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Mechanistic analogies amongst carbohydrate modifying enzymes

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Carbohydrates are known to play essential roles in a myriad of biological processes. The enormous complexity of the various oligosaccharide structures found in biology is derived from a rational orchestration of the enzymatic formation and breakdown of glycosidic linkages achieved by glycosyltransferases, glycosidases and phosphorylases. A detailed understanding of the chemical mechanisms by which these classes of enzymes function not only provides a rational basis for their engineering and application in both the development and synthesis of new classes of therapeutic agents, but also provides insight into the role of convergence in the natural evolution of enzyme function.

Introduction

The oligosaccharides and glycoconjugates present in biological systems carry out a wide array of essential functions ranging from energy storage and utilization to complex information display systems that modulate normal cell function. The number of possible permutations of assemblies of monosaccharides, both in the type and the manner in which individual units are connected, allows for a level of complexity which far exceeds that of DNA and even proteins. Diversity originates from the formation and breakdown of one of the most stable covalent linkages ($\Delta G^{\ddagger} \sim 30$ kcal mol^{-1}) found within natural biopolymers—the glycosidic bond.[1] As such, the mechanistic strategies used by nature's most proficient and sophisticated catalysts, enzymes, to make and break this class of covalent bond with a high degree of both stereo- and regio-selectivity is a topic of considerable interest in various fields of chemistry and chemical biology. The enzymes responsible for the achievement of this formidable task comprise the glycosidases, glycosyltransferases and phosphorylases (Scheme 1).

While the mechanistic strategies used by

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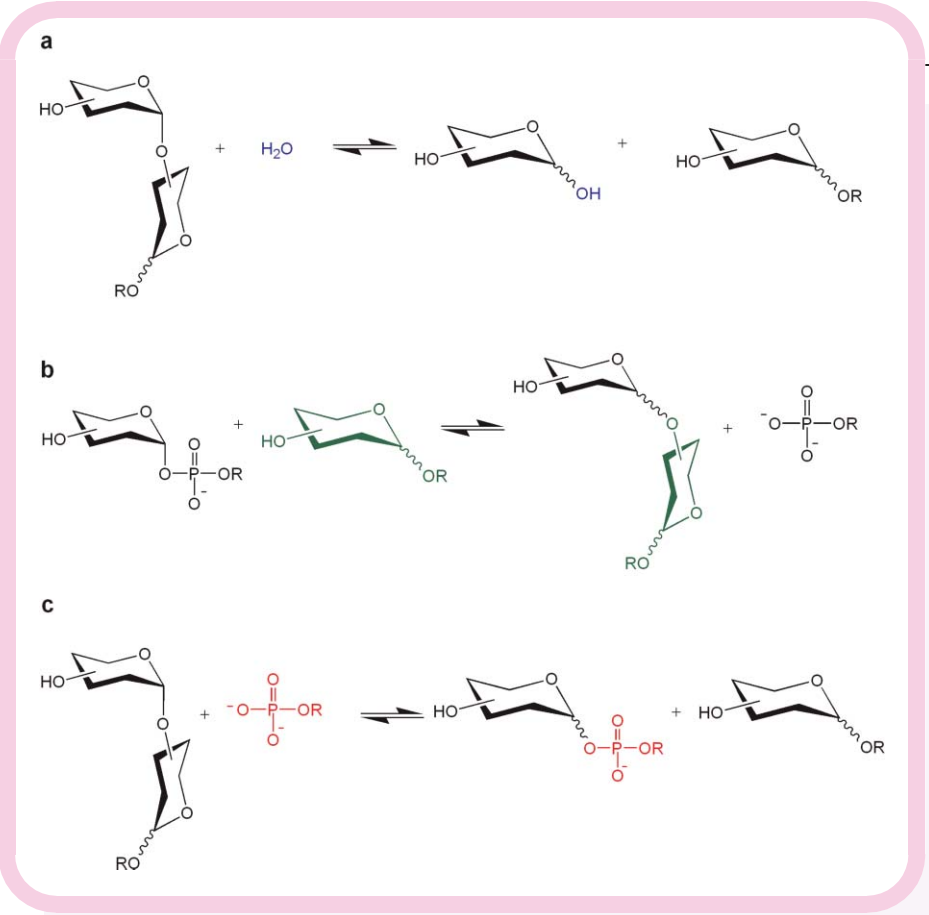

