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Structural Explanation for the Acquisition of Glycosynthase Activity

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Glycosynthases are engineered glycoside hydrolases (GHs) that catalyse the synthesis of glycoside from glycosyl-fluoride donors and suitable acceptors. We have determined five crystal structures of the glycosynthase mutants reducing-end xylose-releasing exo-oligoxylanase, an inverting GH, that exhibit various levels of glycosynthetic activities. At the active site of the Y198F mutant, the most efficient glycosynthase, a water molecule is observed at the same position as nucleophilic water (NW) in the parent enzyme, and the loss of the fixation of the direction of the lone pair of water molecules in the mutant drastically decreases hydrolytic activity. Water molecules were also observed at each active site of the general base mutant, but they were shifted 1.0–3.0 Å from the NW in the wild type. Their positions exhibited a strong correlation with the strength of glycosynthase activity. Here, we propose that a structural prerequisite for the sufficient glycosynthase reaction is the presence of a water molecule at the NW position, and mutation at the NW holder provides a general strategy for inverting GHs. The idea on the position of a water molecule may also be applicable to the design of efficient glycosynthases from retaining GHs.

Keywords: Hehre resynthesis–hydrolysis/inverting glycoside hydrolase/reducing-end xylose-releasing exo-oligoxylanase/structural basis of glycosynthase/X-ray crystallography.

Abbreviations: Abg1, β-glucosidase from *Agrobacterium* sp.; Cel7B, endoglucanase from *Humicola insolens*; GH, glycoside hydrolase; NW, nucleophilic water; pXyl, endo-xylanase from *Pseudoalteromonas haloplanktis*; Rex, reducing-end xylose-releasing exo-oligoxylanase from *Bacillus halodurans*; WT, wild type.

Glycoside hydrolases (GHs) are generally categorized into two types, retaining and inverting enzymes, on the basis of changes in their anomeric configurations during reactions. Their detailed mechanisms have been elucidated, and the active site of both classes of enzyme contains two carboxylic acid residues (*1–5*). In retaining GH, one residue acts as a nucleophile and the other as an acid/base catalyst (Fig. 1A). The reaction proceeds by a double displacement mechanism in which a covalent glycosyl-enzyme intermediate is formed (glycosylation step). Normal hydrolysis occurs when this intermediate reacts with water (deglycosylation step) in a general acid/base catalysed process. If the glycosyl-enzyme is intercepted with a sugar, then the result will be a glycosyl transfer. The inverting reaction mechanism involves two catalytic residues, a general acid and a base catalyst (Fig. 1B). The reaction takes place by a direct attack of nucleophilic water (NW) activated by the general base and is aided by the general acid.

Glycosynthases are mutants of GHs that catalyse the synthesis of a glycoside from a glycosyl-fluoride donor of the opposite anomer and an acceptor. The first of glycosynthase was reported on a β-glucosidase from *Agrobacterium* sp. (Abg1) belonging to GH family 1 (GH1) (*6*, *7*). Most of the glycosynthases have been generated from retaining GHs by mutating the nucleophile residue into an inert one. To date, a number of retaining GHs belonging to various GH families have been converted into glycosynthases (*8–10*). The reaction of GH with the glycosyl fluoride of the opposite anomer with their hydrolytic substrates was first found with an inverting GH. In 1979, Hehre *et al.* (*11*) reported that β-amylase hydrolysed β-maltosyl fluoride into maltose and fluoride ions. Later, various inverting enzymes were shown to hydrolyse the 'wrong' glycosyl fluorides, suggesting that the reaction is common among inverting GHs (*10*, *12–17*). This type of reaction has not been reported in retaining GHs. Instead of simple hydrolysis, the reaction mechanism consists of two steps, resynthesis and hydrolysis. In the first step, the wrong glycosyl fluoride is transferred to the hydroxyl group of the acceptor to synthesize the correct *O*-glycosyl linkage with releasing F^- and so the resynthetic activity is evaluated by the amount of released fluoride ions. The new glycoside is immediately hydrolysed at the same site on the enzyme before it is released from the active centre, which is the normal inverting reaction of GH. The mechanism was verified by the requirement of an acceptor molecule in the trehalase reaction and was later named the 'Hehre resynthesis–hydrolysis mechanism' (Fig. 1C) (*13*). Glycosynthase is developed by exploiting the resynthesis step of the mechanism.

