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Glycosynthesis in a Waterworld: new insight into the molecular basis of transglycosylation in retaining glycoside hydrolases

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Abbreviations: AS, amylosucrases; CAZyme, carbohydrate-active enzyme; CGTase, cyclodextrin glucanotransferase; EG, *endo*-glucanase; ENGase, *endo*-β-*N*-acetylglucosaminidase; FS, fructansucrase; FT, fructosyltransferase; GH, glycoside hydrolase; GP, glycosyl phosphorylase; GS, glucansucrase; GT, glycosyltransferase; KIE, kinetic isotope effect; LG, leaving group; QM/MM, quantum mechanics/molecular mechanics; SA, sialidase; SUH, sucrose hydrolase; TG, transglycosylase; *tr*S, *trans*-sialidase; TS, transition state; TS1, glycosylation step-associated transition state; TS2, deglycosylation step-associated transition state; TST, transition state theory; VI, vacuolar invertase; XEH, xyloglucan *endo*-hydrolase; XET, xyloglucan *endo*-transglycosylase; T/H, transglycosylation/hydrolysis ratio.

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Running title: Glycosynthesis in a Waterworld

Abstract

Carbohydrates are ubiquitous in Nature and play vital roles in many biological systems. Therefore, the synthesis of carbohydrate-based compounds is of considerable interest both for research and commercial purposes. However, carbohydrates are challenging, due to the large number of sugar subunits and the multiple ways in which these can be linked together. Therefore, to tackle the challenge of glycosynthesis, chemists are increasingly turning their attention towards enzymes, which are exquisitely adapted to the intricacy of these biomolecules.

In Nature, glycosidic linkages are mainly synthesized by Leloir glycosyltransferases, but can result from the action of non-Leloir transglycosylases or phosphorylases. Advantageously for chemists, non-Leloir transglycosylases are glycoside hydrolases, enzymes that are readily available and exhibit a wide-range of substrate specificities. Nevertheless, non-Leloir transglycosylases are unusual glycoside hydrolases in as much that they efficiently catalyze the formation of glycosidic bonds, while most glycoside hydrolases favor the mechanistically-related hydrolysis reaction. Unfortunately, because non-Leloir transglycosylases are almost indistinguishable from their hydrolytic counterparts, it is unclear how these enzymes overcome the ubiquity of water, thus avoiding the hydrolytic reaction. Without this knowledge, it is impossible to rationally design non-Leloir transglycosylases using the vast diversity of glycoside hydrolases as protein templates.

In this critical review, a careful analysis of literature data describing non-Leloir transglycosylases and their relationship to glycoside hydrolase counterparts is used to clarify the state of the art knowledge and to establish a new rational basis for the engineering of glycoside hydrolases.

Key words: glycoside hydrolase, transglycosylation, evolution, structure/function, transition state theory

INTRODUCTION

Carbohydrates are ubiquitous in biological systems, being involved in a plethora of life-sustaining or threatening molecular events [1]. Therefore, the *in vitro* synthesis of well-defined complex carbohydrate-based compounds is of considerable importance, both for fundamental research in glycosciences and for the preparation of commercially-valuable products. In this regard, the synthesis of glycosidic bonds by carbohydrate-active enzymes (CAZymes) (i.e. transglycosylation) has been studied for over 60 years [2], being as old as the study of the mechanistically-related hydrolytic reaction. This is because the advantages of enzyme-catalyzed transglycosylation, particularly stereo- and regio-selectivity, have long been recognized by glycochemists, who have increasingly adopted them in order to simplify complex reactions that are usually conducted using more classical organic chemistry methods.

Enzymes available to the synthetic glycochemist

In Nature, the synthesis of glycosidic bonds is mainly performed by glycosyltransferases (GTs), thus it would be quite logical for these to be widely exploited by glycochemists [3,4]. However, this is not the case because these enzymes require nucleotide sugars as donor substrates, which are still not readily available despite recent progress [5,6]. Moreover, experience shows that the heterologous production of GTs is often difficult to achieve, thus limiting the availability of these enzymes. Other CAZymes that are frequently used for glycosynthesis are glycoside hydrolases (GHs), which are more abundant than GTs and cover an extremely wide range of substrate specificities. Nevertheless, although so-called retaining GHs possess inherent ability to catalyze the formation of glycosidic bonds, this mechanistic outcome is usually subordinate to hydrolysis. Therefore, the use of GHs for glycosynthesis often depends on the ability of the glycochemist to suppress the latter activity, for example by acting on the thermodynamic equilibrium of the reaction (e.g. using co-solvents and reducing water activity), thus forcing transglycosylation against hydrolysis [2,7]. However, such techniques are not always easy to implement and the results are often disappointing (e.g. poor selectivity and multiple glycosylations). For this reason, the fundamental basis of the hydrolysis/transglycosylation (H/T) partition in GH-catalyzed reactions has been the subject of much study, and strategies to engineer glycosynthetic enzymes have been developed. Progress in this field is exemplified by the 'glycosynthase concept', first proposed in 1998 [8,9]. This ingenious technique, which has been extensively reviewed elsewhere [10–14], has so far been applied to GHs from a dozen or so different GH families and has benefited from much developmental work. Following the seminal work of Withers et al., a series of review articles dealing with enzyme-catalyzed transglycosylation have either focused on the enzymes [15], on the products [16,17] or on the

catalytic mechanisms involved [18–20].

Transglycosylases – exceptions to the rule

Over the last 15 years, the number of CAZyme-encoding sequences in the CAZy database (www.cazy.org and www.cazypedia.org) has dramatically increased [21–23], reaching more than 210,000 GH modules, assigned to 133 different GH families (14 clans). Among the characterized GHs present in this database, only a few have been described as transglycosylases (TGs), meaning enzymes that mainly (often exclusively) catalyze transglycosylation, even in dilute conditions and aqueous media. Intriguingly, TGs are highly related to hydrolytic GH counterparts, with any single TG being more related to the other members of its GH family, than to TGs from other families. This fact underlines the tight evolutionary relationship between TGs and GHs and implies that transglycosylation in GHs is favored by subtle molecular adjustments rather than major modifications, such as significant structural changes.

A large number of studies have focused on the identification of the molecular determinants that govern acceptor selectivity (i.e. water *vs* sugar moieties) and thus the H/T partition in related GH/TG pairs. Nevertheless, despite some interesting findings the conclusions of these studies fall short of expectations [24,25], since they fail to reveal information of a more generic nature pertaining to the way in which the H/T partition is modulated in GHs. This is unfortunate because the acquisition of such knowledge will allow protein engineers to exploit the vast biodiversity of GHs, conferring efficient glycosynthetic capability to any single GH. In turn, this knowledge gap is preventing wider deployment of TGs in synthetic glycochemistry, an exciting prospect that would revolutionize this field, providing access to hitherto inaccessible sugar structures.

In this review, we invite the reader to revisit the considerable knowledge that has been acquired in recent years, in particular the results pertaining to GH/TG pairs, but also to glycosynthases and pseudo-TGs obtained using protein engineering techniques. The ultimate aim of this review is to discuss this data in terms of the H/T partition and thus provide a much clearer theoretical framework for future work.

TRANSGLYCOSYLATION IN GLYCOSIDE HYDROLASES

A mechanistic description of hydrolysis and transglycosylation in GHs

In 1953, Daniel E. Koshland provided the mechanistic framework to describe how GHs cleave glycosidic linkages via one of two main mechanisms, involving either retention or inversion of the anomeric configuration (from substrate and product) [26]. Regarding retaining GHs, which represent

approximately 60% of GH families, catalysis occurs in two main steps called ‘glycosylation’ and ‘deglycosylation’. Glycosylation begins with the formation of the Michaelis-Menten complex (E.S) and continues up to the formation of the covalent glycosyl-enzyme intermediate (or equivalent oxazolinium ion intermediate), coupled to the release of a leaving group (Figure 1 and Box 1). Deglycosylation involves an acceptor molecule and gives rise to one of two outcomes depending on the nature of the acceptor (Figure 1). If water is the acceptor hydrolysis occurs, whereas the presence of a suitable sugar acceptor will allow transglycosylation to proceed. As mentioned earlier, some retaining GHs are strict TGs, but most are hydrolases that perform hydrolysis and transglycosylation in parallel and at a level defined by the ratio H/T.

Box 1 On the meaning of catalytic constants for retaining GHs

Because k_{cat}, K_M and k_{cat}/K_M values are generally determined to compare wild-type and mutant enzymes, it is pertinent to recall some of the key features of these values [27]. Importantly, in most circumstances the Henri-Michaelis-Menten constant K_M (1913) cannot be equated to the affinity constant ($1/K_d$) [28], especially when considering mutated GHs that display highly modified catalytic capabilities. Indeed, when K_M is rewritten as $[k_3.(k_{-1} + k_2)]/[k_1.(k_2 + k_3)]$ it becomes clear that this constant includes terms that refer to both glycosylation and deglycosylation, whereas the catalytic performance constant $k_{cat}/K_M = k_1.k_2/(k_{-1} + k_2)$ only describes the glycosylation step (enzyme-substrate association and glycosidic bond cleavage) and is thus independent of rate-limiting step considerations (Figure 1). Therefore, while the constant k_{cat}/K_M can be considered as a reliable value to evaluate the impact of a mutation on the glycosylation step, the K_M value should be used with caution. Finally, rewriting the catalytic constant, $k_{cat} = k_2.k_3/(k_2 + k_3)$ reveals that when donors bearing a good leaving group (i.e. usually $pK_a^{LG} < 8.0$) are employed, k_{cat} is approximated by k_3, since the deglycosylation step becomes rate-limiting (i.e. $k_3 << k_2$), a situation that is assumed to be true for most GHs. Therefore, if a suitable donor is used, the measurement of the k_{cat} value provides information about the extent to which mutations affect the deglycosylation step, for example by improving acceptor binding, lowering the TS2 energy barrier or improving product diffusion out of the active site.

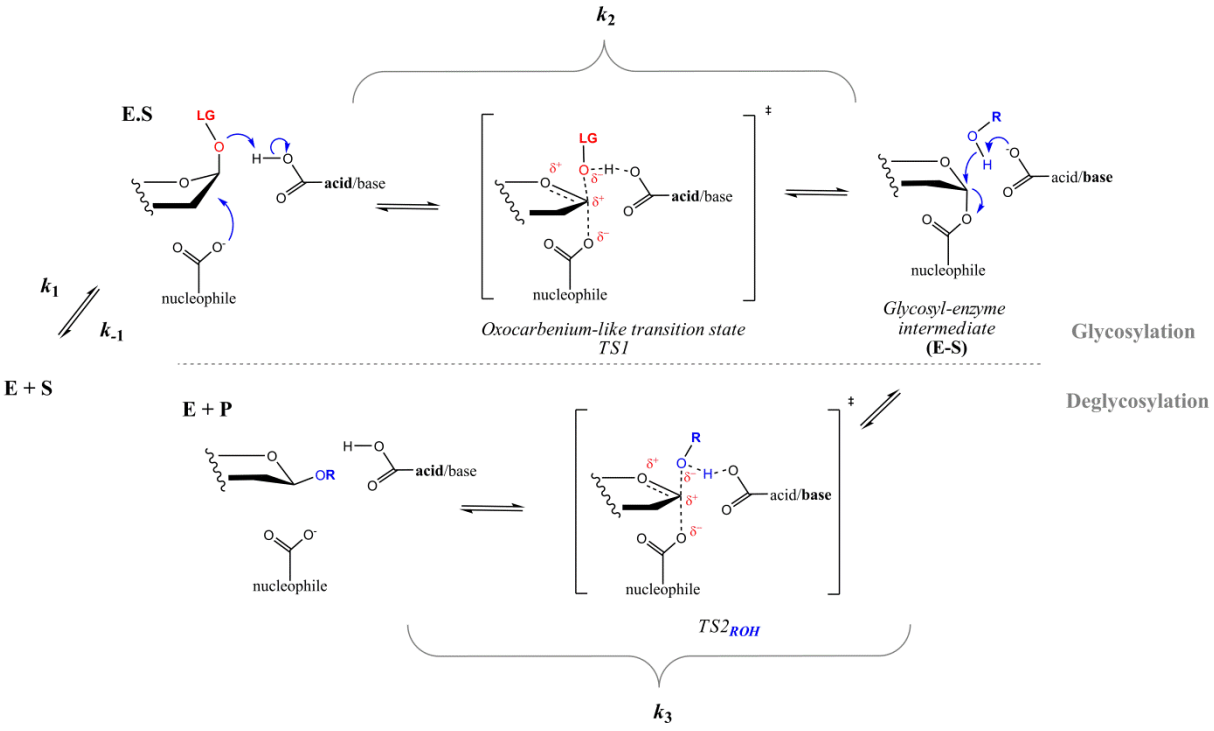

Figure 1 Two-step displacement mechanism of retaining GHs. The donor leaving group (LG) can be either an activated moiety (e.g. *p*NP) or a sugar (e.g. fructose for glucansucrases). Regarding

deglycosylation, the covalent glycosyl-enzyme intermediate can be either attacked by a water molecule (hydrolysis, R = H) or an external acceptor (transglycosylation, R = sugar, alkyl chain, etc.). In the case of secondary hydrolysis the transglycosylation product becomes a donor substrate with a subsequent deglycosylation step involving water as an acceptor.

