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

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10

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

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22

23 **Running title:** Glycosynthesis in a Waterworld

24

1 **Abstract**

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

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

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

21

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

24

25

1 INTRODUCTION

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

12

13 Enzymes available to the synthetic glycochemist

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

1 catalytic mechanisms involved [18–20].

2

3 **Transglycosylases – exceptions to the rule**

4 Over the last 15 years, the number of CAZyme-encoding sequences in the CAZy database
5 (www.cazy.org and www.cazypedia.org) has dramatically increased [21–23], reaching more than
6 210,000 GH modules, assigned to 133 different GH families (14 clans). Among the characterized GHs
7 present in this database, only a few have been described as transglycosylases (TGs), meaning
8 enzymes that mainly (often exclusively) catalyze transglycosylation, even in dilute conditions and
9 aqueous media. Intriguingly, TGs are highly related to hydrolytic GH counterparts, with any single TG
10 being more related to the other members of its GH family, than to TGs from other families. This fact
11 underlines the tight evolutionary relationship between TGs and GHs and implies that
12 transglycosylation in GHs is favored by subtle molecular adjustments rather than major
13 modifications, such as significant structural changes.

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

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

28

29 **TRANSGLYCOSYLATION IN GLYCOSIDE HYDROLASES**

30 **A mechanistic description of hydrolysis and transglycosylation in GHs**

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

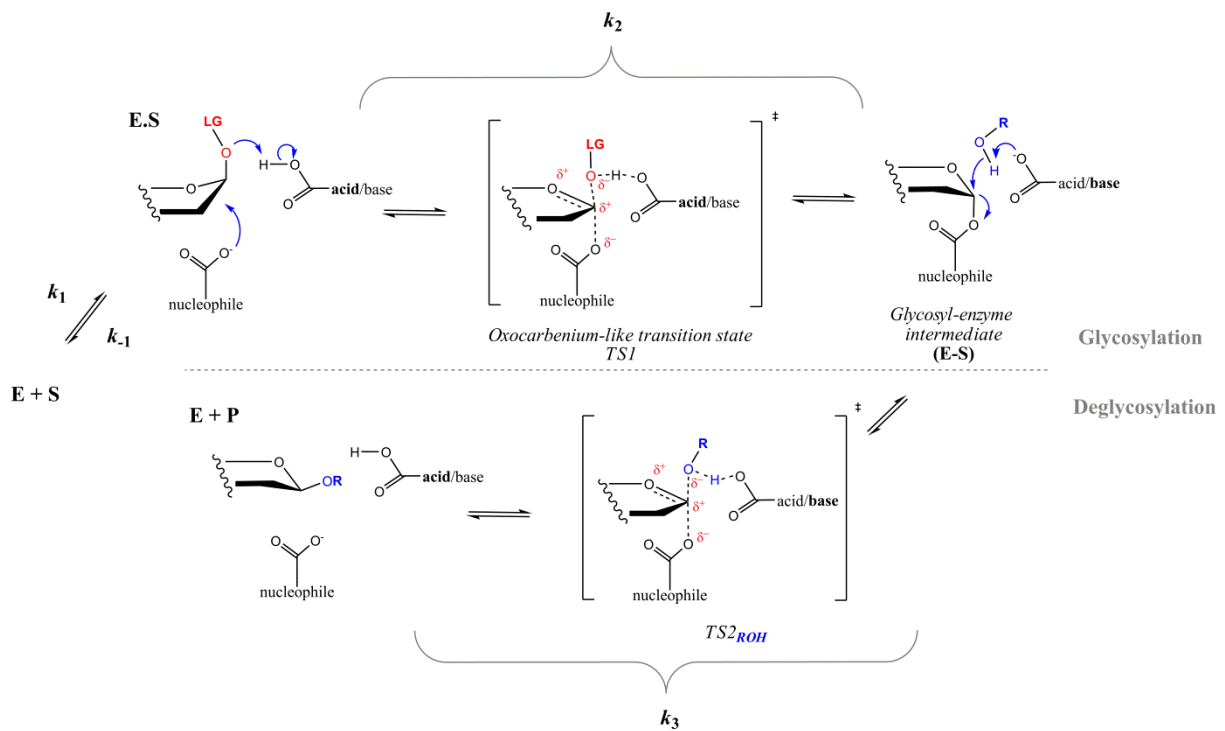
1 approximately 60% of GH families, catalysis occurs in two main steps called 'glycosylation' and
2 'deglycosylation'. Glycosylation begins with the formation of the Michaelis-Menten complex (E.S) and
3 continues up to the formation of the covalent glycosyl-enzyme intermediate (or equivalent
4 oxazolinium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

10

11

1 **Box 1 On the meaning of catalytic constants for retaining GHs**

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



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

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

5

1

2 **From a simple lock to a locked door model**

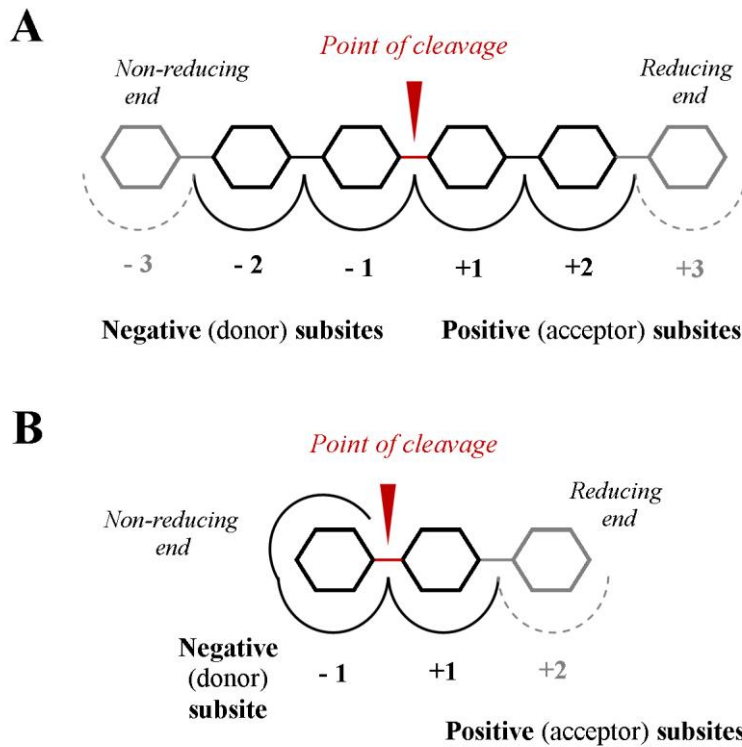
3 As the mechanistic description above indicates, the actual functioning of GHs is far more complex
4 than that illustrated in 1894 by Emil Fisher's original 'lock and key' model [29]. Indeed, as Koshland
5 pointed out, this early model is limited in several ways, but in particular because it omits the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

23

24

1 **Box 2 Simplified view of the GHs' active sites**

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



4

5 **Figure 2 Classification of GHs and nomenclature for sugar-binding subsites.** Following the
6 nomenclature proposed by Davies et al. [31], subsites in GHs can be numbered. Accordingly,
7 subsites located on the non-reducing side of the cleavage point (red triangle) are denoted by
8 negative numbers (i.e. -1, -2, -3, etc.), while those at the reducing side are positively denoted (i.e. +1,
9 +2, +3, etc.). Positive and negative subsites are often designated donor and acceptor subsites
10 respectively, terms that take into account substrate binding over the reaction pathway. However,
11 this nomenclature is ambiguous if one considers that at the beginning of a reaction the donor
12 substrate occupies both negative and positive subsites.

13

1

2 **Transition states in glycoside hydrolases and H/T balance**

3 ***TS: the power of GHs***

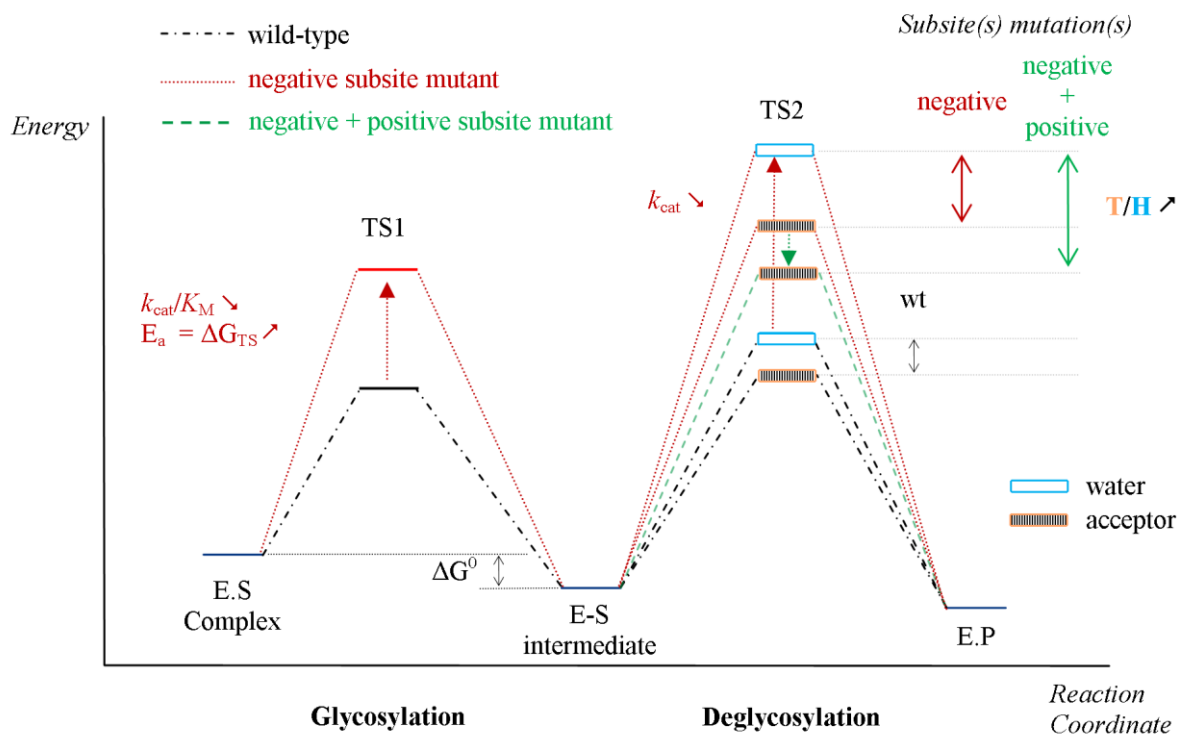
4 Glycosidic bonds are extremely stable and display half-lives of several million years. This can be
5 illustrated by the fact that papyruses from ancient Egypt can still be seen in our museums today.
6 However, in the presence of GHs the half-life of glucosidic bonds in cellulose are reduced to the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

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

16

17

1



2

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

10

1 **Box 3 Basics of enzyme thermodynamics**

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

12 $k = -d[\text{Substrate}]/dt = A.\exp(-E_a/RT)$ (Eq. 1)

13 $k = (k_B.T/h).\exp(-\Delta G_{TS}/RT)$ (Eq. 2)

14 $\Delta E_a = \Delta(\Delta G_{TS}) = -RT.\ln([k_{cat}/K_M]_{mut}/[k_{cat}/K_M]_{wt})$ (Eq. 3)

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

17

1

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

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

12

13 ***On TS properties***

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

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

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

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

26

27 ***Differences between TS1 and TS2***

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

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

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

15 Unfortunately, in the case of retaining GHs, the study of TS is always limited to those developed
16 during hydrolytic reactions, despite the fact that other reagents, such as hydroxylated molecules, can
17 act as acceptors for the deglycosylation step (i.e. transglycosylation). Therefore, in the quest to
18 elucidate the determinants of H/T modulation it is rather evident that water- and carbohydrate-
19 mediated deglycosylation involve different behaviours. Although diffusion issues should be
20 considered as important, thermodynamics are at heart of the enzyme-catalyzed chemical reaction
21 and are probably much more critical, as underlined by *in silico* approaches. Therefore, the key
22 questions regarding the H/T partition appear to concern the properties of the deglycosylation
23 transition state (TS_{2,ROH}) and the impact thereupon of the nature of the reacting acceptor substrate
24 (ROH).

25

26 **NATURALLY-OCCURRING TRANSGLYCOSYLASES: ELUCIDATING NATURE'S** 27 **DESIGN STRATEGY**

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

1 In guise of a general introduction to this section, the reader is referred to Table 1 that underlines
2 the fact that sugar-transferring enzymes are usually catalytically-less efficient (e.g. k_{cat}/K_M values)
3 than hydrolytic counterpart enzymes (85- to 1165-fold lower for GH1 β -glycosidase and GH13
4 sucrose-acting enzymes, respectively). This catalytic sluggishness is likely to be correlated with more
5 energy-demanding TS (for both glycosylation and deglycosylation steps), which lower overall catalytic
6 turnover. In this respect, it is also useful to recall that in a previous study that set out to correlate
7 enzyme and substrate flexibility with catalytic performance, it was proposed that the enzymes we
8 observe today are the result of evolutionary processes that have transformed intrinsically slow,
9 broad specificity prototypes into more efficient catalysts [67]. Of course, this is a rather simplistic
10 view of enzyme evolution and other data suggest that enzymes might have evolved in both directions
11 [68,69], and indeed some GHs (mainly from plants) display both hydrolysis and transglycosylation
12 activities and thus present intermediate cases (i.e. mixed activity) [70–75].

