



# $\alpha$ -Glucosidases and $\alpha$ -1,4-glucan lyases: structures, functions, and physiological actions

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**Abstract**  $\alpha$ -Glucosidases (AGases) and  $\alpha$ -1,4-glucan lyases (GLases) catalyze the degradation of  $\alpha$ -glucosidic linkages at the non-reducing ends of substrates to release  $\alpha$ -glucose and anhydrofructose, respectively. The AGases belong to glycoside hydrolase (GH) families 13 and 31, and the GLases belong to GH31 and share the same structural fold with GH31 AGases. GH13 and GH31 AGases show diverse functions upon the hydrolysis of substrates, having linkage specificities and size preferences, as well as upon transglucosylation, forming specific  $\alpha$ -glucosidic linkages. The crystal structures of both enzymes were determined using free and ligand-bound forms, which enabled us to understand the important structural elements responsible for the diverse functions. A series of mutational approaches revealed features of the structural elements. In particular, amino-acid residues in plus subsites are of significance, because they regulate transglucosylation, which is used in the production of industrially valuable oligosaccharides. The recently solved three-dimensional structure of GLase from red seaweed revealed the amino-acid residues essential for lyase activity and the strict recognition of the  $\alpha$ -(1  $\rightarrow$  4)-glucosidic substrate linkage. The former was introduced to the GH31 AGase, and the resultant mutant displayed GLase activity. GH13 and GH31 AGases hydrate anhydrofructose to produce glucose, suggesting that AGases are involved in the catabolic pathway used to salvage unutilized anhydrofructose.

**Keywords**  $\alpha$ -Glucosidase ·  $\alpha$ -1,4-Glucan lyase · Anhydrofructose · Structural element · Transglucosylation

## Abbreviations

AGase	$\alpha$ -Glucosidase
1,5AnFru	1,5-Anhydrofructose
CNX	Calnexin
CRT	Calreticulin
CtMGAM	C-terminal subunit of maltase-glucoamylase
CtSI	C-terminal subunit of sucrose-isomaltase
DG	Dextran glucosidase
DP	Degree of polymerization
GAA	Human acid $\alpha$ -glucosidase
GH	Glycoside hydrolase family
GLase	$\alpha$ -1,4-Glucan lyase
HaG	<i>Halomonas</i> sp. $\alpha$ -glucosidase
MGAM	Maltase-glucoamylase
NtMGAM	N-terminal subunit of maltase-glucoamylase
NtSI	N-terminal subunit of sucrose-isomaltase
O16G	Oligo-1,6-glucosidase
PL	Polysaccharide lyase
PsGal31A	<i>Pedobacter saltans</i> $\alpha$ -galactosidase
SBG	Sugar beet $\alpha$ -glucosidase
SI	Sucrose-isomaltase
SmDG	<i>Streptococcus mutans</i> dextran glucosidase
SOG	<i>Schwanniomyces occidentalis</i> $\alpha$ -glucosidase

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## Introduction

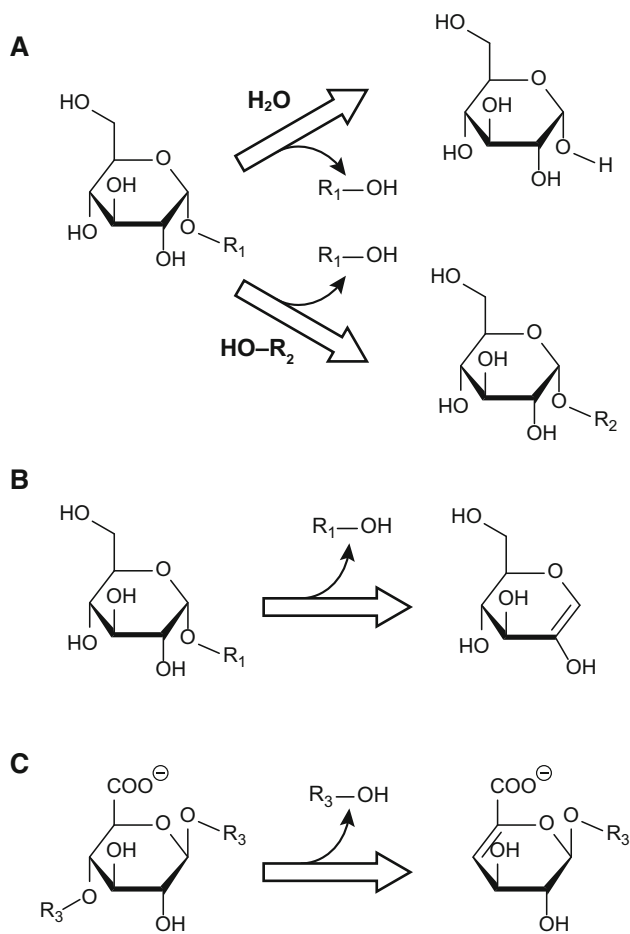
$\alpha$ -Glucosidase (AGase; EC 3.2.1.20) is a typical exohydrolase that reacts with the  $\alpha$ -glucosidic linkage of the non-reducing terminal of substrates and releases  $\alpha$ -glucose [1, 2]. Under high substrate concentrations, the enzyme also catalyzes transglucosylation, synthesizing oligosaccharides

(Fig. 1a). AGases exist in most organisms, indicating the importance of their physiological roles, which generate glucose in the amyolytic metabolism pathway. The reduction in the original AGase activity, caused by inhibition, deletion, or mutation, largely influences plant starch metabolism and animal glycogen metabolism. Enzyme inhibitors prevent the germination of seeds through an accumulation of maltose, which interferes with the AGase-catalyzed conversion from maltose to glucose [3]. In humans, a tissue acid AGase (GAA) deficiency causes a glycogen-storage disease (Pompe disease type II) [4]. These phenomena are indicative of the physiological significance of AGases. Enzymes in the small intestine also supply glucose from  $\alpha$ -amylase-digested starch, followed by an elevation in the blood glucose concentration. Therefore, considerable attention has been given to inhibitors of small intestinal enzymes, such as anti-diabetic

agents [5]. Industrial interests are focused on the AGase-catalyzed production of oligosaccharides having  $\alpha$ -(1  $\rightarrow$  2)-,  $\alpha$ -(1  $\rightarrow$  3)-, or  $\alpha$ -(1  $\rightarrow$  6)-linkages [6, 7]. Thus, it is important to elucidate the structural element(s) that regulate oligosaccharide formation.