From a simple lock to a locked door model

As the mechanistic description above indicates, the actual functioning of GHs is far more complex than that illustrated in 1894 by Emil Fisher's original 'lock and key' model [29]. Indeed, as Koshland pointed out, this early model is limited in several ways, but in particular because it omits the role of water and enzyme flexibility [30]. In the case of GHs, since the mechanism involves both donor and acceptor molecules (which can be water), we would like to extend the lock and key analogy, adding a door handle whose action is linked to the open/close state of the lock. Looking first at the model, one can describe a system in which the door opening process occurs in two steps: unlocking and then handle movement. The first step is achieved using a key and the second step is performed by simply exerting downward pressure on the handle. The looser the door mechanism the easier it is to open the door, even for the weakest of grips, making this type of door locking system accessible to all comers. In GHs the lock is the negative subsite and the key is the donor molecule (Box 2 and Figure 2). The lock is open when a catalytic intermediate is formed and the door handle is actioned by an acceptor or a water molecule, which is followed by product release. A highly efficient GH can be likened to a loose door mechanism that is easy to open and accessible to all-comers. The most frequent door-opener is water, which is ubiquitous (55 M). On the contrary, a stiff door requires a firm grip both to turn the key and exert pressure on the door handle. This type of door can only be opened by a stronger minority. In enzyme catalysis terms, this minority corresponds to acceptor molecules that specifically interact with the enzyme, and the stiffness of the door opening system is determined by how well transition state (TS) interactions are developed during catalysis, with hydrolysis being associated with efficient catalysis and thus highly developed TS interactions.

Box 2 Simplified view of the GHs' active sites

Endo-GHs cleave internal glycosidic bonds (Figure 2A), while *exo*-GHs remove terminal glycosyl moieties, acting generally (but not exclusively) on non-reducing sugars (Figure 2B).

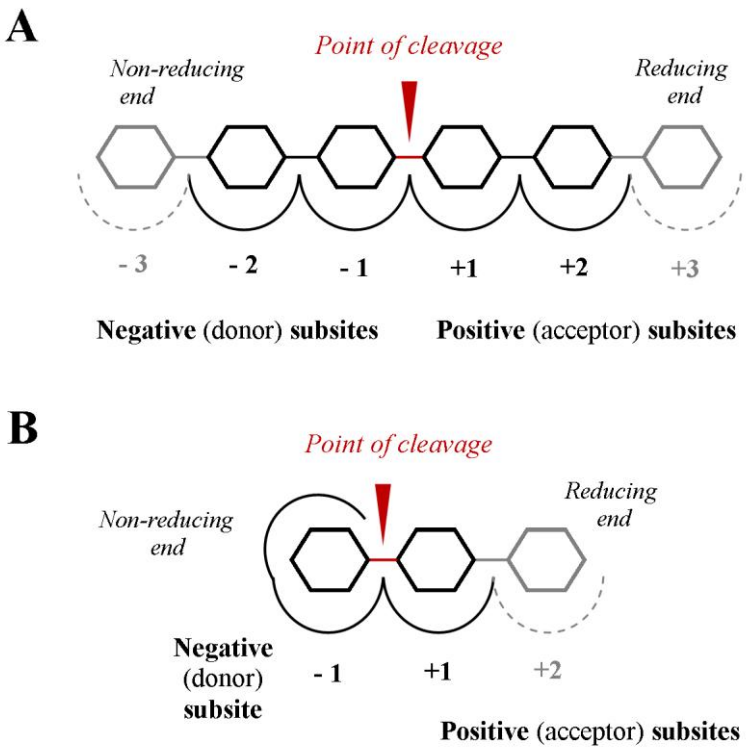

Figure 2 Classification of GHs and nomenclature for sugar-binding subsites. Following the nomenclature proposed by Davies et al. [31], subsites in GHs can be numerbered. Accordingly, subsites located on the non-reducing side of the cleavage point (red triangle) are denoted by negative numbers (i.e. -1, -2, -3, etc.), while those at the reducing side are positively denoted (i.e. +1, +2, +3, etc.). Positive and negative subsites are often designated donor and acceptor subsites respectively, terms that take into account substrate binding over the reaction pathway. However, this nomenclature is ambiguous if one considers that at the beginning of a reaction the donor substrate occupies both negative and positive subsites.

Transition states in glycoside hydrolases and H/T balance

TS: the power of GHs

Glycosidic bonds are extremely stable and display half-lives of several million years. This can be illustrated by the fact that papyruses from ancient Egypt can still be seen in our museums today. However, in the presence of GHs the half-life of glucosidic bonds in cellulose are reduced to the millisecond range [32]. This incredible catalytic potency of GHs and enzymes in general was first rationalized by Linus Pauling in 1946 [33], who proposed that the formation of TS is directly responsible for reaction rate enhancements, which in the case of GHs can be 10^{17}-fold higher than those of uncatalyzed reactions [34].

Retaining GH-catalyzed reactions are characterized by two TS, the first one (TS1) preceding the formation of the glycosyl-enzyme intermediate and the second one (TS2) characterizing disruption of this covalent intermediate and preceding formation of the reaction products (Figures 1 and 3). When compared to the enzyme-free reaction, the enthalpy of activation (ΔH) is significantly lowered and the degree to which it is decreased correlates with the catalytic efficiency of the enzyme (Box 3) [34].

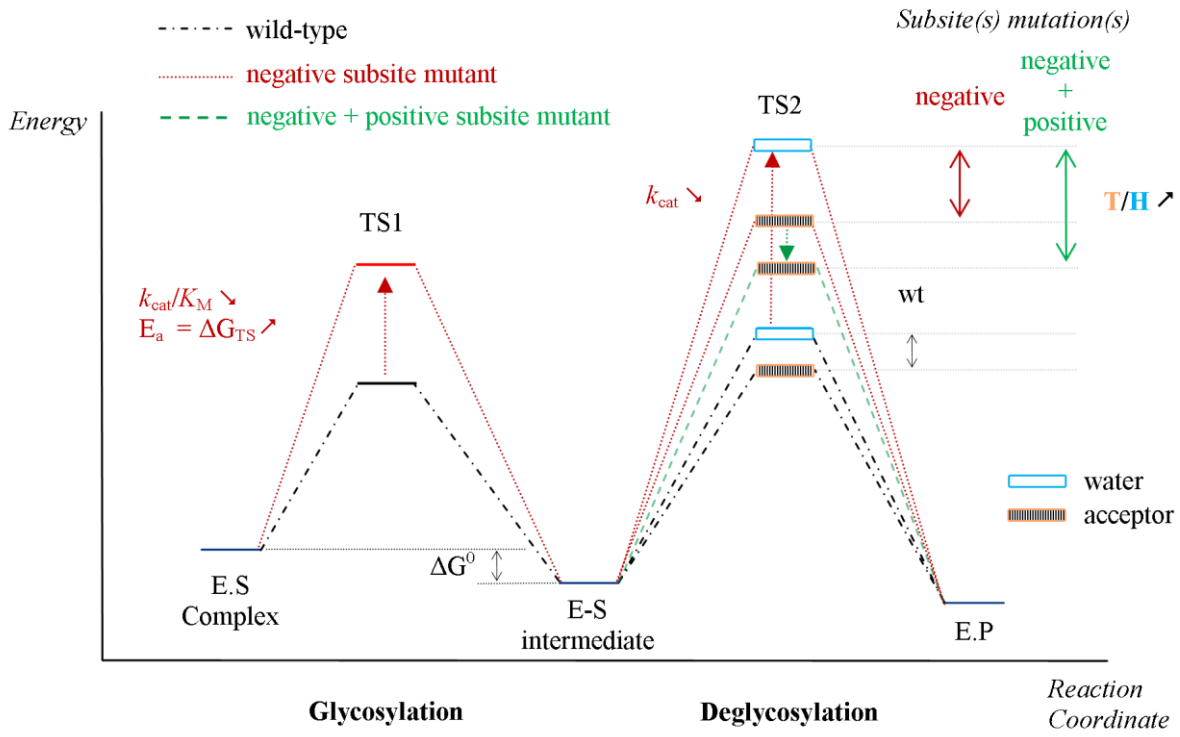

Figure 3 Energy diagram of the two-step displacement mechanism of retaining GHs (black dash-dot) and alternative energetic pathways for evolved transglycosylases (red dot or green dash for negative and negative + positive subsite mutants, respectively). Logically, water-mediated (blue open rectangle) TS2 destabilization coupled to acceptor-mediated (orange dashed rectangle) stabilization will increase the T/H partition. Similarly, increasing the E-S intermediate energy should also favour acceptor-mediated deglycosylation. Since these phenomena are not expected to be mutually exclusive, it is possible that the combination of them will explain the exceptional behavior of TGs.

Box 3 Basics of enzyme thermodynamics

The Arrhenius equation (1889) provides a link between kinetics and thermodynamics, since the rate constant (*k*) can be expressed as a function of the energy of activation (E_a) and temperature (T), where R is the universal gas constant (Equation 1). Similarly, transition state theory, and in particular the Eyring-Polanyi [35] equation (Equation 2), relates the rate to temperature and thermodynamic parameters, such as the Boltzmann (k_B) and Planck (*h*) constants, and the free energy variable (ΔG_{TS}). ΔG_{TS} includes the activation enthalpy (ΔH) and entropy (ΔS) ($\Delta G = \Delta H - T.\Delta S$) and denotes the free energy differences between the ground state (E.S) and TS. When performing site-directed mutagenesis on an enzyme, if the apparent free energy associated with glycosylation is altered for the mutant enzyme relative to the wild-type enzyme, TS can be deduced using these relationships (Equation 3).

$$\boldsymbol{k} = -d[Substrate]/dt = A.\exp(-E_a/RT) \quad \text{(Eq. 1)}$$

$$\boldsymbol{k} = (k_B.T/h).\exp(-\Delta G_{TS}/RT) \quad \text{(Eq. 2)}$$

$$\mathbf{\Delta E_a} = \Delta(\Delta G_{TS}) = -RT.\ln([k_{cat}/K_M]_{mut}/[k_{cat}/K_M]_{wt}) \quad \text{(Eq. 3)}$$

Therefore, these equations provide an evaluation of the impact of mutations or substrate modifications on the global catalytic efficiency with respect to TS destabilization.

The driving force behind enzyme TS is local energy expenditure, which is the price of TS stabilization. This energy is in turn derived from the ability of enzymes to form quite intricate interactions with the substrate donor moieties [36]. Accordingly, tight donor recognition is the result of efficient electron sharing and the formation of strong, low-barrier hydrogen bonds (< 2.5 Å), two factors that are synonymous with efficient enzyme catalysis.

When discussing enzyme catalysis it is also relevant to mention enzyme dynamics because these constitute a key feature of the process [37,38]. Indeed, attempts to investigate catalytic phenomena, such as the modulation of H/T using methods like X-ray crystallography, have often failed to provide any useful information due to the omission of dynamics. Nevertheless, the role of dynamics in TS formation is less clear [39], although it is plausible that they contribute to TS properties.