13

14

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 <i>OsBGlu31</i> (<i>exo</i>)	Ferulic acid (acceptor) ^b	1.21	0.05	25.42	[70]
		<i>p</i> NP- β -D-Glcp (donor) ^{b,c}	1.21	9.33	0.13	
	<i>Agrobacterium</i> β -glucosidase (<i>exo</i>)	<i>p</i> NP- β -D-Glcp	169	0.078	2170	[62]
13	<i>Bc</i> strain 251 CGTase (<i>endo</i>)	β -cyclization	329	-	-	[76]
		hydrolysis	3.9	-	-	
	Barley α - amylase (<i>endo</i>)	Blue starch	248	0.52 (mg.mL ⁻¹)	477 (s ⁻¹ .mL.mg ⁻¹)	[77]
		CNP- β -D-maltoheptaoside ^c	122	1.1	111	[78]
	<i>Np</i> AS (<i>exo</i>)	Sucrose (< 20 mM) ^d	0.55	1.9	3.45	
		Sucrose (> 20 mM)	1.28	50.2	0.0255	
		<i>Xag</i> SUH (<i>exo</i>)	Sucrose	66.5	2.24	29.7
16	<i>Ptt</i> XET16-34 (<i>endo</i>)	XGO _{Glc8} (transglycosylation) ^e	0.08	0.4	0.2	[80]
	<i>Tm</i> 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) (<i>exo</i>)	Sucrose (1-kestose production)	0.78	551	-	[81]
	Wheat VI (<i>exo</i>)	Sucrose (hydrolysis)	608	15	-	
33	<i>Tctr</i> S (<i>exo</i>)	Sialyllactose (transglycosylation)	12.6	1.2	10.5	[82]
	<i>Tctr</i> S (<i>exo</i>)	Sialyllactose	0.18	0.29	0.62	[83]
	<i>Tr</i> SA (<i>exo</i>)	(hydrolyse)	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-Glcp)₈ backbone.

2

1

2 **Xyloglucan *endo*-transglycosylases**

3 In terms of understanding the determinants of the τ/H partition, XETs and their hydrolytic
4 counterparts, xyloglucan *endo*-hydrolases (XEH), are extremely interesting enzymes that are usually
5 referred to as xyloglucan *endo*-transglycosylase/hydrolases or XTHs, even though biochemical
6 evidence reveals that most of them are XETs, displaying very little hydrolytic ability. XTHs are mainly
7 grouped in family GH16 (members of GH-B clan), which also contains other hydrolytic GHs enzymes
8 that display a wide variety of substrate specificities [85,86]. The molecular phylogeny of XTH genes,
9 their catalytic properties and *in vivo* functional differences provide criteria for the definition of three
10 major groups. Members of groups I and II exclusively exhibit XET activity, which is also the
11 predominant feature of group III-B. However, members of group III-A (XEH) are mainly hydrolytic
12 [80,87–89]. With regard to XETs, these are known to be important for plant cell wall remodelling,
13 since they catalyze the non-hydrolytic cleavage and religation of xyloglucan molecules through a
14 ping-pong bi-bi mechanism that is subject to competitive inhibition, since competing substrates can
15 act as both the donor and the acceptor [90,91].

16 As explained earlier, the canonical double-displacement mechanism of glycosyl transfer involves
17 the formation of a covalent glycosyl-enzyme intermediate. In XTHs, glycosylation is rapid (< 2 min)
18 and procures a relatively long-lived glycosyl-enzyme intermediate, whose formation is associated
19 with an estimated free energy change (ΔG^0 , Figure 3) of formation of approximately 1.5-2.0 kcal.mol⁻¹
20 [92–94]. Indeed, the glycosyl-enzyme intermediate of *PttXET16-34* (a XET from hybrid poplar *Populus*
21 *tremula x tremuloides*) is approximately 3 h with a $k_{hydr.} = 1.10^{-4} s^{-1}$. Deglycosylation of the glycosyl-
22 *PttXET16-34* intermediate is brought about by the presence of suitable sugar acceptors, such as
23 xylogluco-oligosaccharides. When this criterion is fulfilled, it has been shown that the xylogluco-
24 oligosaccharyl-XET adduct can be fully deglycosylated in 30 min. In this respect, it is also noteworthy
25 that when *PttXET16-34* was supplied with activated β -D-xyloglucan-oligosaccharidic donors (e.g. LG =
26 2-chloro-4-nitrophenyl or fluoride), no activity (neither transglycosylation nor hydrolysis) was
27 observed [95,96]. This underlines the fact that the energetic barrier of TS1 can only be overcome by
28 the presence of a sugar LG in the positive subsites, as is the case for deglycosylation (TS2). This
29 requirement is removed in the case of the *PttXET16-34*-based glycosynthase, since the reaction only
30 proceeds through the 'pseudo' second step (TS2_{ROH}) of the canonical retaining-mechanism.
31 Moreover, it is remarkable that donor substrate binding is dominated by the higher affinity of the
32 xyloglucan moiety for the positive subsites, an interaction that is driven by the presence of aromatic
33 residues. This increased affinity for the positive subsites is thought to be necessary (though not
34 sufficient *per se*) for transglycosylation [91,95].

1 Despite a lack of sequence identity within family GH16, all of its members display a typical β -
2 jellyroll fold that is composed of two large curved β -sheets, stacked in a sandwich-like manner.
3 However, in the case of XTHs specific structural features reflect the specialization of these enzymes
4 toward their highly branched substrates [95]. Notably, according to Brumer et al., starting from an
5 ancestral (hydrolytic) licheninase active on linear 1,3-1,4- β -glucans [97], the deletion of a loop
6 procured the ability to bind highly branched substrates, such as xyloglucan, a characteristic that is
7 shared by both GH16 *endo*-glucanases (EG) and XTHs that display hydrolytic and/or
8 transglycosylation activities. Examples of this are *PtEG16* from *Populus trichocarpa*, which is able to
9 hydrolyze the xylogluco-oligosaccharide XXXGXXXG, and its counterpart *PttXET16-34*, which performs
10 transglycosylation using the same substrate [90,97]. Moreover, the extension of the C-terminal
11 domain differentiates the XTHs from EGs. This XTH feature provides exclusive specificity for
12 xyloglucan (i.e. branched substrates) to this group of GH16 enzymes [91,97]. Finally, regarding XETs
13 and XEHs, X-ray structure data have revealed that in some cases these differ in two loops located
14 between β -strands β 6 and β 7, and between β 8 and β 9, in the vicinity of the active site [80]. The
15 importance of this last observation has been demonstrated through the creation of a β 8/ β 9 loop
16 deletion in the *Tropaeloum majus* XEH (*TmNXG1*- Δ YNIIG mutant), a loop that forms part of subsite +1
17 and interacts with the D-glucosyl residue. This mutation procured an increased τ / h ratio in the initial
18 phase of the reaction, with a 2-fold increase in transglycosylation rate being coupled to a 5.7-fold
19 decrease in hydrolysis rate.

20 Structural and molecular dynamics work performed on *PttXET16-34* and *TmNXG1* has revealed a
21 correlation between the nature of the principal activity and subsite binding interactions, which are
22 combined with subtle differences in dynamic behavior [98]. As a matter of fact, in XETs, the number
23 of H-bonds formed between the enzyme and the acceptor moiety is greater than in XEHs, whereas in
24 XEHs the number of H-bonds formed with the donor moiety is higher. Moreover, a determinant of
25 transglycosylation in XETs appears to be more flexibility in subsite -1, which is detrimental for overall
26 activity, except when a sugar is present in subsite +1.

27

28 **Sucrase-type enzymes**

29 Sucrases are *exo*-enzymes that include glucansucrases (GS) and fructansucrases (FS). Using sucrose as
30 a substrate, these enzymes are able to synthesize homopolysaccharides composed of D-glucosyl or D-
31 fructosyl subunits respectively, with different linkage specificities [99,100]. GS are classified in both
32 GH13 and GH70 family, with GH13 GS being designated amylosucrases (AS). Transglucosylating AS
33 and GS have been extensively studied both in our group [99,101] and in Lubbert Dijkhuizen's group
34 [100,102]. Although AS, GS and FS act on the same substrate, they actually exhibit different protein

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

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

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

25 Compared to GH70 GS, the data available for GH13 AS is more abundant. These enzymes all
26 display a similar 5-domain structure with a deep pocket at the bottom of which sucrose binds to
27 subsites -1 and +1 [112,113]. Three arginines (R226, R415 and R446), located in positive subsites
28 +2/+3, +4 and +1 respectively, are particularly important in the transglucosylation reaction, since
29 these play a crucial role in the docking and positioning of acceptors [114,115]. In a study of the AS
30 from *Neisseria polysaccharea* (NpAS, subfamily 4 of GH13) the positive subsites were submitted to
31 mutagenesis with the aim of improving transglucosylation using unnatural acceptors. Although quite
32 impressive increases in transglucosylation were achieved (395-fold increase), which were
33 accompanied by decreased apparent K_M values, no evidence of significant structural changes that
34 would alter sucrose binding was detected [116]. Therefore, it was concluded that modified loop

1 flexibility and enzyme dynamics are likely to be the determinants of altered substrate recognition
2 and thus responsible for the establishment of a catalytically-productive state. Overall, this study
3 revealed a certain plasticity of subsite +1, because it was possible to isolate mutants that could
4 glucosylate a series of different acceptors, and suggested that the improvement of transglucosylation
5 using unnatural acceptors was facilitated by improved interactions in the positive subsites. In
6 another study, recognition of D-glucosyl moieties in subsite -1 was investigated. This revealed that
7 despite the fact that AS exhibits slow rates, the D-glucosyl is specifically recognized by a complex
8 network of interactions [117]. To further understand the transglycosylating character of *NpAS*, it is
9 useful to compare this enzyme with a hydrolytic counterpart, such as the sucrose hydrolase from
10 *Xanthomonas axonopodis* pv. *glycines* (*XagSUH*). Although this GH13 member shares 57% sequence
11 identity with *NpAS* and is structurally similar (identical 5-domain structure with rmsd value of 1.78 Å)
12 [79], *XagSUH* catalyzes sucrose hydrolysis and is incapable of catalyzing transglucosylation [118]. One
13 main difference between *XagSUH* and *NpAS* has been revealed by acquiring structural snapshots
14 along the catalytic coordinate. This revealed in *XagSUH* that upon sucrose binding a pocket-shaped
15 active site is formed through rigid-body movements of the B and B' domains towards the active site.
16 Moreover, it is noteworthy that the majority of active site residues are conserved between the two
17 enzymes, except for three arginines (R226, R415 and R446) that are substituted in *XagSUH* by other
18 residues (glycine or leucine). Significantly, as mentioned earlier these arginines are essential in *NpAS*
19 for transglucosylation, although the introduction of homologous arginines in *XagSUH* by mutagenesis
20 failed to confer transglucosylation properties to the enzyme, an observation that is consistent with
21 the fact that improvements in transglycosylation first require diminution of hydrolysis [79], especially
22 given the fact that the value of k_{cat} on sucrose is 120-fold higher than that of *NpAS* (Table 1).
23 Surprisingly, this fact was not evoked by the authors, who suggested that hydrolysis in *XagSUH* might
24 be caused by a collateral effect of D-fructose release, which would disorder the B-domain and thus
25 expose the enzyme-bound D-glucosyl moiety to bulk solvent and thus hydrolysis.

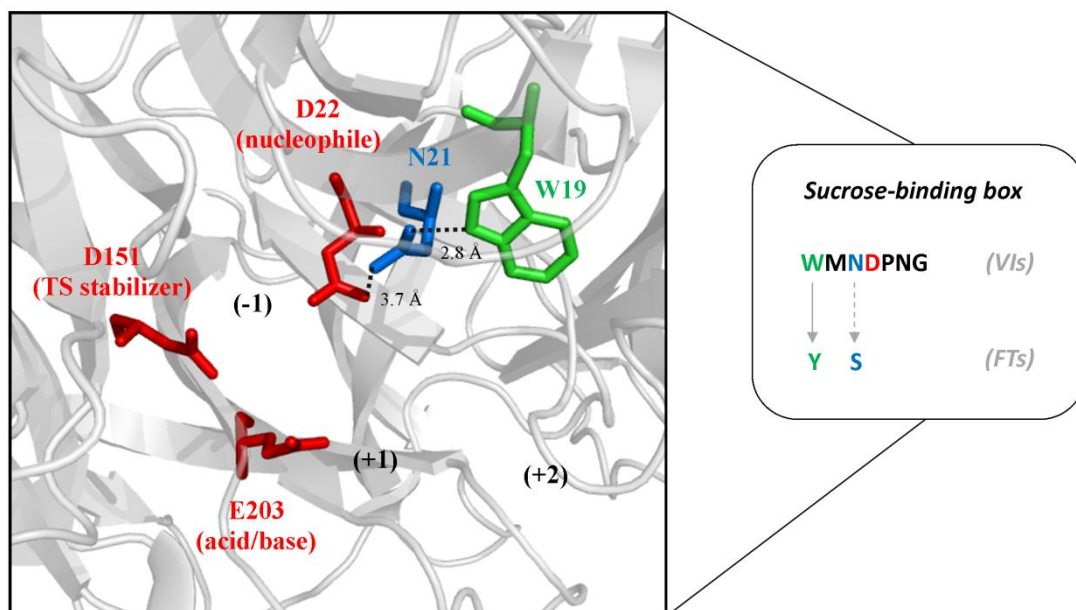
26 In summary, the identification of the key factors that determine the T/H partition in GS has
27 proved to be quite difficult, although it appears evident that interactions in positive subsites play an
28 important role. In this respect, and taking into account the fact that the active site in these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

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

8 Regarding fructose-specific sucrases (FS), which can act on sucrose and/or fructans, these are
9 gathered within families GH68 and GH32 (clan GH-J). The FS in GH68 (i.e. levansucrases,
10 inulosucrases) usually display a dominant hydrolytic activity, accounting for 70-80% of substrate
11 (levan, inulin) conversion. A previous study performed on the single domain levansucrase, SacB from
12 *Bacillus subtilis*, revealed that the addition of transitional and complete C-terminal domains from
13 other FS leads to reductions in hydrolysis (down to 10% of substrate conversion), accompanied by a
14 5-fold increase in transfructosylation. Upon analysis of the chimeric enzymes the authors remarked
15 that the k_{cat} value associated with hydrolysis was unaltered and thus attributed the increase in the
16 τ/H ratio to more favorable positive subsite interactions provided by a structural adjustment in the
17 catalytic site mediated by the addition of extra domains [121].