AGases exhibit diversity in substrate recognition, and Chiba [1] first classified AGases into three groups, type-I, -II, and -III, based on substrate specificity. Type-I enzymes prefer heteroside linkages (e.g., sucrose and aryl  $\alpha$ -glucosides) to homoside linkages (e.g.,  $\alpha$ -glucobioses, maltooligosaccharides, and  $\alpha$ -glucans), while type-II and type-III AGases display an opposite specificity, recognizing homosides rather than heterosides. The classifications imply that the recognition of  $\alpha$ -glucosyl and  $\alpha$ -glucobiose moieties is performed by type-I enzymes and by type-II and type-III enzymes, respectively. The difference between type-II and type-III is the ability to hydrolyze the polysaccharide: the former has a quite low activity on  $\alpha$ -glucan, and the latter has a high activity. In particular, many important AGases originating from plants and animals are type-III AGases, and their activities on starch and glycogen are significant. Rice AGases contribute to the glucose supply in the early germination stage through the direct degradation of starch granules [8, 9]. As stated above, Pompe disease-related human GAA catalyzes the hydrolysis of glycogen in the lysosomes of liver, heart, skeletal muscle, and kidney [1, 10]. Therefore, it is necessary to understand the molecular mechanisms of type-III AGases that recognize long saccharides. Based on structural classifications, type-I is in the glycoside hydrolase family (GH) 13 group, while type-II and type-III are GH31 members.

$\alpha$ -1,4-Glucan lyase (GLase; EC 4.2.2.13) catalyzes the degradation of the non-reducing ends of  $\alpha$ -glucosidic linkages by C2-proton abstraction to generate the 1,2-enol form of anhydrofructose (Fig. 1b) [11, 12]. The produced 1,2-enol is converted into enol and ketone tautomers, which are mainly present as hydrated forms in aqueous solution. The enzymes are found in algae [13, 14] and fungi [15, 16], in which starch and glycogen are catabolized by alternative pathways directly associated with GLase, indicating that this lyase is classified as an amyolytic enzyme. Excellent studies done by Yu revealed that 1,5-anhydrofructose (1,5AnFru) is further metabolized into a series of physiologically important substances by reductase, kinase, dehydratase, monooxygenase, and isomerase activities [12]. This system, termed the “anhydrofructose pathway,” functions under stress conditions through signal transduction [17]. Findings regarding the metabolic members (enzymes and metabolites) and physiological regulation convinced the International Union of Biochemistry and Molecular Biology to acknowledge the anhydrofructose pathway in 2006.



**Fig. 1** Catalytic reactions of AGase (a), GLase (b), and PL (c). AGase  $\alpha$ -glucosidase, GLase  $\alpha$ -1,4-glucan lyase, PL polysaccharide lyase. In a, the upper and lower reactions are hydrolysis and transglucosylation, respectively.  $R_1$ ,  $\alpha$ -glucosaccharide moiety (including  $\alpha$ -glucan) or a glycon for AGase and maltooligosaccharide moiety or  $\alpha$ -glucan for GLase;  $HO-R_2$ , acceptor substrate for transglucosylation; and  $R_3$ , uronic acid-containing polysaccharide moiety. Reaction of PL appears in the section of “ $\alpha$ -1,4-glucan lyase”

The substrates of GLase are glycogen, starch, and maltooligosaccharides, while the enzyme has less activity on an  $\alpha$ -(1  $\rightarrow$  6)-glucosidic linkage [11], indicating that the reaction is terminated at the  $\alpha$ -(1  $\rightarrow$  6)-branched positions of glycogen and starch, and it forms limited amounts of dextrin having  $\alpha$ -(1  $\rightarrow$  6)-glucosidic bonds at the non-reducing ends. For the efficient conversion of these polysaccharides to 1,5AnFru, using waxy starch with less  $\alpha$ -(1  $\rightarrow$  6)-branches and/or the debranching enzyme-assisted reaction is preferable [18]. 1,5AnFru and its metabolites are natural bioactive materials for pharmaceutical purposes, including anti-cariogenic, anti-inflammatory, anti-obesity, and anti-cancer agents, as well as preventers of nosocomial infections, diabetic markers for glycemic regulation, and stimulators of insulin secretion. In addition, they are inhibitors of tumor growth and metastasis by cytotoxic action, and inhibitory agents against human pathogens [19, 20]. Therefore, GLase is one of the most applicable amyolytic enzymes.

Based on their sequence similarities, GLases, which are categorized in GH31, have a  $\sim 22\%$  identity with GH31 AGases, and both enzymes are further labeled as subfamily 2 and subfamily 1, respectively. They share the same three-dimensional structure, but the catalytic reactions are distinct. In this review, we discuss recent research on AGases (of both GH13 and GH31 enzymes) and GLases by focusing on their three-dimensional structures, including free and ligand-bound forms, conformation-based catalytic functions, and physiological actions. Table 1 summarizes the conformations of the oligosaccharides, glucosides, and pseudosaccharides appearing in this review.

## **GH13 $\alpha$ -glucosidases**

### **Overall structure and common structural features of GH13 $\alpha$ -glucosidases**

GH13 AGases have high sequence similarities to oligo-1,6-glucosidase (EC 3.2.1.10; O16G) and dextran glucosidase (glucan 1,6- $\alpha$ -glucosidase; EC 3.2.1.70; DG), and the AGase-type enzymes fall into subfamilies: GH13\_17, GH13\_23, GH13\_30, and GH13\_31 [21]. Several three-dimensional structures have been reported thus far [22–27], and their overall structures are similar to each other, containing three domains, A, B, and C (Fig. 2a, b). Domain A is a catalytic domain formed by a  $(\beta/\alpha)_8$ -barrel fold. The catalytic nucleophile (Asp) and general acid/base catalyst (Glu), included in the conserved regions II and III of the GH13 enzymes [28], are located at the C-terminal ends of the fourth and fifth  $\beta$ -strands of domain A, respectively. Domain B, containing several  $\alpha$ -helices and  $\beta$ -strands, is inserted between the third  $\beta$ -strand and the third  $\alpha$ -helix of