Scheme 1 Overall reactions catalyzed by (a) glycosidases, (b) glycosyltransferases, and (c) phosphorylases.

glycosidases to catalyse glycosidic bond hydrolysis are fairly well understood on both a structural and chemical level,[2,3] the characterization and mechanistic understanding of the glycosyltransferases responsible for glycoside bond formation has lagged far behind. This has mainly been the result of the technical challenges involved in obtaining active forms of these frequently membrane-associated enzymes in sufficient quantities for proper structural and mechanistic investigation. Applications of recombinant DNA technologies have largely overcome this problem and, as such, this field of study has seen significant progress in the past five years and will undoubtedly see tremendous growth in the near future.

Despite a lack of evolutionary relatedness, by simple chemical analogy, glycosyltransferases are thought to use mechanistic strategies that directly parallel those used by glycosidases and transglycosidases,[2] though some distinct differences are becoming apparent. Sandwiched between these two enzyme groups are the phosphorylases; some of which clearly follow a glycosidase type of mechanism while others show much greater mechanistic and structural parallels to the glycosyltransferases. A mechanistic comparison of these two classes of enzymes explores whether a general theme in enzymology and molecular evolution exists. Has nature converged on analogous strategies for catalysing reactions in opposite directions using different unrelated enzymes (*i.e.* glycoside hydrolysis by glycosidases and glycoside formation by glycosyltransferases) and/or what is the nature of this mechanistic continuum?

Glycosidases and transglycosidases

Glycosidases have evolved well-defined and characteristic active sites that allow them to catalyse glycosidic bond hydrolysis with rate constants of up to 1000 s^{-1}. As such they are able to accelerate hydrolysis by factors approaching 10^{17} and therefore are in a class of enzymes described as being some of the most proficient of known catalysts.[1] Primary amino acid sequences are used to classify glycosidases into one of 94 different families.[4] Structural characterization of representatives from a large number of these families has been achieved, revealing an extraordinary degree of diversity in overall fold, despite sharing several identical active site features. This would indicate a convergent evolution of mechanism.

Two stereochemical outcomes are possible for the hydrolysis of a glycosidic bond: the anomeric configuration of the product can either be retained or inverted with respect to the starting material. With the interesting exception of members of GH family 4,[5,6] the enzymes within a given family catalyse hydrolysis with the same stereo-selectivity. The mechanistic strategy employed by inverting glycosidases is that of a direct displacement S_N2-like reaction. A pair of carboxyl groups exist within the active site, typically separated by 7–11 Å, one acting as a general acid (A) protonating the glycosidic oxygen, the second acting as a general base (B) that activates the incoming water nucleophile facilitating a reaction that proceeds *via* an oxocarbenium ion-like transition state (Fig. 1a). The typical mechanism of retaining glycosidases is that of a double-displacement reaction involving a covalently bound glycosyl–enzyme intermediate.[3] Again a pair of carboxylates exist within the active site, in this case 5 Å apart, with one acting as a general acid/base catalyst (A/B) while the other acts as a nucleophile (Nuc), the reaction again proceeding *via* oxocarbenium ion-like transition states (Fig. 1b). Amongst the glycosidases, notable exceptions as to the nature of the catalytic nucleophile exist amongst certain hexosaminidases[7] (in which an *N*-acetamido group acts as an intramolecular nucleophile) and a recently characterized *trans*-sialidase[8] (in which a tyrosine plays the role). Evidence supporting an S_N2-like mechanism involving enzymatic nucleophilic catalysis comes from the observation of normal secondary α deuterium KIEs for *both* transition states (indicating rehybridization of C1 from sp^3 to sp^2),[9] as well as the observation of primary ^{13}C KIEs > 1.01 (indicating reaction coordinate motion contributions from both the nucleophile and the leaving group) consistent with S_N2 pathways.[10,11] Additionally, the covalent glycosyl–enzyme intermediates from representatives of multiple families have been isolated and characterized using mass spectrometry and X-ray crystallography. This includes the characterization of the intermediate of lysozyme, a result that contradicts the classical textbook mechanism involving an oxocarbenium ion intermediate.[12] This detailed mechanistic understanding has been exploited in successful engineering efforts that have converted retaining glycosidases into useful tools for the synthesis of oligosaccharides (glycosynthases) and thio-linked oligosaccharide analogues (thioglycoligases and thioglycosynthases).[13–15]