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

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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.

1

2 Importantly, within the sucrose-binding box, W is always replaced by a Y in FTs from the same
3 subgroup. Likewise, the first N is very often substituted in FTs by S. Engineering of these alternative
4 residues into VIs demonstrated that the disruption of the hydrogen bond network involving the
5 nucleophilic aspartate (i.e. W23Y and N25S) enhanced transglycosylation up to 17-fold when
6 compared to wild-type VIs [81]. Similarly, other studies performed on VIs from yeast [125] or onion
7 [69] led to similar conclusions, although the increase in transglycosylation was more modest.
8 Furthermore, a shift of optimum pH from 3.8-4.8 to 4.8-5.7 was observed for a yeast VI mutant
9 (W19Y-N21S), consistent with an alteration of the ionization state of the catalytic residues [125].
10 Interestingly, the reverse experiment involving the substitution of Y by W in two different FTs failed
11 to procure a more hydrolytic VI-like enzyme [126,127], which suggests that it is much easier to
12 disrupt rather than create a hydrogen bond network !

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

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

30

31 **Cyclodextrin glucanotransferases and α -amylases**

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

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

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

14 In addition to the synthesis of cyclodextrins, CGTases have also been shown to be capable of
15 hydrolysis or to perform the transfer of the bound glycosyl intermediate onto another α -glucan chain
16 (i.e. disproportionation) [130]. In order to prevent hydrolysis, it appears that CGTases have acquired
17 positive subsites that favor sugar recognition. This is illustrated by mutagenesis work that was
18 performed on the positive subsites (+2 and +3) of the *Bacillus circulans* 251 CGTase (*BcCGTase*). The
19 substitution of F183 and F259 in *BcCGTase* by N or S resulted in a 10- to 300-fold decrease in
20 transglycosylation activity (β -cyclization) and a 3- to 20-fold increase in hydrolysis [131]. Similarly, the
21 simultaneous mutation of equivalent residues (F184Q and F260W) in the CGTase from
22 *Thermoanaerobacterium thermosulfurigenes* strain EM1 (Tabium CGTase) and the addition of a third
23 mutation (A231V) converted this enzyme into an α -amylase-like hydrolytic enzyme [132].
24 Impressively, this mutant no longer displayed detectable CGTase activity, with the τ/H ratio being
25 0.0012 (compared to 5 for the parental CGTase). Consistent with these results, another study
26 focusing on the positive subsites in liquefying (hydrolytic) and maltogenic (transglycosylating) α -
27 amylases revealed that increased hydrophobicity in subsites +2/+3 of the α -amylase from *Bacillus*
28 *licheniformis* (BLA) increased the τ/H ratio, reducing the hydrolysis rate (associated with a higher K_M
29 value) on starch by one third [133]. Likewise, the sequence comparison of hydrolytic and maltogenic
30 α -amylases revealed the presence in subsite +1 of a conserved histidine or glutamate residue,
31 respectively [134]. The introduction of the substitution H235E in BLA created a transglycosylation
32 activity, which is undetectable in the wild-type enzyme, but did not drastically affect the efficiency of
33 hydrolysis (72% residual) [135]. Overall, mutations in the positive subsites of CGTases generally
34 provoke a diminution of transglycosylation activity [130], whereas negative subsite mutations mostly

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

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

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

27

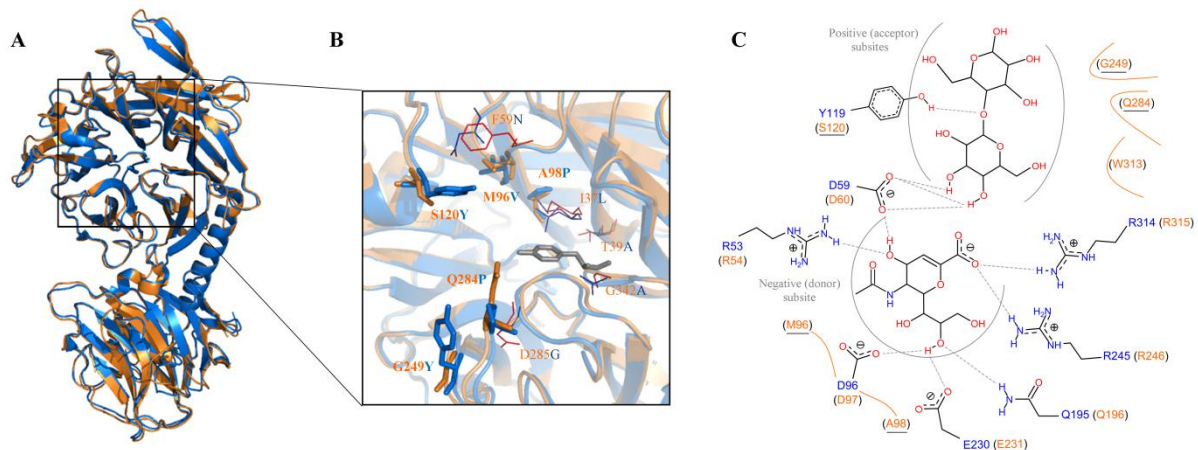
28 **Transferring vs hydrolyzing sialidases**

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

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

10

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1

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

11

1

2 It has been suggested that subtle structural differences are likely to be responsible for the
3 different selectivities of hydrolysis and transglycosylation reactions catalysed by *Trypanosoma*
4 *rangeli* SA (*TrSA*) and *TctrS* respectively. Although these enzymes share 70% amino acid identity, their
5 active sites display distinctive features [144,147–149]. *TctrS* exhibits a narrower, more hydrophobic
6 substrate-binding pocket. This implies that the reactive center is less solvent-exposed and results in
7 an alternative hydrogen bonding pattern with the sialyl donor moiety. Additionally, residue Q284 in
8 *TrSA* is replaced by P283 in *TctrS* (Figure 5B and C), a substitution that alters the conformation of the
9 neighboring W312 residue (W313 in *TrSA*). In *TctrS*, W312 and Y119 (S120 in *TrSA*) form the two
10 lateral walls of the acceptor binding site, providing the basis for stacking interactions with the sugar
11 acceptor. Moreover, it is noteworthy *TctrS* appears to display greater active site flexibility than *TrSA*
12 [83,147,148,151], a point that is exemplified by the study of the inherent motions of Y119 and the
13 strictly conserved Y342 (catalytic nucleophile) residues. According to Demir and Roitberg, structural
14 rearrangements that are triggered by ‘allosteric’ binding of the sialyl-conjugate donor forming a
15 covalent sialyl-enzyme intermediate lead to the creation of a productive acceptor sugar binding site
16 [151].

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

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

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

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

23

24 **ENGINEERED TRANSGLYCOSYLASES**

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

29

30 **Modification of negative subsite interactions**

31 *Enzyme engineering*

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

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

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

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

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

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

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

21 Regarding rational engineering work focused on the nucleophile catalyst, several studies have
22 revealed that modifications of the latter can also have drastic effects on the ability of water to
23 deglycosylate the glycosyl-enzyme intermediate. Recently, it was reported that the introduction of a
24 sulfinate function (i.e. SOO⁻), to replace the catalytic nucleophile of the GH13 dextran glucosidase,
25 provoked a drastic drop in k_{cat} (0.27%), an acidic pK_a shift (from 3.9 to 1.5) and an increase in
26 transglucosylation yields [164]. According to the authors of this work, the observed effects can be
27 attributed to differences in the TS energy barriers between water and acceptor-mediated enzyme
28 deglycosylation. In this respect, shortening or lengthening (E78D or carboxymethylation of the
29 mutant E78C) of the nucleophile residue in the GH11 xylanase from *Bacillus circulans* was also shown
30 to be detrimental for global catalytic efficiency, nucleophile shortening having a greater impact
31 (1600-5000-fold decrease) than lengthening (16-100-fold) [165]. However, in this study no
32 information concerning the impact on the τ/H ratio was reported. Nevertheless, in a very recent
33 study, nucleophile shortening (E134D) in a GH16 EG was shown to introduce glycosynthase-like
34 activity [166]. The resulting enzyme, which retained 2% residual hydrolytic activity and displayed a

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

5

6 **Substrate modifications**

7 The previous section described how modifications in enzyme negative subsites can favor
8 transglycosylation. In a similar manner, several authors have revealed that substrate modifications
9 can procure the same overall effect (i.e. altering the TS energy barrier for water-mediated
10 deglycosylation). An excellent example of this was reported for the GH51 α -L-arabinofuranosidase
11 from *Thermobacillus xylanilyticus* (TxAbf). This enzyme was shown to display much better
12 transglycosylation yields in the presence of the non-natural donor sugar *p*NP- β -D-Galf (75% when
13 using Bn- α -D-Xyl*p* as the acceptor) than with *p*NP- α -L-Araf (7%) [168,169]. Compared to *p*NP- α -L-Araf,
14 *p*NP- β -D-Galf possesses an extra hydroxymethyl moiety at position C5, a difference that is sufficient
15 to decrease by 100-fold the hydrolytic rate and radically increase the K_M value (> 50 mM, compared to
16 0.72 mM on *p*NP- α -L-Araf), changes that are clearly indicative of modified glycosylation and
17 deglycosylation steps. It is noteworthy, that similar results were subsequently observed for the GH51
18 Abf from *Clostridium thermocellum* [170].

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

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

1 be hydrolyzed by the enzyme, since this is catalytically-impotent, being unable to perform
2 glycosylation. However, this rather neat account of how glycosynthases work fails to explain why
3 water does not compete with the acceptor. In fact, the rather elusive answer to this question
4 concerns the donor-borne fluorine atom [171]. This atom is likely to extensively perturb the donor-
5 enzyme interaction that is necessary to overcome the TS energy barrier and thus only counter-
6 interactions provided by the presence of a sugar in the positive subsite are sufficient to decrease the
7 energy of TS2 [25]. In this respect, it is also interesting to note that a correlation has been observed
8 between the ability to create efficient glycosynthases from GHs and the behavior of the
9 corresponding parental GH with 2-fluorosugars [25]. When high k_{trans} and $k_{\text{trans}}/k_{\text{H}_2\text{O}}$ were measured in
10 the presence of such inhibitors, it was predicted that the enzyme would form the basis of a good
11 glycosynthase. Actually, it is highly probable that, in both cases, 'equivalent' TS destabilizations are
12 responsible for this improved selectivity for sugar acceptors over water molecules.

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

18

19 **Positive subsite interactions and impact on the deglycosylation step**

20 Work performed on lysozyme in the 1970's provided the first evidence that acceptor specificity might
21 influence transglycosylation [172]. The role of residues in positive subsites +1/+2 (originally denoted
22 E and F) were shown to be important for transglycosylation activity by contributing to higher binding
23 free energy of the incoming acceptor [173,174]. Similarly, a hypothetical link between
24 transglycosylation and acceptor binding interactions was proposed on the basis of work performed
25 on the GH10 xylanase from *Streptomyces lividans* (XlnA) [175]. In this work, the mutation of a subsite
26 +3 residue (N173D) decreased transglycosylation activity in the presence of xylo-oligosaccharide (DP
27 > 3) acceptors, thus revealing the sensitivity of this reaction to changes in acceptor binding. In
28 another study involving a GH10 xylanase (Xyn10A from *Pseudomonas cellulosa*) the introduction of
29 alanines in subsites +2 (N182), +3 (Y255) and +4 (Y220) provoked a strong decrease in
30 transglycosylation activity [176]. This loss of activity was correlated with increased k_{cat} and K_{M} on
31 xylan (21- and 22-fold, respectively), suggesting that decreased 'affinity' in the positive subsite region
32 accelerates the leaving group departure and facilitates the access of water to the active site.

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

1 (1,4)-mannanases have been subjected to positive subsite engineering. The results of this work
2 revealed that stacking interactions (subsite +1 of Man C) and hydrogen bonding with the acceptor
3 moiety (revealed by the deleterious effect of R171K in subsite + 2 of *TrMan5A*) are critical features
4 for efficient transglycosylation [177,178].

