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X-ray crystallography and QM/MM investigation on the oligosaccharide synthesis mechanism of rice BGlu1 glycosynthases

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A B S T R A C T
Nucleophile mutants of retaining β-glycosidase can act as glycosynthases to efficiently catalyze the synthesis of oligosaccharides. Previous studies proved that rice BGlu1 mutants E386G, E386S and E386A catalyze the oligosaccharide synthesis with different rates. The E386G mutant gave the fastest transglycosylation rate, which was approximately 3- and 19-fold faster than those of E386S and E386A. To account for the differences of their activities, in this paper, the X-ray crystal structures of BGlu1 mutants E386S and E386A were solved and compared with that of E386G mutant. However, they show quite similar active sites, which implies that their activities cannot be elucidated from the crystal structures alone. Therefore, a combined quantum mechanical/molecular mechanical (QM/MM) calculations were further performed. Our calculations reveal that the catalytic reaction follows a single-step mechanism, i.e., the extraction of proton by the acid/base, E176, and the formation of glycosidic bond are concerted. The energy barriers are calculated to be 19.9, 21.5 and 21.9 kcal/mol for the mutants of E386G, E386S and E386A, respectively, which is consistent with the order of their experimental relative activities. But based on the calculated activation energies, 1.1 kcal/mol energy difference may translate to nearly 100 fold rate difference. Although the rate limiting step in these mutants has not been established, considering the size of the product and the nature of the active site, it is likely that the product release, rather than chemistry, is rate limiting in these oligosaccharides synthesis catalyzed by BGlu1 mutants.

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1. Introduction

The synthesis of carbohydrates is challenging since there are several functional groups and chiral centers presenting on the monosaccharides [1,2]. Although chemical methods can be used to synthesize oligosaccharides, their analogues, and glycoconjugates with exceptional flexibility, stereochemical control required during the synthesis process must be achieved by sequential protection and deprotection of the functional groups. These complex procedures often result in low yields of oligosaccharides. Enzymatic oligosaccharide synthesis approaches provide alternative tools to obtain the desired oligosaccharide by the use of substrate specificity and stereoselectivity of glycosidases and glycosyltransferases.

Enzymatic oligosaccharide synthesis involves two main classes of carbohydrate enzymes: glycosidases and glycosyltransferases. The oligosaccharide synthesis reactions promoted by glycosidases may be achieved either with a large excess of acceptors (thermodynamically controlled synthesis) or with activated glycosyl donors (kinetically controlled transglycosylation) [3,4]. However, the typical retaining glycosidases usually give poor synthetic yields (10–40%) due to hydrolysis of substrates and products. Glycosyltransferases can catalyze the glycosyl transfer from a saccharide donor to the acceptor with strict regiospecificity and stereospecificity without hydrolysis [5]. However, although glycosyltransferases have been used for oligosaccharide synthesis, their poor availability and high cost of substrates limit their exploitation.

In 1998, Withers and colleagues discovered that a mutant of the retaining Agrobacterium sp. β-glucosidase (E.C. 3.2.1.21) in which the catalytic nucleophile was mutated to a nonnucleophilic amino acid residue (Glu358Ala) could be used for transglycosylation reactions [6]. This enzyme, denoted as a glycosynthase, could efficiently catalyze the synthesis of oligosaccharides with high yields (>80%) without detectable hydrolysis. Since then, engineering a retaining glycosidase into a glycosynthase by the mutation of the catalytic nucleophile to a smaller nonnucleophilic residue, to form a hydroltically inactive enzyme has become a popular strategy for synthesizing various oligosaccharides [7]. When the glycosyl fluorides of inverted configuration relative to

Abbreviations: QM/MM, quantum mechanical/molecular mechanical; α-GlcF, alpha-glucosyl fluoride; pNPC2, p-nitrophenyl-α-D-glucoside; RMD, root-mean-square deviation; MD, molecular dynamics; HB, hydrogen bond; R, reactant; TS, transition state; P, product
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the natural substrates are used, these hydrolytically inactive enzymes can be used to synthesize oligosaccharides on a large scale in the presence of suitable glycosyl acceptor, giving products that accumulate in a very high yield.

