyl galactosaminyltransferases show a small preference for binding their ligands in the *tg* conformations. A study of the bound conformations of

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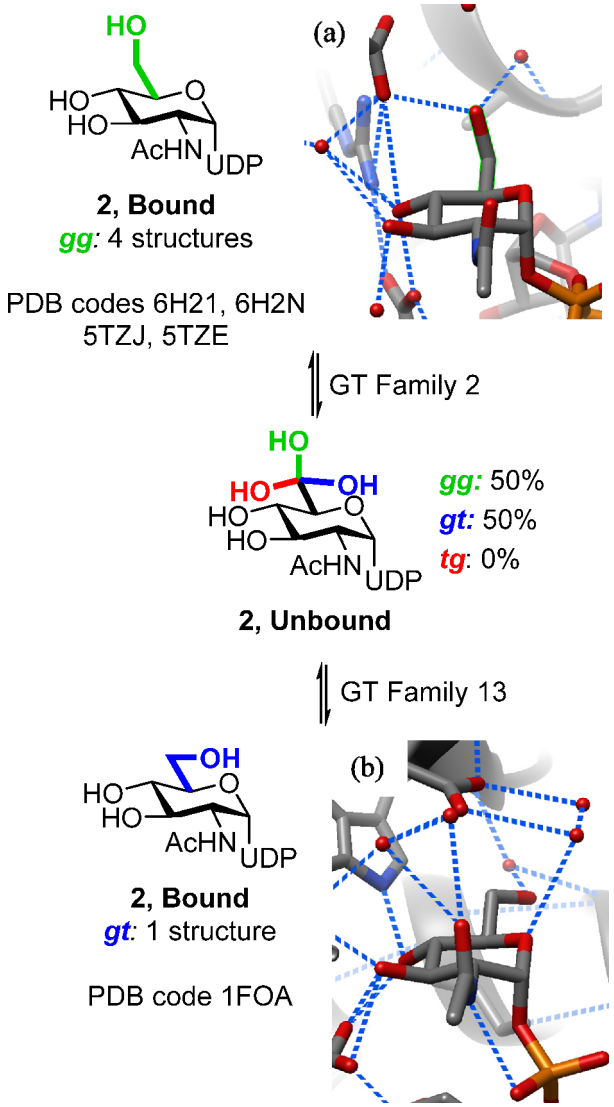
Scheme 2. Bound and Unbound Side Chain Conformations of UDP-α-*N*-acetyl Glucosamine (2), as Exemplified in the *S. aureus* TarP and Rabbit *N*-Acetyl Glucosaminyltransferase 1 (PDB Structures (a) 6H21 and (b) 1FOA)



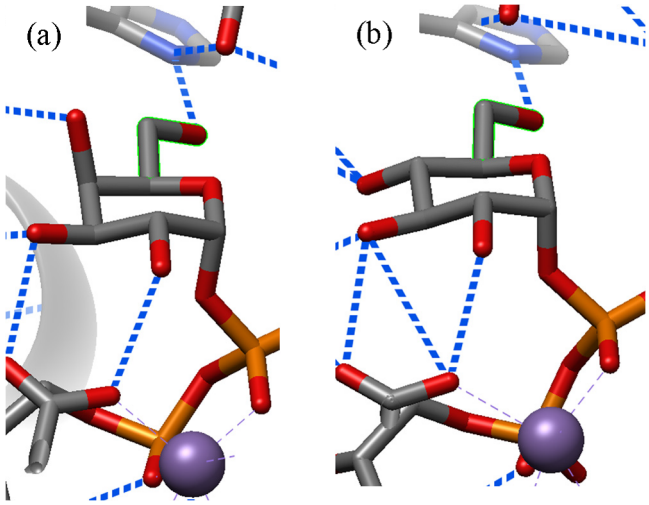


Figure 4. Partial structures of (a) UDP-α-galactopyranose (PDB ID 5C1G) and (b) UDP-α-glucopyranose (PDB ID 5C8R) in the donor site of GTB with the tucked-under conformation of the pyrophosphate and the side chain restricted to the *gt* conformation by H-bonding.