5 The question of acceptor affinity has also been addressed in the case of the aforementioned
6 chitinase, *SmChiA* [161]. By further mutating the transglycosylating variant, *SmChiA*-D313N,
7 introducing the subsite +2 mutation F396W, it was possible to further enhance transglycosylation by
8 several fold (not numerically quantified). Unfortunately, in this study the effect of the mutation
9 F396W alone was not studied, making it impossible to state whether it needs to be combined with
10 D313N in order to observe an effect on transglycosylation. Nevertheless, this example is particularly
11 interesting, because it underlines the importance of the aromatic surface area. The mutation F396W
12 increases this parameter, which might explain the higher rate of the transglycosylation reaction
13 (compared to D313N alone) and the lower catalytic efficiency (the relative k_{cat}/K_M for D313N/F396W
14 and D313N are 1.4 and 4.9% respectively) of this enzyme [161]. Consistent with this result are
15 independent observations that the removal of aromatic residues located in the positive subsites of
16 other chitinases, such as that of *Serratia proteamaculans* (*SpChiD*), *Aspergillus fumigatus* (*AfChiB1*) or
17 *Cycas revolute* (*CrChi-A*), decreases or annihilates transglycosylation activity [159,160,179]. In the
18 case of *AfChiB1*, computational simulation suggested that the complete loss of transglycosylation
19 coupled to the maintenance of hydrolysis in the mutant W137E would be due to the loss of a
20 stacking interaction between W137 and the acceptor sugar. According to the authors, this interaction
21 would be necessary for the efficient attack of the oxazolinium ion intermediate.

22 Another example of how modifications in the acceptor binding site of GHs can lead to τ/H
23 modulation in favor of transglycosylation has been provided for family GH85 *endo*- β -*N*-
24 acetylglucosaminidases (ENGase). The enhancement of transglycosylation, procured by either
25 increased hydrophobicity in the positive subsite (achieved by the mutation Y217F in the ENGase from
26 *Mucor hiemalis*) or mutation of so-called 'gate keeper' amino acids (W216 and W244 in the ENGase
27 from *Arthrobacter protophormiae*), was partially attributed to alterations in active site dynamics
28 [180,181].

29 Although the introduction of aromatic side-chains substitutions into the acceptor binding regions
30 of GHs is frequently used to modulate the τ/H ratio, this is neither an exclusive nor a general strategy.
31 Indeed, the introduction of other mutations (P402D and F328A) into the +1 region of the active site
32 of a family GH36 α -D-galactosidase (subsite +1) actually led to an enlargement of the entrance to the
33 active site and consequently influenced the orientation of the bound acceptor. Such mutations led to
34 4- to 16-fold increases in the yield of transglycosylation products, although in absolute terms the

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

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

12

13 **Water ‘activation’ and channels**

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

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

24 In other studies, tyrosine residues have been identified as water binding determinants. For
25 example, the introduction of a tyrosine near to the active site (V286Y) of the *Bacillus licheniformis* α -
26 amylase led to a 5-fold increase in hydrolytic activity on starch compared to the wild-type enzyme,
27 consistent with the hypothesis that the tyrosine OH group favors water access to the active site
28 [133]. Similarly, the key role of tyrosine residues has been demonstrated in other GH13 α -amylases
29 [185,186] and in *endo*-xylanases [187,188], with the elimination (mutation to F, A, S or N) of tyrosine
30 generally leading to the loss of anchoring points for water molecules, coupled to increased
31 hydrophobicity and thus overall lower hydrolysis. It is also noteworthy, that the mutation of the
32 water-binding tyrosine (Y198F) in a GH8 *exo*-acting, inverting xylanase (Rex) led to the creation of an
33 unusual glycosynthase that displays a high (4.7 s^{-1}) fluoride release rate when fed with α -xylobiosyl

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

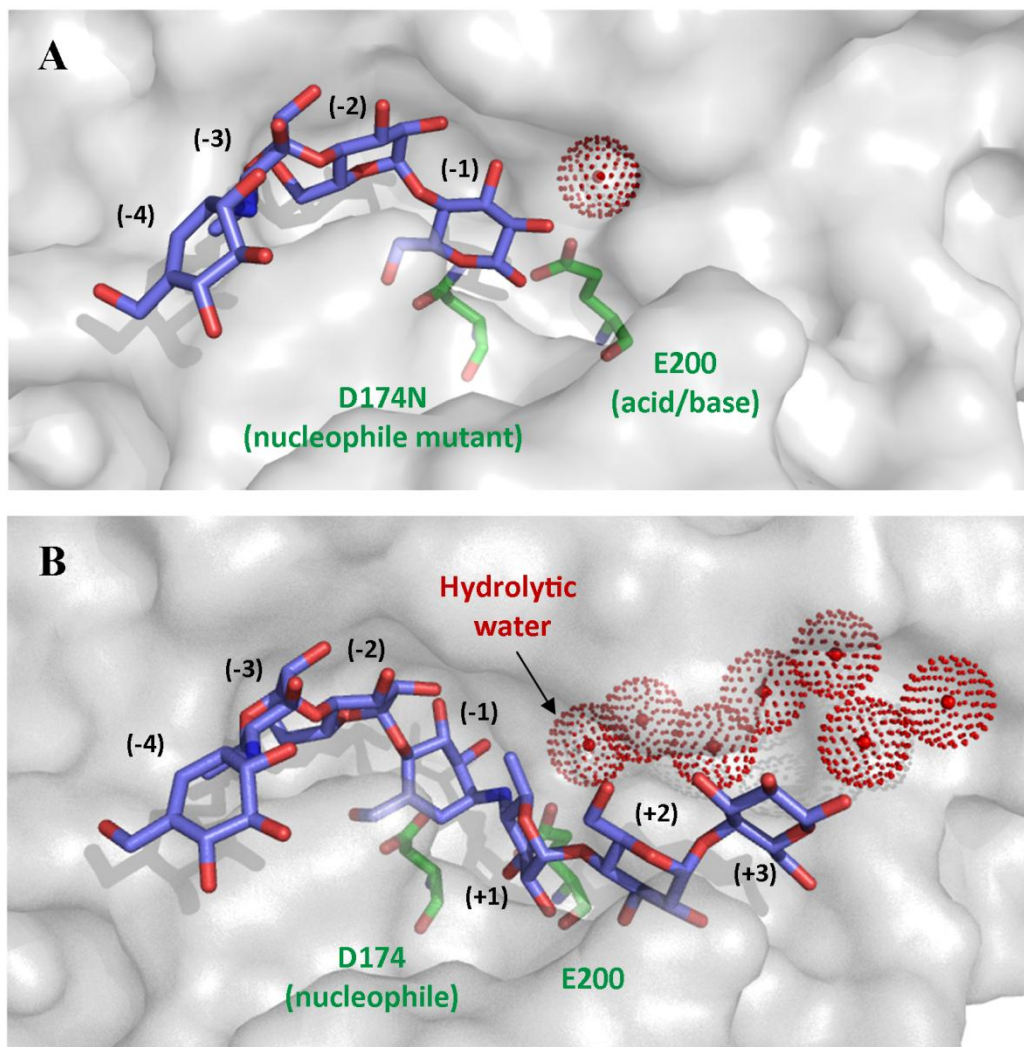
5 Tyrosines that provide catalytic assistance have also been revealed in GH85 enzymes, in
6 particular in the *Arthrobacter protophormiae* ENGase A. The creation in this enzyme of mutations
7 Y205F (negative subsite) and Y299F (acceptor region) led to 2.5- and 3-fold increases respectively in
8 transglycosylation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

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

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

1 surface to the active site [196].

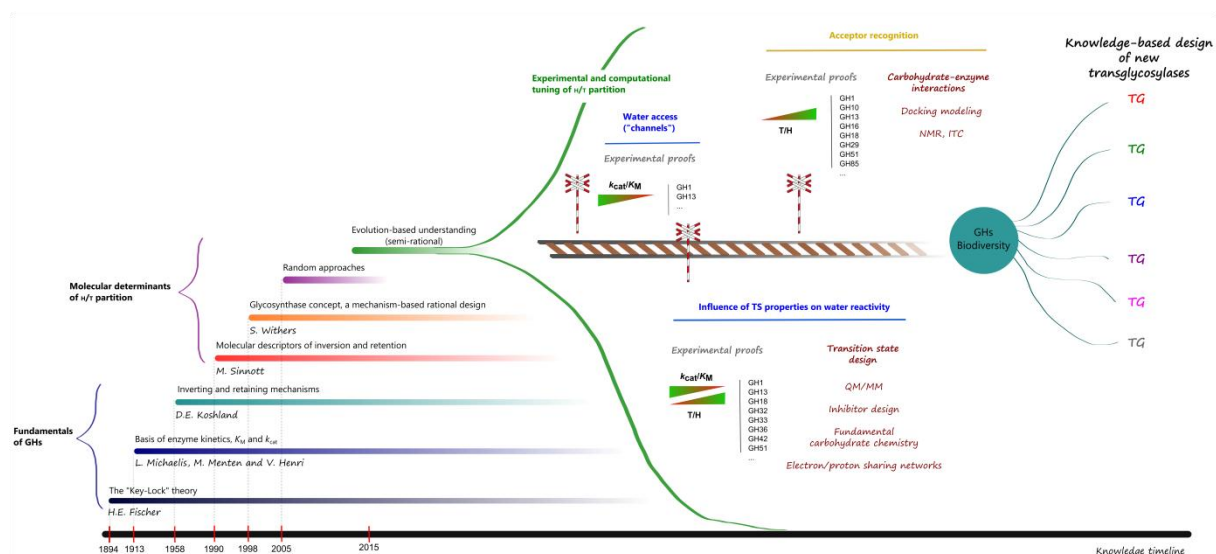
2



1
 2 **Figure 6 Substrate-induced water channel formation.** (A) Complex of the inactive
 3 *Pseudoalteromonas haloplanktis* α -amylase D174N with acarbose bound in non-productive mode
 4 (PDB ID: 1KXH [191]) and (B) complex with a heptasaccharide (a transglycosylation product) showing
 5 7 well-ordered water molecules running from the surface to the catalytic center (PDB ID: 1G94
 6 [191]). The graphic was prepared using PyMOL Molecular Graphics System, v0.99 (Schrödinger, LLC).
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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.



9

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

12

13 **Conclusions**

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

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

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

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

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

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

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

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

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

11

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16 **REFERENCES**

- 17 1 Varki, A., Cummings, R. D., Esko, J. D., Freeze, H. H., Stanley, P., Bertozzi, C. R., Hart, G. W. and
18 Etzler, M. E. (2009) In *Essentials of Glycobiology*, Cold Spring Harbor Lab Press
- 19 2 Edelman, J. (1956) The formation of oligosaccharides by enzymic transglycosylation. *Adv.*
20 *Enzym. Relat. Areas Mol. Biol.* **17**, 189–232
- 21 3 Breton, C., Fournel-Gigleux, S. and Palcic, M. M. (2012) Recent structures, evolution and
22 mechanisms of glycosyltransferases. *Curr. Opin. Struct. Biol.* **22**, 540–549
- 23 4 Lairson, L. L., Henrissat, B., Davies, G. J. and Withers, S. G. (2008) Glycosyltransferases:
24 structures, functions, and mechanisms. *Annu. Rev. Biochem.* **77**, 521–555
- 25 5 Field, R. A. (2011) Glycobiology: challenging reaction equilibria. *Nat. Chem. Biol.* **7**, 658–659
- 26 6 Gantt, R. W., Peltier-Pain, P., Cournoyer, W. J. and Thorson, J. S. (2011) Using simple donors to
27 drive the equilibria of glycosyltransferase-catalyzed reactions. *Nat. Chem. Biol.* **7**, 685–691
- 28 7 Cote, G. L. and Tao, B. Y. (1990) Oligosaccharide synthesis by enzymatic transglycosylation.
29 *Glycoconjugate J.* **7**, 145–162
- 30 8 Mackenzie, L. F., Wang, Q., Warren, R. A. J. and Withers, S. G. (1998) Glycosynthases: mutant
31 glycosidases for oligosaccharide synthesis. *J. Am. Chem. Soc.* **120**, 5583–5584
- 32 9 Malet, C. and Planas, A. (1998) From β -glucanase to β -glucansynthase: glycosyl transfer to α -
33 glycosyl fluorides catalyzed by a mutant endoglucanase lacking its catalytic nucleophile. *FEBS*
34 *Lett.* **440**, 208–212
- 35 10 Moracci, M., Trincone, A. and Rossi, M. (2001) Glycosynthases: new enzymes for
36 oligosaccharide synthesis. *J. Mol. Catal. B Enzym.* **11**, 155–163
- 37 11 Perugino, G., Trincone, A., Rossi, M. and Moracci, M. (2004) Oligosaccharide synthesis by
38 glycosynthases. *Trends Biotechnol.* **22**, 31–37
- 39 12 Faijes, M. and Planas, A. (2007) In vitro synthesis of artificial polysaccharides by glycosidases
40 and glycosynthases. *Carbohydr. Res.* **342**, 1581–1594