Transglycosidases are enzymes that share homologous structure, catalytic machinery and mechanistic strategies with various glycosidases and are therefore classified amongst the glycosidase families. However, instead of catalysing the hydrolysis of glycosidic linkages between sugars, they facilitate the transfer of the

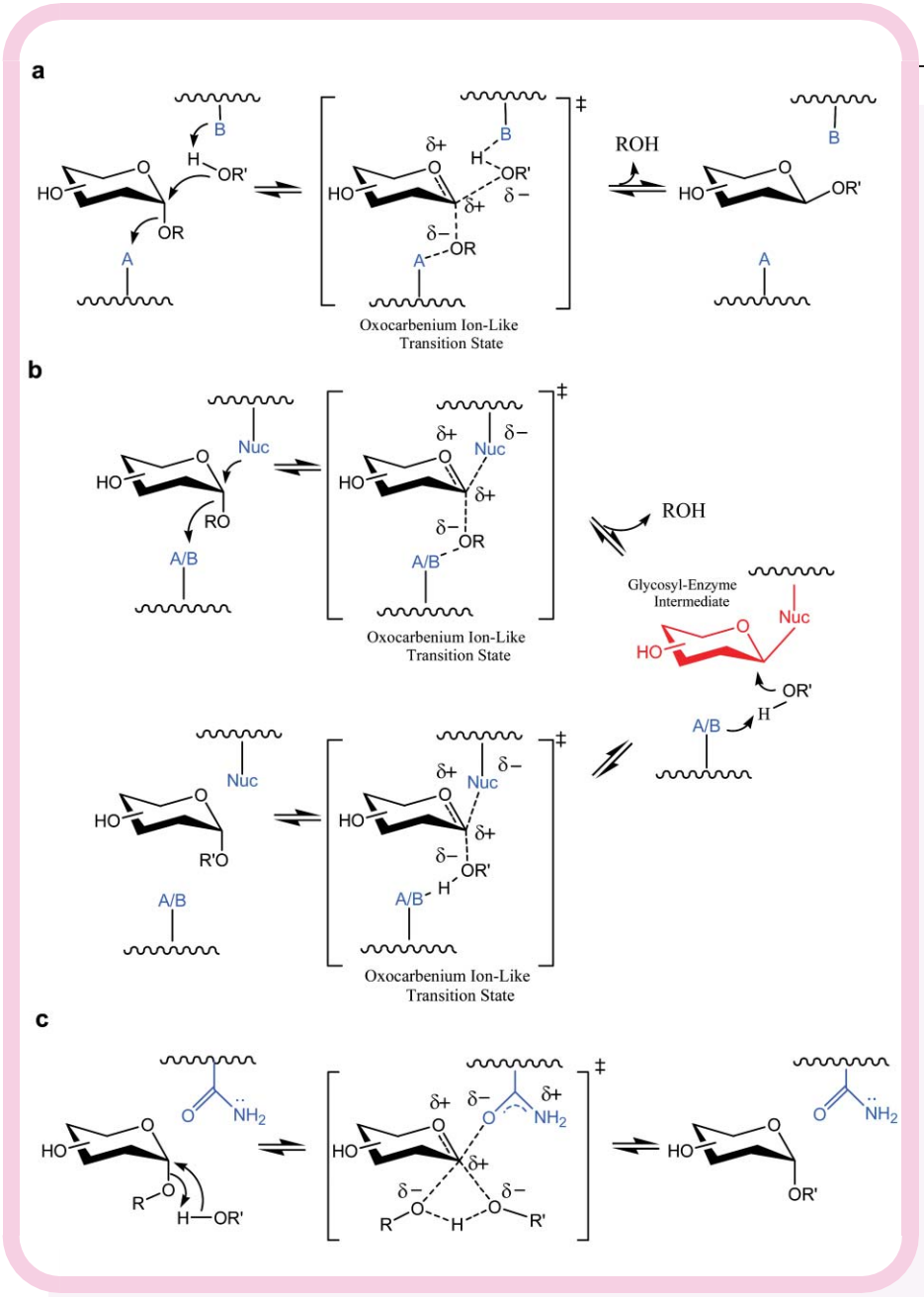

Fig. 1 Established and proposed mechanisms for glycosidases/transglycosidases and glycosyltransferases, respectively. For glycosidases, R = a carbohydrate derivative and R'OH = H_2O or phosphate (phosphorylases classified as glycosidases). For transglycosidases, R = a carbohydrate derivative and R'OH = another sugar. For glycosyltransferases, R = a nucleoside diphosphate (*e.g.* UDP, GDP), a lipid phosphate, or phosphate (phosphorylases classified as glycosyltransferases) and R'OH = an acceptor group (*e.g.* another sugar or a protein). (a) A direct displacement S_N2-like reaction results in inverted anomeric configuration *via* a single oxocarbenium ion-like transition state. (b) The double displacement mechanism proceeds *via* two oxocarbenium ion-like transition states with the intermediate formation of a discrete covalently bound glycosyl–enzyme species, resulting in overall retention of anomeric configuration. (c) S_Ni-like mechanism proposed for retaining glycosyltransferases and glycogen phosphorylase involving a direct front-side displacement resulting in retention of anomeric configuration.

glycone moiety to the hydroxyl of another sugar (transglycosylation) (Fig. 1b R'OH = a sugar). Well-characterised representatives of this group are the cyclodextrin glycosyltransferases (CGTases) from GH family 13. These enzymes use α-linked glucose polymers as substrates for the formation of cyclic oligoglucosides by first cleaving the polysaccharide chain 5–7 glucose residues from the non-reducing end, with formation of an oligoglucosyl–enzyme intermediate. The free, non-reducing 4-hydroxyl then attacks this intermediate to form the cyclic oligosaccharide. Again the active site of CGTase contains a suitably positioned pair of side chain carboxylates to catalyse the reaction *via* a double displacement mechanism.[16,17]