On TS properties

In the case of β-glycopyranosidases, the structures adopted by TS along the reaction pathway have been comprehensively described by Davies et al. [40], whereas nothing is yet known about the TS developed by furanosidases. For these latter, the only relevant information available is that of the quite extensive work performed by the Lowary group on furanose conformations [41,42]. On the basis of this current knowledge, it is clear that the TS along reaction pathways display coplanar geometry between C5, O5, C1 and C2 in pyranoses (or C4, O4, C1 and C2 in furanoses), which infers the formation of an oxocarbenium ion-like state (sp^2-hybridization) [43]. In this case, the anomeric carbon is subject to electrophilic migration (Figure 1) towards the nucleophile catalyst [44,45]. To favor orbital overlap between the electron lone pair of the endocyclic oxygen and C1 (necessary for cationic character establishment) the sugar undergoes ring distortion, moving away from the lowest energy chair conformation [46], as illustrated by structural [47,48] and computational [49] analyses. Recently, *in silico* approaches have been employed to demonstrate that maximum charge development and TS coordinate points do not necessarily occur at the same time point [50].

Regarding the energetic properties of TS, the contribution of the 2-hydroxyl group is a well-known feature of retaining β-glycosidases (5-10 $kcal.mol^{-1}$, compared to < 2 $kcal.mol^{-1}$ for other hydroxyl groups) [18,51,52]. This is because in β-glycosidases the 2-hydroxyl group hydrogen bonds to the catalytic nucleophile, thus favoring a greater share of positive charge and directly affecting oxocarbenium cation formation [53], though to different extents depending on the GH family [19]. In the case of retaining α-glycosidases and α-glycosyltransferases [54], this contribution plays a lesser role (5.2 and 1.9 $kcal.mol^{-1}$), probably because of different electronic patterns within the trio

constituted by the nucleophile's carboxylic acid function, the endocyclic oxygen and the anomeric carbon of the sugar moiety [18]. In retaining β-glycosidases, the nucleophile carboxyl oxygen establishes a *syn* interaction with the 2-hydroxyl group and the anomeric center, whereas in retaining α-glycosidases the equivalent *syn* interaction involves the endocyclic oxygen and the anomeric carbon center. A direct consequence of this in retaining β- or α-glycosidases is a greater share of positive charge localized either on the anomeric center or on the endocyclic oxygen respectively. Taking this difference into account, when considering TS electronic patterns it is plausible that this feature could be a key determinant of the principal activity displayed by any given glycosidase. Indeed, it is noteworthy that many 'true' non-Leloir TGs are α-retaining enzymes (e.g. glucansucrases, CGTases), which form a β-linked covalent intermediate that displays inherently greater reactivity compared to its α-counterpart [55]. Furthermore, α-retaining GHs are all equipped with *anti*-protonators, which means that unlike *syn*-protonators the interaction of the acid/base catalyst with the lone pair of the endocyclic oxygen is impossible [56,57]. In principle, the absence of this interaction is detrimental for TS stabilization, although some GHs display compensatory interactions (e.g. provided by conserved tyrosines in some β-retaining glycosidases) [57,58]. In other work, it has been shown that the presence of a hydrophobic platform within the subsite -1, present in almost all GHs (α or β, retaining or inverting), might play a critical role in TS stabilization [58]. Therefore, for any given GH the study of the impact of charge distribution at TS and the anomery of the glycosyl-enzyme intermediate on the selectivity between water and sugar acceptors should be a useful source of information on the enzyme's H/T partition.

From a temporal point of view, TS are highly transient displaying lifetimes estimated to be within a single bond vibration timescale (i.e. approximately 10 fs, or 10^{-15} s) [59], far lower than the global k_{cat}, which occurs on a millisecond timescale in most GHs. Regarding water molecules, their diffusion occurs over approximately 1 ps (10^{-12} s) and does not constitute a rate-limiting step, unlike bond breaking and formation that are much more critical (see below).

Differences between TS1 and TS2

From a practical point of view the kinetic isotope effect (KIE), also called 'isotope fractionation' [60], has so far proved to be the only experimental approach that can provide details about TS formation and properties (i.e. geometry and electronic environment) [59,61]. Using this technique it has been shown that in the reaction catalyzed by *Agrobacterium sp.* β-glucosidase the oxocarbenium ion character is stronger for the deglycosylation TS than for that of the glycosylation step [62], despite the fact that the TS1 and TS2 in retaining GHs are usually considered to share very similar features. Nevertheless, it is clear that the study of TS, in particular the activation barrier of TS2, is hampered

by the lack of experimental approaches that can provide sound data.

Recently, Quantum Mechanics/Molecular Mechanics (QM/MM) approaches have been developed to provide insight into the properties of TS and the extent of bond breaking at each individual step [63,64]. Accordingly, based on the findings of QM/MM it has been postulated that TS2 is more dissociative, meaning that the (C1-nucleophile) bond is almost broken before the nascent C1-OR bond (with OR from acceptor HOR, with R= H for water) is formed [65]. This provides interesting insight into the enzyme-catalyzed chemistry of the second reaction step and is consistent with the fact that water-mediated deglycosylation is rate-limiting. Moreover, QM/MM has revealed that conserved, non-catalytic active site residues, which are involved in hydrogen bonding with the sugar moiety, contribute to TS stabilization to different extents, this being dependent on the exact position of the hydroxyl moiety and the reaction step under consideration [66]. This is consistent with previous experimental findings that reported on the different contributions of the sugar hydroxyl groups [52]. Despite these encouraging results, QM and MM are still in their infancy and thus findings need to be more extensively corroborated by experimental data.

Unfortunately, in the case of retaining GHs, the study of TS is always limited to those developed during hydrolytic reactions, despite the fact that other reagents, such as hydroxylated molecules, can act as acceptors for the deglycosylation step (i.e. transglycosylation). Therefore, in the quest to elucidate the determinants of H/T modulation it is rather evident that water- and carbohydrate-mediated deglycosylation involve different behaviours. Although diffusion issues should be considered as important, thermodynamics are at heart of the enzyme-catalyzed chemical reaction and are probably much more critical, as underlined by *in silico* approaches. Therefore, the key questions regarding the H/T partition appear to concern the properties of the deglycosylation transition state ($TS2_{ROH}$) and the impact thereupon of the nature of the reacting acceptor substrate (ROH).

NATURALLY-OCCURRING TRANSGLYCOSYLASES: ELUCIDATING NATURE'S DESIGN STRATEGY

In the following section, naturally-occurring TGs are defined as retaining GHs that display a dominant or exclusive ability to transfer glycosyl units onto acceptor sugars (e.g. xyloglucan *endo*-transglycosylases or XET, sucrase-type enzymes, cyclodextrin glucanotransferases or CGTases and *trans*-sialidases or *tr*S). For practical reasons, in the specific case of TGs the partition between hydrolysis and transglycosylation is described by the ratio T/H rather than the more usual H/T ratio. Moreover, herein we only discuss enzymes for which there is a sufficient amount of knowledge concerning structure-function relationships.

In guise of a general introduction to this section, the reader is referred to Table 1 that underlines the fact that sugar-transferring enzymes are usually catalytically-less efficient (e.g. k_{cat}/K_M values) than hydrolytic counterpart enzymes (85- to 1165-fold lower for GH1 β-glycosidase and GH13 sucrose-acting enzymes, respectively). This catalytic sluggishness is likely to be correlated with more energy-demanding TS (for both glycosylation and deglycosylation steps), which lower overall catalytic turnover. In this respect, it is also useful to recall that in a previous study that set out to correlate enzyme and substrate flexibility with catalytic performance, it was proposed that the enzymes we observe today are the result of evolutionary processes that have transformed intrinsically slow, broad specificity prototypes into more efficient catalysts [67]. Of course, this is a rather simplistic view of enzyme evolution and other data suggest that enzymes might have evolved in both directions [68,69], and indeed some GHs (mainly from plants) display both hydrolysis and transglycosylation activities and thus present intermediate cases (i.e. mixed activity) [70–75].

1 **Table 1 Comparison of catalytic constants between glycosynthetic and hydrolytic natural GHs**

GH family	Enzyme	Substrate	k_{cat}[a] (s^{-1})	K_M[a] (mM)	k_{cat}/K_M[a] ($s^{-1}.mM^{-1}$)	Reference
1	Rice *Os*BGlu31 (*exo*)	Ferulic acid (acceptor)[b]	1.21	0.05	25.42	[70]
		*p*NP-β-D-Glc*p* (donor)[b,c]	1.21	9.33	0.13	
	Agrobacterium β-glucosidase (*exo*)	*p*NP-β-D-Glc*p*	169	0.078	2170	[62]
13	*Bc* strain 251 CGTase (endo)	β-cyclization	329	-	-	[76]
		hydrolysis	3.9	-	-	
	Barley α-amylase (endo)	Blue starch	248	0.52 ($mg.mL^{-1}$)	477 ($s^{-1}.mL.mg^{-1}$)	[77]
		CNP-β-D-maltoheptaoside[c]	122	1.1	111	
	*Np*AS (*exo*)	Sucrose (< 20 mM)[d]	0.55	1.9	3.45	[78]
		Sucrose (> 20 mM)	1.28	50.2	0.0255	
	*Xag*SUH (exo)	Sucrose	66.5	2.24	29.7	[79]
16	*Ptt*XET16-34 (endo)	XGO_{Glc8} (transglycosylation)[e]	0.08	0.4	0.2	[80]
	*Tm*NXG1 (XEH)	XGO_{Glc8} (hydrolysis)[e]	0.071	0.08	0.85	
		XGO_{Glc8} (transglycosylation)[e]	0.015	0.5	0.028	
32	Wheat FT (1-SST) (*exo*)	Sucrose (1-kestose production)	0.78	551	-	[81]
	Wheat VI (*exo*)	Sucrose (hydrolysis)	608	15	-	
33	*Tc*trS (*exo*)	Sialyllactose (tranglycosylation)	12.6	1.2	10.5	[82]
	*Tc*trS (*exo*)	Sialyllactose (hydrolyse)	0.18	0.29	0.62	[83]
	*Tr*SA (*exo*)		151.4	0.27	554.7	

[a] Determined in the optimal operating conditions for each enzyme. Specific activity is provided when the k_{cat} value is unavailable.

[b] Kinetic parameters were determined either for the acceptor (with 30 mM donor) or for the donor (with 0.25 mM acceptor)

[c] *p*NP, 4-nitrophenyl; and CNP, 2-chloro-4-nitrophenyl.

[d] For low sucrose concentration (< 20 mM), hydrolysis is dominant.

[e] Xyloglucan-oligosaccharides mixture composed of XXXG, XLXG, XXLG, and XLLG moieties (using the nomenclature developed by Fry et al. [84]) and based on $(D\text{-Glc}p)_8$ backbone.

2

Xyloglucan *endo*-transglycosylases

In terms of understanding the determinants of the T/H partition, XETs and their hydrolytic counterparts, xyloglucan *endo*-hydrolases (XEH), are extremely interesting enzymes that are usually referred to as xyloglucan *endo*-transglycosylase/hydrolases or XTHs, even though biochemical evidence reveals that most of them are XETs, displaying very little hydrolytic ability. XTHs are mainly grouped in family GH16 (members of GH-B clan), which also contains other hydrolytic GHs enzymes that display a wide variety of substrate specificities [85,86]. The molecular phylogeny of XTH genes, their catalytic properties and *in vivo* functional differences provide criteria for the definition of three major groups. Members of groups I and II exclusively exhibit XET activity, which is also the predominant feature of group III-B. However, members of group III-A (XEH) are mainly hydrolytic [80,87–89]. With regard to XETs, these are known to be important for plant cell wall remodelling, since they catalyze the non-hydrolytic cleavage and religation of xyloglucan molecules through a ping-pong bi-bi mechanism that is subject to competitive inhibition, since competing substrates can act as both the donor and the acceptor [90,91].

As explained earlier, the canonical double-displacement mechanism of glycosyl transfer involves the formation of a covalent glycosyl-enzyme intermediate. In XTHs, glycosylation is rapid (< 2 min) and procures a relatively long-lived glycosyl-enzyme intermediate, whose formation is associated with an estimated free energy change (ΔG^0, Figure 3) of formation of approximately 1.5-2.0 kcal.mol^{-1} [92–94]. Indeed, the glycosyl-enzyme intermediate of *Ptt*XET16-34 (a XET from hybrid poplar *Populus tremula x tremuloides*) is approximately 3 h with a $k_{hydr.}$ = 1.10^{-4} s^{-1}. Deglycosylation of the glycosyl-*Ptt*XET16-34 intermediate is brought about by the presence of suitable sugar acceptors, such as xylogluco-oligosaccharides. When this criterion is fulfilled, it has been shown that the xylogluco-oligosaccharyl-XET adduct can be fully deglycosylated in 30 min. In this respect, it is also noteworthy that when *Ptt*XET16-34 was supplied with activated β-D-xyloglucan-oligosaccharidic donors (e.g. LG = 2-chloro-4-nitrophenyl or fluoride), no activity (neither transglycosylation nor hydrolysis) was observed [95,96]. This underlines the fact that the energetic barrier of TS1 can only be overcome by the presence of a sugar LG in the positive subsites, as is the case for deglycosylation (TS2). This requirement is removed in the case of the PttXET16-34-based glycosynthase, since the reaction only proceeds through the 'pseudo' second step ($TS2_{ROH}$) of the canonical retaining-mechanism. Moreover, it is remarkable that donor substrate binding is dominated by the higher affinity of the xyloglucan moiety for the positive subsites, an interaction that is driven by the presence of aromatic residues. This increased affinity for the positive subsites is thought to be necessary (though not sufficient *per se*) for transglycosylation [91,95].