- 1 13 Rakić, B. and Withers, S. G. (2009) Recent developments in glycoside synthesis with
2 glycosynthases and thioglycoligases. *Aust. J. Chem.* **62**, 510–520
- 3 14 Cobucci-Ponzano, B. and Moracci, M. (2012) Glycosynthases as tools for the production of
4 glycan analogs of natural products. *Nat. Prod. Rep.* **29**, 697–709
- 5 15 Schmaltz, R. M., Hanson, S. R. and Wong, C.-H. (2011) Enzymes in the synthesis of
6 glycoconjugates. *Chem. Rev.* **111**, 4259–4307
- 7 16 Kobayashi, S. and Makino, A. (2009) Enzymatic polymer synthesis: an opportunity for green
8 polymer chemistry. *Chem. Rev.* **109**, 5288–5353
- 9 17 Kadokawa, J. (2011) Precision polysaccharide synthesis catalyzed by enzymes. *Chem. Rev.*
10 **111**, 4308–4345
- 11 18 Zechel, D. L. and Withers, S. G. (2000) Glycosidase mechanisms: anatomy of a finely tuned
12 catalyst. *Acc. Chem. Res.* **33**, 11–18
- 13 19 Vasella, A., Davies, G. J. and Böhm, M. (2002) Glycosidase mechanisms. *Curr. Opin. Chem.*
14 *Biol.* **6**, 619–629
- 15 20 Vuong, T. V and Wilson, D. B. (2010) Glycoside hydrolases: catalytic base/nucleophile
16 diversity. *Biotechnol. Bioeng.* **107**, 195–205
- 17 21 Henrissat, B. (1991) A classification of glycosyl hydrolases based sequence similarities amino
18 acid. *Biochem. J.* **280**, 309–316
- 19 22 Henrissat, B. and Davies, G. (1997) Structural and sequence-based classification of glycoside
20 hydrolases. *Curr. Opin. Struct. Biol.* **7**, 637–644
- 21 23 Lombard, V., Golaconda Ramulu, H., Drula, E., Coutinho, P. M. and Henrissat, B. (2014) The
22 carbohydrate-active enzymes database (CAZy) in 2013. *Nucleic Acids Res.* **42**, D490–D495
- 23 24 Street, I. P., Kempton, J. B. and Withers, S. G. (1992) Inactivation of a β -glucosidase through
24 the accumulation of a stable 2-deoxy-2-fluoro- α -D-glucopyranosyl-enzyme intermediate: a
25 detailed investigation. *Biochemistry* **31**, 9970–9978
- 26 25 Ducros, M.-A., Tarling, C. A., Zechel, D. L., Brzozowski, A. M., Frandsen, T. P., Ossowski, I. Von,
27 Withers, S. G. and Davies, G. J. (2003) Anatomy of glycosynthesis : structure and kinetics of
28 the *Humicola insolens* Cel7B E197A and E197S glycosynthase mutants. *Chem. Biol.* **10**, 619–
29 628
- 30 26 Koshland Jr., D. E. (1953) Stereochemistry and the mechanism of enzymatic reactions. *Biol.*
31 *Rev.* **28**, 416–436
- 32 27 Eienthal, R., Danson, M. J. and Hough, D. W. (1997) Catalytic efficiency and k_{cat}/K_M : a useful
33 comparator? *Trends Biotechnol.* **25**, 247–249
- 34 28 Johnson, K. A. (2013) A century of enzyme kinetic analysis, 1913 to 2013. *FEBS Lett.* **587**,
35 2753–2766.
- 36 29 Fischer, E. (1894) Einfluss der configuration auf die wirkung der enzyme. *Ber. Dtsch. Chem.*
37 *Ges.* **27**, 2985–2993
- 38 30 Koshland, D. E. (1995) The key–lock theory and the induced fit theory. *Angew. Chem. Int. Ed.*
39 **33**, 2375–2378
- 40 31 Davies, G. J., Wilson, K. S. and Henrissat, B. (1997) Nomenclature for sugar-binding subsites in
41 glycosyl hydrolases. *Biochem. J.* **321**, 557–559
- 42 32 Wolfenden, R. and Snider, M. J. (2001) The depth of chemical time and the power of enzymes
43 as catalysts. *Acc. Chem. Res.* **34**, 938–945
- 44 33 Pauling, L. (1946) Molecular architecture and biological reactions. *Chem. Eng. News* **24**, 1375–
45 1377
- 46 34 Wolfenden, R., Lu, X. and Young, G. (1998) Spontaneous hydrolysis of glycosides. *J. Am. Chem.*
47 *Soc.* **120**, 6814–6815
- 48 35 Eyring, H. (1935) The activated complex in chemical reactions. *J. Chem. Phys.* **3**, 107–115
- 49 36 Warshel, A. (1981) Electrostatic basis of structure-function correlation in proteins. *Acc. Chem.*
50 *Res.* **14**, 284–290
- 51 37 Tokuriki, N. and Tawfik, D. S. (2009) Protein dynamism and evolvability. *Science* **324**, 203–207
- 52 38 Chouard, T. (2011) Breaking the protein rules. *Nature* **471**, 151–153

- 1 39 Glowacki, D. R., Harvey, J. N. and Mulholland, A. J. (2012) Taking Ockham's razor to enzyme
2 dynamics and catalysis. *Nat. Chem.* **4**, 169–176
- 3 40 Davies, G. J., Ducros, V. M.-A., Varrot, A. and Zechel, D. L. (2003) Mapping the conformational
4 itinerary of β -glycosidases by X-ray crystallography. *Biochem. Soc. Trans.* **31**, 523–527
- 5 41 Taha, H. A., Richards, M. R. and Lowary, T. L. (2013) Conformational analysis of furanoside-
6 containing mono- and oligosaccharides. *Chem. Rev.* **113**, 1851–1876
- 7 42 Houseknecht, J. B., Altona, C., Hadad, C. M. and Lowary, T. L. (2002) Conformational analysis
8 of furanose rings with PSEUROT: parametrization for rings possessing the arabino, lyxo, ribo,
9 and xylo stereochemistry and application to arabinofuranosides. *J. Org. Chem.* **67**, 4647–4651
- 10 43 Sinnott, M. L. (1990) Catalytic mechanism of enzymic glycosyl transfer. *Chem. Rev.* **90**, 1171–
11 1202
- 12 44 Vocadlo, D. J. and Davies, G. J. (2008) Mechanistic insights into glycosidase chemistry. *Curr.*
13 *Opin. Chem. Biol.* **12**, 539–555
- 14 45 Hovel, K., Shallom, D., Niefind, K., Belakhov, V., Shoham, G., Baasov, T., Shoham, Y. and
15 Schomburg, D. (2003) Crystal structure and snapshots along the reaction pathway of a family
16 51 α -L-arabinofuranosidase. *EMBO J.* **22**, 4922–4932
- 17 46 Davies, G. J., Planas, A. and Rovira, C. (2012) Conformational analyses of the reaction
18 coordinate of glycosidases. *Acc. Chem. Res.* **45**, 308–316
- 19 47 Sulzenbacher, G., Driguez, H., Henrissat, B., Schülein, M. and Davies, G. J. (1996) Structure of
20 the *Fusarium oxysporum* endoglucanase I with a nonhydrolyzable substrate analogue:
21 substrate distortion gives rise to the preferred axial orientation for the leaving group.
22 *Biochemistry* **35**, 15280–15287
- 23 48 Davies, G. J., Mackenzie, L., Varrot, A., Dauter, M. and Brzozowski, A. M. (1998) Snapshots
24 along an enzymatic reaction coordinate: analysis of a retaining β -glycosidase. *Biochemistry*
25 **37**, 11707–11713
- 26 49 Biarnés, X., Nieto, J., Planas, A. and Rovira, C. (2006) Substrate distortion in the Michaelis
27 complex of *Bacillus* 1,3-1,4- β -glucanase. Insight from first principles molecular dynamics
28 simulations. *J. Biol. Chem.* **281**, 1432–1441
- 29 50 Biarnés, X., Ardèvol, A., Iglesias-Fernández, J., Planas, A. and Rovira, C. (2011) Catalytic
30 itinerary in 1,3-1,4- β -glucanase unraveled by QM/MM metadynamics. Charge is not yet fully
31 developed at the oxocarbenium ion-like transition state. *J. Am. Chem. Soc.* **133**, 20301–20309
- 32 51 Zechel, D. L., Reid, S. P., Stoll, D., Nashiru, O., Warren, R. A. J. and Withers, S. G. (2003)
33 Mechanism, mutagenesis, and chemical rescue of a β -mannosidase from *Cellulomonas fimi*.
34 *Biochemistry* **42**, 7195–7204
- 35 52 Namchuk, M. N. and Withers, S. G. (1995) Mechanism of *Agrobacterium* β -glucosidase: kinetic
36 analysis of the role of noncovalent enzyme/substrate interactions. *Biochemistry* **34**, 16194–
37 16202
- 38 53 Notenboom, V., Birsan, C., Nitz, M., Rose, D. R., Warren, R. A. J. and Withers, S. G. (1998) Insights
39 into transition state stabilization of the β -1,4-glycosidase Cex by covalent intermediate
40 accumulation in active site mutants. *Nat. Struct. Biol.* **5**, 812–818
- 41 54 Sierks, M. R. and Svensson, B. (1992) Kinetic identification of a hydrogen bonding pair in the
42 glucoamylase-maltose transition state complex. *Protein Eng.* **5**, 185–188
- 43 55 Kim, Y.-W., Zhang, R., Chen, H. and Withers, S. G. (2010) O-glycoligases, a new category of
44 glycoside bond-forming mutant glycosidases, catalyse facile syntheses of isoprimeverosides.
45 *Chem. Commun.* **46**, 8725–8727
- 46 56 Heightman, T. D. and Vasella, A. T. (1999) Recent insights into inhibition, structure, and
47 mechanism of configuration-retaining glycosidases. *Angew. Chem. Int. Ed.* **38**, 750–770
- 48 57 Nerinckx, W., Desmet, T., Piens, K. and Claeysens, M. (2005) An elaboration on the *syn-anti*
49 proton donor concept of glycoside hydrolases: electrostatic stabilisation of the transition state
50 as a general strategy. *FEBS Lett.* **579**, 302–312
- 51 58 Nerinckx, W., Desmet, T. and Claeysens, M. (2003) A hydrophobic platform as a
52 mechanistically relevant transition state stabilising factor appears to be present in the active

- 1 centre of all glycoside hydrolases. FEBS Lett. **538**, 1–7
- 2 59 Schramm, V. L. (2011) Enzymatic transition states, transition-state analogs, dynamics,
3 thermodynamics, and lifetimes. Annu. Rev. Biochem. **80**, 703–732
- 4 60 Bigeleisen, J. and Mayer, M. G. (1947) Calculation of equilibrium constants for isotopic
5 exchange reactions. J. Chem. Phys. **15**, 261–267
- 6 61 Cleland, W. W. (2003) The use of isotope effects to determine enzyme mechanisms. J. Biol.
7 Chem. **278**, 51975–51984
- 8 62 Kempton, J. B. and Withers, S. G. (1992) Mechanism of *Agrobacterium* β -glucosidase: kinetic
9 studies. Biochemistry **31**, 9961–9969
- 10 63 Wang, J., Hou, Q., Sheng, X., Gao, J., Liu, Y. and Liu, C. (2013) Theoretical study on the
11 deglycosylation mechanism of rice BGlu1 β -glucosidase. Int. J. Quantum Chem. **113**, 1071–
12 1075
- 13 64 Wang, J., Hou, Q., Dong, L., Liu, Y. and Liu, C. (2011) QM/MM studies on the glycosylation
14 mechanism of rice BGlu1 β -glucosidase. J. Mol. Graph. Model. **30**, 148–152
- 15 65 Brás, N. F., Ramos, M. J. and Fernandes, P. A. (2010) DFT studies on the β -glycosidase catalytic
16 mechanism: the deglycosylation step. J. Mol. Struct. THEOCHEM **946**, 125–133
- 17 66 Badieyan, S., Bevan, D. R. and Zhang, C. (2012) Probing the active site chemistry of β -
18 glucosidases along the hydrolysis reaction pathway. Biochemistry **51**, 8907–8918
- 19 67 Demetrius, L. (1998) Role of enzyme-substrate flexibility in catalytic activity: an evolutionary
20 perspective. J. Theor. Biol. **194**, 175–194
- 21 68 del-Rio, G., Morett, E. and Soberon, X. (1997) Did cyclodextrin glycosyltransferases evolve
22 from α -amylases? FEBS Lett. **416**, 221–224
- 23 69 Ritsema, T., Hernández, L., Verhaar, A., Altenbach, D., Boller, T., Wiemken, A. and Smeekens,
24 S. (2006) Developing fructan-synthesizing capability in a plant invertase via mutations in the
25 sucrose-binding box. Plant J. **48**, 228–237
- 26 70 Luang, S., Cho, J.-I., Mahong, B., Opassiri, R., Akiyama, T., Phasai, K., Komvongsa, J., Sasaki, N.,
27 Hua, Y., Matsuba, Y., Ozeki, Y., Jeon, J.-S. and Cairns, J. R. K. (2013) Rice Os9BGlu31 is a
28 transglucosidase with the capacity to equilibrate phenylpropanoid, flavonoid, and
29 phytohormone glycoconjugates. J. Biol. Chem. **288**, 10111–10123
- 30 71 Schröder, R., Atkinson, R. G. and Redgwell, R. J. (2009) Re-interpreting the role of endo- β -
31 mannanases as mannan endotransglycosylase/hydrolases in the plant cell wall. Ann. Bot. **104**,
32 197–204
- 33 72 Johnston, S. L., Prakash, R., Chen, N. J., Kumagai, M. H., Turano, H. M., Cooney, J. M.,
34 Atkinson, R. G., Paull, R. E., Cheetamun, R., Bacic, A., Brummell, D. A. and Schröder, R. (2013)
35 An enzyme activity capable of endotransglycosylation of heteroxylan polysaccharides is
36 present in plant primary cell walls. Planta **237**, 173–187
- 37 73 Franková, L. and Fry, S. C. (2011) Phylogenetic variation in glycosidases and glycanases acting
38 on plant cell wall polysaccharides, and the detection of transglycosidase and trans- β -xylanase
39 activities. Plant J. **67**, 662–681
- 40 74 Franková, L. and Fry, S. C. (2012) Trans- α -xylosidase and trans- β -galactosidase activities,
41 widespread in plants, modify and stabilize xyloglucan structures. Plant J. **71**, 45–60
- 42 75 Fry, S. C. (2004) Primary cell wall metabolism: tracking the careers of wall polymers in living
43 plant cells. New Phytol. **161**, 641–675
- 44 76 van der Veen, B. A., Uitdehaag, J. C., Penninga, D., van Alebeek, G. J., Smith, L. M., Dijkstra, B.
45 W. and Dijkhuizen, L. (2000) Rational design of cyclodextrin glycosyltransferase from *Bacillus*
46 *circulans* strain 251 to increase α -cyclodextrin production. J. Mol. Biol. **296**, 1027–1038
- 47 77 Mori, H., Bak-Jensen, K. S. and Svensson, B. (2002) Barley α -amylase Met53 situated at the
48 high-affinity subsite -2 belongs to a substrate binding motif in the $\beta \rightarrow \alpha$ loop 2 of the catalytic
49 (β/α)₈-barrel and is critical for activity and substrate specificity. Eur. J. Biochem. **269**, 5377–
50 5390
- 51 78 Potocki de Montalk, G., Remaud-Simeon, M., Willemot, R. M., Sarçabal, P., Planchot, V. and
52 Monsan, P. (2000) Amylosucrase from *Neisseria polysaccharea*: novel catalytic properties.