Catalytic nucleophile mutants of retaining glycosidases have been used as glycosynthases to synthesize various glycosides and oligosaccharides [8–10]. Although glycosynthases were originally derived from retaining β-glycosidases, their sources were recently expanded to retaining α-glycosidases and inverting glycosidases [11,12]. Furthermore, the catalytic activity and substrate specificity of the glycosynthase has been improved greatly by additional mutations of their catalytic sites, while the regioselectivity of glycosidic linkages could be enhanced by changing the stereocchemistry of the acceptor substrate [13]. Thus, glycosynthase technology has become a versatile tool for the synthesis of novel glycosides and oligosaccharides [14,15]. So far, a variety of novel glycosynthases have been derived from different glycosidase families, including endoglycosidases and exoglycosidases [16–21].

Rice (Oryza sativa) BGlu1 β-glucosidase, belonging to the glycoside hydrolase family 1 enzyme, shows hydrolysis activities towards β-(1,4)- and short β-(1,3)-linked gluco-oligosaccharides [22–24], and glycosides such as pNP-β-glycoside. The hydrolysis mechanism of rice BGlu1 β-glucosidase has been studied by the combined quantum mechanical/molecular mechanical (QM/MM) method. As the energy barriers were 15.7 kcal/mol for the glycosylation step [25] and 21.4 kcal/mol for the deglycosylation step [26], the hydrolysis rate may proceed quickly. Therefore, although the wild-type BGlu1 enzyme displays transglycosylation activity on oligosaccharide and glycoside substrates, the yields of products are relatively low and the newly formed products are subsequently hydrolyzed at the same time [22,27].

Because the aglycone-binding site of rice BGlu1 β-glucosidase was relatively long, the ability of its glycosynthase to synthesize the long oligomeric saccharides was explored [28]. Hommalai et al. reported that the mutations of rice BGlu1 β-glucosidase at glutamate residue 386 (the residue was numbered according to its position in the BGlu1 precursor, as E414 in that paper) destroyed the hydrolytic activities of the enzyme. When the catalytic activities of three candidate mutants, E386G, E386S and E386A, were compared with α-glucosyl fluoride (α-GlcF) as donor and pNP-celllobioside (pNPC2) as acceptor, all these mutants displayed transglycosylation activities to synthesize mixed length oligosaccharides (see Fig. 1). This enzymatic oligosaccharide synthesis reaction was proposed to be a concerted single-step mechanism [6]. The acid/base residue abstracts a proton from one hydroxyl group of substrate pNPC2, as this deprotonated hydroxyl group attacks on the anomeric carbon of α-GlcF, and then the fluorine atom departs. The BGlu1 E386G mutant gave the fastest transglycosylation rate, which was approximately 3- and 19-fold faster than those of E386S and E386A, respectively, and gave yields of up to 70–80% insoluble products.

Recently, the structure of the BGlu1 E386G glycosynthase mutant alone and in complexes with α-GlcF, cellotetraose or cellopentaose were reported [29]. However, those structures could not explain the relative activities of the BGlu1 E386G, E386S and E386A glycosynthases. Therefore, in this paper we solved the structures of the E386S and E386A glycosynthases complexed with α-GlcF for comparison and explored the dynamic issues contributing to these relative activities by the QM/MM method.

Using the Gaussian frequency data, we also determined the kinetic isotope effect (KIE) of the proton transfer process in the E386 mutants based on two expressions [30]. One expression is the semi-classical Eyring equation, where the KIE is given as

$$\frac{k_{H}}{k_{D}} = \exp \left( - \frac{G_H - G_D}{RT} \right)$$

(1)

The other one is a simple “quantum correction” equation with the semiclassical $\left(k_{H}/k_{D}\right)$, multiplying the Wigner quantum correction $Q^{\text{corr}}$ factor

$$\frac{k_{H}}{k_{D}} = Q^{\text{corr}} \left(\frac{k_{H}}{k_{D}}\right)$$

(2)

$$Q^{\text{corr}} = 1 + \frac{\nu^2}{24}$$

(3)

$$u_i = \frac{\hbar^2 k_i}{k_0^2}$$

(4)

$$u_f = \frac{\hbar^2 k_f}{k_0^2}$$

(5)

where $\nu$ is the imaginary frequency of the transition state.