We previously reported the conversion of an inverting GH into glycosynthase by selectively blocking the

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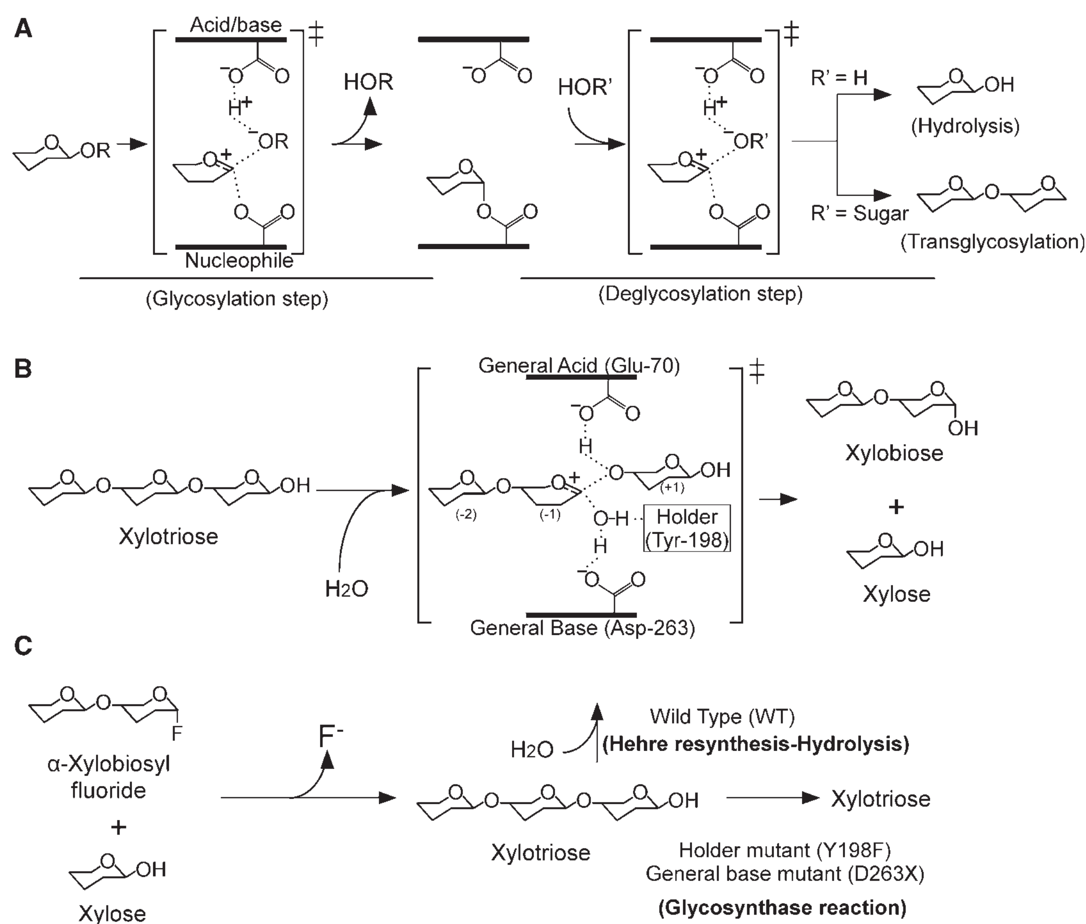

Fig. 1 Proposed reaction mechanisms of GHs. (A) Typical reaction mechanism of retaining GH. (B) The reaction mechanism of an inverting GH, Rex. (C) The reaction mechanism of the Hehre resynthesis–hydrolysis reaction catalysed by Rex.