Despite a lack of sequence identity within family GH16, all of its members display a typical β-jellyroll fold that is composed of two large curved β-sheets, stacked in a sandwich-like manner. However, in the case of XTHs specific structural features reflect the specialization of these enzymes toward their highly branched substrates [95]. Notably, according to Brumer et al., starting from an ancestral (hydrolytic) licheninase active on linear 1,3-1,4-β-glucans [97], the deletion of a loop procured the ability to bind highly branched substrates, such as xyloglucan, a characteristic that is shared by both GH16 *endo*-glucanases (EG) and XTHs that display hydrolytic and/or transglycosylation activities. Examples of this are *Pt*EG16 from *Populus trichocarpa*, which is able to hydrolyze the xylogluco-oligosaccharide XXXGXXXG, and its counterpart *Ptt*XET16-34, which performs transglycosylation using the same substrate [90,97]. Moreover, the extension of the C-terminal domain differentiates the XTHs from EGs. This XTH feature provides exclusive specificity for xyloglucan (i.e. branched substrates) to this group of GH16 enzymes [91,97]. Finally, regarding XETs and XEHs, X-ray structure data have revealed that in some cases these differ in two loops located between β-strands β6 and β7, and between β8 and β9, in the vicinity of the active site [80]. The importance of this last observation has been demonstrated through the creation of a β8/β9 loop deletion in the *Tropaeloum majus* XEH (*Tm*NXG1-ΔYNIIG mutant), a loop that forms part of subsite +1 and interacts with the D-glucosyl residue. This mutation procured an increased T/H ratio in the initial phase of the reaction, with a 2-fold increase in transglycosylation rate being coupled to a 5.7-fold decrease in hydrolysis rate.

Structural and molecular dynamics work performed on *Ptt*XET16-34 and *Tm*NXG1 has revealed a correlation between the nature of the principal activity and subsite binding interactions, which are combined with subtle differences in dynamic behavior [98]. As a matter of fact, in XETs, the number of H-bonds formed between the enzyme and the acceptor moiety is greater than in XEHs, whereas in XEHs the number of H-bonds formed with the donor moiety is higher. Moreover, a determinant of transglycosylation in XETs appears to be more flexibility in subsite -1, which is detrimental for overall activity, except when a sugar is present in subsite +1.

Sucrase-type enzymes

Sucrases are *exo*-enzymes that include glucansucrases (GS) and fructansucrases (FS). Using sucrose as a substrate, these enzymes are able to synthesize homopolysaccharides composed of D-glucosyl or D-fructosyl subunits respectively, with different linkage specificities [99,100]. GS are classified in both GH13 and GH70 family, with GH13 GS being designated amylosucrases (AS). Transglucosylating AS and GS have been extensively studied both in our group [99,101] and in Lubbert Dijkhuizen's group [100,102]. Although AS, GS and FS act on the same substrate, they actually exhibit different protein

folds, with AS and GS being characterized by a $(\beta/\alpha)_8$-barrel architecture and belonging to clan GH-H (α-amylase superfamily), which is divided into 40 subfamilies [103], and FS belonging to clan GH-J (5-bladed-β-propeller). Nevertheless, all three enzyme groups operate through a retaining mechanism.

Until recently, the structure of GS remained elusive [104–106], thus hampering progress in the understanding of structure-function relationships in these enzymes [107]. Nevertheless, using sequence-based approaches it was possible to identify transition state stabilizers (histidines) that are present in both GH13 and GH70, being conserved in α-amylases, CGTases and GS. These residues are essential for overall catalysis (hydrolysis and transglycosylation) and their mutation is often highly detrimental for activity (< 0.5% residual activity) [107].

Concerning GH70 GS, the analysis of the impact of mutations of key catalytic residues and others located in the positive subsites has led to the conclusion that such mutations can be grouped into one of three categories: those affecting *(i)* D-glucosidic linkage specificity, *(ii)* glucan solubility and *(iii)* overall enzyme activity [100]. Structural data analysis revealed that subsite +1 residues form H-bonds with the D-fructosyl moiety, as do residues in subsite +2 with the D-glucosyl moiety, these latter playing an important role in determining the linkage ratio [107]. Results from the study of a reuteransucrase (GH70) from *Lactobacillus reuteri* suggest that steric hindrances play a major role in chain elongation, since the deletion of a variable N-terminal domain procured an increase in transglycosylation (3- to 4-fold) at the expense of hydrolysis [108]. Similarly, the creation of a single point mutation (N1179E) within the same subgroup of enzymes led to a T/H ratio increase [109]. In another study, it was reported that a GH70 4,6-α-glucanotransferase is able to perform a disproportionation reaction on α-(1,4)-linked malto-oligosaccharides, but is unable to use sucrose as a substrate, despite the high energy (6.6 kcal.mol^{-1}) associated with its glycosidic linkage [110]. In the light of this observation it was proposed that this enzyme represents an evolutionary intermediate between GH13 and GH70 [111].

Compared to GH70 GS, the data available for GH13 AS is more abundant. These enzymes all display a similar 5-domain structure with a deep pocket at the bottom of which sucrose binds to subsites -1 and +1 [112,113]. Three arginines (R226, R415 and R446), located in positive subsites +2/+3, +4 and +1 respectively, are particularly important in the transglucosylation reaction, since these play a crucial role in the docking and positioning of acceptors [114,115]. In a study of the AS from *Neisseria polysaccharea* (*Np*AS, subfamily 4 of GH13) the positive subsites were submitted to mutagenesis with the aim of improving transglucosylation using unnatural acceptors. Although quite impressive increases in transglucosylation were achieved (395-fold increase), which were accompanied by decreased apparent K_M values, no evidence of significant structural changes that would alter sucrose binding was detected [116]. Therefore, it was concluded that modified loop

flexibility and enzyme dynamics are likely to be the determinants of altered substrate recognition and thus responsible for the establishment of a catalytically-productive state. Overall, this study revealed a certain plasticity of subsite +1, because it was possible to isolate mutants that could glucosylate a series of different acceptors, and suggested that the improvement of transglucosylation using unnatural acceptors was facilitated by improved interactions in the positive subsites. In another study, recognition of D-glucosyl moieties in subsite -1 was investigated. This revealed that despite the fact that AS exhibits slow rates, the D-glucosyl is specifically recognized by a complex network of interactions [117]. To further understand the transglycosylating character of *Np*AS, it is useful to compare this enzyme with a hydrolytic counterpart, such as the sucrose hydrolase from *Xanthomonas axonopodis* pv. g*lycines* (*Xag*SUH). Although this GH13 member shares 57% sequence identity with *Np*AS and is structurally similar (identical 5-domain structure with rmsd value of 1.78 Å) [79], *Xag*SUH catalyzes sucrose hydrolysis and is incapable of catalyzing transglucosylation [118]. One main difference between *Xag*SUH and *Np*AS has been revealed by acquiring structural snapshots along the catalytic coordinate. This revealed in *Xag*SUH that upon sucrose binding a pocket-shaped active site is formed through rigid-body movements of the B and B' domains towards the active site. Moreover, it is noteworthy that the majority of active site residues are conserved between the two enzymes, except for three arginines (R226, R415 and R446) that are substituted in *Xag*SUH by other residues (glycine or leucine). Significantly, as mentioned earlier these arginines are essential in *Np*AS for transglucosylation, although the introduction of homologous arginines in *Xag*SUH by mutagenesis failed to confer transglucosylation properties to the enzyme, an observation that is consistent with the fact that improvements in transglycosylation first require diminution of hydrolysis [79], especially given the fact that the value of k_{cat} on sucrose is 120-fold higher than that of *Np*AS (Table 1). Surprisingly, this fact was not evoked by the authors, who suggested that hydrolysis in *Xag*SUH might be caused by a collateral effect of D-fructose release, which would disorder the B-domain and thus expose the enzyme-bound D-glucosyl moiety to bulk solvent and thus hydrolysis.

In summary, the identification of the key factors that determine the T/H partition in GS has proved to be quite difficult, although it appears evident that interactions in positive subsites play an important role. In this respect, and taking into account the fact that the active site in these enzymes is often buried, it has been suggested that the presence of an acceptor group in sucrases during catalysis protects the covalent intermediate from water-mediated attack [119]. This could be true if sucrose was able to bind in subsites -1 and +1 in the presence of the acceptor (implying that the acceptor is bound elsewhere). However, this hypothesis assumes that upon formation of the covalent intermediate the acceptor is somehow displaced towards subsite +1 and that there is considerable flexibility within the active site, allowing for example the unhindered departure of the

D-fructose LG. Unfortunately, at least in the case of AS the 'U-shaped' active site structure does not appear to allow for this possibility. Moreover, even if the active site of all AS were highly flexible and accessible, the hypothesis would not explain how water-mediated deglycosylation is avoided, especially in an enzyme such as the AS from *Deinococcus radiodurans*, which despite its open active site topology, still mainly performs transglucosylation [119,120]. Therefore, alternative hypotheses are required, not to explain how water is prevented from entering active sites, but rather to explain how the presence of water is rendered irrelevant with respect to deglycosylation.

Regarding fructose-specific sucrases (FS), which can act on sucrose and/or fructans, these are gathered within families GH68 and GH32 (clan GH-J). The FS in GH68 (i.e. levansucrases, inulosucrases) usually display a dominant hydrolytic activity, accounting for 70-80% of substrate (levan, inulin) conversion. A previous study performed on the single domain levansucrase, SacB from *Bacillus subtilis*, revealed that the addition of transitional and complete C-terminal domains from other FS leads to reductions in hydrolysis (down to 10% of substrate conversion), accompanied by a 5-fold increase in transfructosylation. Upon analysis of the chimeric enzymes the authors remarked that the k_{cat} value associated with hydrolysis was unaltered and thus attributed the increase in the T/H ratio to more favorable positive subsite interactions provided by a structural adjustment in the catalytic site mediated by the addition of extra domains [121].

The GH32 family comprises both fructan-acting (β-D-fructofuranosidases and inulinases) and sucrose-acting enzymes, and compared to GH68 FS contains an additional β-sandwich domain. The GH32 sucrases or invertases (as they are often known) are able to transfer the D-fructosyl moiety of sucrose either onto water (hydrolysis) leading to the production of fructose (i.e. inverted sugar), or onto a sucrose acceptor (transglycosylation) thus catalyzing the synthesis of fructan. In the latter case, the enzymes are designated as fructosyltransferases (FTs). Within the plant kingdom, sucrose can be degraded by vacuolar (VIs) or cell wall invertases, and from a phylogenetic standpoint FTs and VIs belong to the same GH32 subgroup, sharing high sequence identity (ca. 65%) and structural homology. Using phylogenetic tree analysis, it has been proposed that FTs have evolved from ancestral VIs [69]. Among the different VIs, it is noteworthy that three amino acid sequence motifs are highly conserved: *(i)* the sucrose-binding box motif WMNDPNG, which contains the catalytic nucleophile D, *(ii)* the EC motif, which includes the catalytic acid/base E and *(iii)* the RDP motif, with D being identified as a TS-stabilizing residue [122,123]. The first N in the sucrose-binding box is involved in a hydrogen bond network, forming links with the nucleophile D and W (Figure 4).