2. Methods

2.1. Protein X-ray crystal structure determination

The recombinant proteins of BGlu1 E386A and BGlu1 E386S [28] were expressed and purified as previously described for wild type BGlu1 [22,27]. Crystallization of BGlu1 E386S and E386A without and with 10 mM α-GlcF (kindly provided by Prof. Stephen G. Withers) was optimized by hanging drop vapor diffusion with microseeding around the conditions used for crystallization of BGlu1 [22,27], varying the concentrations of polyethylene glycol monomethyl ether (PEG MME) 5000 over the range of 16–26%, (NH4)2SO4 between 0.12 and 0.26 M, and protein between 2 and 6 mg/ml in 0.1 M MES, pH 6.7, at 288 K. Before flash cooling in liquid nitrogen, the crystals with 10 mM α-GlcF were soaked...
in cryo solution (18% (v/v) glycerol in precipitant solution) containing 10 mM α-GlcF for 1–5 min.

Preliminary diffraction experiments were done with a Cu Kα rotating anode X-ray source mounted on a MicroSTAR generator operating at 45 kV and 60 mA connected to Rayonix SX-165 CCD detector at the Synchrotron Light Research Institute (Public Organization) (SLRI, Nakhon Ratchasima, Thailand). Promising crystals were used to diffract 1.0 Å wavelength X-rays on the BL13B1 beamline at the National Synchrotron Radiation Research Center (NSRRC in Hsinchu, Taiwan), and reflections were recorded with an ADSC Quantum 315 CCD detector. The crystals were maintained at 105 K during diffraction with a nitrogen cold stream (Oxford Instruments). Data were processed and scaled with the HKL-2000 package [31]. Structures were solved by molecular replacement with wild type BGlu1 (PDB code: 2RGL), refined, and validated as previously described for BGlu1 E386G and its complex with α-GlcF [29].

2.2. Automated docking setup

Recently, we published the structure of the E386G mutant and its complex with α-GlcF (PDB code: 3SCO) [29]. Since the crystal of rice BGlu1 E386G mutant was only complexed with the donor α-GlcF, and the transglucosylation activities were detected under the acceptor pNPC2, the pNPC2 was docked into the binding pocket using the Autodock 4.0 program [32]. Before docking, the pNPC2 substrate was optimized at the B3LYP/6-31 + G(d) level with the Gaussian 03 package [33]. When docking, the grid scale was set as 60 Å × 60 Å × 60 Å based on a grid module, with a spacing of 0.375 Å between the grid points. Gasteiger charges [34] were set for both the ligand and protein. Fifty independent docking runs were performed. In the whole calculation, the protein was kept rigid, and all the torsional bonds of the ligand were kept free. Based on a root-mean-square deviation (RMSD) criterio

2.3. Computational model

The obtained docking ternary complex structures of BGlu1 E386G, E386S and E386A glycosynthases were used as the initial structures in molecular dynamics (MD) simulations. For the glycosyltransferase mechanism of these mutants, the catalytic acid/base (E176) acts as a base to extract a proton from the substrate, and was modeled in its deprotonated state. The protonation/deprotonation states of other ionizable residues were altered on the basis of PROPKA method [35,36]. The Mulliken charge parameters of the substrates (α-GlcF and pNPC2) were achieved based on the QM (at the B3LYP/6-31 + G(d) level) calculations. All the hydrogen atoms were added with the HBUILD facility of the CHARMM package [37]. The crystallographic water molecules were kept in their original positions and an extra 4,648 water molecules were used to solvate each of the three systems with a 37.9 Å water sphere centered on E176. Nine Cl− ions were also added at random positions to neutralize each system. To equilibrate the prepared systems, a minimization followed by a 1000 ps MD simulation was performed with the CHARMM22 force field [38]. To confirm the equilibrium of the initial model, the RMSD curves for the mutants of E386G, E386S and E386A have been checked, which are given in supporting information (Fig. S1–S3).