UDP-α-galactopyranose, UDP-α-glucopyranose, and UDP-α-*C*-galactopyranose in the vicinity of the donor sites of the homologous glycosyltransferases α-(1→3)-*N*-acetyl galactosaminyltransferase (GTA) and α-(1→3)-galactosyltransferase (GTB), and their chimera, is revealing.[49] Lowary and coworkers have shown that, for each of these enzymes, the donor binds in a stepwise manner, anchoring first by the UDP group and then by rotating the pyranoside ring to place it directly above the pyrophosphate moiety in the conformation required for catalytic activity, denoted the tucked-under conformation. As is clear from Figure S1 and from Table S5, which show the side chain conformations of ligands bound in all non-tucked-under conformations, only when UDP-α-galactopyranose is correctly placed in the donor site in the tucked-under conformation does the enzyme restrict side chain conformation by H-bonding (Figure 4a). Four structures containing UDP-α-*C*-galactopyranose are not correctly located in the donor site for transfer, do not have the tucked-under conformation of the pyrophosphate, and do not exhibit protein−side chain H-bonding. Four structures with bound UDP-α-glucopyranose hold the side chain in the *gg* conformation by H-bonding, but in an alternate orientation within the binding pocket; a further structure has UDP-α-glucopyranose correctly located in the binding pocket for transfer, with the tucked-under conformation of the pyrophosphate, and the side chain held in the *gt* conformation consistent with the pattern for a galactose-processing enzyme (Figure 4b). Clearly, GTB has evolved to capture the side chain in a suitable conformation for transition-state stabilization once the natural substrate is correctly poised for glycosyl transfer in the catalytically active tucked-under conformation.

In accordance with the binding preferences exhibited by the β-galactosidases, all eight crystal structures of β-galactosyltransferases hold the side chain in the *tg* conformation (Table 1), standing in stark contrast to the high selectivity for the *gt* conformation observed for galactosyl substrates bound to alternate sites and to the corresponding lectins (vide infra). The switch in the side chain conformation from *gt* to *tg* on going from the retaining α-galactosyltransferases to the inverting β-galactosyltransferases is illustrated in Scheme 3 for the common natural substrate UDP-α-galactose (**3**).

Finally, the sialyltransferases show a high preference for binding of the substrate side chain in the *gg* conformation, which is the predominant conformation in free solution. This raises the possibility that these enzymes have simply evolved to bind the most stable conformation, which coincidentally provides optimal stabilization to the TS. Lectins for other higher-carbon sugars are also known to bind their ligands with the side chain in the conformation that is strongly preferred in solution.[33,50] It is possible that, when suitable structures eventually become available, GTs for the synthesis of sialic and/or ulosonic acid containing bacterial capsular polysaccharides, particularly those carrying pseudaminic acids or Kdo residues,[51−53] may bind their ligands in conformations different from the strongly disarming *tg* conformation preferred in solution (Figure 5).[54,55] In this respect, it is of interest that docking of CMP β-Kdo into the crystallographically characterized active site of the β-Kdo GT from the multidomain