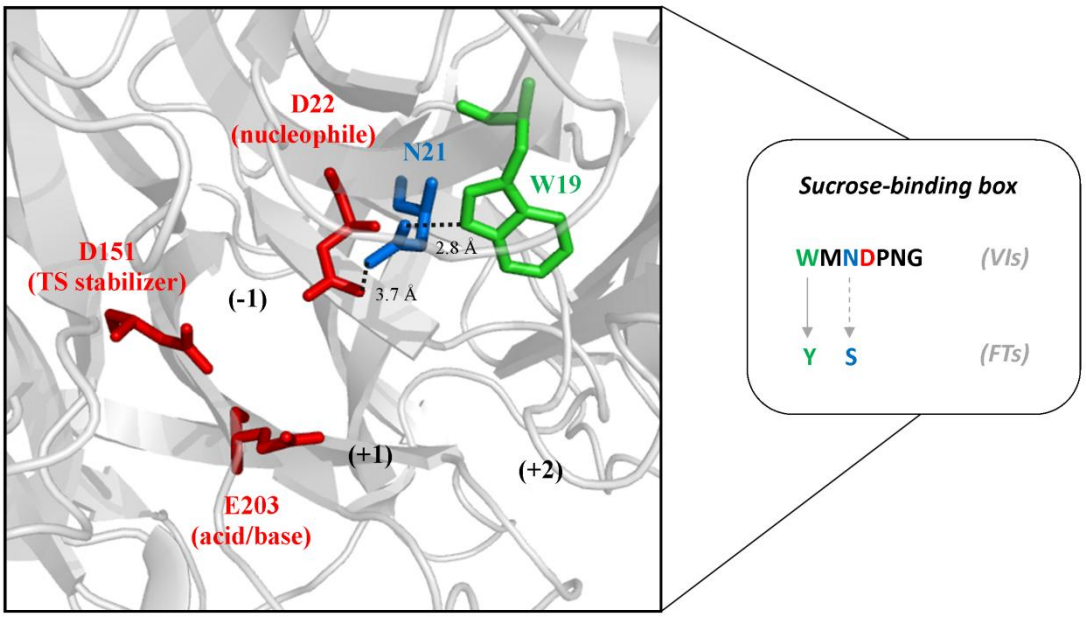

Figure 4 Cartoon representation of the X-ray structure of the active site of the *Saccharomyces* invertase (PDB ID: 4EQV [124]), showing the catalytic triad (red) and the H-bond network of the sucrose-binding box. **This motif in VIs is shown along with the key amino acid substitutions that characterize FTs.** PyMOL Molecular Graphics System, v0.99 (Schrödinger, LLC) was used to prepare the figure.

Importantly, within the sucrose-binding box, W is always replaced by a Y in FTs from the same subgroup. Likewise, the first N is very often substituted in FTs by S. Engineering of these alternative residues into VIs demonstrated that the disruption of the hydrogen bond network involving the nucleophilic aspartate (i.e. W23Y and N25S) enhanced transglycosylation up to 17-fold when compared to wild-type VIs [81]. Similarly, other studies performed on VIs from yeast [125] or onion [69] led to similar conclusions, although the increase in transglycosylation was more modest. Furthermore, a shift of optimum pH from 3.8-4.8 to 4.8-5.7 was observed for a yeast VI mutant (W19Y-N21S), consistent with an alteration of the ionization state of the catalytic residues [125]. Interestingly, the reverse experiment involving the substitution of Y by W in two different FTs failed to procure a more hydrolytic VI-like enzyme [126,127], which suggests that it is much easier to disrupt rather than create a hydrogen bond network !

From a kinetic point of view, compared to FTs (Table 1) VIs are more efficient catalysts. FTs do not display a saturation profile (i.e. K_M of hundreds of mM relative to 2-20 mM range for VIs), but are nonetheless very good at transfructosylation (70-80% substrate conversion) compared to VIs (2-5% of substrate conversion). When considering mutated VIs, these can be seen as intermediate cases, since for most of the available examples K_M values were increased from 4- to 34-fold [69,81,125], resulting in severely reduced k_{cat}/K_M values, an alteration that is indicative of higher TS1 energy levels.

Acceptor substrate selectivity among GH32 has also been investigated using a mutagenesis approach to modify residues located in subsites +1 or +2. However, this type of mutation has so far failed to confer significant transglycosylation ability to invertases, although in at least one case both regioselectivity (β-(2,6)/β-(2,1)) and catalytic efficiency were significantly altered [125,128]. On the other hand, the mutagenesis of putative positive subsite residues in a FT proved to be quite detrimental for transglycosylation [127]. Therefore, based on available data on FT/VIs it is possible to conclude that the modification of positive subsite determinants can be used to improve acceptor recognition and positioning for transglycosylation, but this is insufficient to destabilize water-mediated deglycosylation (i.e. $TS2_{water}$) in invertases. To achieve this, it is much better to target the proton network in the negative subsite (Figure 3).

Cyclodextrin glucanotransferases and α-amylases

Involved in starch depolymerization, CGTases and their hydrolytic counterparts, α-amylases, belong to GH13 and thus to clan GH-H. These enzymes share a common structural architecture, which is

defined by three domains, A, B and C, although CGTases possess two extra domains D and E. It has been proposed that CGTases have evolved from α-amylases, since the latter display greater sequence diversity and are more widespread through the different taxonomic groups [68]. Regarding the natural function of CGTases, it is likely that by providing cyclodextrins of defined size (i.e. α, β or γ), CGTases procure 'tailored' substrates for α-amylases and thus accelerate starch saccharification.

Crystallographic analysis of CGTases has revealed an extensive active site structure, extending from at least a subsite -7 to a subsite +3. Due to their architecture, these *endo*-enzymes are able to catalyze intra-molecular transglycosylation (β-cyclization) through the transfer of the covalently bound sugar unit onto the 4-hydroxyl group of the non-reducing end of the same donor molecule [129]. Compared to GS (*exo*-enzymes with only one negative subsite), negative subsite interactions in CGTases are much more developed and, taking into account the high transfer rates that characterize these enzymes (10^2-10^3 IU.mg^{-1}, Table 1), it is probable that the transition state energy barrier is lower than that of GS.

In addition to the synthesis of cyclodextrins, CGTases have also been shown to be capable of hydrolysis or to perform the transfer of the bound glycosyl intermediate onto another α-glucan chain (i.e. disproportionation) [130]. In order to prevent hydrolysis, it appears that CGTases have acquired positive subsites that favor sugar recognition. This is illustrated by mutagenesis work that was performed on the positive subsites (+2 and +3) of the *Bacillus circulans* 251 CGTase (*Bc*CGTase). The substitution of F183 and F259 in *Bc*CGTase by N or S resulted in a 10- to 300-fold decrease in transglycosylation activity (β-cyclization) and a 3- to 20-fold increase in hydrolysis [131]. Similarly, the simultaneous mutation of equivalent residues (F184Q and F260W) in the CGTase from *Thermoanerobacterium thermosulfurigenes* strain EM1 (Tabium CGTase) and the addition of a third mutation (A231V) converted this enzyme into an α-amylase-like hydrolytic enzyme [132]. Impressively, this mutant no longer displayed detectable CGTase activity, with the T/H ratio being 0.0012 (compared to 5 for the parental CGTase). Consistent with these results, another study focusing on the positive subsites in liquefying (hydrolytic) and maltogenic (transglycosylating) α-amylases revealed that increased hydrophobicity in subsites +2/+3 of the α-amylase from *Bacillus licheniformis* (BLA) increased the T/H ratio, reducing the hydrolysis rate (associated with a higher K_M value) on starch by one third [133]. Likewise, the sequence comparison of hydrolytic and maltogenic α-amylases revealed the presence in subsite +1 of a conserved histidine or glutamate residue, respectively [134]. The introduction of the substitution H235E in BLA created a transglycosylation activity, which is undetectable in the wild-type enzyme, but did not drastically affect the efficiency of hydrolysis (72% residual) [135]. Overall, mutations in the positive subsites of CGTases generally provoke a diminution of transglycosylation activity [130], whereas negative subsite mutations mostly

alter cyclodextrin specificity (α, β and γ ratio, for cyclodextrins composed of 6, 7 and 8 glucose units respectively). In this respect, it is noteworthy that a five-residue loop localized in subsites -3/-4 of α-amylases has been described as a key determinant (steric hindrance) of the T/H partition, since it is absent in CGTases. To test this hypothesis, the loop in the α-amylase Novamyl (residues 191 to 195) was deleted and positive subsite mutations (F189L/T190Y) were introduced. These modifications procured CGTase-like behavior [136], but the reverse experiment (i.e. introduction of a loop in US132 CGTase) failed, since it yielded a mutant that was unable to catalyze hydrolysis or even initial β-cyclization [137]. This failure once again underlines the complexity of the phenomenon and supports the notion that hydrolysis is driven by optimized interactions in the negative subsites, which in turn contribute to the formation of TS. In this respect, it is interesting to mention that the successful conversion of the aforementioned Tabium CGTase into a hydrolase was almost certainly facilitated by the fact that the parental enzyme already displays unusually high hydrolytic ability. This implies that in Tabium CGTase the donor interactions required for hydrolysis are already in place and thus it is simply a case of deleting the determinants of transfer activity.

More generally, these studies highlight the role of aromatic/hydrophobic residues in positive subsites. Notably, it appears obvious that the presence of aromatic residues provides both a stacking platform for better acceptor docking [138] and a hydrophobic barrier, which limits the presence of water in the active site, with both of these factors favoring transglycosylation. Similarly, such features were also suggested to be part of an evolutionary relationship between α-amylases and 4-α-glucanotransferase within family GH57 [139].

Assuming that CGTases are indeed the consequence of the evolution of α-amylases, presumably the former have somehow dealt with the well-developed negative subsite interactions that favor hydrolysis [140]. Theoretically, the existence of intermediate CGTases that display high 'residual' hydrolytic activity, such as the GH13 Tabium CGTase [132] or the one from *Bacillus* sp. SK 13.002 strain [141], should provide clues as to how this has been achieved. However, in reality unravelling subtle molecular differences might actually prove to be a considerable challenge [142].

Transferring *vs* hydrolyzing sialidases

Sialidases (SA) and *trans*-sialidases (*tr*S) are members of family GH33 and belong to the GH-E clan. These enzymes catalyze either the hydrolysis or the synthesis of sialyl-glycoconjugates respectively, operating via a classical ping-pong bi-bi mechanism with acid/base catalysis [143–145]. *tr*S exhibits both activities, although when a suitable acceptor is available transglycosylation is approximately 10-fold higher than hydrolysis [146]. Moreover, in the case of *Tctr*S, the *tr*S from *Trypanosoma cruzi*, the

K_M value for the acceptor is lower than that of the donor (10 μM and in the millimolar range for the lactose and sialic acid moieties respectively) [82,144]. Both SA and *tr*S possess similar catalytic domains that display six-bladed β-propeller topology, which are connected via a long α-helix and a large hydrophobic interface to a domain displaying a β-sandwich fold and lectin-like topology (Figure 5A). This latter does not appear to be directly involved in transglycosylation activity [147]. The molecular architecture of the active sites of these enzymes displays several common features, including eight strictly invariant residues and a hydrophobic pocket that binds the *N*-acetyl group of the sialic acid moiety, suggesting a mutual evolutionary origin and a similar mode of action for the entire family [83,144,146].