During the subsequent QM/MM calculations, the QM region included residues E176, Y315, E440, the mutated nucleophile residue X386, the substrates α-GlcF, pNPC2 (terminal glucosyl residue) and one order water Wat1, where X referred to Gly, Ser and Ala for the E386G, E386S and E386A mutants, respectively (see Fig. 3). Fig. 3 shows the labels to indicate the serial numbers of relevant atoms in the substrate rings. The total number of QM atoms were 85, 86 and 88 for the E386G, E386S and E386A mutants, respectively. The remainder of the enzyme and waters was set as the MM region. Any residue with at least one atom within 10 Å of E176 (including the QM region and part of the MM region) was kept loose, while the remaining part was kept frozen. In the geometry optimizations, the QM region was treated with quantum mechanics by the Turbomole module [39] and the MM part with molecular mechanics under the CHARMM22 force field by the DL-POLY program [40]. For the whole QM/MM optimizations, calculations were performed at the B3LYP/6-31G(d,p)//CHARMM22 level. The electrostatic interactions between the QM and MM regions were described by the standard electronic embedding scheme [41]. To avoid the hyperpolarization effect during the QM treatment, the MM atomic partial charges were incorporated into the one-electron Hamiltonian of the QM calculation. The charge shift model with hydrogen linked atoms was used to simulate covalent bonds across the QM/MM boundary [42]. The ChemShell package [43], incorporating

![Fig. 2. (A) The overall obtained docking structure of rice BGlu1 E386G mutant (PDB code: 3SCO), (B) The corresponding residues in the active site.](image)
the Turbomole and DL-POLY programs, were used to perform the QM/MM calculations. A geometry optimizer of hybrid delocalized internal coordinates (HDLIC) [44] was adopted for the geometry optimizations. Stationary points were searched by the quasi-Newton limited memory Broyden–Fletcher–Goldfarb–Shanno (L-BFGS) algorithm [45,46], and transition states with the algorithm of partitioned rational function optimization (P-RFO) [47,48]. The vibrational frequencies of the QM region atoms were determined at the same level by using the Gaussian 03 package [33]. After all the stationary points were located, high level single point electronic energy calculations were performed at a larger basis set 6-31+G(d,p) to obtain accurate energies.

3. Results and discussion

3.1. 3D Structures of three glycosynthases in complexes with α-GlcF

To assess the structural basis for the differing glycosynthase efficiencies of BGlu1 E386G, E386S and E386A, the structures of the E386S and E386G glycosynthases and those of their complexes with α-GlcF were determined by X-ray crystallography and compared with the corresponding structure of E386G. Crystals of the apo glycosyltransferase enzymes and of their complexes were isomorphous with wild type BGlu1 crystals [22,27]. Diffraction data parameters are shown in Table S1 and model parameters for the structures of the glycosynthases are summarized in Table 1. The resolution limits of 1.85 Å for BGlu E386S and 2.10 Å for E386A and free residual (Rfree) values of 20.5% for E386S and 20.6% for E386A in complexes with α-GlcF are comparable to the previously reported values for the corresponding complex of E386G of 1.95 Å and 20.7% [29]. However, the temperature (B) factors reported for the E386G protein (16.6 Å²) and α-GlcF (14.0 Å²) in the E386G complex are significantly higher than those in the E386S (9.5 and 5.7 Å², respectively) and E386A (10.8 Å² and 8.2 Å², respectively) complexes. These differences may support the supposition of a greater flexibility in the E386G protein and its complex, although we cannot exclude other factors contributing to the higher crystal structure disorder.