domain A, and forms part of the wall of the active site pocket. Domain C is formed by anti-parallel  $\beta$ -sheets, and follows domain A. GH13 AGase-type enzymes have two extra  $\alpha$ -helices on  $\beta \rightarrow \alpha$  loop 8 of domain A, which are the main parts of domain B' (the domain nomenclature was established by Møller et al. [27]) that interact with the substrate and domain B. Because of the presence of domain B', they have pocket-shaped substrate-binding sites, unlike GH13 endo-type enzymes that have cleft-shaped substrate-binding sites [29–32]. As in GH13 AGase-type enzymes, other GH13 exo-type enzymes have pocket-shaped substrate-binding sites owing to the presence of a B'-like domain. Two additional  $\alpha$ -helices on the  $\beta \rightarrow \alpha$  loop 8 of domain A form an additional domain in trehalulose synthase similar to that observed in AGase-type enzymes [33], whereas in sucrose phosphorylase, amylosucrase, and trehalose synthase, long  $\beta \rightarrow \alpha$  loops 7 and 8 of domain A form an additional domain [34–36].

As commonly observed in GH13 enzymes, the conserved His (Fig. 3, region I), Arg (region II), and His (region IV) residues of GH13 AGase-type enzymes form hydrogen-bond interactions with the non-reducing end of a glucosyl residue in the substrate at subsite  $-1$ , and the Tyr on  $\beta \rightarrow \alpha$  loop 2 of domain A stacks onto the glucosyl residue [23, 26] (Fig. 2c). The Asp and Arg, which form a salt-bridge, recognize the non-reducing end of the substrate through an interaction with the 4OH of the glucosyl residue. These equivalent Asp and Arg residues are also found in the other exo-type enzymes in GH13 [33, 37, 38].

Hondoh et al. [26] found a row of three water molecules that connected the bottom of the active-site pocket to the surface of the enzyme on the opposite side of the active pocket entrance in the DG structure. Rows of water molecules are also seen in the other exo-type enzymes having pocket-shaped substrate-binding sites [37–39]. Because the exo-type GH13 enzymes share this water path, it might be important for the enzyme reactions. Water molecules occupying the substrate-binding site need to be displaced by the substrate when it enters an active site. However, the structural analysis of DG suggested that the active-site pocket is too narrow to accommodate both the incoming substrate and the water molecules occupying the active site [26]. This indicates the possibility of another path for draining the water molecules from the active site. The water path observed in DG and other exo-type enzymes could work as the water drain. Substrate molecule entering the active site could push the water molecules through this water drain.

Some GH13 AGases possess a calcium ion bound to the  $\beta \rightarrow \alpha$  loop 1 of domain A [22, 26, 27, 39]. Similarly, some other GH13 enzymes, such as neopullulanase [40], cyclodextrin glucanotransferase [32], trehalulose synthase [33], and  $\alpha$ -amylase [41], also have a calcium ion at the

**Table 1** Conformations of oligosaccharides, glucosides, and pseudosaccharides in this review

Sugar	Structure	Remark
Isomaltose	$\alpha$ -D-Glcp-(1 $\rightarrow$ 6)-D-Glcp	$\alpha$ -Glucobiose
Maltose	$\alpha$ -D-Glcp-(1 $\rightarrow$ 4)-D-Glcp	$\alpha$ -Glucobiose
Nigerose	$\alpha$ -D-Glcp-(1 $\rightarrow$ 3)-D-Glcp	$\alpha$ -Glucobiose
Trehalose	$\alpha$ -D-Glcp-(1 $\leftrightarrow$ 1)- $\alpha$ -D-Glcp	$\alpha$ -Glucobiose; $\alpha,\alpha$ -trehalose
Isomaltulose	$\alpha$ -D-Glcp-(1 $\rightarrow$ 6)-D-Fruf	Fructose-containing disaccharide
Sucrose	$\alpha$ -D-Glcp-(1 $\leftrightarrow$ 2)- $\beta$ -D-Fruf	Fructose-containing disaccharide
Trehalulose	$\alpha$ -D-Glcp-(1 $\rightarrow$ 1)-D-Fruf	Fructose-containing disaccharide
Centose	$\alpha$ -D-Glcp-(1 $\rightarrow$ 4)-[ $\alpha$ -D-Glcp-(1 $\rightarrow$ 2)]-D-Glcp	Trisaccharide
Panose	$\alpha$ -D-Glcp-(1 $\rightarrow$ 6)- $\alpha$ -D-Glcp-(1 $\rightarrow$ 4)-D-Glcp	Trisaccharide
Erllose	$\alpha$ -D-Glcp-(1 $\rightarrow$ 4)- $\alpha$ -D-Glcp-(1 $\leftrightarrow$ 2)- $\beta$ -D-Fruf	Trisaccharide
Isomaltooligosaccharide	[6]- $\alpha$ -D-Glcp-(1 $\rightarrow$ ) <sub>n</sub>	Isomaltotriose: $n = 3$
Maltooligosaccharide	[4]- $\alpha$ -D-Glcp-(1 $\rightarrow$ ) <sub>n</sub>	Maltotriose: $n = 3$
Nigerooligosaccharide	[3]- $\alpha$ -D-Glcp-(1 $\rightarrow$ ) <sub>n</sub>	Non-reducing $\alpha$ -nigerosyl unit-having $\alpha$ -glucooligosaccharides also belong in this category
Cycloamylose DP26	Cyclo[4]- $\alpha$ -D-Glcp-(1 $\rightarrow$ ) <sub>26</sub>	Cyclic saccharide
Alkyl $\alpha$ -glucoside	$\alpha$ -D-Glcp-(1 $\rightarrow$ )-AL	AL: curcumin, ethanol, gingerol, glycerol, or <i>l</i> -menthol unit
Aryl $\alpha$ -glucoside	$\alpha$ -D-Glcp-(1 $\rightarrow$ )-AR	AR: (+)-catechin, hydroquinone, <i>p</i> -nitrophenol, or phenol unit
$\alpha$ -Glucosyl fluoride	$\alpha$ -D-Glcp-(1 $\rightarrow$ )-F	
$\alpha$ -Glucose 1-phosphate	$\alpha$ -D-Glcp-(1 $\rightarrow$ )-OPO <sub>3</sub> <sup>2-</sup>	
Acarviosyl-maltooligosaccharide	Acv-[ $\alpha$ -D-Glcp-(1 $\rightarrow$ 4)] <sub>n</sub> -D-Glcp	Acv: acarviosyl unit, of which structure is shown in Fig. 6b (located at subsite -1 and +1); acarbose: $n = 1$ ; acarviosyl-maltohexaose: $n = 5$