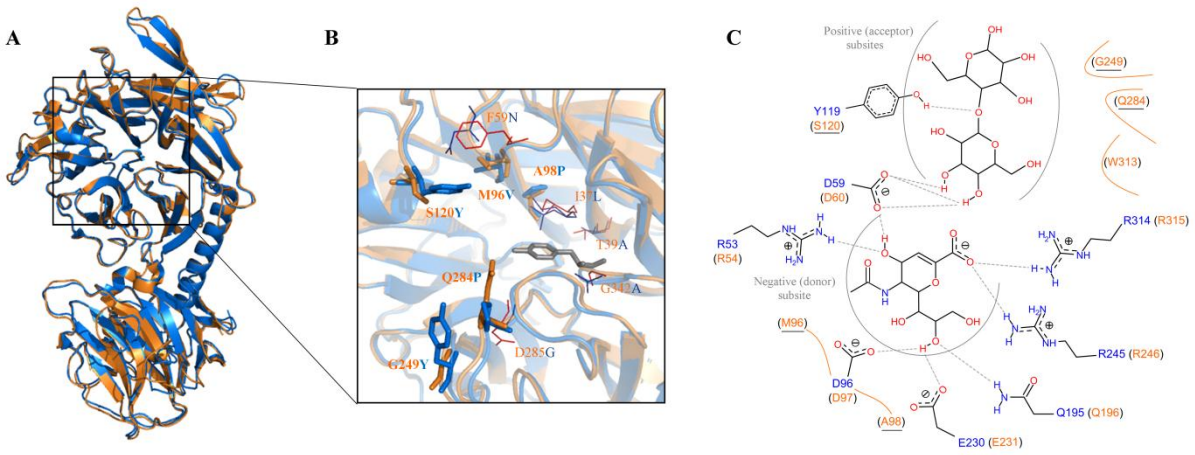

Figure 5 (A) Superimposition of the structures of the sialidase from *T. rangeli* (*Tr*SA - orange, PDB ID: 1N1T [148]) **and the *trans*-sialidase from *T. cruzi*** (*Tctr*S - blue, PDB ID: 1MS3 [149]); **(B) zoom on the residues that are mutated, creating *Tr*SA$_{10mut}$ (*Tr*SA numbering) and (C) hydrogen bonding of *Tctr*S (blue) and *Tr*SA (orange) with DANA and lactose** (PDB ID: 1MS0 [149]). Bold/cartoon (B), or underlined (C) amino acids are those that are mutated to create *Tr*SA$_{5mut}$. (B) The additional substitutions are those introduced (*in silico* only) to create *Tr*SA$_{10mut}$ and are close to the nucleophilic tyrosine (Y343, *Tr*SA numbering), drawn with grey sticks. (C) The base catalyst (D60, *Tr*SA numbering) is depicted, but Y343 is not shown. Graphics were prepared using PyMOL Molecular Graphics System, v0.99 (Schrödinger, LLC) and PoseView [150].

It has been suggested that subtle structural differences are likely to be responsible for the different selectivities of hydrolysis and transglycosylation reactions catalysed by *Trypanosoma rangeli* SA (*Tr*SA) and *Tctr*S respectively. Although these enzymes share 70% amino acid identity, their active sites display distinctive features [144,147–149]. *Tctr*S exhibits a narrower, more hydrophobic substrate-binding pocket. This implies that the reactive center is less solvent-exposed and results in an alternative hydrogen bonding pattern with the sialyl donor moiety. Additionally, residue Q284 in *Tr*SA is replaced by P283 in *Tctr*S (Figure 5B and C), a substitution that alters the conformation of the neighboring W312 residue (W313 in *Tr*SA). In *Tctr*S, W312 and Y119 (S120 in *Tr*SA) form the two lateral walls of the acceptor binding site, providing the basis for stacking interactions with the sugar acceptor. Moreover, it is noteworthy *Tctr*S appears to display greater active site flexibility than *Tr*SA [83,147,148,151], a point that is exemplified by the study of the inherent motions of Y119 and the strictly conserved Y342 (catalytic nucleophile) residues. According to Demir and Roitberg, structural rearrangements that are triggered by 'allosteric' binding of the sialyl-conjugate donor forming a covalent sialyl-enzyme intermediate lead to the creation of a productive acceptor sugar binding site [151].

Overall, finely-tuned enzyme-donor substrate interactions, conformational flexibility (notably loops), solvent exposure and the presence of an acceptor sugar-binding site are all crucial to obtain *trans*-sialidase activity. Therefore, to switch between hydrolysis and transglycosylation, *Tr*SA has been submitted to mutagenesis, introducing five mutations designed to modify the structure and dynamics of the negative subsite and to create a suitable positive subsite. This work provided *Tr*SA_{5mut}, a mutant that displayed detectable *trans*-sialidase activity, although this was only 1% of that exhibited by the true *trans*-sialidase, *Tctr*S [83]. Further mutation of *Tr*SA_{5mut}, introducing either I37L or G342A (Figure 5B), which affect the negative subsite, procured a higher transglycosylation rate, which was 11% of that exhibited by *Tctr*S. Therefore, it appears that the acquisition of improved *trans*-sialidase activity requires alterations in the negative subsite, notably to alter the flexibility of the tyrosine nucleophile residue and thus diminish hydrolytic activity. In this respect, it is also significant that while *Tr*SA is inhibited by DANA (K_i = 1.5 μM for *Tr*SA), a structural analog of the transition state sialic acid oxocarbenium ion (Figure 5C), the mutated *Tr*SA described above is less sensitive to inhibition (K_i = 1.54 mM) [83], as is the case for *Tctr*S (K_i = 12.3 mM). This implies that the acquisition of *trans*-sialidase activity may involve a modification of the TS that is developed during the glycosylation step.

More recently, using QM/MM approaches Roitberg et al. evaluated the free energy profiles for the conversion of the Michaelis complex to the covalent glycosyl-enzyme intermediate in *Tr*SA,

*Tr*SA$_{5mut}$ and *Tctr*S [152,153]. In SA enzymes, the free energy barrier (ΔG^{TS1}) to reach the glycosylated-enzyme intermediate (15.2 and 15.0 kcal.mol^{-1} for *Tr*SA and *Tr*SA$_{5mut}$, respectively) is approximately 5 kcal.mol^{-1} lower than that of *Tctr*S (20.8 kcal.mol^{-1}). Moreover, the change in free energy (ΔG^{0}, Figure 3) associated with the glycosylation step of the *Tctr*S-catalyzed reaction is close to zero (-0.89 kcal.mol^{-1}), compared to -10.9 and -9.8 kcal.mol^{-1} for *Tr*SA and *Tr*SA$_{5mut}$ respectively, these values being linked to the higher stability of the glycosyl-enzyme intermediates. However, the deglycosylation step appears to be favorable for *tr*S-like enzymes, with the difference being approximately 5 kcal.mol^{-1} (i.e. 21.6, 24.8 and 26.1 kcal.mol^{-1} for *Tctr*S, *Tr*SA$_{5mut}$ and *Tr*SA, respectively). Based on these findings further *in silico* design of an efficient *trS* was performed, giving rise to the hypothetical mutant *Tr*SA$_{10mut}$, which contains five additional substitutions (Figure 5B). According to the *in silico* results, in *Tr*SA$_{10mut}$ residues I37L and G342A (*Tr*SA numbering), both located in the vicinity of the catalytic nucleophile tyrosine (Y343), would be responsible for the predicted increased T/H ratio. Moreover, it was speculated that *Tr*SA$_{10mut}$ would only weakly stabilize the covalent intermediate and when compared to *Tctr*S would display a lower free energy barrier for deglycosylation step (-3.2 and 19.1 kcal.mol^{-1}, respectively). However, regarding the free energy barrier of the glycosylation step, it was predicted that this would be similar (ΔG^{TS1} = 16.0 kcal.mol^{-1}) to that of a typical hydrolytic SA.

Finally, it is noteworthy that on the edge of its acceptor substrate binding cleft *Tctr*S displays a seven-amino acid loop (VTNKKKQ) whose composition, physico-chemical properties and dynamics differ from the equivalent loop (IADMGGR) in *Tr*SA. Using an enzyme engineering approach it was shown that the loop in *Tctr*S promotes transglycosylation, increasing product yield and reduces hydrolysis, effects that were attributed to a perturbation of the water binding network [154].

ENGINEERED TRANSGLYCOSYLASES

Although TGs have only been identified in a few GHs families, hydrolytic GHs from other families have been submitted to protein engineering in order to modify their H/T balance. In the following section, the different strategies that have been adopted are described along with the results that have been obtained.

Modification of negative subsite interactions

Enzyme engineering

One of the very first protein engineering studies aimed specifically at increasing the T/H ratio was performed on a GH1 β-glycosidase from *Thermus thermophilus* using a random

mutagenesis/screening methodology. The mutation of two conserved residues F401 and N282 in this enzyme increased K_M values (> 6-fold) and significantly improved transglycosylation (up to 78% synthesis yield compared to 8% for the wild-type enzyme) [155]. In a follow-up study, using a site-directed approach the same authors probed the importance of conserved residues in the donor (-1) subsite [156,157] and revealed that these play an important role in TS stabilization (29- to 3577-fold decrease k_{cat}/K_M values), but do not induce major structural changes.

Working on AMY1, a GH13 α-amylase (clan GH-H), it was shown that the mutation of a subsite -2 residue (M53W) leads to increased lifetime of the glycosyl-enzyme and thus to the acquired ability to perform transglycosylation using *p*NP-α-D-maltoheptaose as the donor [77]. It is also noteworthy that the introduction of a range of mutations at position 53 procured k_{cat}/K_M values that were 59- to 5000-fold lower than that of the parental enzyme (mainly due to up to a 20-fold increase in K_M values). Likewise, it is significant that the presence of tryptophan at position 53 is a common occurrence in GH13 CGTases, which is consistent with the impact of the mutation M53W in AMY1.

The mutation of conserved negative subsites residues produces a similar effect to the one described above in other GH families. This is exemplified by protein engineering work performed on a GH18 chitinase from *Serratia marcescens* (*Sm*ChiA). The latter possesses a long active site cleft positioned at the top of a $(\beta/\alpha)_8$ barrel, the negative subsite of which was targeted with the aim of prolonging the retention time of the donor glycosyl moiety [158]. The introduction of the mutation W167A (subsite -3) procured a higher transglycosylation yield (45% of the substrate converted into transglycosylation products, compared to 8% for wild-type *Sm*ChiA) and subsequent determination of the 3D structure of the mutated enzyme revealed that repositioning of D313 (subsite -1) had occurred. This is significant because D313 is involved in the stabilization of the oxazolinium intermediate and interacts with E315, a residue that is putatively responsible for water molecule activation during hydrolysis. Therefore, the mutation W167A might both prolong residency of the donor glycosyl moiety and/or diminish hydrolysis. Similar examples of such a coupled effect (i.e. improved transglycosylation and diminished hydrolysis) are provided by work performed on chitinases from *Serratia proteamaculans* (*Sp*ChiD) [159] and *Aspergillus fumigatus* (*Af*ChiB) [160], with mutations being introduced at the catalytic center and in subsite -1 respectively. Furthermore, QM/MM calculations performed on a hyper-transglycosylating variant (D142N) of ChiB from *S. marcescens* (*Sm*ChiB) predicted that the mutation, which is within a highly conserved DxDxE motif, would affect both TS stabilization and the catalytic water molecule [161].

A further example concerns two homologous α-galactosidases (AgaA and AgaB) from family GH36 (clan GH-D) [162]. Despite being highly related (97% identity), AgaA displays a relatively low K_M value for raffinose (K_M = 3.8 mM) and exhibits high hydrolytic activity and no detectable ability to

catalyze transglycosylation. On the other hand, AgaB displays a higher K_M value (200 mM) for raffinose and exhibits the ability to catalyze autocondensation reactions (i.e. transglycosylation). In this context, the mutation of residue 355 (Ala in AgaA and Glu in AgaB) provides the means to switch between the two phenotypes, with for example the substitution A355E in AgaA procuring AgaB-like behavior and *vice versa*. Although residue 355 is located far from the active site (20 Å), structural analysis revealed that the presence of a Glu at position 355 provokes the displacement of the conserved W336, which is present in subsite -1 where it provides the basis for sugar stacking. This modification widens the active site and thus probably disturbs the binding of raffinose.

Regarding another example of a galactose-acting enzyme family, random mutagenesis and screening performed on the GH42 β-galactosidase from *Geobacillus stearothermophilus* (BgaB) pinpointed a residue (R109) for subsequent site-saturation mutagenesis. This ultimately procured a mutant (R109W) that displayed improved ability (23% yield compared to 2% for the parental enzyme) to transfer D-galactosyl moieties onto lactose [163]. R109 is a highly conserved amino acid among GH42 β-galactosidases that according to 3D structure analyses is involved in hydrogen bonding with the D-galactosyl moiety. Therefore, mutation of this residue probably leads to the destabilization of donor binding in subsite -1 (K_M values on lactose increase from 1.8 to 114 mM), coupled to decreased hydrolysis (15% residual) and thus alterations in the T/H ratio that favor transglycosylation. Overall, in terms of TS it is likely that the mutation R109W increases the TS energy barriers for glycosylation and deglycosylation, thus rendering water-mediated deglycosylation less competitive.