The overall structures of the three BGlu1 glycosyltransferase mutants and the positions of residues and α-GlcF in the active site are very similar. In each structure, the α-GlcF binds to the glycosynthases in a relaxed 4C1 chair conformation stacked onto the indole ring of W433 at the —1 subsite, in the same position as the 2-deoxy-2-fluoro-α-D-glucosyl moiety (G2F) in its covalent complex with BGlu1 (PDB code: 2RGM) [22,27], with the same hydrogen bonds as observed for that complex. As shown in Fig. 4, the active site residue N313, as well as the fluorine and O2 of α-GlcF, interact with a single ordered water molecule in all three mutant complexes. An ordered water molecule is also found in the same position in the structure of the covalent intermediate of BGlu1 with G2F, and this is also the position of the nucleophile carbonyl oxygen of apo wild type BGlu1 (PDB code: 2RGL) [22,27]. As seen in Fig. 4, the water is closer to the α-fluorine in the E386S structure, while in the E386A structure and is in an intermediate position in the BGlu1 E386G structure, but these differences may not be significant.

Although the determination of the structures of the BGlu1 E386G, E386A and E386S glycosynthases in apo and α-GlcF-bound forms is the most thorough structural investigation of one enzyme’s glycosyltransferase mutants to date, the structures alone cannot easily explain the relative activities of BGlu1 E386G, E386S and E386A. The previous idea that a Gly nucleophile may be a better catalyst than a Ser nucleophile due to

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Structure refinement statistics for E386A and E386S X-ray structures.</th>
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<tr>
<td>Carbohydrate atoms</td>
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</tr>
<tr>
<td>Subsite −1 α-GlcF (A/B)</td>
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</tr>
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</table>

* Rfree = (Σ|Fo|−|Fc|)/Σ|Fo|.
* Based on 5% of the unique observations not included in the refinement.
* Ramachandran values were determined from PROCHECK [51].
* Based on Rfree values, as calculated by Refmac 5.5.0110 [52].
* Twelve per protein molecule in the asymmetric unit.

Fig. 3. The selected quantum mechanics (QM) region in QM/MM calculations; X refers to the mutated nucleophile residue in the active site.
a water taking the place of the Ser hydroxyl but with more optimal geometry [49], since only the one water molecule was observed in this area in both structures. As described above, this water molecule appears in a critical position to stabilize the leaving group in all three structures, and S386 points toward it to stabilize its position in the E386S mutant, which could be used to explain the higher activity of E386S than E386A. However, this gives little insight into the high relative activity of the E386G mutant. As noted by Ducros et al. [50], X-ray structures, such as those described here of initial Michaelis complex structures, do not account for changes in the ternary complex with the acceptor and at the transition state, which may be more critical for determining the relative activities. Therefore, a combined quantum mechanical/molecular mechanical (QM/MM) calculations were performed to account for the difference of the relative activities of the reactions.

3.2. Docking structure

Since no acceptor density was seen in crystals containing α-GlcF and pNPC2 (data not shown), docking was used to produce the ternary complex structures. The overall structure of rice BGlul E386G mutant in complex with α-GlcF and docked pNPC2 is shown in Fig. 2A, and the pocket residues are given in Fig. 2B. The donor α-GlcF is fixed by two hydrogen bonds (HBS), with its fluorine atom (F1, as labeled in Fig. 3) to the phenolic hydroxyl of residue Y315 (2.79 Å) and the C4-OH group to the E440 carboxyl (2.82 Å). The important crystal water molecule Wat1 lies near the F1 atom with a distance of 2.79 Å. The acceptor pNPC2 locates in an appropriate position to facilitate the reaction, and forms one strong HB with the acid/base residue E176 with a distance of 2.14 Å. Furthermore, the distance between C1 atom of α-GlcF and O4' of C4'-OH group of pNPC2 is close enough for the formation of the glycosidic bond with a distance of 2.44 Å. Thus, the docking structure appears reasonable for the following QM/MM calculations.

3.3. Analyses of reaction pathways

For the mutants of E386G, E386S and E386A, the optimized geometries of the reactants, transition states and products obtained at the 6-31G(d,p) level are shown in Figs. 5–7, respectively. The bond distances which are formed or broken in each state are colored magenta. Furthermore, the single point energies were calculated at the 6-31++G(d,p) level, and the obtained energy profiles for each mutant are shown in the corresponding figures.