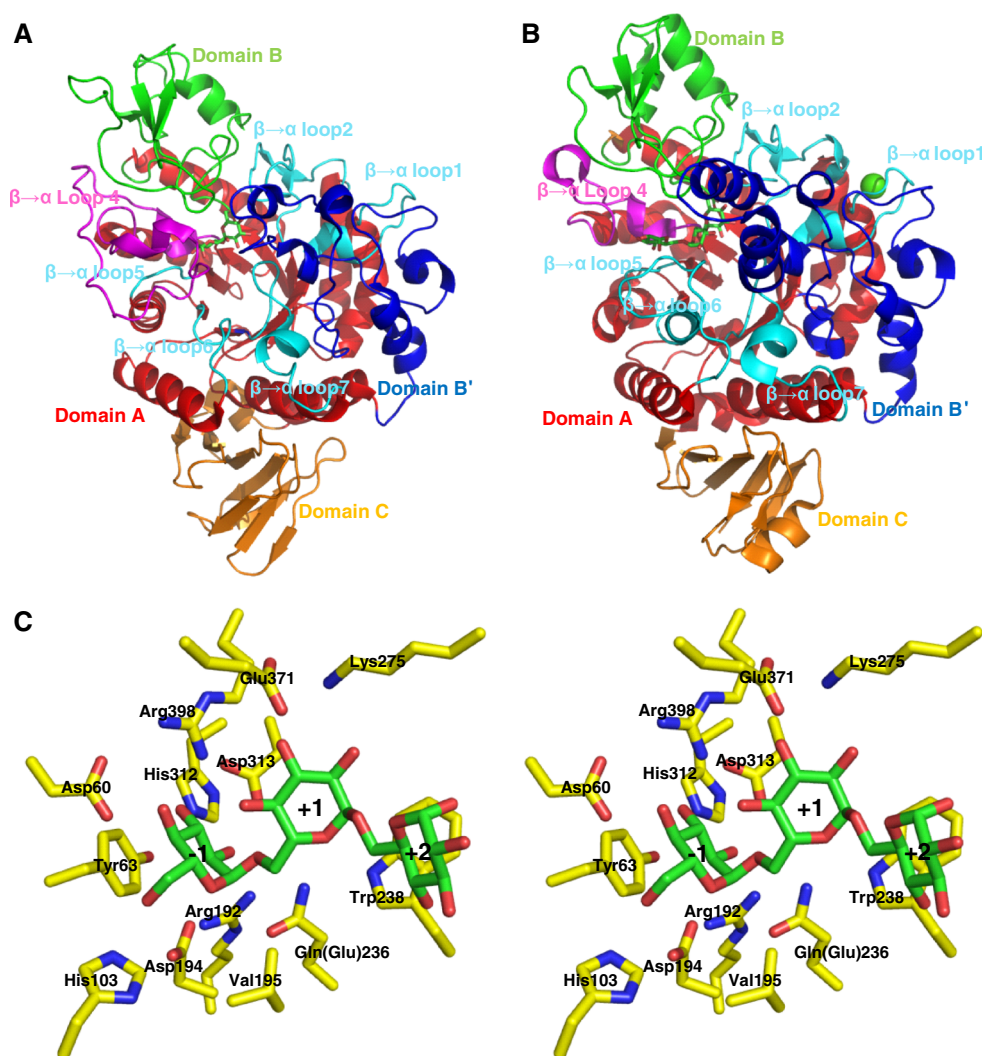
equivalent position. This calcium ion binding site is different from that generally found in  $\alpha$ -amylase, in which a calcium ion binds to domain B, maintaining the active site's structure [42]. Together with the kinetic analysis conducted using *Streptococcus mutans* DG (*SmDG*), which demonstrated a calcium ion-enhanced thermal stability [43], the evidence suggests that the calcium ion bound to  $\beta \rightarrow \alpha$  loop 1 is involved in enzyme stabilization at high temperatures.

### Glucosidic linkage specificity of GH13 $\alpha$ -glucosidases

Although some enzymes have high hydrolytic activities on sucrose and/or trehalose [44, 45], GH13 AGase-type enzymes are roughly divided into two groups based on their specificity for scissile glucosidic linkages:  $\alpha$ -(1  $\rightarrow$  4)-glucosidic linkage-specific enzyme, such as AGase [46–48], and  $\alpha$ -(1  $\rightarrow$  6)-glucosidic linkage-specific enzymes, such as DG and O16G [27, 49–51]. GH13\_31 contains both types of enzymes [21], indicating that a small number of amino-acid residues can determine the specificity for the glucosidic linkage. Comparisons of amino-acid sequences between  $\alpha$ -(1  $\rightarrow$  4)- and  $\alpha$ -(1  $\rightarrow$  6)-glucosidic linkage-specific glucosidases demonstrated that

the amino-acid residue next to the catalytic nucleophile is differently conserved depending on the glucosidic linkage specificity: Ala or Thr was found at the position in  $\alpha$ -(1  $\rightarrow$  4)-linkage-specific enzymes, and Val was conserved in the  $\alpha$ -(1  $\rightarrow$  6)-linkage-specific enzymes (Fig. 3). A structural analysis of *SmDG* complexed with isomaltotriose, covering subsite -1 to +2, showed that Val195, next to the catalytic nucleophile, made hydrophobic contact with the  $\alpha$ -(1  $\rightarrow$  6)-linked substrate, and this interaction is favorable for binding the substrate [26]. This Val residue is predicted to cause a steric hindrance upon binding to an  $\alpha$ -(1  $\rightarrow$  4)-linked substrate. In fact, the substitution of the Val residue in several  $\alpha$ -(1  $\rightarrow$  6)-linkage-specific enzymes resulted in increasing maltase activity [52–54]. However, enzymes with Val mutated still retained hydrolytic activity on the  $\alpha$ -(1  $\rightarrow$  6)-glucosidic linkage (the V195A mutant enzyme showed only 3.9-fold lower  $k_{cat}/K_m$  for isomaltose); therefore, other amino-acid residues must contribute to the recognition of this linkage (Table 2). Lys275 and Glu371 of *SmDG*, located on the  $\beta \rightarrow \alpha$  loop 6 and second  $\alpha$ -helix of domain B', respectively, form hydrogen-bond interactions with the 2OH and 3OH groups of the glucosyl residue at subsite +1 [26]. Equivalent amino-acid residues are found in almost all of the  $\alpha$ -(1  $\rightarrow$  6)-glucosidic linkage-specific glucosidases