Regarding rational engineering work focused on the nucleophile catalyst, several studies have revealed that modifications of the latter can also have drastic effects on the ability of water to deglycosylate the glycosyl-enzyme intermediate. Recently, it was reported that the introduction of a sulfinate function (i.e. SOO^-), to replace the catalytic nucleophile of the GH13 dextran glucosidase, provoked a drastic drop in k_{cat} (0.27%), an acidic pK_a shift (from 3.9 to 1.5) and an increase in transglucosylation yields [164]. According to the authors of this work, the observed effects can be attributed to differences in the TS energy barriers between water and acceptor-mediated enzyme deglycosylation. In this respect, shortening or lengthening (E78D or carboxymethylation of the mutant E78C) of the nucleophile residue in the GH11 xylanase from *Bacillus circulans* was also shown to be detrimental for global catalytic efficiency, nucleophile shortening having a greater impact (1600-5000-fold decrease) than lengthening (16-100-fold) [165]. However, in this study no information concerning the impact on the T/H ratio was reported. Nevertheless, in a very recent study, nucleophile shortening (E134D) in a GH16 EG was shown to introduce glycosynthase-like activity [166]. The resulting enzyme, which retained 2% residual hydrolytic activity and displayed a

modified pK_a value (5.8 instead of 7.0 for the parental enzyme) for its acid/base catalytic residue, was described as a hydrolase-glycosynthase intermediate [167]. Unfortunately, no information regarding the reactivity of the glycosyl-enzyme covalent intermediate towards water or sugar acceptors was reported.

Substrate modifications

The previous section described how modifications in enzyme negative subsites can favor transglycosylation. In a similar manner, several authors have revealed that substrate modifications can procure the same overall effect (i.e. altering the TS energy barrier for water-mediated deglycosylation). An excellent example of this was reported for the GH51 α-L-arabinofuranosidase from *Thermobacillus xylanilyticus* (*Tx*Abf). This enzyme was shown to display much better transglycosylation yields in the presence of the non-natural donor sugar *p*NP-β-D-Gal*f* (75% when using Bn-α-D-Xyl*p* as the acceptor) than with *p*NP-α-L-Ara*f* (7%) [168,169]. Compared to *p*NP-α-L-Ara*f*, *p*NP-β-D-Gal*f* possesses an extra hydroxymethyl moiety at position C5, a difference that is sufficient to decrease by 100-fold the hydrolytic rate and radically increase the K_M value (> 50 mM, compared to 0.72 mM on *p*NP-α-L-Ara*f*), changes that are clearly indicative of modified glycosylation and deglycosylation steps. It is noteworthy, that similar results were subsequently observed for the GH51 Abf from *Clostridium thermocellum* [170].

Another example, described over 20 years ago, concerns the GH1 β-glucosidase from *Agrobacterium faecalis* (Abg). When acting on *p*NP-β-D-Xyl*p*, the value of k_{cat}/K_M was divided by approximately 140-fold compared to that obtained with *p*NP-β-D-Fuc*p* (a substrate that contains an extra methyl group at C5), while the T/H ratio for the autocondensation reaction was 4.3 [62]. Moreover, it was shown that when Abg acts on dNP-3-deoxy-3-fluoro-glucoside, the reaction mechanism is altered and is described by a biphasic profile of V_i = f(S) plot, with transglycosylation (autocondensation) occurring above a certain threshold concentration [52]. Through the measurement of k_{cat} values (Box 3) it was possible to demonstrate that this effect was due to an increase of TS energy barriers associated with glycosylation (6.4 kcal.mol^{-1}) and deglycosylation (12.0 kcal.mol^{-1}) steps, respectively.

Glycosynthases, which are crippled enzymes in which the catalytic nucleophile is usually mutated into A, S or G, are also good examples of how the donor substrate can diminish the potency of water. In the glycosynthase-mediated reaction, glycosyl fluorides displaying inverted anomery are used as donors. These mimic the covalent glycosyl-enzyme intermediate and, in the presence of an acceptor, allow deglycosylation to occur. Importantly, once the transglycosylation product is formed, it cannot

be hydrolyzed by the enzyme, since this is catalytically-impotent, being unable to perform glycosylation. However, this rather neat account of how glycosynthases work fails to explain why water does not compete with the acceptor. In fact, the rather elusive answer to this question concerns the donor-borne fluorine atom [171]. This atom is likely to extensively perturb the donor-enzyme interaction that is necessary to overcome the TS energy barrier and thus only counter-interactions provided by the presence of a sugar in the positive subsite are sufficient to decrease the energy of TS2 [25]. In this respect, it is also interesting to note that a correlation has been observed between the ability to create efficient glycosynthases from GHs and the behavior of the corresponding parental GH with 2-fluorosugars [25]. When high k_{trans} and k_{trans}/k_{H2O} were measured in the presence of such inhibitors, it was predicted that the enzyme would form the basis of a good glycosynthase. Actually, it is highly probable that, in both cases, 'equivalent' TS destabilizations are responsible for this improved selectivity for sugar acceptors over water molecules.

In summary, although very few studies have actually measured catalytic efficiencies of GHs in the presence of different donors and compared transglycosylation rates, it appears likely that the destabilization of the hydrogen bonding network in negative subsites is responsible for increased transglycosylation, irrespective of whether this is achieved through enzyme mutagenesis or donor substrate modification.

Positive subsite interactions and impact on the deglycosylation step

Work performed on lysozyme in the 1970's provided the first evidence that acceptor specificity might influence transglycosylation [172]. The role of residues in positive subsites +1/+2 (originally denoted E and F) were shown to be important for transglycosylation activity by contributing to higher binding free energy of the incoming acceptor [173,174]. Similarly, a hypothetical link between transglycosylation and acceptor binding interactions was proposed on the basis of work performed on the GH10 xylanase from *Streptomyces lividans* (XlnA) [175]. In this work, the mutation of a subsite +3 residue (N173D) decreased transglycosylation activity in the presence of xylo-oligosaccharide (DP > 3) acceptors, thus revealing the sensitivity of this reaction to changes in acceptor binding. In another study involving a GH10 xylanase (Xyn10A from *Pseudomonas cellulosa*) the introduction of alanines in subsites +2 (N182), +3 (Y255) and +4 (Y220) provoked a strong decrease in transglycosylation activity [176]. This loss of activity was correlated with increased k_{cat} and K_M on xylan (21- and 22-fold, respectively), suggesting that decreased 'affinity' in the positive subsite region accelerates the leaving group departure and facilitates the access of water to the active site.

To better understand the determinants of transglycosylation in members of family GH5, *endo*-β-

(1,4)-mannanases have been subjected to positive subsite engineering. The results of this work revealed that stacking interactions (subsite +1 of Man C) and hydrogen bonding with the acceptor moiety (revealed by the deleterious effect of R171K in subsite + 2 of *Tr*Man5A) are critical features for efficient transglycosylation [177,178].

The question of acceptor affinity has also been addressed in the case of the aforementioned chitinase, *Sm*ChiA [161]. By further mutating the transglycosylating variant, *Sm*ChiA-D313N, introducing the subsite +2 mutation F396W, it was possible to further enhance transglycosylation by several fold (not numerically quantified). Unfortunately, in this study the effect of the mutation F396W alone was not studied, making it impossible to state whether it needs to be combined with D313N in order to observe an effect on transglycosylation. Nevertheless, this example is particularly interesting, because it underlines the importance of the aromatic surface area. The mutation F396W increases this parameter, which might explain the higher rate of the transglycosylation reaction (compared to D313N alone) and the lower catalytic efficiency (the relative k_{cat}/K_M for D313N/F396W and D313N are 1.4 and 4.9% respectively) of this enzyme [161]. Consistent with this result are independant observations that the removal of aromatic residues located in the positive subsites of other chitinases, such as that of *Serratia proteamaculans* (*Sp*ChiD), *Aspergillus fumigatus* (*Af*ChiB1) or *Cycas revolute* (*Cr*Chi-A), decreases or annihilates transglycosylation activity [159,160,179]. In the case of *Af*ChiB1, computational simulation suggested that the complete loss of transglycosylation coupled to the maintenance of hydrolysis in the mutant W137E would be due to the loss of a stacking interaction between W137 and the acceptor sugar. According to the authors, this interaction would be necessary for the efficient attack of the oxazolinium ion intermediate.

Another example of how modifications in the acceptor binding site of GHs can lead to T/H modulation in favor of transglycosylation has been provided for family GH85 *endo*-β-*N*-acetylglucosaminidases (ENGase). The enhancement of transglycosylation, procured by either increased hydrophobicity in the positive subsite (achieved by the mutation Y217F in the ENGase from *Mucor hiemalis*) or mutation of so-called 'gate keeper' amino acids (W216 and W244 in the ENGase from *Arthrobacter protophormiae*), was partially attributed to alterations in active site dynamics [180,181].

Although the introduction of aromatic side-chains substitutions into the acceptor binding regions of GHs is frequently used to modulate the T/H ratio, this is neither an exclusive nor a general strategy. Indeed, the introduction of other mutations (P402D and F328A) into the +1 region of the active site of a family GH36 α-D-galactosidase (subsite +1) actually led to an enlargement of the entrance to the active site and consequently influenced the orientation of the bound acceptor. Such mutations led to 4- to 16-fold increases in the yield of transglycosylation products, although in absolute terms the

amounts produced were modest [182]. Another example reveals that the presence of aromatic residues in the positive subsite does not automatically favor transglycosylation. Indeed, the introduction of the mutation F116A into the positive subsite of the GH39 β-xylosidase from *Bacillus halodurans* (BhXyl39) revealed that the effect of this mutation on transglycosylation was dependent on the nature of the acceptor molecule, with transglycosylation being increased when octanol was used, but decreased in the case of pentanol [183].

In summary, generally-speaking one can affirm that although certain mutations in the positive subsites of GHs enhance the T/H ratio, such mutations are much less likely to drastically affect hydrolysis. Consequently, positive subsite mutations mostly exhibit a limited potential to increase transglycosylation, since any beneficial effects that maybe associated with positive subsite mutations are masked to some extent by the persistence of hydrolysis.

Water 'activation' and channels

Evidently, water is a key external element in hydrolytic reactions catalyzed by GHs. Therefore, in order to better diminish hydrolysis, it is quite logical that scientists have attempted to elucidate the molecular determinants of water access to the active site and the way in which 'catalytic water' is bound in a productive manner for catalysis.

In the case of T4 lysozyme, which is actually an inverting GH, the O^{γ} of T26 was identified as a solvent binding determinant. Consistent with this hypothesis, mutation of T26 to histidine (T26H) procured transglycosylase activity, with a T/H ratio of 10:1 being observed after a 60 min reaction period [184]. This quite surprising feat (considering that the parental enzyme is an inverting one) is explicable if one considers that the N^{ε} of H26, which lies close to the putative catalytic water binding site, permits the formation of a covalent glycosyl-enzyme intermediate.

In other studies, tyrosine residues have been identified as water binding determinants. For example, the introduction of a tyrosine near to the active site (V286Y) of the *Bacillus licheniformis* α-amylase led to a 5-fold increase in hydrolytic activity on starch compared to the wild-type enzyme, consistent with the hypothesis that the tyrosine OH group favors water access to the active site [133]. Similarly, the key role of tyrosine residues has been demonstrated in other GH13 α-amylases [185,186] and in *endo*-xylanases [187,188], with the elimination (mutation to F, A, S or N) of tyrosine generally leading to the loss of anchoring points for water molecules, coupled to increased hydrophobicity and thus overall lower hydrolysis. It is also noteworthy, that the mutation of the water-binding tyrosine (Y198F) in a GH8 *exo*-acting, inverting xylanase (Rex) led to the creation of an unusual glycosynthase that displays a high (4.7 s^{-1}) fluoride release rate when fed with α-xylobiosyl

fluoride despite the fact that the enzyme's general base was present [189]. However, in this case the mutation did not remove the nucleophilic water *per se*, but rather led to the non-productive orientation of the water's lone electron pair, due to the absence of the H-bond ordinarily furnished by Y198 [171].

Tyrosines that provide catalytic assistance have also been revealed in GH85 enzymes, in particular in the *Arthrobacter protophormiae* ENGase A. The creation in this enzyme of mutations Y205F (negative subsite) and Y299F (acceptor region) led to 2.5- and 3-fold increases respectively in transglycosylation yield, although hydrolysis was not affected in the same way. Indeed mutation of Y205 significantly reduced hydrolysis (44% residual activity), consistent with the postulate that the hydroxyl moiety of Y205 activates the catalytic water molecule. However, mutation of Y299 did not affect hydrolysis [181], an observation that implies that the substitution Y299F mainly improves acceptor binding and thus transglycosylation.

Overall, the mutation of residues that interact with catalytic water in GHs appears to be a good strategy to reduce hydrolysis and thus modulate the H/T ratio. Attractively, such a strategy reduces the risk of major active site perturbations, while targeting a critical element of hydrolysis.