3.3.1. E386G mutant

Fig. 5 gives the structures of reactant (R1), transition state (TS1), product (P1) and energy profile of the oligosaccharide synthesis process catalyzed by E386G Mutant.
In R1 (Fig. 5A), both of the donor α-GlcF and acceptor pNPC2 are located in favorable positions for the reaction to occur. Compared with the docking structure in Fig. 2B, the donor α-GlcF is still hydrogen bonded with residues E440 and Y315, but the interactions of these HBs have been strengthened. For example, the distance between F1 atom and hydroxyl group of Y315 decreases to 1.76 Å. A new HB forms between E440 and C6–OH of α-GlcF with a distance of 1.62 Å. Furthermore, the crystal water Wat1 comes closer to F1 atom, establishing a new HB (1.91 vs 3.13 Å in Fig. 2B). The acceptor pNPC2 moves a little further away from α-GlcF, but it still remains at a reasonable distance (3.35 Å) for the formation of glycosidic bond. In addition, the HB length between the C4′-OH group and side chain of acid/base E176 decreases from 2.14 Å in Fig. 2B to 1.73 Å, which is feasible for the nucleophilic attack of E176.

At TS1 (Fig. 5B), the pNPC2 O4′-H4′ bond has almost broken (bond length distance of 1.09 Å). The proton H4′ is much closer to the acid/base residue E176 with a distance of 1.37 Å. As E176 extracts the proton from the pNPC2 C4′–OH group, the distance between the donor and acceptor is also strengthened. The distance between the C1 and O4′ atoms decreases to 1.90 Å (3.35 Å in R1). The leaving F1 atom moves away from α-GlcF with a distance of 2.44 Å and forms two strong HBs with water Wat1 and Y315.

In the structure of P1 (Fig. 5C), the O4′-H4′ bond in pNPC2 has been further elongated to 1.59 Å and the H4′ atom has formed a covalent H-O bond with E176 (1.01 Å). The glycosidic bond between C1 and O4′ has formed with a distance of 1.48 Å. The departed F1 atom has moved far away from C1 (2.85 Å), but still forms two strong HBs with Wat1 and Y315 with distances of 1.56 and 1.32 Å, respectively. Obviously, the water Wat1 and residue Y315 play an important role in stabilizing the departed F1 atom. Fig. 5D shows that the energy barrier is 19.9 kcal/mol, and the reaction is endothermic with the reaction energy of 9.7 kcal/mol.

3.3.2. E386S mutant

Fig. 6 gives the optimized structures of reactant (R2), transition state (TS2), product (P2) and energy profile of E386S mutant enzyme. In R2 (Fig. 6A), the binding modes of substrates α-GlcF and pNPC2 are similar with that of R1 in Fig. 5A. The side chain of E176 still forms one strong HB with C4′-OH group of pNPC2, while the α-GlcF is H-bonded with E440, Y315 and water Wat1. The main difference is the newly formed HB between F1 atom and side chain of acid/base E176 decreases from 2.14 Å in Fig. 2B to 1.73 Å, which is feasible for the nucleophilic attack of E176.

The pNPC2 O4′-H4′ bond has been weakened, but it remains bonded to O4′ (1.03 vs 0.99 Å) in R2. With the weakness of the O4′-H4′ bond, the interaction between the donor and acceptor has been strengthened: the distance between the C1 and O4′ atoms decreases to 1.90 Å (3.35 Å in R1). The leaving F1 atom moves away from α-GlcF with a distance of 2.44 Å and forms two strong HBs with water Wat1 and Y315.

In the structure of P1 (Fig. 5C), the O4′-H4′ bond in pNPC2 has been further elongated to 1.59 Å and the H4′ atom has formed a covalent H-O bond with E176 (1.01 Å). The glycosidic bond between C1 and O4′ has formed with a distance of 1.48 Å. The departed F1 atom has moved far away from C1 (2.85 Å), but still forms two strong HBs with Wat1 and Y315 with distances of 1.56 and 1.32 Å, respectively. Obviously, the water Wat1 and residue Y315 play an important role in stabilizing the departed F1 atom. Fig. 5D shows that the energy barrier is 19.9 kcal/mol, and the reaction is endothermic with the reaction energy of 9.7 kcal/mol.
In the product, P2 (Fig. 6C), the acid/base E176 has extracted the H4' proton from the C4'-OH group of pNPC2 forming a new H-O bond (distance of which is 1.00 Å). The donor of α-GlcF has connected with pNPC2, establishing a new glycosidic bond of 1.49 Å. At the same time, the leaving F1 atom moves further away (3.07 Å from the C1 atom) but still interacts with the surrounding residues (Y315 and S386) and water Wat1. All these indicate that the oligosaccharide synthesis process is accomplished at this state.