Glycosyltransferases

Glycosyltransferases catalyse the transfer of glycosyl moieties from activated donor sugars to an acceptor. The activating group of the donor is a nucleoside diphosphate (NDP) or monophosphate, phosphate, or a lipid phosphate and the acceptor is a hydroxyl group from another sugar, a lipid, a serine or threonine residue or the amide of an asparagine residue in a protein. As with the glycosidases, glycosyltransferases are classified as either retaining or inverting depending on the stereochemical outcome at the anomeric centre relative to that of the donor sugar and are also classified into sequence similarity-based families.[4]

A recent burst of reported glycosyltransferase structures has revealed an interesting difference from those of the glycosidases as only two general folds, called GT-A and GT-B, have been observed for all structures to date.[18,19] Further, threading analysis has revealed that the majority of uncharacterized families are predicted to adopt one of these two folds. This finding is probably the result of the structural constraints of a nucleotide-binding motif and may indicate that all transferases have evolved from a small number of progenitor sequences.

Catalysis by inverting glycosyltransferases is believed to parallel that of inverting hydrolases wherein a general base (B) deprotonates the incoming nucleophile of the acceptor facilitating direct S_N2 displacement of the nucleoside diphosphate (Fig. 1a). Indeed, structural and mechanistic studies clearly point to such a mechanism with the major difference being that a metal ion often plays the role of acid catalyst (A) in many transferases.[20,21]