Table 1. Properties of mutants of Rex.

	Kinetic parameters related to glycosynthase activity[a]				Water molecule around the nucleophile site				
	α-xylobiosyl fluoride consumption[b]	F^--release (F; s^{-1})	xylotriose hydrolysis (H; s^{-1})	F/H ratio	B-factor (Å^2)	Distance from original NW (Å)	Hydrogen bond distance (Å) D195	Y198	D263X
WT	Strong	3.1	31.2	0.1	13.4	–	–	2.7	2.7
Y198F	Strong	4.7	0.06	78	24.6	0.4	–	–	2.6
D263N	Moderate	0.32	0.09	3.6	23.7	1.0	2.8	2.5	2.9
D263C	Moderate	0.29	0.014	21	23.8	1.3	2.5	2.9	–
D263S	Weak				22.2	1.8	2.3	–	2.7
D263G	Weak				9.2	3.0	2.4	–	–
Y198F/D263N	Moderate	0.23	0.0024	96					
Y198F/D263C	Weak	0.03	0.00086	35					

[a]Values taken from (*18*, *19*). [b]Estimated by thin-layer chromatography.

hydrolysis step in the Hehre resynthesis–hydrolysis mechanism (*18*). Saturation mutagenesis was performed at the general base residue of a GH8 enzyme, reducing-end xylose-releasing exo-oligoxylanase (Rex, EC 3.2.1.156), and two mutants, D263C and D263N, were found to exhibit glycosynthase activity. These mutants accumulate significant amounts of xylotriose from α-xylobiosyl fluoride and xylose. However, the mutations concomitantly caused significant decreases in the resynthetic (F^--releasing) activity and faint hydrolytic activities remained after the mutation (Table 1). Next, we performed mutation analysis of a tyrosine residue (Tyr198) that supports the NW molecule in concert with the catalytic base residue (see Fig. 2A), expecting to eliminate the hydrolytic activity (*19*). The single mutation, Y198F, caused a decrease in the hydrolytic activity to a level similar to the catalytic base mutants. Interestingly, the Y198F mutation caused a slight increase in the rate of the resynthesis step, producing an efficient glycosynthase (Table 1). In contrast, the double mutations with Asp263 caused significant decreases in the resynthetic

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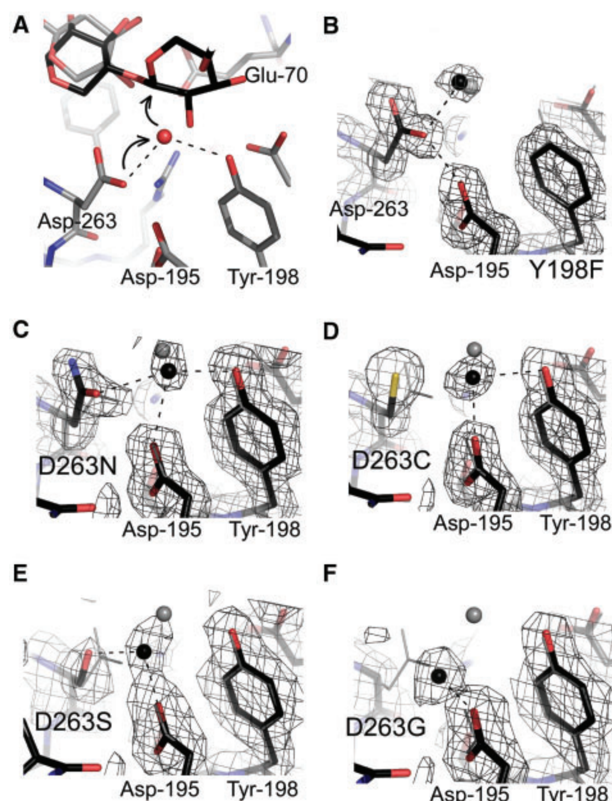