In the product, P2 (Fig. 6C), the acid/base E176 has extracted the H4' proton from the C4'-OH group of pNPC2 forming a new H-O bond (distance of which is 1.00 Å). The donor of α-GlcF has connected with pNPC2, establishing a new glycosidic bond of 1.49 Å. At the same time, the leaving F1 atom moves further away (3.07 Å from the C1 atom) but still interacts with the surrounding residues (Y315 and S386) and water Wat1. All these indicate that the oligosaccharide synthesis process is accomplished at this state.

In the reactant R1 (Fig. 5A), the side chain of A386 is a methyl group, which is bigger than that of residue G386 in E386G. The sterically hindered methyl group occupies the active site, which is relatively unfavorable for the binding of α-GlcF. The distances between the F1 atom and the surrounding Wat1 and Y315 are 2.01 and 1.81 Å, respectively, which are longer than those of the distances in R1 (lengths of which are 1.91 and 1.76 Å, respectively). Besides, the extra methyl group also pushes the donor away from the acceptor: the distance between C1 and O4' atoms is increased from 3.35 Å in Fig. 5A to 3.41 Å in Fig. 7A.

In TS3 (Fig. 7B), the pNPC2 O4’-H4’ bond has been partially broken (distance of which is 1.07 vs. 0.99 Å in R3) and the proton H4’ has partially moved to the carboxyl oxygen of E176 (1.41 vs. 1.73 Å in R3). With the weakness of the O4’-H4’ bond, the C1 atom of α-GlcF has come closer to the O4’ atom of pNPC2 with a distance of 1.93 Å. Therefore, the interaction between the donor and acceptor has been strengthened and the glycosidic bond of C1-O4’ has been partially established. With the formation of the C1-O4’ bond, the C1-F1 bond becomes weakened, and its bond length increases to 2.52 Å. Additionally, the leaving group F1 atom is still hydrogen bonded with Wat1 and Y315 with distances of 1.60 and 1.42 Å, respectively, which are favorable for the departure of F1 atom.

In product P3 (Fig. 7C), the pNPC2 O4’-H4’ covalent bond has broken with a long distance of 1.56 Å, and the H4’ atom has formed a covalent H-O bond with the side chain of E176 (distance of 1.01 Å). The glycosidic bond C1-O4’ is completely established (length of 1.48 Å).
at this state. Since the residue A386 has a methyl group on its side chain, it has no HB interaction with the leaving F1 atom, which is different from residue S386 in the E386S mutant. Similar to the structure of P2, the departed F1 atom also forms two strong HBs with Wat1 and Y315 with distances of 1.59 and 1.40 Å, respectively, indicating that the water Wat1 and Y315 are important in stabilization of the leaving group. Owing to the steric hindrance of A386, the activity of this mutant is lower than that of the previous two mutants, which can be reflected from the energy barrier. Fig. 7D shows that the energy barrier of this reaction is 21.9 kcal/mol, while for E386G this barrier is 19.9 kcal/mol and for E386S is 21.5 kcal/mol. The order of the energy barriers is consistent with the experimental relative activities of these enzymes [28]: the experimental $k_{cat}$ value of Gly mutant is about 6 per minute, and 2 per minute for Ser, and about 0.3 per minute for Ala. But based on calculated activation energies (19.9, 21.5, and 21.9 kcal/mol for Gly, Ser and Ala mutants, respectively), 1.1 kcal/mol energy difference translates to more than 100 fold rate difference with Gly mutant. Although the rate limiting step in these mutants has not been established, Shoham has suggested that product release is likely a rate limiting step in some glycosynthases [53]. Considering the size of the product and the nature of the active site, it is likely that the product release, rather than chemistry, is rate limiting in BGlu1 mutants. Glycine may well be the fastest as it presents little steric hindrance for the departure of the trisaccharide from the active site.