Again by direct comparison to retaining glycosidases, the mechanism of retaining glycosyltransferases has been proposed to be that of a double displacement mechanism involving a covalently bound glycosyl–enzyme intermediate (Fig. 1b), demanding the existence of an appropriately positioned nucleophile (Nuc) within the active site.[2] If such a mechanism is followed, a divalent cation would presumably play the role of a Lewis acid, while the leaving diphosphate group itself probably plays the role of a general base activating the incoming acceptor hydroxyl group for nucleophilic attack. However, the mechanistic characterization of this class of enzymes has proven to be a challenging task. The conclusive identification of a catalytic nucleophile and observation of a covalent intermediate has yet to be reported for any retaining transferase despite exhaustive studies using techniques that have been successfully applied to the characterization of retaining glycosidases. Although this may be interpreted as evidence against the double displacement, it could also be the result of the inapplicability of these techniques to

the study of transferases due to inherent differences in the nature of the substrates being studied. The most successful approach used for the characterization of retaining glycosidases has involved the use of fluorinated substrate analogues.[22] The introduction of an electronegative fluorine at either the 2 or 5 position of a pyranose ring inductively destabilizes the oxocarbenium ion-like transition states through which both steps of the double displacement reaction proceed and in some cases also removes key hydrogen-bonding interactions, resulting in a significant decrease in the rate of the overall reaction. By introducing a good leaving group (*e.g.* dinitrophenol or fluoride), the first step is "rescued", resulting in the accumulation of the intermediate species with a significant lifetime that allows mass spectrometric and X-ray crystallographic characterization. However, because of the strict requirement of the glycosyltransferases for their NDP leaving group, the relative leaving group ability cannot be manipulated, thus the relative rates of the glycosylation and deglycosylation steps cannot be altered.[23] This has rendered the fluoro-sugar approach ineffective in the study of retaining transferases.[24]

To date the 3-dimensional structures of representatives from 6 families of retaining transferases have been determined crystallographically. The majority of these do not have intact donor substrates bound in the active site, limiting the identification of candidate nucleophiles to modelling attempts. Recent acquisitions of crystal structures with bound intact donor substrates were therefore anticipated with great excitement within the field. However, the resulting information has not provided great support for the double displacement mechanism. The structure of the *Neisseria meningitidis* α-galactosyltransferase LgtC with stable donor and acceptor substrate analogues bound revealed an active site in which the only functional group appropriately positioned to act as a nucleophile was that of the side chain amide of glutamine 189 (Fig. 2a).[25] Replacement of this residue with alanine, however, yielded an enzyme that retained sufficient activity (~3%) to shed considerable doubt on its role as a nucleophile.

In light of these structural and mutagenesis results, an alternative mechanism termed S_Ni-like was proposed.[25] This involves a single transition state in which attack by the incoming nucleophile of the acceptor and departure of the leaving group of the donor occur on the same face (Fig. 1c). The single exploded transition state for such a process is cyclic and late with highly developed oxocarbenium ion character. In such a mechanism, the enzyme acts as a scaffold that precisely orients substrates in close proximity, decreasing the energy of the transition state by stabilizing the oxocarbenium ion-like species and activating the leaving group. To prevent antibonding interactions and to allow for the development of density in the σ* orbital between the anomeric carbon and the leaving group of the donor, departure of the leaving group and front side attack must occur in an asynchronous fashion. Chemical precedent for this type of a mechanism comes from detailed kinetic and conformational studies of the solvolysis of glucose derivatives in mixtures of ethanol and trifluoroethanol by Sinnott and Jencks.[26] The S_Ni-like mechanism has since been proposed for other structurally defined retaining transferases based on the lack of appropriately positioned nucleophiles in their active sites,[27,28] and had previously been proposed for the structurally similar glycogen phosphorylase,[29] as discussed later.

However, the recent observation of a catalytically relevant steady state population of a glycosyl–enzyme intermediate on