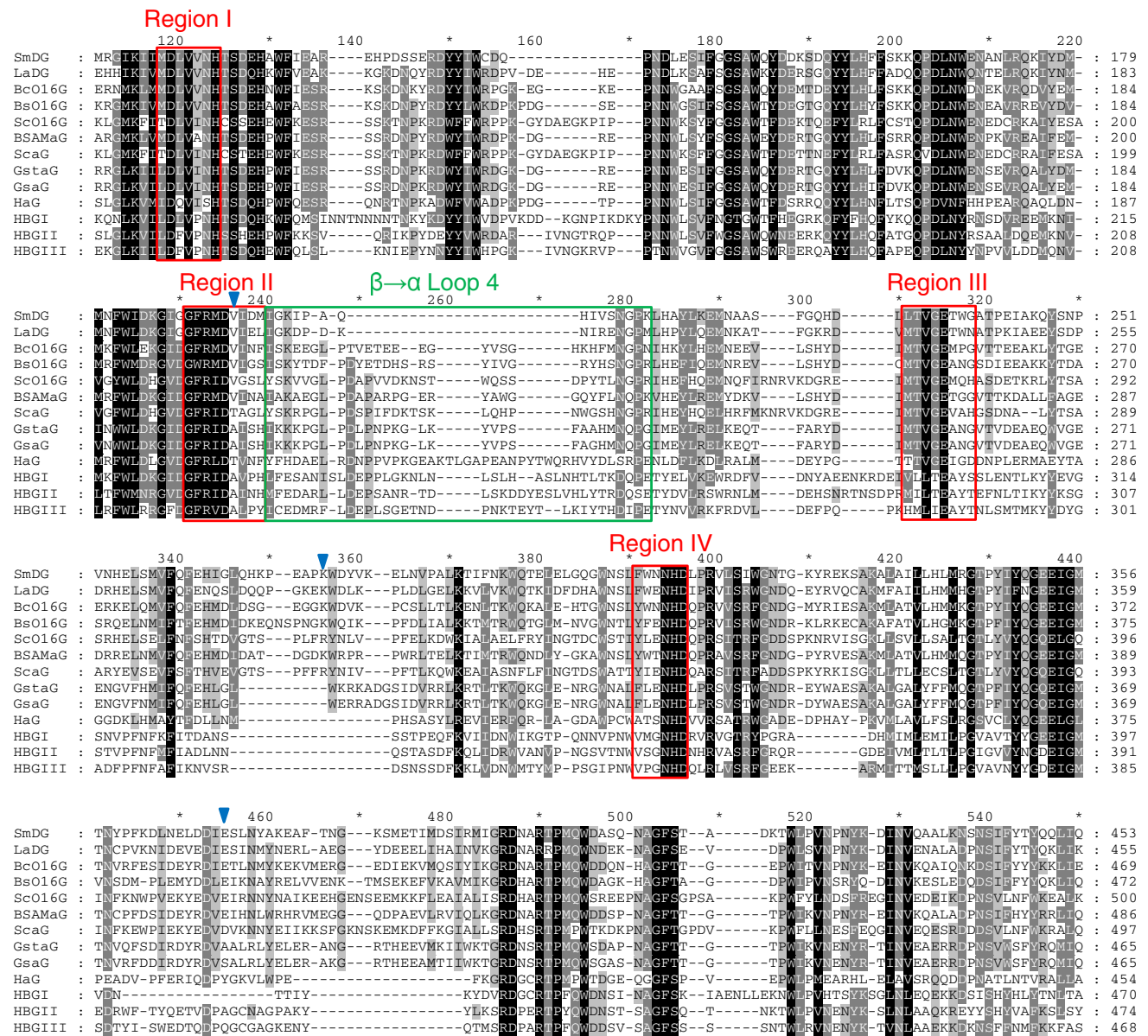
**Fig. 2** Three-dimensional structures of GH13 AGases. **a** Overall structure of HaG and maltose complex (PDB, 3WY4). **b** Overall structure of *Sm*DG and isomaltotriose complex (PDB, 2ZID). Domain A red; domain B green; domain C orange; domain B' blue;  $\beta \rightarrow \alpha$  loop 1, 2, 5, 6, and 7, cyan; and  $\beta \rightarrow \alpha$  loop 4, magenta. The substrates bound to the enzymes are shown by green stick. Calcium ion bound to the  $\beta \rightarrow \alpha$  loop 1 of domain A is indicated by a green sphere. It is thought to regulate the thermostability based on the kinetic experiment [43]; **c** close-up view of the *Sm*DG active site. Isomaltotriose covers from subsite -1 to +2. The inactive mutant enzyme (E236Q) was used to trap the Michaelis complex



(Fig. 3). One exception is Arg315 of yeast isomaltase, which corresponds to Lys275 of *Sm*DG [55]. Single mutations at Lys275 and Glu371 of *Sm*DG (K275A and E371A) lowered activities on both isomaltose and maltose, but the decrease was more severe for the former, indicating that these amino-acid residues are important for  $\alpha$ -(1  $\rightarrow$  6)-glucosidic linkage recognition (Table 2) [54]. Combining K275A and/or E371A mutations with the V195A mutation significantly enhanced  $\alpha$ -(1  $\rightarrow$  4)-glucosidic linkage selectivity. The triple mutant *Sm*DG (V195A/K275A/E371A) had a 73-fold higher  $k_{\text{cat}}/K_{\text{m}}$  for maltose than isomaltose, whereas the  $k_{\text{cat}}/K_{\text{m}}$  of wild-type for maltose is  $1.07 \times 10^4$ -fold lower than that for isomaltose. In *Saccharomyces cerevisiae*'s isomaltase, Gln279 is situated two residues after the general acid/base catalyst and causes a steric hindrance upon binding to maltose [55]. Conversely, the substitution of the corresponding amino-acid residue, Gly273, in *Bacillus* sp. SAM1606 AGase, which shows the structural features of an  $\alpha$ -(1  $\rightarrow$  6)-

glucosidic linkage-specific glucosidase but has relatively high maltase activity [44], with a bulky residue decreased maltase activity more severely than isomaltase activity [56, 57]. This Gly residue is also important for the high activity of this enzyme on trehalose [56]. These observations suggest that having a bulky amino-acid residue located two residues after the general acid/base catalyst is unfavorable for the hydrolysis of  $\alpha$ -(1  $\rightarrow$  4)-linked substrates.