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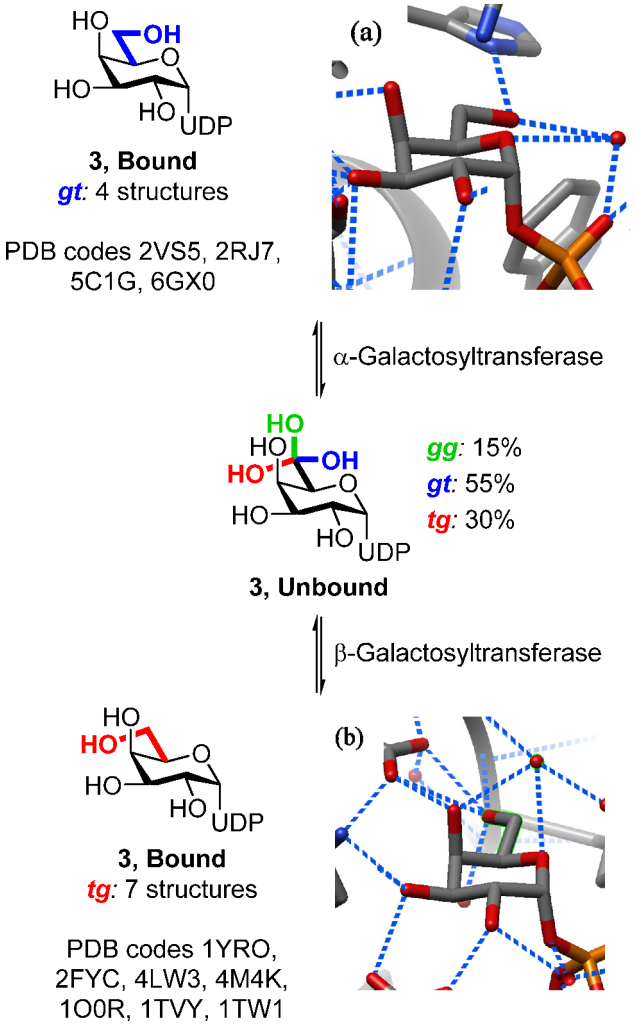
Scheme 3. Contrasting Binding Preferences of α- and β-Galactosyltransferases, as Exemplified by (a) Cow α-Galactosyltransferase and (b) Mouse β-Galactosyltransferase (PDB Structures (a) 2VS5 and (b) 1YRO)



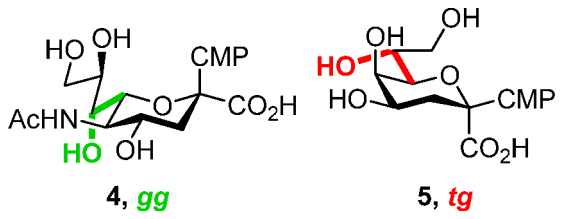


Figure 5. Solution side chain conformations of CMP-*N*-acetylneuraminic acid (**4**) and CMP-Kdo (**5**).

WbbB protein involved in the biosynthesis of the *Raoultella terrigena* lipopolysaccharide *O*-antigen favors a $^{2,6}B$ conformation of the pyranose ring with the side chain held in the *gt* conformation by H-bonding to the protein.[56]

Overall, the data suggest that restriction of the side chain conformation by the GTs takes place on binding of the substrate in a conformation primed for transfer and presumably continues through the transition state to product release. This pattern of initial side chain conformational restriction at the level of the enzyme–substrate complex, with maintenance through the transition state, is directly analogous to that seen in the GHs.[37] Because of the relative absence of true transition-state inhibitors for the GTs, snapshots of the processing of substrates by GTs, while crystallography is employed to reveal the enzyme–substrate and enzyme–product complexes, use computations to visualize the transition-state complex.[57,58] Such computations typically do not focus on the conformation of the substrate side chain or reveal the extent to which it is restricted by hydrogen bonding to the GT.[38,57−60] Nevertheless, crystallographic studies on cognate enzyme–substrate and enzyme–product complexes are informative, particularly as both reveal a common conformation of the side chain. For example, such a study of the human *O*-GlcNAc transferase, an inverting GT, by the Vocadlo and Walker groups,[61] reveals that both the substrate (Figure 6a) and product (Figure 6b) analogues based on the 5-thia-GlcNAc skeleton are bound with the side chain in the *gg* conformation.

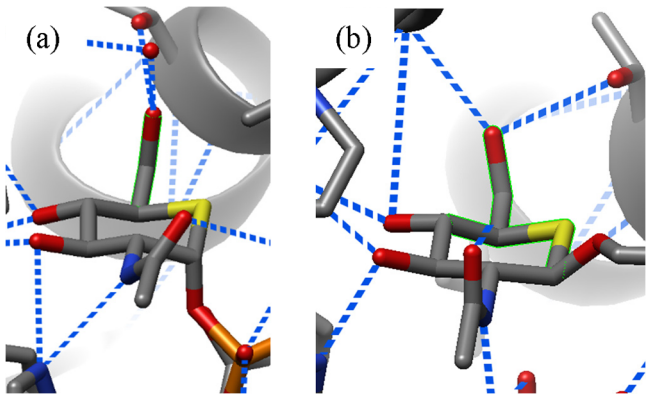


Figure 6. Partial structures of (a) UDP-5*S*-GlcNAc donor (PDB ID 4GYY) and (b) a thioglycopeptide product (PDB ID 4GZ3) complexed to human *O*-GlcNAc transferase.