Fig. 2 Structural changes of Rex induced by mutations at Asp263 (general base) and Tyr198 (holder of the NW). The NW and hydrogen bonds are shown as a sphere model and broken lines, respectively. A residue interacting with the general base or the NW (Asp195) is also labelled. (A) WT structure. Bound xylose in subsite −1 is shown as a stick model. A model substrate (black) is constructed by reference to the cellopentaose structure of endoglucanase CelA from *Clostridium thermocellum* (*34*). (B–F) Mutant structures with the $2|F_o| - |F_c|$ electron density maps (1.0 σ). Each mutant structure is shown as stick model (black) superimposed on that of WT (grey).

activity with drastic decreases in the hydrolytic activity, producing less active glycosynthases. Therefore, we concluded that mutations at the residue holding the NW, not at the catalytic base residue, produce better glycosynthases from inverting GHs, because such mutations remove the hydrolytic activity without decreasing the resynthetic activity.

In contrast to inverting GHs, retaining GHs generally do not possess resynthetic activity in their native form (*20*). Mutation at the nucleophile residue (Glu or Asp) to residues with small side chains (e.g. Ala, Cys, Gly and Ser) eliminates the hydrolytic activity and concomitantly endows resynthetic activity. The resynthetic activity varies with the pattern of substitution and GH families. For example, the substitution of Glu with Ser resulted in quite different levels of resynthetic activity among GH families. In cases of GH7 endoglucanase from *Humicola insolens* (Cel7B) (*21*) and GH2 β-mannosidase from *Cellulomonas fimi* (*22*), the best glycosynthase was obtained by the substitution. On the other hand, the Gly mutants of GH1 Abg1 and GH26 β-mannanase from *Cellvibrio japonicus* (Man26A) showed 2–4-fold higher glycosynthase activity than Ser mutants (*23*, *24*). The substitution of Glu with Ser on GH10 xylanases (*20*) and GH17 barley β-(*1,3*)-D-glucan endohydrolase (*25*) caused almost no glycosynthase activity but substitution with Gly caused some activity.

The structural basis of the acquisition of high resynthetic activity has been discussed for the nucleophile mutants (E197A and E197S) of Cel7A (*21*) and glycine mutant, E320G, of Man26A (*23*). In the case of Cel7A, although there is no direct interaction between the serine hydroxyl and the axial O1 of the donor glucoside at subsite −1 in the complex structure, Ducros *et al*. suggested that the difference in resynthetic activities between E197A and E197S, which is 35 times more efficient, comes from a possible stabilizing interaction between the serine hydroxyl and departing fluoride during the transition state. Jahn *et al*. also proposed that a water molecule in Man26A that takes the place of the nucleophile residue plays a similar role to the hydroxyl of the serine glycosynthase. However, a general structural explanation for the resynthetic activity is still required.

In this study, we report the crystal structures of a series of Rex mutants in which the hydrolytic and resynthetic activities have been already investigated. Here, we show a possible structural prerequisite for exhibiting sufficient resynthetic activity that can be expanded to retaining GHs.

Materials and methods

Structure determination and refinement

The Rex mutant proteins were expressed and purified as described earlier (*26*, *27*). Crystals of the mutant enzymes were grown and stored in cryoprotectant as described for the wild type (WT) (*28*). X-ray diffraction data sets were collected using synchrotron radiation (Beamlines BL-6A, BL-17A and NW12A; Photon Factory, Tsukuba, Japan). Data processing, model correction and refinement were performed with the programs HKL2000 (*29*), Coot (*30*) and Refmac5 (*31*). The statistics for data collection, processing and refinements are given in Table 2. The figures were prepared using PyMol (DeLano Scientific). Pairwise structural comparisons were carried out using the whole structure.