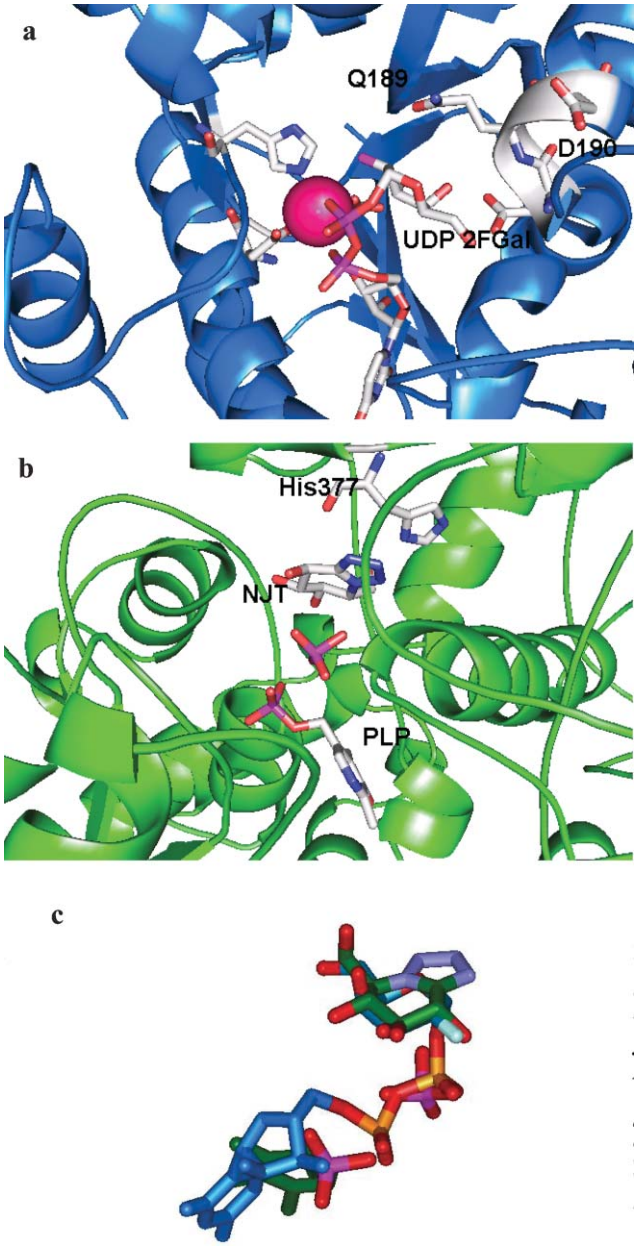

Fig. 2 Common structural features of retaining glycosyltransferases and retaining phosphorylases classified as glycosyltransferses. (a) UDP 2-deoxy-2-fluoro galactose bound to the active site of LgtC (pdb accession code 1GA8). (b) Pyridoxal phosphate, phosphate (PLP) and nojirimycin tetrazole (NJT) bound to the active site of glycogen phosphorylase (pdb accession code 1NOJ). (c) Overlay of the bound ligands from (a) and (b). The carbons of the nucleoside and galactose moieties are coloured blue and the phosphates orange for UDP 2-deoxy-2-fluoro galactose. The carbons of NJT and PLP are coloured green and the pyridoxal and inorganic phosphates are coloured magenta. Notice the near identical conformation of the phosphates tucked under the plane of the sugar rings.

Table 1 Classification and characteristics of carbohydrate-utilizing phosphorylase enzymes

Class	Family	Mechanism	Activities	3D Fold
GT[a]	4	Retaining	Trehalose phosphorylase	GT-B[c]
GT	35	Retaining	Glycogen and starch phosphorylases	GT-B
GH[b]	13	Retaining	Sucrose phosphorylase	$(\alpha/\beta)_8$
GH	65	Inverting	Maltose, kojibiose, and trehalose phosphorylases	$(\alpha/\alpha)_6$
GH	94	Inverting	Cellobiose, cellodextrin, and chitobiose phosphorylases	$(\alpha/\alpha)_6$[d]

[a] GT = glycosyltransferase. [b] GH = glycoside hydrolase (glycosidase). [c] Predicted from threading analysis. [d] Members of this family were classified as GT 36 until the recently determined structure of chitobiose phosphorylase from *Vibrio protelyticus* was reported[35] revealing an $(\alpha/\alpha)_6$ fold that has previously been observed amongst the hydrolases. This may either be interpreted as evidence supporting the notion of a mechanistic continuum between the two classes of enzymes or may simply be the result of inherent limitations of the classification system.