Turning to the glycoside phosphorylases, which catalyze both phosphorolytic cleavage of glycosidic linkages and glycosylation of anomeric phosphate-bearing donors, we located 136 crystal structures of hexopyranoside ligands bound to the donor subsite. In every nonambiguous crystal structure located, the ligand side chain is restricted to the *gg* conformation through hydrogen bonding with the enzyme (Table 2), which is in stark contrast with the side chain populations of glucosides observed in free solution (Scheme 4). Clearly these enzymes have evolved to take advantage of

Table 2. Side Chain Conformations of Ligands Bound to Glycoside Phosphorylases[a]

enzyme function	*gg*	*gt*	*tg*	eclipsed	total	H-bonds to O6
α-glucoside phosphorylase	126 (66)				126 (66)	126 (66)
β-glucoside phosphorylase	3 (2)				4 (2)[b]	4 (2)
α-mannoside phosphorylase	6 (5)				6 (5)	6 (5)
total	135 (73)				136 (73)	136 (73)

[a]Data are listed at the 2.50 Å cutoff, with those for the 2.00 Å cutoff given in parentheses. [b]One structure bears an ambiguous side-chain conformation.

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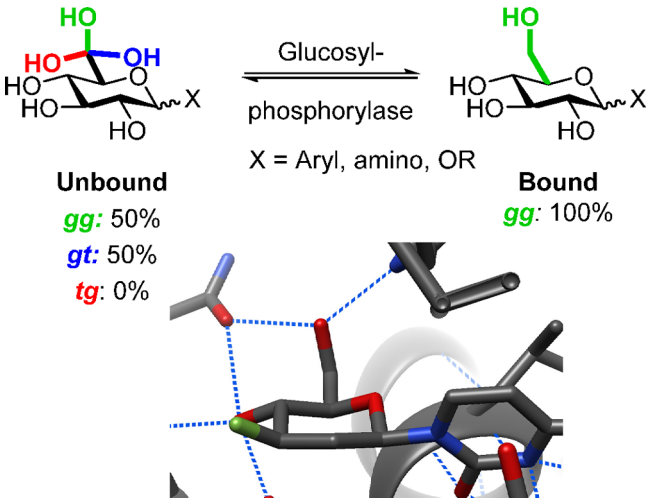
the increased reactivity imposed by the *gg* conformation as an adjunct to catalysis.

Scheme 4. Bound and Unbound Side Chain Populations of Nonhydrolyzable Glucose Derivatives, as Exemplified by Rabbit Muscle Glycogen Phosphorylase (PDB ID 3L7D)



Our CAZy-assisted search of the PDB for transglycosidases carrying substrates at the −1 site, conducted analogously with the same constraints as those for GT-substrate complexes, unearthed a total of 54 structures. These are comprised of mainly α-glucosyl transglycosidases with a smaller number of β-glucosyl transglycosidases and *trans*-sialidases, with high preference (71%, 74%, and 100% respectively) for binding the ligand with the *gg* conformation of the side chain. Only five structures were identified bound with the *gt* conformation of the side chain, three with an eclipsed conformation, and none with the *tg* conformation (Table 3). Not surprisingly in view of their evolutionary relationship, this pattern closely resembles that found in the α- and β-glucosidases and differs significantly from that in the free sugars (Figure 1).