Protein data bank accession codes

The atomic coordinates of Rex-Y198F, D263N, D263C, D263S and D263G have been deposited in the Research Collaboratory for Structural Bioinformatics Protein Data Bank under accession codes 3A3V, 2DRR, 2DRO, 2DRS and 2DRQ, respectively.

Results

Location of the important residues of the Rex mutants

We determined the crystal structures of Y198F mutant of Rex as well as four general base mutants (D263N, D263C, D263S and D263G). There were no remarkable differences in the overall structures of the mutants from that of the WT (*28*); the rmsd for C^{α} atoms between all pairs was within 0.15 Å. In Y198F, side chains of Phe198 and general base Asp263 overlap with their equivalents in that of WT (Fig. 2B). In both structures, the general base residue forms a short hydrogen bond (2.5 Å) with Asp195. The short hydrogen bond may be due to the interaction of deprotonated Asp263 with protonated Asp195 in a highly hydrophobic pocket. It should be noted that the general acid residue, Glu70, of the mutant had

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Table 2. Data collection statistics.

Crystal	Y198F	D263N	D263C	D263S	D263G
Data collection					
Wavelength (Å)	1.0000	1.0000	1.0000	1.0000	1.0000
Beamline	BL17A	NW12A	NW12A	BL-6A	BL-6A
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters					
a (Å)	53.2	53.2	53.2	52.2	52.2
b (Å)	86.2	86.2	86.4	85.0	85.3
c (Å)	87.7	87.6	87.7	86.8	87.0
Resolution (Å)	50–1.39 (1.44–1.39)	61.4–1.60 (1.66–1.60)	61.6–1.70 (1.76–1.70)	60.7–2.10 (2.18–2.10)	60.9–2.10 (2.18–2.10)
Unique reflections	81,959	53,617	44,431	22,673	22,498
Redundancy	3.7 (3.4)	5.0 (4.4)	5.0 (4.8)	4.5 (4.5)	4.1 (4.1)
Completeness (%)	99.7 (97.6)	99.4 (98.8)	98.5 (99.6)	97.3 (99.2)	96.2 (98.1)
Mean I/σ	32.1 (3.5)	27.9 (3.1)	27.5 (4.1)	15.2 (5.9)	20.5 (8.8)
R_{merge} (%)	7.9 (38.4)	6.1 (23.4)	6.4 (35.8)	9.9 (24.3)	6.9 (12.8)
Refinement					
PDB ID	3A3V	2DRR	2DRO	2DRS	2DRQ
Resolution range (Å)	45.50–1.39	40.23–1.60	39.07–1.70	43.40–2.10	44.77–2.10
No. of protein atoms	3,084	3,085	3,083	3,106	3,088
No. of solvent atoms	516	545	457	437	436
No. of heteroatoms					
Ni	1	1	1	1	1
Glycerol	24	18	12	6	6
Average B-factor (Å^2)	15.0	16.7	18.7	10.3	11.4
Root mean square deviations					
Bond lengths (Å)	0.009	0.01	0.011	0.015	0.015
Bond angles (deg)	1.2	1.2	1.2	1.4	1.3
R-factor/R_{free}[a] (%)	17.7 (19.8)	16.2 (18.1)	16.1 (18.7)	14.0 (21.0)	14.2 (19.4)

Numbers in parentheses correspond to the shell of data at the highest resolution. [a]$R_{free} = \sum_{hkl}||F_{obs}| - |F_{calc}||/\sum_{hkl}|F_{obs}|$, where the crystallographic R-factor was calculated including and excluding refinement reflections. The free reflections constituted 5% of the total number of reflections.