the Gln189Glu mutant of LgtC has rekindled the mechanistic debate surrounding this enzyme, which has served as a model system for the study of retaining glycosyltransferases.[30] It was fully expected that the site of labelling would be Glu189. However, surprisingly, the sugar was found attached to a sequentially adjacent residue within the active site, Asp190, implicating this residue as an alternative candidate catalytic nucleophile. The remote positioning ($\sim$9 Å) of the side chain carboxylate of this residue from the anomeric reaction centre in the available ground state crystal structure suggests that a significant conformational change would have to occur during catalysis if this residue were to act as the nucleophile in a double displacement mechanism. Evidence suggesting that the surprising site of labelling was not simply a structural artefact arising from the creation of an active site mutant comes from the crystallographic analysis of the Gln189Glu mutant with a bound donor substrate analogue, revealing no significant perturbation of active site conformation or mode of substrate binding compared to the wild type enzyme.[30] Additionally, mutation of Asp190 to alanine resulted in a 3000-fold decrease in observed catalytic activity. However, the evidence to date does not unequivocally support either the double displacement or the S_Ni-like mechanism and more experimental evidence is clearly needed before a lucid understanding of the mechanism of retaining glycosyltransferases will be obtained.

Phosphorylases

Depending on the direction of the reaction being catalysed, phosphorylase enzymes serve to either degrade or polymerize oligosaccharide substrates. The degradation process proceeds *via* phosphorolysis of a glycosidic linkage, while in the synthetic direction a sugar phosphate acts as the donor substrate. Two distinct groups of phosphorylase enzymes that act on carbohydrate substrates exist, one that displays significant structural and mechanistic relatedness to glycosidases and the other to glycosyltransferases. Indeed, the phosphorylases are characterized as belonging to several families of both classes (Table 1) and therefore provide a direct link between the previously described classes of enzymes. Also, while the mechanistic characteristics of members classified as glycosidases are fairly well established, great ambiguity remains for the retaining phosphorylases classified as glycosyltransferases. In fact, the isolation and characterization of the glucosyl–enzyme intermediate of a double displacement mechanism was described for the glycosidase-like sucrose phosphorylase (GH family 13) in an elegant and classic study over 30 years ago.[31] In marked contrast, despite exhaustive studies spanning multiple decades, the mechanism of the transferase-like glycogen phosphorylase (GT family 35) remains elusive.

Recent findings with retaining glycosyltransferases have revealed several interesting mechanistic and structural parallels with what has been seen for glycogen phosphorylase. In fact, the S_Ni-like mechanism was first proposed for glycogen phosphorylase, in response to the apparent lack of an appropriately positioned nucleophile.[29] Since then, on the basis of structures of enzyme–inhibitor complexes, a proposal has been made that the main chain amide of His377 could function in this role (Fig. 2b),[32,33] foreshadowing the identification of a similar role for the side chain amide of Gln189 in LgtC. Other parallels include the similarly unusual "tucked under" conformation of the phosphate moiety attached to, or attacking the sugar in each case (Fig. 2). Such a conformation could provide some ground state destabilization as well as assisting acid/base catalysis of glycosyl transfer. Indeed the very similar overall disposition of the UDP galactose moiety in LgtC to that of the pyridoxal phosphate (PLP) cofactor plus glucose-1-phosphate in glycogen phosphorylase (Fig. 2c) not only suggests mechanistic parallels but has also led to the proposal that the PLP dependence of glycogen phosphorylase is a vestige of the evolutionary origin of this enzyme as an NDP–sugar dependent glycosyltransferase.[34]

Summary

Chemical intuition may lead one to the assumption that selective pressure would force the evolution of mechanistic strategies amongst unrelated enzymes that obey the law of microscopic reversibility. This would require that a single parsimonious reaction coordinate for a given transformation should be converged upon by *unrelated* catalysts to enhance the rate of a given class of reaction in both directions. On the other hand it is also possible that, during the evolution of mechanism, local energy minima on the strategic landscape are sufficient to provide selective advantage and are thus perpetuated through time. Indeed, this would be the hope of protein engineers with goals of improving the catalytic efficiency of enzymes.

The study and comparison of the enzymes responsible for making and breaking glycosidic linkages provides insight into the nature of mechanistic evolution. A direct analogy between the straightforward mechanisms of inverting glycosidases and glycosyl transferases is apparent. However, when it comes to a comparison between the mechanisms of retaining enzymes, while recent insights may serve to provide a key, it has not yet been turned to unlock the door and a distinct relation remains unclear.

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