Like the retaining GHs, the transglycosidases offer the possibility of characterization of the covalent enzyme-bound substrate intermediate between the two steps. Cyclodextrin glycosyltransferase, for which the covalently trapped intermediate has been studied crystallographically by Dijkstra and co-workers, is a case in point.[62] In the covalently bound 4-deoxymaltotriose the residue β-linked to the nucleophilic aspartate in the −1 site has the side chain held in the *gg* conformation by hydrogen bonding to the protein (PDB 1CXL). Sufficiently well resolved structures at the level of the enzyme substrate complex employ γ-cyclodextrin (PDB 1D3C)[63] and maltobiose (PDB 1PJ9),[64] whose side chains are both bound in the *gg* conformation in the −1 site. An acarbose-derived inhibitor is also bound in the −1 site with the side chain in a conformation that is formally analogous to the *gg* conformation in a saturated pyranose ring (PDB 1KCK).[65] Thus, reinforcing the general notion that side chain restriction begins at the level of the enzyme substrate complex, inverting cyclodextrin glycosyltransferases bind their substrates, transition states, and covalent intermediates all with the *gg* conformation (Figure 7).

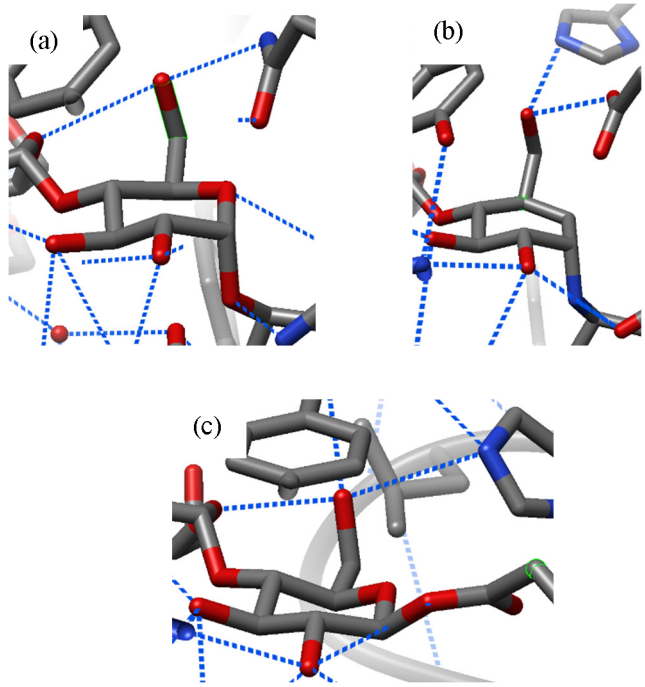


Figure 7. Partial structures of (a) a cyclodextrin substrate, (b) an inhibitor, and (c) a covalent intermediate in the −1 site of cyclodextrin glycosyltransferases, all with *gg* side chain conformations.

As a control, analogously to the preceding GH study, we examined the side chain conformations of ligands bound to alternate subsites of the GTs, glycoside phosphorylases, and transglycosidases (Table 4), again curating the data by Privateer analysis. Although the data set is more limited than that developed for the GHs, it is readily apparent that differences in global side chain populations exist for ligands bound in GT donor sites and alternate sugar binding sites for α- and β-glucosides as well as for the β-galactosides. Together with the obvious differences in side chain population between the donor-site-bound ligands and free sugars (Figure 1),[28,66−68] it is clear that GTs, glycoside phosphorylases, and transglycosidases employ restriction of donor side chain conformation as a means of transition-state optimization. In passing we note the more diverse populations adopted by ligand side chains in alternate GT binding sites do not preclude any other site from preferentially binding a specific

Table 3. Side Chain Conformations of Ligands Bound to Transglycosidases[a]

enzyme function	*gg*	*gt*	*tg*	eclipsed	total	H-bonds to O6
α-glucoside transglycosidase	29 (15)	4 (2)		2 (-)	41 (21)[b]	29 (16)
β-glucoside transglycosidase	7 (6)	1 (1)		1 (1)	9 (8)	6 (5)
trans-sialidase	4 (4)				4 (4)	2 (2)
total	40 (25)	5 (3)		3 (1)	54 (33)	37 (23)

[a]Data are listed at the 2.50 Å cutoff, with those for the 2.00 Å cutoff given in parentheses [b]Six structures bear ambiguous side chain conformations