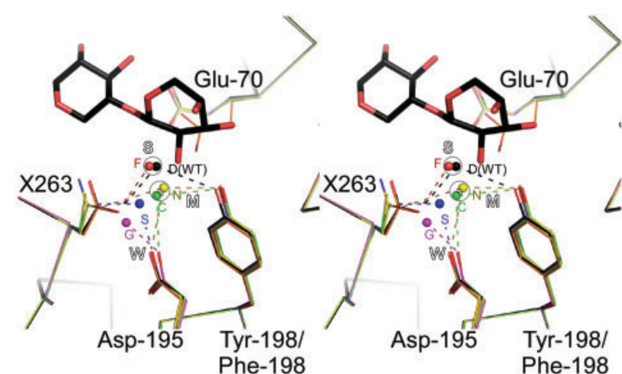

Fig. 3 Stereographic figure showing superimposition of Rex mutants at the active site. Y198F (orange), D263N (yellow), D263C (green), D263S (blue), D263G (magenta) and WT (black) are shown. Side chains of Glu70 (general acid), Asp195, Tyr198 and Asp263 are shown as a wireframe model. Water molecules in each structure are shown as sphere models. (S), (M), (W) in open face indicates the categories based on the strength of resynthetic activity; Strong, Moderate and Weak. A model substrate (black) is constructed by reference to CelA as in Figure 2.

a different conformation from that of the WT (Fig. 3). This distinctive conformation of the residue was also detected in the structures of endo-xylanase from *Pseudoalteromonas haloplanktis* (pXyl), which shares 32.6% amino acid sequence identity with Rex (*32*, *33*). The conformation of the general acid residue of pXyl (Glu78) was presumed to be inactive and substrate binding induced the repositioning of the Glu78 into a more catalytically competent position, corresponding to Glu70 in Rex-WT. This suggested that the conformation of Glu70 in Rex Y198F was possibly an artefact of the crystallization process and that it is altered to the 'active' form according to substrate binding. Such conformational differences of the general acid residue do not affect the conclusions of this paper.

In the structure of D263N, the corresponding distance between Asn263 and Asp195 is longer than in the WT (2.9 Å) and the side chain of Asn263 takes a different conformation from the Asp263 of WT (Fig. 2C). A similar difference in the distance caused by the mutation at the general base residue was also observed with pXyl (*32*). In the case of pXyl, the distance between Asp(Asn)281 and Asp200 was 2.5 and 2.74 Å for the WT and D281N mutant, respectively. In the structures of D263C and D263S of Rex, side chains of Cys263 and Ser263 take similar conformations to that of Asn263 rather than Asp263 (Figs. 2D, 2E and 3). The $\chi1$ angles of Cys263 (−63.3°) and Ser263 (−71.6°) were similar to that of Asn263 (−65.9°) rather than Asp263 (−83.8°). It should be noted that the mutations at Asp263 did not significantly affect the conformation of Asp195 and Tyr198, the residues interacting with Asp263 in the WT structure.

Location of a water molecule near the active site in the Rex mutants

In the WT structure, a water molecule is held by two hydrogen bonds from the catalytic base residue Asp263 and a conserved Tyr residue, Tyr198

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(Fig. 2A) (*28*). The water corresponds to the NW that is activated by the catalytic base residue. In the active site of each mutant, the positions of the water molecule were obviously different (Fig. 2 and Table 1). A water molecule was detected in the structure of Y198F at a position that was almost identical to the position of NW in WT (Fig. 2B). The water molecule retains a hydrogen bond with Asp263 while another hydrogen bond with Tyr198 is eliminated (discussed later). The atomic displacement parameter (B-factor) of the water molecule (24.6 Å^2) was higher than the NW of the WT (13.4 Å^2) whereas the overall B-factors for Y198F (15.0 Å^2) and WT (13.8 Å^2) were similar.

The water molecules in the D263C and D263N structures were detected at similar positions. They were shifted ~1 Å toward Asp195 from the NW position in the WT and form hydrogen bonds with Asp195 and Tyr198. The water molecule in D263N also forms a hydrogen bond with Asn263. No water molecules were detected within 1.5 Å of the NW position in the structures of D263S and D263G. In the structure of D263S, a water molecule that had hydrogen bonds with Asp195 and Ser263 was observed ~1.8 Å from the NW position in the WT. As a result, the hydrogen bond with Tyr198 was broken. In the structure of D263G, a water molecule that had formed a hydrogen bond with Asp195 was detectable at a position corresponding to the O$^\delta$ atom of Asp263 in WT.