The crystal structures of E386G, E386S and E386A (Fig. 4) shows that the mutant structures look very similar and one critical water molecule (Wat1) appears in the active site. The calculations were also performed with the absence of Wat1 in the QM regions at the same level. The corresponding optimized structures (reactants, transition states and products) and energy profiles were shown in the Supporting Information (Fig. S4–S6). The geometries of these structures are similar with those structures with the presence of the water Wat1 in the QM regions (see Figs. 5–7). Differences can be seen in the energy barriers. In the absence of Wat1, the energy barrier is calculated to be 22.4 kcal/mol for catalytic reaction of E386G mutant (Fig. S4), 25.7 kcal/mol for E386S (Fig. S5) and 28.2 kcal/mol for E386A (Fig. S6). We can see that all the energy barrier are higher than those of corresponding mutants with considering the water Wat1 in the QM regions, but they are all consistent with the order of activities [28]. The lower barriers indicate that this crystal water performs an assist role in the catalytic reaction of three mutants. Obviously, the hydrolysis reactions of E386 mutants are endothermic, the extra energy may be abstracted from the surroundings.

3.3.4. Kinetic Isotope Effect

To approximately evaluate the nuclear quantum dynamical effect on the proton transfer in E386 mutants, we calculated the semiclassical kinetic isotope effect (KIE) based on the QM region defined in the above QM/MM calculation [54,55]. The required free energy of activation and vibrational frequencies were determined at the B3LYP/6-31 G(d,p) level by using the Gaussian 03 package [33]. Detailed formulations of the calculations are shown in the Supporting Information. Both the semiclassical KIE values and the derived Wigner-corrected KIE values for the proton-transfer processes are listed in Table 2. It exhibits that the proton-transfer processes of all the three E386 mutants give the small KIE and Wigner tunneling corrections.
meaning that the quantum mechanical tunneling effect is insigni
cificant for E386 mutants.

4. Conclusion

The oligosaccharide synthesis mechanism catalyzed by rice BGlu1 E386 mutants (E386G, E386S and E386A) has been studied by crystallographic and QM/MM approaches. The structures of these glycosyltransferases were quite similar, so the computational approach was necessary to account for the dynamic issues leading to the differences in their activities. In the dynamic model, the extraction of the proton-fluoride in the glycine mutant and the

Table 2

<table>
<thead>
<tr>
<th>TS</th>
<th>Frequencies (cm⁻¹)</th>
<th>H/D KIE</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3 (E386G)</td>
<td>129.3</td>
<td>-227.2</td>
</tr>
<tr>
<td>T2 (E386S)</td>
<td>109.2</td>
<td>1.40</td>
</tr>
<tr>
<td>T1 (E386A)</td>
<td>300.6</td>
<td>-292.4</td>
</tr>
</tbody>
</table>

meaning that the quantum mechanical tunneling effect is insignifi-
cant for E386 mutants.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

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Appendix A Supplementary data

Diffraction data parameters for E386S and E386A mutants (Table S1); the RMSD curves for the mutants of E386G, E386S and E386A (Fig. S1–3); the optimized structures (reactants, transition states and products) and energy profiles with the presence of Wat1 in the MM regions (Fig. S4–6). This material is available free of charge via the Internet. Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadapa.2012.11.003.

References

[11] Y. Honda, M. Kitaoka, The first glycosynthase derived from an inverting glycosidase allowing steric constraints on this departure imposed by the Ser hydroxymethyl side chain. We also demonstrate that, the presence of Wat1 is able to assist the catalytic reaction for each mutant. Although the rate limiting step in these mutants has not been established, considering the size of the product and the nature of the active site, it is likely that the product release, rather than chemistry, is rate limiting in BGlu1 mutants.

Accession Numbers

Coordinates and structure factors have been deposited in the Protein Data-bank with accession codes 3SCR, 3SCS,3SCP and 3CQ.