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Table 4. Comparison of Side Chain Conformations at Donor Sites of Leloir GTs, Glycoside Phosphorylases, and Transglycosidases with Those at Alternate Sites[a]

sugar	position	*gg*	*gt*	*tg*
β-galactosides	Leloir donor site			8 (3)
	alt site		16 (14)	
α-glucosides	Leloir donor site	30 (15)	3 (3)	1 (−)
	phos donor site	126 (66)		
	transglycos −1 site	29 (15)	4 (2)	
	alt site	46 (19)	28 (18)	1 (−)
β-glucosides	Leloir donor site	10 (6)		1 (1)
	phos donor site	3 (2)		
	transglycos −1 Site	7 (6)	1 (1)	
	alt site	26 (21)	18 (11)	2 (2)
α-mannosides	Leloir donor site			1 (−)
	phos donor site	6 (5)		
	alt site	1 (1)		
β-mannosides	Leloir donor site	2 (2)		
	alt site	2 (1)		

[a]Data are listed at the 2.50 Å cutoff, with those for the 2.00 Å cutoff given in parentheses.

conformation. Thus, Hindsgaul and co-workers previously demonstrated preferential recognition of acceptor alcohols on conformational restriction of the side chain to the *gg* conformation by *N*-acetylglucosamine transferase V.[69,70]

Side chain restriction at the level of the enzyme−substrate complex by the GHs, GTs, glycoside phosphorylases, and transglycosidases is consistent with Namchuk and Withers' kinetic analysis of *A. faecalis β*-glucosidase, which showed that enzyme−ligand H-bonding interactions involving the side-chain hydroxymethyl group provide substantial ground-state stabilization in contrast to H-bonding interactions with the ring hydroxyl groups, which mainly affect the transition state.[71] In effect, the entropic penalty for restricting the conformation of the side chain is paid in the form of ligand preorganization at the level of the enzyme−substrate complex, such that the contribution of the C6−O6 bond to increased transition-state affinity is mainly enthalpic, consistent with Wolfenden's proposals.[44,72,73] Note that substrate preorganization in this manner is distinct from the more familiar concept of substrate binding in a high-energy conformation in which the reactive groups are preorganized for bond formation.[74−77] With the side chain preorganized into the *gg* and to a lesser extent the *gt* conformation, it can be expected to stabilize the transition state for glycosyl transfer comparably to the axial C4−O4 bond in galactosyl substrates.

The recognition of side chain conformation as a contributing factor to transition-state stabilization by GTs opens up a new avenue for the development of a next generation of inhibitors designed to take advantage of the inherent binding preference of the glycosyltransferases. A clue as to how this might be achieved is provided by the *E. coli* heptosyltransferase WaaC, which processes the natural substrate[78] ADP-L-*glycero*-*β*-D-*manno*-heptose (**6**) 10 times more effectively than the side-chain isomer ADP-D-*glycero*-*β*-D-*manno*-heptose (**7**).[79] Crystallographic studies by Ducruix and co-workers revealed that the substrate analogue ADP-2-deoxy-2-fluoro-L-*glycero*-*β*-D-*gluco*-heptose, whose side chain is predicted to take up the *gg* conformation in free solution,[80] is bound in the active site with its side chain held in the *gg* conformation by hydrogen bonding to lysine 192 (Figure 8).[81] The less effective substrate

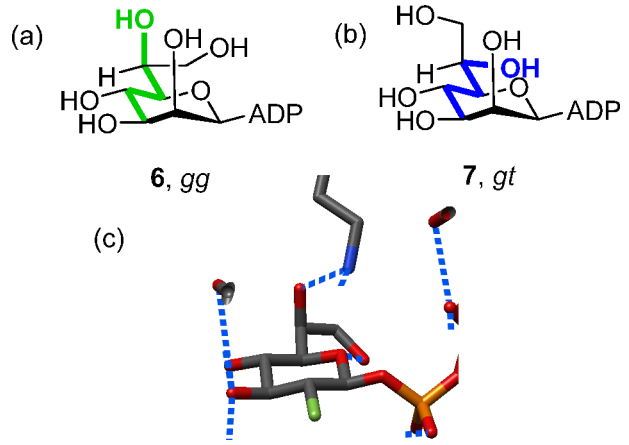

Figure 8. Solution conformations of (a) ADP-L-*glycero*-*β*-D-*manno*-heptose (**6**), (b) ADP-D-*glycero*-*β*-D-*manno*-heptose (**7**), and (c) ADP-2-deoxy-2-fluoro-L-*glycero*-*β*-D-*gluco*-heptose bound to *E. coli* WaaC (PDB ID 2H1H) in the *gg* conformation.