Compared with  $\alpha$ -(1  $\rightarrow$  6)-glucosidic linkage-specific glucosidases, less is understood regarding the molecular basis for the specificity of  $\alpha$ -(1  $\rightarrow$  4)-glucosidic linkage. In  $\alpha$ -(1  $\rightarrow$  4)-glucosidic linkage-specific AGases, only the enzyme-substrate complex of *Halomonas* sp. AGase (HaG; GH13\_23) has been determined thus far [23]. From the structure of the HaG E271Q mutant complexed with maltose, the Phe297 residue of HaG on  $\beta \rightarrow \alpha$  loop 6 is a determinant of the glucosidic linkage specificity. The orientation of this Phe residue's side-chain is different from



**Fig. 3** Multiple alignment of GH13 AGases. Amino-acid sequences of GH13 AGases were aligned using the MAFFTash program [70]. SmDG, *Streptococcus mutans* DG (GenBank ID: BAE79634.1); LaDG, *Lactobacillus acidophilus* DG (GenBank ID: AAV42157.1); BcO16G, *Bacillus cereus* O16G (GenBank ID: CAA37583.1); BsO16G, *Bacillus subtilis* O16G (GenBank ID: CAB15461.1); ScO16G, *Saccharomyces cerevisiae* isomaltase (GenBank ID: BAA07818.1); BSAMaG, *Bacillus* sp. SAM1606 AGase (GenBank ID: CAA54266.1); ScaG, *S. cerevisiae* maltase (GenBank ID: CAA85264.1); GstaG, *Geobacillus stearothermophilus* AGase (GenBank ID: BAA12704.1); GsaG, *Geobacillus* sp. HTA426 AGase (GenBank ID: BAE48285.1); HaG, *Halomonas* sp. H11 AGase

(GenBank ID: BAL49684.1); HBGI, *Apis mellifera* AGase I (GenBank ID: BAE86926.1); A. mellifera AGase II (GenBank ID: BAE86927.1); and A. mellifera AGase III (GenBank ID: BAE11466.1). SmDG, LaDG, BcO16G, BsO16G, and ScO16G are specific to α-(1 → 6)-glucosidic linkage, and ScaG, GstaG, GsaG, HaG, HBGI, HBGII, and HBGIII have high activities toward α-(1 → 4)-linked substrates. BSAMaG has a high activity toward both α-(1 → 6)- and α-(1 → 4)-linked substrates. Conserved regions and β → α loop 5 are indicated by red and green boxes, respectively. Amino-acid residues responsible for the α-(1 → 6)-glucosidic-linkage specificity are indicated by inverted triangles

that of Phe262 in SmDG and Phe303 in *S. cerevisiae* isomaltase, which are at the corresponding position, and the Phe residue is predicted to cause a steric hindrance upon isomaltose binding. The contribution of the Phe residue to

the recognition of the α-(1 → 4)-linked substrate in HaG has not been biochemically confirmed, and thus, mutational analyses are required to better understand α-(1 → 4)-glucosidic linkage specificity.

**Table 2** Amino-acid residues responsible for substrate specificity and transglucosylation activity in GH13 AGases

Enzyme	Amino acid	$\beta \rightarrow \alpha$ loop	Subsite	Function
<i>SmDG</i>	Val195	4	+1	Specificity to $\alpha$ -(1 $\rightarrow$ 6)-glucosidic linkage
	Trp238	5	+1 and +2	Selectivity to long-chain substrates and transglucosylation activity
	Phe262	6	None <sup>a</sup>	Regulating orientation of Trp238
	Lys275	6	+1	Specificity to $\alpha$ -(1 $\rightarrow$ 6)-glucosidic linkage
	Glu371	8 <sup>b</sup>	+1	Specificity to $\alpha$ -(1 $\rightarrow$ 6)-glucosidic linkage
<i>L. acidophilus</i> DG	Arg212	4	+2	Selectivity to long-chain substrates
	Asn243	5	+2	Selectivity to long-chain substrates
<i>Bacillus</i> sp. SAM1606 AGase	Gly273	5	+1	Selectivity to trehalose and maltose
<i>S. cerevisiae</i> isomaltase	Gln279	5	+1	Specificity to $\alpha$ -(1 $\rightarrow$ 6)-glucosidic linkage
<i>Halomonas</i> sp. AGase	Phe297	6	+1	Specificity to $\alpha$ -(1 $\rightarrow$ 4)-glucosidic linkage
HBG-I	Pro233	4	+1	Selectivity to $\alpha$ -(1 $\rightarrow$ 4)-glucosidic linkage in transglucosylation
	His234	4	+1	High selectivity to maltose
HBG-II	Asn226	4	+1	Selectivity to $\alpha$ -(1 $\rightarrow$ 6)-glucosidic linkage in transglucosylation
	His227	4	+1	High selectivity to maltose
HBG-III	Pro226	4	+1	Selectivity to $\alpha$ -(1 $\rightarrow$ 4)-glucosidic linkage in transglucosylation
	Tyr227	4	+1	High selectivity to sucrose

<sup>a</sup> Phe262 has no direct interaction with the substrate

<sup>b</sup> Corresponds to domain B'

### Substrate chain-length specificity of GH13 $\alpha$ -glucosidases

The substrate chain-length specificity of GH13 exo-glucosidases is diverse, as observed in GH31 AGases. AGases and O16G are highly selective for short-chain substrates (in many cases, the catalytic efficiency of trisaccharide is the highest among a series of oligosaccharides) [47, 48, 50, 58, 59]. DG has the highest activity on isomaltotriose among isomaltooligosaccharides, similar to O16G, but this enzyme shows a higher preference than O16G for long-chain substrates, isomaltoheptaose, and dextran T2000 [27, 51]. HaG has high disaccharide selectivity [48]. This enzyme has only 5 % of the hydrolytic activity on maltotriose compared with the activity on maltose, and does not have measurable hydrolytic activity on maltooligosaccharides longer than maltotriose.