- 1 FEBS Lett. **471**, 219–223
- 2 79 Kim, M.-I., Kim, H.-S., Jung, J. and Rhee, S. (2008) Crystal structures and mutagenesis of
3 sucrose hydrolase from *Xanthomonas axonopodis* pv. *glycines*: insight into the exclusively
4 hydrolytic amylosucrase fold. *J. Mol. Biol.* **380**, 636–647
- 5 80 Baumann, M. J., Eklöf, J. M., Michel, G., Kallas, A. M., Teeri, T. T., Czjzek, M. and Brumer, H.
6 (2007) Structural evidence for the evolution of xyloglucanase activity from xyloglucan *endo*-
7 transglycosylases: biological implications for cell wall metabolism. *Plant Cell* **19**, 1947–1963
- 8 81 Schroeven, L., Lammens, W., Van Laere, A. and Van den Ende, W. (2008) Transforming wheat
9 vacuolar invertase into a high affinity sucrose: sucrose 1-fructosyltransferase. *New Phytol.*
10 **180**, 822–831
- 11 82 Ribeirão, M., Pereira-Chioccola, V. L., Eichinger, D., Rodrigues, M. M. and Schenkman, S.
12 (1997) Temperature differences for *trans*-glycosylation and hydrolysis reaction reveal an
13 acceptor binding site in the catalytic mechanism of *Trypanosoma cruzi* *trans*-sialidase.
14 *Glycobiology* **7**, 1237–1246
- 15 83 Paris, G., Ratier, L., Amaya, M. F., Nguyen, T., Alzari, P. M. and Frasch, A. C. C. (2005) A
16 sialidase mutant displaying *trans*-sialidase activity. *J. Mol. Biol.* **345**, 923–934
- 17 84 Fry, S. C., York, W. S., Albersheim, P., Darvill, A., Hayashi, T., Joseleau, J.-P., Kato, Y., Lorences,
18 E. P., Maclachlan, G. A., McNeil, M., Mort, A. J., Reid, J. S. G., Seitz, H. U., Selvendran, R. R.,
19 Voragen, A. G. J. and White A. R. (1993) An unambiguous nomenclature for xyloglucan-derived
20 oligosaccharides. *Physiol. Plant.* **89**, 1–3
- 21 85 Gilbert, H. J., Stålbrand, H. and Brumer, H. (2008) How the walls come crumbling down: recent
22 structural biochemistry of plant polysaccharide degradation. *Curr. Opin. Plant Biol.* **11**, 338–
23 348
- 24 86 Strohmeier, M., Hrmova, M., Fischer, M., Harvey, A. J., Fincher, G. B. and Pleiss, J. (2004)
25 Molecular modeling of family GH16 glycoside hydrolases: Potential roles for xyloglucan
26 transglucosylases/hydrolases in cell wall modification in the poaceae. *Protein Sci.* **13**, 3200–
27 3213
- 28 87 Campbell, P. and Braam, J. (1999) Xyloglucan endotransglycosylases: diversity of genes,
29 enzymes and potential wall-modifying functions. *Trends Plant. Sci.* **4**, 361–366
- 30 88 Rose, J. K. C., Braam, J., Fry, S. C. and Nishitani, K. (2002) The XTH family of enzymes involved
31 in xyloglucan endotransglucosylation and endohydrolysis: current perspectives and a new
32 unifying nomenclature. *Plant Cell Physiol.* **43**, 1421–1435
- 33 89 Eklöf, J. M. and Brumer, H. (2010) The XTH gene family: an update on enzyme structure,
34 function, and phylogeny in xyloglucan remodeling. *Plant Physiol.* **153**, 456–466
- 35 90 Saura-Valls, M., Fauré, R., Ragàs, S., Piens, K., Brumer, H., Teeri, T. T., Cottaz, S., Driguez, H.
36 and Planas, A. (2006) Kinetic analysis using low-molecular mass xyloglucan oligosaccharides
37 defines the catalytic mechanism of a *Populus* xyloglucan endotransglycosylase. *Biochem. J.*
38 **395**, 99–106
- 39 91 Saura-Valls, M., Fauré, R., Brumer, H., Teeri, T. T., Cottaz, S., Driguez, H. and Planas, A. (2008)
40 Active-site mapping of a *Populus* xyloglucan *endo*-transglycosylase with a library of xylogluco-
41 oligosaccharides. *J. Biol. Chem* **283**, 21853–21863
- 42 92 Piens, K., Fauré, R., Sundqvist, G., Baumann, M. J., Saura-Valls, M., Teeri, T. T., Cottaz, S.,
43 Planas, A., Driguez, H. and Brumer, H. (2008) Mechanism-based labeling defines the free
44 energy change for formation of the covalent glycosyl-enzyme intermediate in a xyloglucan
45 *endo*-transglycosylase. *J. Biol. Chem.* **283**, 21864–21872
- 46 93 Steele, N. M. and Fry, S. C. (1999) Purification of xyloglucan endotransglycosylases (XETs): a
47 generally applicable and simple method based on reversible formation of an enzyme–
48 substrate complex. *Biochem. J.* **340**, 207–211
- 49 94 Sulová, Z., Takáčová, M., Steele, N. M., Fry, S. C. and Farkaš, V. (1998) Xyloglucan
50 endotransglycosylase: evidence for the existence of a relatively stable glycosyl-enzyme
51 intermediate. *Biochem. J.* **330**, 1475–1480
- 52 95 Johansson, P., Brumer, H., Baumann, M. J., Kallas, A. M., Henriksson, H., Denman, S. E., Teeri,

- 1 T. T. and Jones, T. A. (2004) Crystal structures of a poplar xyloglucan endotransglycosylase
2 reveal details of transglycosylation acceptor binding. *Plant Cell* **16**, 874–886
- 3 96 Fauré, R., Saura-Valls, M., Brumer, H., Planas, A., Cottaz, S. and Driguez, H. (2006) Synthesis of
4 a library of xylogluco-oligosaccharides for active-site mapping of xyloglucan *endo*-
5 transglycosylase. *J. Org. Chem.* **71**, 5151–5161
- 6 97 Eklöf, J. M., Shojania, S., Okon, M., McIntosh, L. P. and Brumer, H. (2013) Structure-function
7 analysis of a broad specificity *Populus trichocarpa* *endo*- β -glucanase reveals an evolutionary
8 link between bacterial licheninases and plant *XTH* gene products. *J. Biol. Chem.* **288**, 15786–
9 15799
- 10 98 Mark, P., Baumann, M. J., Eklöf, J. M., Gullfot, F., Michel, G., Kallas, A. M., Teeri, T. T., Brumer,
11 H. and Czjzek, M. (2009) Analysis of nasturtium *TmNXG1* complexes by crystallography and
12 molecular dynamics provides detailed insight into substrate recognition by family GH16
13 xyloglucan *endo*-transglycosylases and *endo*-hydrolases. *Proteins* **75**, 820–836
- 14 99 Monsan, P., Remaud-Siméon, M. and André, I. (2010) Transglucosidases as efficient tools for
15 oligosaccharide and glucoconjugate synthesis. *Curr. Opin. Microbiol.* **13**, 293–300
- 16 100 van Hijum, S. A. F. T., Kralj, S., Ozimek, L. K., Dijkhuizen, L. and van Geel-schutten, I. G. H.
17 (2006) Structure-Function relationships of glucansucrase and fructansucrase enzymes from
18 lactic acid bacteria. *Microbiol. Mol. Biol. Rev.* **70**, 157–176
- 19 101 Monchois, V., Willemot, R. M. and Monsan, P. (1999) Glucansucrases: mechanism of action
20 and structure-function relationships. *FEMS Microbiol. Rev.* **23**, 131–151
- 21 102 Leemhuis, H., Pijning, T., Dobruchowska, J. M., van Leeuwen, S. S., Kralj, S., Dijkstra, B. W. and
22 Dijkhuizen, L. (2013) Glucansucrases: three-dimensional structures, reactions, mechanism, α -
23 glucan analysis and their implications in biotechnology and food applications. *J. Biotechnol.*
24 **163**, 250–272
- 25 103 Stam, M. R., Danchin, E. G. J., Rancurel, C., Coutinho, P. M. and Henrissat, B. (2006) Dividing
26 the large glycoside hydrolase family 13 into subfamilies: towards improved functional
27 annotations of α -amylase-related proteins. *Protein Eng. Des. Sel.* **19**, 555–562
- 28 104 Vujicic-Zagar, A., Pijning, T., Kralj, S., López, C. A. and Eeuwema, W. (2010) Crystal structure of
29 a 117 kDa glucansucrase fragment provides insight into evolution and product specificity of
30 GH70 enzymes. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 21406–21411
- 31 105 Ito, K., Ito, S., Shimamura, T., Weyand, S., Kawarasaki, Y., Misaka, T., Abe, K., Kobayashi, T.,
32 Cameron, A. D. and Iwata, S. (2011) Crystal structure of glucansucrase from the dental caries
33 pathogen *Streptococcus mutans*. *J. Mol. Biol.* **408**, 177–186
- 34 106 Brison, Y., Pijning, T., Malbert, Y., Fabre, É., Mourey, L., Morel, S., Potocki-Véronèse, G.,
35 Monsan, P., Tranier, S., Remaud-Siméon, M. and Dijkstra, B. W. (2012) Functional and
36 structural characterization of α -(1 \rightarrow 2) branching sucrose derived from DSR-E glucansucrase. *J.*
37 *Biol. Chem.* **287**, 7915–7924
- 38 107 Leemhuis, H., Pijning, T., Dobruchowska, J. M., Dijkstra, B. W. and Dijkhuizen, L. (2012)
39 Glycosidic bond specificity of glucansucrases: on the role of acceptor substrate binding
40 residues. *Biocatal. Biotransfor.* **30**, 366–376
- 41 108 Kralj, S., van Geel-Schutten, G. H., van der Maarel, M. J. E. C. and Dijkhuizen, L. (2004)
42 Biochemical and molecular characterization of *Lactobacillus reuteri* 121 reuteransucrase.
43 *Microbiol.* **150**, 2099–2112
- 44 109 Kralj, S., van Leeuwen, S. S., Valk, V., Eeuwema, W., Kamerling, J. P. and Dijkhuizen, L. (2008)
45 Hybrid reuteransucrase enzymes reveal regions important for glucosidic linkage specificity
46 and the transglucosylation/hydrolysis ratio. *FEBS J.* **275**, 6002–6010
- 47 110 Goldberg, R. N., Tewaril, Y. B. and Ahluwaliaq, J. C. (1989) Thermodynamics of the hydrolysis
48 of sucrose. *J. Biol. Chem.* **264**, 9901–9904
- 49 111 Kralj, S., Grijpstra, P., van Leeuwen, S. S., Leemhuis, H., Dobruchowska, J. M., van der Kaaij, R.
50 M., Malik, A., Oetari, A., Kamerling, J. P. and Dijkhuizen, L. (2011) 4,6- α -Glucanotransferase, a
51 novel enzyme that structurally and functionally provides an evolutionary link between
52 glycoside hydrolase enzyme families 13 and 70. *Appl. Environ. Microbiol.* **77**, 8154–8163