The WT structures and Rex mutants can be classified into three groups based on the position of the water molecules (Fig. 3). Interestingly, the position of the water molecules is likely related to the strength of their resynthetic activity (Table 1). The groups are therefore: Strong (S), WT and Y198F; Moderate (M), D263C and D263N and Weak (W), D263S and D263G. The water molecule in the structures of group S was located at a position very close to the NW in the WT. The water molecules in group M were found ~1 Å from the NW position in the WT, forming a hydrogen bond with Asp195. No water molecules were observed within 1.8 Å from the NW position in the structures of group W.

Discussion

A critical role of Y198F as the NW holder in inverting hydrolysis

The tyrosine residue corresponding to Tyr198 of Rex is conserved in all GH8 enzymes and forms a hydrogen bond with NW in all known crystal structures (*28*, *32–34*). The role of Tyr203 in the pXyl, which corresponds to Tyr198 of Rex, has been reported in detail (*32*). Although the Y203F mutation of pXyl caused a drastic decrease in the hydrolytic activity (0.03%), the similar pH-dependences of Y203F and the WT pXyl strongly suggested that the tyrosine residue is not a general base. Collins *et al.* predicted that the residue was important for the positioning of the NW in a catalytically productive orientation. In our previous study, we expected that the elimination of the hydrogen bond with Tyr198 would remove the NW from the active site (*19*) because the mutation results in large decrease in the hydrolytic activity (0.2%). In this study, however, we detected a water molecule at the NW position in the crystal structure of Y198F (Fig. 2B), strongly supporting the prediction of pXyl. Hence, here we focused on the possible directions of the lone pairs on the oxygen atom of NW. Assuming that two hydrogen atoms of NW are directed toward Asp263 and Tyr198, one of the lone pairs of oxygen should be directed to the anomeric carbon of xyloside (Supplemental Fig. S1A). When the hydrogen bond with Tyr198 is broken by mutating Phe, the water molecule can rotate and lose the direction of the lone pair. Therefore, the loss of the hydrolytic activity could be explained by the loss of the fixation of the direction of the lone pair of the water molecule. A similar level of inactivation in the mutations at the NW holders of Rex and pXyl strongly support this explanation. This concept is likely to be generally applicable to other inverting GHs. We investigated all known crystal structures of inverting GHs that contain both possible NW and substrate (or analogue) and all appeared to have the same holding mechanism to position a lone pair on the oxygen into the anomeric carbon atom (see Supplemental Fig. S1 and Supplemental Information for details) (*35–41*). Today, investigations of inverting GHs are strongly biased to the two catalytic residues, general acid and base. We propose that the holding mechanism of NW should be added to the general explanation of the reaction scheme of inverting GHs.

Correlation between resynthetic activity and position of a water molecule

As described in the Results section, there is a significant correlation between the resynthetic activity and the position of the water molecule in the active site. The resynthetic activity tends to decrease as deviation of the water molecule from the NW position in the WT increases. The concept can also explain the difference in the resynthetic activity of the double mutants, Y198F/D263N and Y198F/D263C (*19*), although crystallization of these double mutants was not successful in this study. In case of D263N mutants, the double mutant Y198F/D263N exhibited a similar resynthetic activity to the single mutant D263N. However, the resynthetic activity of Y198F/D263C was much less than the single mutant D263C. Since the water molecule in D263N is held by Asn263, Asp195 and Tyr198, the water molecule in D263N/Y198F should still be fixed with Asn263 and Asp195 at the same position. Therefore, the resynthetic activity of Y198F/D263N may also be similar to that of D263N. The water molecule in D263C should be held by Asp195 and Tyr198, but should not interact with Cys263. In the additional Y198F mutations the water molecule is only supported by Asp195, moving it away from the original position.