Another way to alter the hydrolytic potency of GHs is to actually modify water access to the active site. Although protein are generally tightly packed and surrounded by bulk water, internal water molecules are observed in cavities and channels, which in some cases may provide access to the active site, following paths that are determined by a combination of hydrogen bonding, electrostatic interactions and hydrophobic effects [190]. An elegant example of such a channel was found in a substrate-complexed form of the GH13 α-amylase from *Pseudoalteromonas haloplanktis*. X-ray crystallography revealed a series of seven well-ordered water molecules that followed a path from the surface to the enzyme's active site (Figure 6) [191]. Similarly, evidence that the alteration of water channels can provide the means to modulate the H/T ratio was shown in the case of the neopullulanase from *Bacillus stearothermophilus*. Here, the introduction of a hydrophobic residue at the entry point of water into the catalytic active site was sufficient to increase transglycosylation. Inversely, the introduction of hydrophilicity produced the opposite effect, increasing hydrolysis [192]. Likewise, in another α-amylase (human salivary type), a water channel composed of aromatic residues was also demonstrated to be relevant for catalysis [193]. Two recent studies probed the role of water channels with respect to the H/T balance in GH1 β-glucosidases from *Thermotoga maritima* and *Thermus thermophilus* respectively [194,195]. In the latter, internal water dynamics were extensively studied using hydrogen-deuterium exchange mass spectrometry (DXMS) and molecular dynamics techniques [195]. Finally, structural analysis of a 1,3-α-3,6-anhydro-L-galactosidase from family GH117 has also revealed the presence of a putative water channel that runs from the protein's

surface to the active site [196].

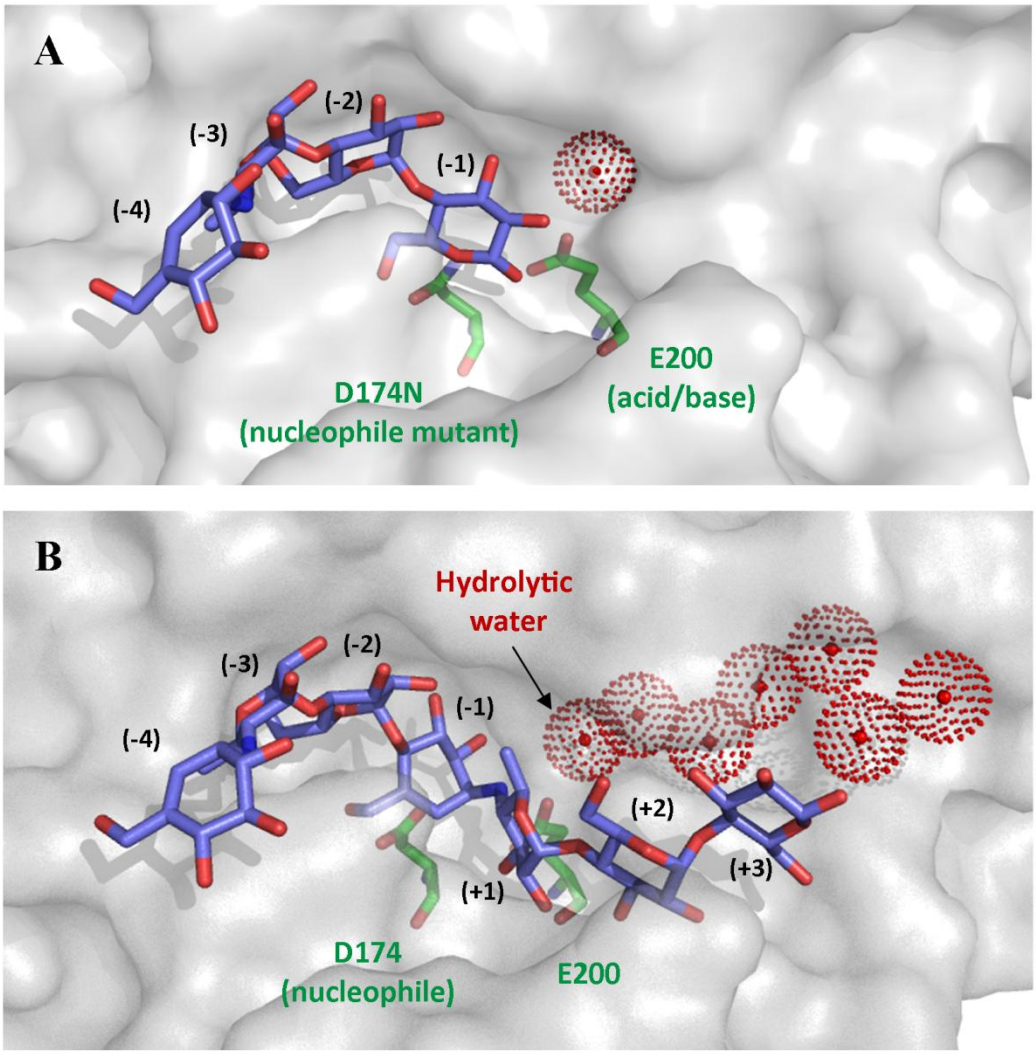

Figure 6 Substrate-induced water channel formation. (A) Complex of the inactive *Pseudoalteromonas haloplanktis* α-amylase D174N with acarbose bound in non-productive mode (PDB ID: 1KXH [191]) and **(B)** complex with a heptasaccharide (a transglycosylation product) showing 7 well-ordered water molecules running from the surface to the catalytic center (PDB ID: 1G94 [191]). The graphic was prepared using PyMOL Molecular Graphics System, v0.99 (Schrödinger, LLC).

From an evolutionary perspective, the presence of specific water binding determinants (e.g. water-binding tyrosine residues) or water delivery systems within the active site can be considered as extra features that contribute to the enzyme's hydrolytic potency. Nevertheless, the presence of such features is manifestly facultative for activity, because TS stabilization is the key element for catalysis to occur. Indeed, the lack of specific water interactions during the deglycosylation step may explain why this step is usually rate-limiting in the majority of GHs.

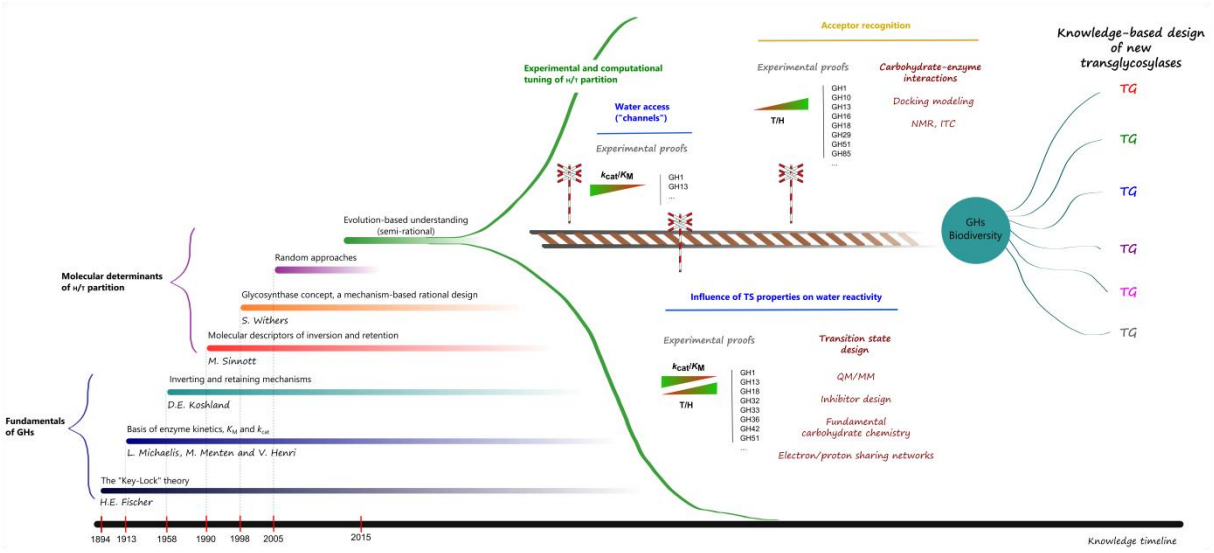

Figure 7 Over a century of knowledge pertaining to glycoside hydrolases and their ability to catalyse both hydrolysis and transglycosylation.

Conclusions

Hopefully, this quite extensive review of available literature allows the reader to better perceive a certain number of generic features that allow GHs to perform transglycosylation in adverse reaction conditions, dominated by the presence of water (Figure 7).

Unsurprisingly, the first lesson that can be drawn from the examples that are described herein is that TGs are probably characterized by modified transition states when compared to their GH counterparts, as illustrated by our 'locked door' analogy. This leads to the conclusion that the first condition that must be met if a GH is to be converted into an efficient TG is that new transition state structures or electronic displacement systems must be developed that lower the efficiency of water-mediated deglycosylation, thus radically reducing the inherent advantage of ubiquitous water over other suitably-nucleophilic acceptors. The analysis of available data indicates that the destabilization of $TS2_{water}$ is generally brought about by altering interactions in the negative subsite, which inevitably

results in higher transition state energy barriers (c.f. enhanced stiffness of the lock mechanism) for both deglycosylation and glycosylation (TS2 and TS1 respectively), since they display similar properties. In turn, this translates into less efficient catalysis (i.e. low values of k_{cat}/K_M).

The second lesson that can be drawn from the cumulative knowledge presented here is that transglycosylation is also promoted by the presence of substrate-specific interactions in the positive subsites (c.f. the better grip on the door handle). These are useful, because they partially compensate for the destabilization of the negative subsite interactions, possibly providing a situation where $TS2_{acceptor}$ energy is lower than that of $TS2_{water}$. Moreover, these can be very specific for certain acceptor molecules, thus their presence does not necessarily imply more general access for acceptors, including water. In this respect, it is noteworthy that the conversion of *endo*-GHs into TG (i.e. *endo*-TGs) might not be accompanied by significantly lowered k_{cat}/K_M values, because in these enzymes both donor and acceptor regions are involved in donor substrate recognition, with relative contributions probably being GH family-dependent. Consequently, understanding the evolutionary pathway that has provided *endo*-TGs may prove to be more complicated than the study of *exo*-TGs.

Finally, despite the fact that the natural solvent for most enzymes is water, most of this remains as bulk water. For catalysis many GHs display specific features that either fix specific water molecules within the vicinity of the catalytic center and/or deliver water to the active site via channels. In both cases, these water-specific molecular determinants can be modified in order to reduce the presence of catalytic water, thus further promoting transglycosylation.

In the case of natural TGs, evolutionary processes have no doubt resulted in the combination of the aforementioned features. However, it is certain that the foremost of these must be the destabilization of $TS2_{water}$, a modification that we consider to be the main driving force of the GH→TG transition. Unfortunately, from an enzyme engineering perspective, the rational modification of TS structures (geometry and/or electronic features) remains impossible due to insufficient knowledge of these. Nevertheless, in this respect, the use of combined approaches, associating QM/MM and experimental validation (e.g. the specific design of transition state inhibitors for GHs and TG counterparts), should provide new information in the near future. Moreover, with the increasing integration of knowledge in the fields of chemistry and biology, it is likely that new comprehension of carbohydrate reactivity in chemically-catalyzed glycosylation will rapidly contribute to better understanding of the enzyme-substrate interactions that are necessary for transglycosylation to occur.

From an evolutionary standpoint, it appears that TGs have mostly evolved from their hydrolytic counterparts, although *endo*-acting XTH is an exception to this general rule [97]. Therefore, it is intriguing to speculate upon the circumstances that might have led to the conversion of catalytically-

optimized GHs into today's relatively inefficient TGs. Logically, glycosynthesis should have been left to the dedicated glycosynthetic enzymes (i.e. GTs) that we know today. Unless evolutionary divergence between GHs and TGs occurred at a point when these were not available?

Despite the long history of carbohydrate chemistry, the fact that some fundamental features of glycosylation (e.g. the factors that influence anomeric center reactivity and stereo-electronic effects) are still subject to research [197,198] underlines the complexity of this important field and reveals the fact that there are still considerable opportunities for progress. In the case of synthetic glycochemistry, the extension of the available palette of TGs is undoubtedly an attractive grail for enzyme engineers. From this point of view, the knowledge that is presented herein should provide a rational starting point for would-be enzyme engineers wishing to convert GHs into TGs.

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