with the D-*glycero*-D-*manno* configuration, on the other hand, is predicted to adopt the *gt* conformation of the side chain in solution,[80] which, if it is maintained at the transition state, necessarily places the hydroxymethyl group uncomfortably close to Lys 192. Alternatively, rotation of the side chain in the D-*glycero*-D-*manno* isomer into the *gg* conformation so as to pick up the H-bond to Lys 192 and provide additional transition-state stabilization would place the hydroxymethyl group in a disfavored 1,5-*syn* relationship to the C4−O4 bond and in proximity to Asp 261.

A further example of the effectiveness of side chain conformational control in Nature is provided by the natural glycosidase inhibitors castanospermine (**8**) and 1-deoxynojirimycin (**9**) (Figure 9). The former, whose side chain is locked

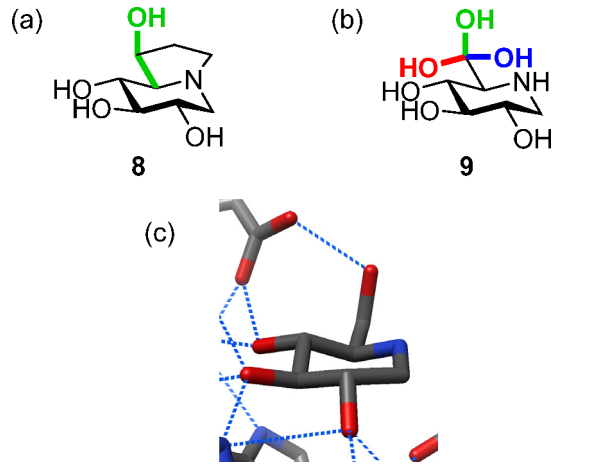

Figure 9. Glycosidase inhibitors (a) castanospermine **8**, (b) 1-deoxynojiromcyin **9**, and (c) **9** bound to *T. maritima β*-glucosidase in the *gg* conformation (PDB ID 2J77).

in the *gg* conformation by the ethylene bridge, has 4-fold greater affinity for the *Thermotoga maritima β*-glucosidase than the latter,[82] whose side chain is freely rotating in the solution phase but which is restricted to the *gg* conformation when it is bound to glycosidases.[37,83] Clearly, depending on the targeted enzyme, it should be possible to design improved and possibly more selective inhibitors by incorporation of side chain

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conformation biasing elements such as the fused ring in **8**, or even by adding a simple methyl or substituted methyl group as in **6**.

CONCLUSION

We demonstrate that GTs, glycoside phosphorylases, and transglycosidases mimic their GH counterparts in the restriction of substrate side chain conformations. Glucosyltransferases show a strong tendency for binding their substrates in the *gg* conformation, thereby maximizing transition-state stabilization. α-Galactosyltransferases restrict their substrates predominantly to the secondmost stabilizing *gt* conformation, avoiding the energetic penalty of the *gg* conformation in galactosyl pyranosides, while β-galactosyltransferases bind their substrates in the least reactive *tg* conformation. The restriction of side chain conformation in the course of glycosidic bond formation and/or cleavage by the respective enzymes, although not without occasional exceptions, is therefore a broad general principle, the consideration of which should inform the design of improved and more selective inhibitors. For example, the locking of the side chain in a given inhibitor to the *gg* conformation will enhance selectivity for glucose- and mannose-processing enzymes over galactose-processing enzymes, while restriction to either the *gt* or *tg* conformations will achieve the opposite effects and could even provide selectivity between the different classes of galactose-processing enzymes.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscatal.1c00896.

Additional information on Privateer analysis, raw crystal structure data, bound side chain conformations for each family, and families encompassed by each enzyme category (PDF)

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Notes

The authors declare no competing financial interest.

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