A comparison of amino-acid sequences and three-dimensional structures of GH13 exo-glucosidases showed that the length of  $\beta \rightarrow \alpha$  loop 4 of domain A differs depending on the substrate chain-length specificity: the enzymes with high and low preference for long-chain substrates have short and long  $\beta \rightarrow \alpha$  loop 4 lengths, respectively (Fig. 3). The long  $\beta \rightarrow \alpha$  loop 4 of HaG covers a major part of the active site entrance and disturbs the formation of subsite +2 [23]. Similarly, Ser222, His224, and Met228 on  $\beta \rightarrow \alpha$  loop 4 of *Bacillus cereus* O16G are predicted to cause steric hindrance upon binding to long-chain substrates [25, 26]. In contrast to these enzymes, the short  $\beta \rightarrow \alpha$  loop 4 of *SmDG* provides space

for long-chain substrates. The contribution of  $\beta \rightarrow \alpha$  loop 4 to substrate chain-length specificity was confirmed by the biochemical analyses [51, 60]. Shortening the  $\beta \rightarrow \alpha$  loop 4 of AGase from *Bacillus* sp. SAM1606 broadened the substrate chain-length specificity, and exchanging the short  $\beta \rightarrow \alpha$  loop 4 of *SmDG* with the corresponding long loop of *Bacillus subtilis* O16G decreased the preference for long-chain substrates.

*SmDG* has a bulky amino-acid residue, Trp238, at the C-terminal end of  $\beta \rightarrow \alpha$  loop 5 [26]. This amino-acid residue spans subsites +1 and +2. Most other GH13 AGases and related enzymes have small amino-acid residues at this position, while GH13\_20 neopullulanases and GH13\_36  $\alpha$ -amylases possess Trp or aromatic residues as the equivalent [61]. The substitution of Trp238 in *SmDG* with a smaller amino-acid residue decreases catalytic efficiency for isomaltooligosaccharides (longer than isomaltose) relative to that for isomaltose (Table 2) [51]. The lower preference of Trp238-mutated *SmDG* for long-chain substrates indicates that this residue, together with the short  $\beta \rightarrow \alpha$  loop 4, is important for the hydrolytic activity on long-chain substrates. Trp238 is thought to lead a long-chain substrate to the space formed by the short  $\beta \rightarrow \alpha$  loop 4. In DG from *Lactobacillus acidophilus*, which has a higher preference for long-chain substrates than *SmDG*, the additional interactions of Arg212 and Asn243 are predicted to form hydrogen bonds with the glucosyl residue at subsite +2 and contribute to the high activity on long-chain substrates [27].

### Transglucosylation of GH13 $\alpha$ -glucosidases

AGases also catalyze transglucosylation, in which the glucosyl residue at the non-reducing end of the substrate is transferred to a hydroxy group of the acceptor molecule and are utilized as biocatalysts for the production of oligosaccharides and glucosides. HaG is an efficient enzyme that produces  $\alpha$ -glucosides using maltose as a glucosyl donor, because this enzyme does not use maltose as an acceptor substrate owing to its high disaccharide specificity and rarely produces trisaccharides through the transglucosylation [48]. The efficient production of  $\alpha$ -glucosyl glycerol and  $\alpha$ -glucosyl 6-gingerol using HaG has been reported [48, 62]. The latter was first synthesized using HaG. Similarly, an AGase from *Xanthomonas campestris* belonging to GH13\_23 efficiently synthesizes glucosides through transglucosylation, in which L-menthol, (+)-catechin, and hydroquinone are used as acceptor substrates [63–65]. AGase from *Geobacillus* sp. HTA426, belonging to GH13\_31, utilizes various alkyl and aromatic alcohols as acceptors in transglucosylation. In the reaction with curcumin as the acceptor, mono-, di-, and, tri-glucosyl compounds are generated [22].

Amino-acid residues forming substrate-binding sites can regulate the transglucosylation activity. The substitution of Trp238 in *SmDG* with non-aromatic amino-acid residues (Asn, Pro, and Ala), which formed subsites +1 and +2, decreases transglucosylation activity on the substrate *p*-nitrophenyl  $\alpha$ -glucoside [51]. A structural analysis of the glucosyl-enzyme intermediate of *SmDG*, captured using the general acid/base mutant E236Q and  $\alpha$ -glucosyl fluoride as a substrate with a good leaving group, reveals that Trp238's side chain turns away from the active-site pocket in the intermediate [66], even though this Trp forms an aromatic stacking interaction with the glucosyl residues in subsites +1 and +2 in the Michaelis complex [26]. This structural change may be due to the elimination of the hydrogen bonding interaction between Trp238 and the general acid/base catalyst Glu236 that is induced by the covalent bond formation between the anomeric carbon of glucose and the catalytic nucleophile Asp194 in subsite -1. This structural information suggests that the Trp238 side chain is flexible, and that the conformation of the side chain is altered during the reaction process. Because the Phe262 side chain in *SmDG* interacts with the Trp238 side chain through aromatic stacking [26], Phe262 is predicted to regulate the orientation of the Trp238 side chain. To assess the importance of the Trp238 side chain's flexibility in transglucosylation, Phe262-mutated *SmDGs* in which the Phe262 of *SmDG* was substituted with Ala and Trp to increase and decrease the flexibility of Trp238, respectively, were analyzed [66]. F262A and F262W mutant *SmDGs* demonstrate stronger and weaker preferences,

respectively, for transglucosylation than the wild-type enzyme, indicating that the high flexibility of the Trp238 side chain is important for the high transglucosylation activity of *SmDG*.