- 1 112 Skov, L. K., Mirza, O., Henriksen, A., De Montalk, G. P., Remaud-Siméon, M., Sarçabal, P.,
2 Willemot, R. M., Monsan, P. and Gajhede, M. (2001) Amylosucrase, a glucan-synthesizing
3 enzyme from the α -amylase family. *J. Biol. Chem.* **276**, 25273–25278
- 4 113 Guérin, F., Barbe, S., Pizzut-Serin, S., Potocki-Véronèse, G., Guieysse, D., Guillet, V., Monsan,
5 P., Mourey, L., Remaud-Siméon, M., André, I., and Tranier, S. (2012) Structural investigation of
6 the thermostability and product specificity of amylosucrase from the bacterium *Deinococcus*
7 *geothermalis*. *J. Biol. Chem.* **287**, 6642–6654
- 8 114 Albenne, C., Skov, L. K., Mirza, O., Gajhede, M., Feller, G., D’Amico, S., André, G., Potocki-
9 Véronèse, G., van der Veen, B., Monsan, P., and Remaud-Simeon, M. (2004) Molecular basis
10 of the amylose-like polymer formation catalyzed by *Neisseria polysaccharea* amylosucrase. *J.*
11 *Biol. Chem.* **279**, 726–734
- 12 115 Cambon, E., Barbe, S., Pizzut-Serin, S., Remaud-Simeon, M. and André, I. (2014) Essential role
13 of amino acid position 226 in oligosaccharide elongation by amylosucrase from *Neisseria*
14 *polysaccharea*. *Biotechnol. Bioeng.* **111**, 1719–1728
- 15 116 Champion, E., Guérin, F., Moulis, C., Barbe, S., Tran, T. H., Morel, S., Descroix, K., Monsan, P.,
16 Mourey, L., Mulard, L., Tranier, S., Remaud-Siméon, M. and André, I. (2012) Applying pairwise
17 combinations of amino acid mutations for sorting out highly efficient glycosylation tools for
18 chemo-enzymatic synthesis of bacterial oligosaccharides. *J. Am. Chem. Soc.* **134**, 18677–
19 18688
- 20 117 Daudé, D., Champion, E., Morel, S., Guieysse, D., Remaud-Siméon, M. and André, I. (2013)
21 Probing substrate promiscuity of amylosucrase from *Neisseria polysaccharea*. *ChemCatChem*
22 **5**, 2288–2295
- 23 118 Kim, H., Park, H., Heu, S. and Jung, J. (2004) Molecular and functional characterization of a
24 unique sucrose hydrolase from *Xanthomonas axonopodis* pv. *glycines*. *J. Bacteriol.* **186**, 411–
25 418
- 26 119 Skov, L. K., Pizzut-Serin, S., Remaud-Siméon, M., Ernst, H. A., Gajhede, M. and Mirza, O. (2013)
27 The structure of amylosucrase from *Deinococcus radiodurans* has an unusual open active-site
28 topology. *Acta Crystallogr. Sect. F* **69**, 973–978
- 29 120 Pizzut-Serin, S., Potocki-Véronèse, G., van der Veen, B. A., Albenne, C., Monsan, P. and
30 Remaud-Siméon, M. (2005) Characterisation of a novel amylosucrase from *Deinococcus*
31 *radiodurans*. *FEBS Lett.* **579**, 1405–1410
- 32 121 Olvera, C., Centeno-Leija, S., Ruiz-Leyva, P. and López-Munguía, A. (2012) Design of chimeric
33 levansucrases with improved transglycosylation activity. *Appl. Environ. Microbiol.* **78**, 1820–
34 1825
- 35 122 Lammens, W., Le Roy, K., Schroeven, L., Van Laere, A., Rabijns, A. and Van den Ende, W.
36 (2009) Structural insights into glycoside hydrolase family 32 and 68 enzymes: functional
37 implications. *J. Exp. Bot.* **60**, 727–740
- 38 123 Van den Ende, W., Lammens, W., Van Laere, A., Schroeven, L. and Le Roy, K. (2009) Donor and
39 acceptor substrate selectivity among plant glycoside hydrolase family 32 enzymes. *FEBS J.*
40 **276**, 5788–5798
- 41 124 Sainz-Polo, M. A., Ramírez-Escudero, M., Lafraya, A., González, B., Marín-Navarro, J., Polaina,
42 J. and Sanz-Aparicio, J. (2013) Three-dimensional structure of *Saccharomyces* invertase: role
43 of a non-catalytic domain in oligomerization and substrate specificity. *J. Biol. Chem.* **288**,
44 9755–9766
- 45 125 Lafraya, Á., Sanz-Aparicio, J., Polaina, J. and Marín-Navarro, J. (2011) Fructo-oligosaccharide
46 synthesis by mutant versions of *Saccharomyces cerevisiae* invertase. *Appl. Environ. Microbiol.*
47 **77**, 6148–6157
- 48 126 Ritsema, T., Verhaar, A., Vijn, I. and Smeekens, S. (2005) Using natural variation to investigate
49 the function of individual amino acids in the sucrose-binding box of fructan:fructan 6G-
50 fructosyltransferase (6G-FFT) in product formation. *Plant Mol. Biol.* **58**, 597–607
- 51 127 Altenbach, D., Rudiño-Pinera, E., Olvera, C., Boller, T., Wiemken, A. and Ritsema, T. (2009) An
52 acceptor-substrate binding site determining glycosyl transfer emerges from mutant analysis of

1 a plant vacuolar invertase and a fructosyltransferase. *Plant Mol. Biol.* **69**, 47–56

2 128 Álvaro-Benito, M., Sainz-Polo, M. A., González-Pérez, D., González, B., Plou, F. J., Fernández-
3 Lobato, M. and Sanz-Aparicio, J. (2012) Structural and kinetic insights reveal that the amino
4 acid pair Gln-228/Asn-254 modulates the transfructosylating specificity of *Schwanniomyces*
5 *occidentalis* β -fructofuranosidase, an enzyme that produces prebiotics. *J. Biol. Chem.* **287**,
6 19674–19686

7 129 Uitdehaag, J. C. M. (1999) The cyclization mechanism of cyclodextrin glycosyltransferase
8 (CGTase) as revealed by a γ -cyclodextrin-CGTase complex at 1.8-Å resolution. *J. Biol. Chem.*
9 **274**, 34868–34876

10 130 Leemhuis, H., Kelly, R. M. and Dijkhuizen, L. (2010) Engineering of cyclodextrin
11 glucanotransferases and the impact for biotechnological applications. *Appl. Microbiol.*
12 *Biotechnol.* **85**, 823–835

13 131 Van der Veen, B. A., Leemhuis, H., Kralj, S., Uitdehaag, J. C., Dijkstra, B. W. and Dijkhuizen, L.
14 (2001) Hydrophobic amino acid residues in the acceptor binding site are main determinants
15 for reaction mechanism and specificity of cyclodextrin-glycosyltransferase. *J. Biol. Chem.* **276**,
16 44557–44562

17 132 Kelly, R. M., Leemhuis, H. and Dijkhuizen, L. (2007) Conversion of a cyclodextrin
18 glucanotransferase into an α -amylase: assessment of directed evolution strategies.
19 *Biochemistry* **46**, 11216–11222

20 133 Rivera, M. H., López-Munguía, A., Soberón, X. and Saab-Rincón, G. (2003) α -Amylase from
21 *Bacillus licheniformis* mutants near to the catalytic site: effects on hydrolytic and
22 transglycosylation activity. *Protein Eng.* **16**, 505–514

23 134 Kim, T., Park, C., Cho, H., Cha, S., Kim, J., Lee, S., Moon, T., Kim, J., Oh, B. and Park, K. (2000)
24 Role of the glutamate 332 residue in the transglycosylation activity of *Thermus* maltogenic
25 amylase. *Biochemistry* **39**, 6773–6780

26 135 Tran, P. L., Cha, H.-J., Lee, J.-S., Park, S.-H., Woo, E.-J. and Park, K.-H. (2014) Introducing
27 transglycosylation activity in *Bacillus licheniformis* α -amylase by replacement of His235 with
28 Glu. *Biochem. Biophys. Res. Commun.* **451**, 541–547

29 136 Beier, L., Svendsen, A., Andersen, C., Frandsen, T. P., Borchert, T. V and Cherry, J. R. (2000)
30 Conversion of the maltogenic α -amylase Novamyl into a CGTase. *Protein Eng.* **13**, 509–513

31 137 Jemli, S., Ben-Ali, M., Ben-Hlima, H., Khemakhem, B. and Bejar, S. (2012) Mutations affecting
32 the activity of the cyclodextrin glucanotransferase of *Paenibacillus pabuli* US132: insights into
33 the low hydrolytic activity of cyclodextrin glucanotransferases. *Biologia* **67**, 636–643

34 138 Asensio, J. L., Ardá, A., Cañada, F. J. and Jiménez-Barbero, J. (2013) Carbohydrate-aromatic
35 interactions. *Acc. Chem. Res.* **46**, 946–954

36 139 Janeček, S. and Blesák, K. (2011) Sequence-structural features and evolutionary relationships
37 of family GH57 α -amylases and their putative α -amylase-like homologues. *Prot. J.* **30**, 429–435

38 140 Janeček, S., Svensson, B. and MacGregor, E. A. (2003) Relation between domain evolution,
39 specificity, and taxonomy of the α -amylase family members containing a C-terminal starch-
40 binding domain. *Eur. J. Biochem.* **270**, 635–645

41 141 Sun, T., Letsididi, R., Pan, B. and Jiang, B. (2013) Production of a novel cyclodextrin
42 glycosyltransferase from *Bacillus* sp. SK13.002. *Afr. J. Microbiol. Res.* **7**, 2311–2315

43 142 Bornscheuer, U. T. and Kazlauskas, R. J. (2004) Catalytic promiscuity in biocatalysis: using old
44 enzymes to form new bonds and follow new pathways. *Angew. Chem., Int. Ed.* **43**, 6032–6040

45 143 Watts, A. G., Damager, I., Amaya, M. L., Buschiazzo, A., Alzari, P., Frasch, A. C. and Withers, S.
46 G. (2003) *Trypanosoma cruzi* trans-sialidase operates through a covalent sialyl-enzyme
47 intermediate: tyrosine is the catalytic nucleophile. *J. Am. Chem. Soc.* **125**, 7532–7533

48 144 Amaya, M. F., Watts, A. G., Damager, I., Wehenkel, A., Nguyen, T., Buschiazzo, A., Paris, G.,
49 Frasch, A. C., Withers, S. G. and Alzari, P. M. (2004) Structural insights into the catalytic
50 mechanism of *Trypanosoma cruzi* trans-sialidase. *Structure* **12**, 775–784

51 145 Damager, I., Buchini, S., Amaya, M. F., Buschiazzo, A., Alzari, P., Frasch, A. C., Watts, A. and
52 Withers, S. G. (2008) Kinetic and mechanistic analysis of *Trypanosoma cruzi* trans-sialidase

1 reveals a classical ping-pong mechanism with acid/base catalysis. *Biochemistry* **47**, 3507–3512
2 146 Montagna, G., Cremona, M. L., Paris, G., Amaya, M. F., Buschiazzo, A., Alzari, P. M. and Frascch,
3 A. C. C. (2002) The *trans*-sialidase from the african trypanosome *Trypanosoma brucei*. *Eur. J.*
4 *Biochem.* **269**, 2941–2950
5 147 Buschiazzo, A., Tavares, G. A., Campetella, O., Spinelli, S., Cremona, M. L., Paris, G., Amaya, M.
6 F., Frascch, A. C. and Alzari, P. M. (2000) Structural basis of sialyltransferase activity in
7 trypanosomal sialidases. *EMBO J.* **19**, 16–24
8 148 Amaya, M. F., Buschiazzo, A., Nguyen, T. and Alzari, P. M. (2003) The high resolution
9 structures of free and inhibitor-bound *Trypanosoma rangeli* sialidase and its comparison with
10 *T. cruzi trans*-sialidase. *J. Mol. Biol.* **325**, 773–784
11 149 Buschiazzo, A., Amaya, M. F., Cremona, M. L., Frascch, A. C. and Alzari, P. M. (2002) The crystal
12 structure and mode of action of *trans*-sialidase, a key enzyme in *Trypanosoma cruzi*
13 pathogenesis. *Mol. Cell* **10**, 757–768
14 150 Stierand, K. and Rarey, M. (2011) Consistent two-dimensional visualization of protein-ligand
15 complex series. *J. Cheminform., Chemistry Central Ltd* **3**, 21
16 151 Demir, O. and Roitberg, A. E. (2009) Modulation of catalytic function by differential plasticity
17 of the active site: case study of *Trypanosoma cruzi trans*-sialidase and *Trypanosoma rangeli*
18 sialidase. *Biochemistry* **48**, 3398–3406
19 152 Pierdominici-Sottile, G., Horenstein, N. A. and Roitberg, A. E. (2011) A free energy study of the
20 catalytic mechanism of *Trypanosoma cruzi trans*-sialidase. From the Michaelis complex to the
21 covalent intermediate. *Biochemistry* **50**, 10150–10158
22 153 Pierdominici-Sottile, G., Palma, J. and Roitberg, A. E. (2013) Free energy computations identify
23 the mutations required to confer *trans*-sialidase activity into *Trypanosoma rangeli* sialidase.
24 *Proteins* **82**, 424–435
25 154 Jers, C., Michalak, M., Larsen, D. M., Kepp, K. P., Li, H., Guo, Y., Kirpekar, F., Meyer, A. S. and
26 Mikkelsen, J. D. (2014) Rational design of a new *Trypanosoma rangeli trans*-sialidase for
27 efficient sialylation of glycans. *PLoS One* **9**, e83902
28 155 Feng, H.-Y., Drone, J., Hoffmann, L., Tran, V., Tellier, C., Rabiller, C. and Dion, M. (2005)
29 Converting a β -glycosidase into a β -transglycosidase by directed evolution. *J. Biol. Chem.* **280**,
30 37088–37097
31 156 Teze, D., Hendrickx, J., Czjzek, M., Ropartz, D., Sanejouand, Y.-H., Tran, V., Tellier, C. and Dion,
32 M. (2014) Semi-rational approach for converting a GH1 β -glycosidase into a β -
33 transglycosidase. *Protein Eng. Des. Sel.* **27**, 13–19
34 157 Teze, D., Daligault, F., Ferrières, V., Sanejouand, Y.-H. and Tellier, C. (2014) Semi-rational
35 approach for converting a GH36 α -glycosidase into an α -transglycosidase. *Glycobiology*,
36 doi:10.1093/glycob/cwu124
37 158 Aronson, N. N., Halloran, B. A., Alexeyev, M. F., Zhou, X. E., Wang, Y., Meehan, E. J. and Chen,
38 L. (2006) Mutation of a conserved tryptophan in the chitin-binding cleft of *Serratia*
39 *marcescens* chitinase A enhances transglycosylation. *Biosci., Biotechnol., Biochem.* **70**, 243–
40 251
41 159 Madhuprakash, J., Tanneeru, K., Purushotham, P., Guruprasad, L. and Podile, A. R. (2012)
42 Transglycosylation by chitinase D from *Serratia proteamaculans* improved through altered
43 substrate interactions. *J. Biol. Chem.* **287**, 44619–44627
44 160 Lü, Y., Yang, H., Hu, H., Wang, Y., Rao, Z. and Jin, C. (2009) Mutation of Trp137 to glutamate
45 completely removes transglycosyl activity associated with the *Aspergillus fumigatus* AfChiB1.
46 *Glycoconjugate J.* **26**, 525–534
47 161 Zakariassen, H., Hansen, M. C., Jøranli, M., Eijsink, V. G. H. and Sørli, M. (2011) Mutational
48 effects on transglycosylating activity of family 18 chitinases and construction of a
49 hypertransglycosylating mutant. *Biochemistry* **50**, 5693–5703
50 162 Merceron, R., Foucault, M., Haser, R., Mattes, R., Watzlawick, H. and Gouet, P. (2012) The
51 molecular mechanism of thermostable α -galactosidases AgaA and AgaB explained by X-ray
52 crystallography and mutational studies. *J. Biol. Chem.* **287**, 39642–39652