General concept for converting GH to glycosynthase

We propose a structural prerequisite for effective resynthesis based on the observations described here: a water molecule should be located at a similar position to the NW in the parent enzyme. This explains why inverting GHs exhibit the Hehre

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resynthesis-hydrolysis reaction and retaining GHs do not. The active site of inverting GHs satisfies the requirement because it possesses NW at the nucleophile site in the state without substrate. In contrast, no water molecules are found at the position in the retaining GH because this position is occupied by the nucleophile residue. The apparent relationship between the water molecule position and the resynthetic activity can be explained by one of the following two reasons. The fluorine atom of the donor substrate in the glycosynthase reaction is considered to be located at a position similar to the NW in the hydrolytic reaction of the WT enzyme when it is in the transition state. The structural conditions that accept the water molecule at the position may be also favourable to accepting the fluorine atom at the same position. Therefore, such structural conditions likely support the transition states of both hydrolysis and resynthesis, enhancing these reactions. Another possibility is that the water molecule at the position donates a proton to activate the abstraction of the fluorine atom.

The strategy of converting an inverting GH into glycosynthase by mutating the NW holder residue can be expanded to inverting GHs in other families because most of them adopt a similar NW holding mechanism. Since the mutation at the holder has little effect on the active site structure, we propose that this residue is the most promising target for converting GHs to glycosynthase.

The strategy of targeting a residue other than the general base was also successful in producing a glycosynthase from an inverting GH95 α-1,2-fucosidase that has an unusual reaction mechanism (*38*, *42*). The unusual general base residue (Asn423) is suggested to be activated by Asp766 and a NW molecule is held by Asn423 and Asn421. Although the mutation at the NW holder (Asn421) did not result an effective glycosynthase activity, the mutation at Asp766, which was not the general base, produced the best glycosynthase mutant in this case. The mutation drastically reduced hydrolytic activity by removing indirect activation of the water molecule, but probably did not affect the location of the NW.

The presence of a water molecule at the nucleophile site would be an indicator when designing an efficient glycosynthase from both inverting GHs and retaining ones. At the active site of the substrate free structure of Cel7B-E197A and E197S (*21*), a water molecule is located very close to the original nucleophile residue site, which is held by the residues that originally interact with the nucleophile residue (Supplemental Fig. S2A and B). In the E197S structure, the Ser residue takes a significantly different rotamer conformation from that of WT Glu and the water molecule is bound at slightly different position from that of E197A (Supplementary Fig. S2C). The difference might be related to the strength of their resynthetic activity as in Rex. Today, several mutants of retaining GHs that show improved glycosynthase activity have been obtained by a directed evolution approach and such mutations are often introduced apart from the nucleophile residue (*43*, *44*). The mutations might influence the position of the water molecule, resulting in the difference of resynthetic activities. Because the water molecule forms a hydrogen bond with a residue that originally interacts with the nucleophile residue in the parent enzyme (*e.g.* Asp199 of Cel7B), mutations with such a residue would be alternative candidates for glycosynthase engineering.

Conclusion

In this article, a significant correlation between the resynthetic activity and the position of a water molecule was found by determining a series of glycosynthase mutant structures of an inverting GH, Rex. We suggest that mutation at the NW holder, not the catalytic base residue, is a general strategy for producing efficient glycosynthase from inverting GHs because most have a similar water holding mechanism with multiple hydrogen-bonding residues. Such mutations eliminate the hydrolytic activity due to loss of the water fixation, but retain the resynthetic activity because they have little effect on the active site structure as compared with the catalytic base mutants. The position of a water molecule is a good indicator of the expected resynthetic activity. This idea may also be applicable to designing efficient glycosynthases from retaining GHs.

Supplementary data

Supplementary Data are available at *JB* Online.

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Conflict of interest

None declared.

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