- 1 163 Placier, G., Watzlawick, H., Rabiller, C. and Mattes, R. (2009) Evolved β -galactosidases from
2 *Geobacillus stearothermophilus* with improved transgalactosylation yield for galacto-
3 oligosaccharide production. *Appl. Environ. Microbiol.* **75**, 6312–6321
- 4 164 Saburi, W., Kobayashi, M., Mori, H., Okuyama, M. and Kimura, A. (2013) Replacement of the
5 catalytic nucleophile aspartyl residue of dextran glucosidase by cysteine sulfinate enhances
6 transglycosylation activity. *J. Biol. Chem.* **288**, 31670–31677
- 7 165 Lawson, S. L., Wakarchuk, W. W. and Withers, S. G. (1996) Effects of both shortening and
8 lengthening the active site nucleophile of *Bacillus circulans* xylanase on catalytic activity.
9 *Biochemistry* **35**, 10110–10108
- 10 166 Andrés, E., Aragunde, H. and Planas, A. (2014) Screening glycosynthase libraries with a
11 fluoride chemosensor assay independently of enzyme specificity: identification of a
12 transitional hydrolase to synthase mutant. *Biochem. J.* **458**, 355–363
- 13 167 Aragunde, H., Castilla, E., Biarnés, X., Faijes, M. and Planas, A. (2014) A transitional hydrolase
14 to glycosynthase mutant by Glu to Asp substitution at the catalytic nucleophile in a retaining
15 glycosidase. *Carbohydr. Res.* **389**, 85–92
- 16 168 Rémond, C., Plantier-Royon, R., Aubry, N., Maes, E., Bliard, C. and O'Donohue, M. J. (2004)
17 Synthesis of pentose-containing disaccharides using a thermostable α -L-arabinofuranosidase.
18 *Carbohydr. Res.* **339**, 2019–2025
- 19 169 Rémond, C., Plantier-Royon, R., Aubry, N. and O'Donohue, M. J. (2005) An original
20 chemoenzymatic route for the synthesis of β -D-galactofuranosides using an α -L-
21 arabinofuranosidase. *Carbohydr. Res.* **340**, 637–644
- 22 170 Chlubnova, I., Filipp, D., Spiwok, V., Dvorakova, H., Daniellou, R., Nugier-Chauvin, C., Kralova,
23 B. and Ferrières, V. (2010) Enzymatic synthesis of oligo-D-galactofuranosides and L-
24 arabinofuranosides: from molecular dynamics to immunological assays. *Org. Biomol. Chem.* **8**,
25 2092–2102
- 26 171 Hidaka, M., Fushinobu, S., Honda, Y., Wakagi, T., Shoun, H. and Kitaoka, M. (2010) Structural
27 explanation for the acquisition of glycosynthase activity. *J. Biochem.* **147**, 237–244
- 28 172 Pollock, J. J. and Sharon, N. (1970) Acceptor specificity of the lysozyme-catalyzed
29 transglycosylation reaction. *Biochemistry* **9**, 3913–3925
- 30 173 Toshima, G., Kawamura, S., Araki, T. and Torikata, T. (2003) Histidine-114 at subsites E and F
31 can explain the characteristic enzymatic activity of guinea hen egg-white lysozyme. *Biosci.,*
32 *Biotechnol., Biochem.* **67**, 540–546
- 33 174 Kawamura, S., Eto, M., Imoto, T., Ikemizu, S., Araki, T. and Torikata, T. (2004) Functional and
34 structural effects of mutagenic replacement of Asn37 at subsite F on the lysozyme-catalyzed
35 reaction. *Biosci., Biotechnol., Biochem.* **68**, 593–601
- 36 175 Moreau, A., Shareck, F., Kluepfel, D. and Morosoli, R. (1994) Alteration of the cleavage mode
37 and of the transglycosylation reactions of the xylanase A of *Streptomyces lividans* 1326 by
38 site-directed mutagenesis of the Asn173 residue. *Eur. J. Biochem.* **219**, 261–266
- 39 176 Armand, S., Andrews, S. R., Charnock, S. J. and Gilbert, H. J. (2001) Influence of the aglycone
40 region of the substrate binding cleft of *Pseudomonas* xylanase 10A on catalysis. *Biochemistry*
41 **40**, 7404–7409
- 42 177 Rosengren, A., Hägglund, P., Anderson, L., Pavon-Orozco, P., Peterson-Wulff, R., Nerinckx, W.
43 and Stålbrand, H. (2012) The role of subsite +2 of the *Trichoderma reesei* β -mannanase
44 TrMan5A in hydrolysis and transglycosylation. *Biocatal. Biotransform.* **30**, 338–352
- 45 178 Dilokpimol, A., Nakai, H., Gotfredsen, C. H., Baumann, M. J., Nakai, N., Abou Hachem, M. and
46 Svensson, B. (2011) Recombinant production and characterisation of two related GH5 endo- β -
47 1,4-mannanases from *Aspergillus nidulans* FGSC A4 showing distinctly different
48 transglycosylation capacity. *BBA-Proteins Proteom., Elsevier B.V.* **1814**, 1720–1729
- 49 179 Taira, T., Fujiwara, M., Denhart, N., Hayashi, H., Onaga, S., Ohnuma, T., Letzel, T., Sakuda, S.
50 and Fukamizo, T. (2010) Transglycosylation reaction catalyzed by a class V chitinase from
51 cycad, *Cycas revoluta*: a study involving site-directed mutagenesis, HPLC, and real-time ESI-
52 MS. *BBA-Proteins Proteom.* **1804**, 668–675

- 1 180 Umekawa, M., Huang, W., Li, B., Fujita, K., Ashida, H., Wang, L.-X. and Yamamoto, K. (2008)
2 Mutants of *Mucor hiemalis* endo- β -N-acetylglucosaminidase show enhanced
3 transglycosylation and glycosynthase-like activities. *J. Biol. Chem.* **283**, 4469–4479
- 4 181 Yin, J., Li, L., Shaw, N., Li, Y., Song, J. K., Zhang, W., Xia, C., Zhang, R., Joachimiak, A., Zhang, H.-
5 C., Wang, L.-X., Liu, Z.-J. and Wang, P. (2009) Structural basis and catalytic mechanism for the
6 dual functional endo- β -N-acetylglucosaminidase A. *PLoS One* **4**, e4658
- 7 182 Bobrov, K. S., Borisova, A. S., Eneyskaya, E. V., Ivanen, D. R., Shabalin, K. A., Kulminskaya, A. A.
8 and Rychkov, G. N. (2013) Improvement of the efficiency of transglycosylation catalyzed by α -
9 galactosidase from *Thermotoga maritima* by protein engineering. *Biochemistry (Moscow)* **78**,
10 1112–1123
- 11 183 Ochs, M., Belloy, N., Dauchez, M., Muzard, M., Plantier-Royon, R. and Rémond, C. (2013) Role
12 of hydrophobic residues in the aglycone binding subsite of a GH39 β -xylosidase in alkyl
13 xylosides synthesis. *J. Mol. Catal. B: Enzym.* **96**, 21–26
- 14 184 Kuroki, R., Weaver, L. H. and Matthews, B. W. (1999) Structural basis of the conversion of T4
15 lysozyme into a transglycosidase by reengineering the active site. *Proc. Natl. Acad. Sci. U.S.A.*
16 **96**, 8949–8954
- 17 185 Matsui, I., Yoneda, S., Ishikawa, K., Miyairi, S., Fukui, S., Umeyama, H. and Honda, K. (1994)
18 Roles of the aromatic residues conserved in the active center of *Saccharomycopsis* α -amylase
19 for transglycosylation and hydrolysis activity. *Biochemistry* **33**, 451–458
- 20 186 Mizuno, M., Tonozuka, T., Uechi, A., Ohtaki, A., Ichikawa, K., Kamitori, S., Nishikawa, A. and
21 Sakano, Y. (2004) The crystal structure of *Thermoactinomyces vulgaris* R-47 α -amylase II (TVA
22 II) complexed with transglycosylated product. *Eur. J. Biochem.* **271**, 2530–2538
- 23 187 Collins, T., De Vos, D., Hoyoux, A., Savvides, S. N., Gerday, C., Van Beeumen, J. and Feller, G.
24 (2005) Study of the Active Site Residues of a Glycoside Hydrolase Family 8 Xylanase. *J. Mol.*
25 *Biol.* **354**, 425–435
- 26 188 Wakarchuk, W. W., Campbell, R. L., Sung, W. L., Davoodi, J. and Yaguchi, M. (1994) Mutational
27 and crystallographic analyses of the active site residues of the *Bacillus circulans* xylanase.
28 *Protein Sci.* **3**, 467–475
- 29 189 Honda, Y., Fushinobu, S., Hidaka, M., Wakagi, T., Shoun, H., Taniguchi, H. and Kitaoka, M.
30 (2008) Alternative strategy for converting an inverting glycoside hydrolase into a
31 glycosynthase. *Glycobiology* **18**, 325–330
- 32 190 Purkiss, A., Skoulakis, S. and M., G. J. (2001) The protein–solvent interface: a big splash. *Phil.*
33 *Trans. R. Soc. Lond. A* **359**, 1515–1527
- 34 191 Aghajari, N., Roth, M. and Haser, R. (2002) Crystallographic evidence of a transglycosylation
35 reaction: ternary complexes of a psychrophilic α -amylase. *Biochemistry* **41**, 4273–4280
- 36 192 Kuriki, T., Kaneko, H., Yanase, M., Takata, H., Shimada, J., Handa, S., Takada, T., Umeyama, H.
37 and Okada, S. (1996) Controlling substrate preference and transglycosylation activity of
38 neopullulanase by manipulating steric constraint and hydrophobicity in active center. *J. Biol.*
39 *Chem.* **271**, 17321–17329
- 40 193 Ramasubbu, N., Rangunath, C., Sundar, K., Mishra, P. J. and Kandra, L. (2005) Structure-function
41 relationships in human salivary α -amylase: role of aromatic residues. *Biologia* **60**, 47–56
- 42 194 Frutuoso, M. A. and Marana, S. R. (2013) A single amino acid residue determines the ratio of
43 hydrolysis to transglycosylation catalyzed by β -glucosidases. *Protein Pept. Lett.* **20**, 102–106
- 44 195 Teze, D., Hendrickx, J., Dion, M., Tellier, C., Woods, V. L., Tran, V. and Sanejouand, Y.-H. (2013)
45 Conserved water molecules in family 1 glycosidases: a DXMS and molecular dynamics study.
46 *Biochemistry* **52**, 5900–5910
- 47 196 Rebuffet, E., Groisillier, A., Thompson, A., Jeudy, A., Barbeyron, T., Czjzek, M. and Michel, G.
48 (2011) Discovery and structural characterization of a novel glycosidase family of marine origin.
49 *Environ. Microbiol.* **13**, 1253–1270.
- 50 197 Jensen, H. H. and Bols, M. (2006) Stereoelectronic substituent effects. *Acc. Chem. Res.* **39**,

- 1 259–265
- 2 198 Mydock, L. K. and Demchenko, A. V. (2010) Mechanism of chemical *O*-glycosylation: from
- 3 early studies to recent discoveries. *Org. Biomol. Chem.* **8**, 497–510
- 4