The replacement of the catalytic carboxylate of inverting glycosidases, including glucoamylase and cellulase, with cysteine sulfinate ( $-\text{SOO}^-$ ) alters enzymatic properties. It increases the catalytic activity of glucoamylase and raises cellulase activity at acidic pH levels [67, 68]. The introduction of cysteine sulfinate as the catalytic nucleophile in *SmDG* reduces significantly the catalytic activity on *p*-nitrophenyl  $\alpha$ -glucoside, but enhances significantly the preference for transglucosylation [69]. The mutant enzyme with cysteine sulfinate as the catalytic nucleophile shows a higher transglucosylation activity not only on *p*-nitrophenyl  $\alpha$ -glucoside but also on a natural substrate, isomaltooligosaccharide. Thus, this approach may be useful for enhancing the transglucosylation efficiency rather than using synthetic substrates with good leaving groups.

### GH13 enzymes acting on sucrose

Some AGases act on sucrose and cleave its  $\alpha$ -glucosidic linkages. However, no three-dimensional structures of the enzyme proteins in a complex with sucrose have been determined yet. GH13 includes some other sucrose-acting enzymes, such as amylosucrase (EC 2.4.1.4; GH13\_4), sucrose phosphorylase (EC 2.4.1.7; GH13\_18), and sucrose isomerase (isomaltulose synthase, trehalulose synthase, EC 5.4.99.11; GH13\_31) [21]. Structures of sucrose complexes with the acid-base mutants of the enzymes have been determined and provide insights into their recognition of sucrose as a substrate [33–35, 37, 38]. The enzymes catalyze glucosyl transfers, phosphorolysis, and isomerization, but their catalytic domains are ( $\beta/\alpha$ )<sub>8</sub>-barrel fold in shape, and the active sites are well conserved as observed in GH13 enzymes. Seven highly conserved amino-acid residues, including catalytic residues and residues involved in the formation of subsite -1, are found in the structures. The conservation of five additional residues (Asp in  $\beta \rightarrow \alpha$  loop 2, two Phe and Gln in domain B, and Arg in the first extra helix in  $\beta \rightarrow \alpha$  loop 8) in the sucrose-acting enzymes, together with oligo-1,6-glucosidases and AGases, has been pointed out by Ravaud et al. [33].

The first Asp in loop 2 and the last Arg in loop 8 form a salt bridge and prevent substrates from binding toward the subsite -2. The glucose moiety of sucrose is well accommodated in subsite -1, as was in general found in GH13 enzymes, through interactions with the highly conserved residues. The salt bridge-forming Asp and Arg also provide hydrogen bonds with O4, as observed in AGases and related enzymes. However, the binding modes of the



fructose moiety are diverse. Amylosucrase [34] and sucrose phosphorylase [35] possess a long insertion in  $\beta \rightarrow \alpha$  loop 7 at the position just following the third catalytic Asp of conserved region IV. This insertion in loop 7 forms domain B'. The same denotation is used in dextran 6- $\alpha$ -glucosidase for loop 8 [27], but domain B' in amylosucrase and sucrose phosphorylase is in loop 7. Domain B' of amylosucrase forms a part of the pocket and the active site is at the bottom. Amino acids directly interacting with the fructosyl residues are found only in domain B'. Asp394 and Arg446 in the amylosucrase of *Neisseria polysaccharia* mediated the hydrogen bonds to O6 at the fructosyl residue [37]. In the sucrose phosphorylase from *Bifidobacterium adolescentis*, Asp342 and Gln345 in domain B' directly interact with fructosyl O4 and O5, respectively [38]. Interestingly, the two residues are on one of the flexible loops, and the drastic change in the orientation of the side chains has been suggested to better accommodate the inorganic phosphate to produce glucose 1-phosphate ( $\alpha$ -Glc1P) in the second half of the reaction process. Sucrose isomerase (trehalulose synthase) from *Pseudomonas mesoacidophila* has no equivalent insertion in loop 7, but a pair of Phe residues form an aromatic clamp, which is probably involved in product specificity, and in the entry and release of molecules [33]. One of them, Phe256, which occurs two residues after the acid/base catalyst in the sequence and is equivalent to Trp238 in *SmDG* mentioned above, hydrophobically interacts with C3 of the fructose moiety. In addition, Arg414, which is one of the five additional conserved residues and forms a salt bridge in subsite -1, has an extra hydrogen bond with fructosyl O6.

### Honeybee $\alpha$ -glucosidases

In the European honeybee (*Apis mellifera*), three AGase isozymes, HBG-I, HBG-II, and HBG-III, have been found [45, 71]. The three enzymes are expressed in different organs at different stages of the bees' life [72]. HBG-I is localized in the ventriculus. HBG-II is present in the ventriculus and the hemolymph. HBG-III is secreted from the hypopharyngeal glands into the nectar and is directly involved in honey formation through the hydrolysis of sucrose in the nectar. In fact, the AGase present in honey is identical to HBG-III in enzymatic properties and N-terminal sequences as determined by protein sequencing [72]. The expression level of the gene encoding HBG-III is limited only in worker bees after age-dependent change of the roles of the hypopharyngeal glands, which produce royal jelly in nurse bees [73]. The functional/physiological transition of the hypopharyngeal glands occurs through the actions of juvenile hormones and ecdysone. The HBG-III

gene's expression level is used as an "indicator" of the age-dependent roles of bees [74].

The three AGases from honeybee are clearly different in substrate specificity, ability to catalyze transglucosylation, and regioselectivity in transglucosylation. HBG-I prefers maltose to sucrose. Maltotriose is the best substrate, and nigerose, isomaltose, and soluble starch are poor substrates [75]. HBG-I catalyzes transglucosylation at higher rates than the others with  $\alpha$ -1  $\rightarrow$  4 regioselectivity, to produce erlose [ $\alpha$ -Glc-(1  $\rightarrow$  4)- $\alpha$ -Glc-(1 $\leftrightarrow$ 2)- $\beta$ -Fru] from sucrose, and maltotriose and even longer maltooligosaccharides from maltose [76]. HBG-II also prefers maltose to sucrose, like HBG-I, but glucobioses having  $\alpha$ -(1  $\rightarrow$  2)-,  $\alpha$ -(1  $\rightarrow$  3)-, and  $\alpha$ -(1  $\rightarrow$  6)-glucosidic linkages, phenyl  $\alpha$ -glucoside, and sucrose are substrates of HBG-II, in addition to maltooligosaccharides [77]. HBG-II even hydrolyses  $\alpha$ -Glc1P and soluble starch to some degree. HBG-I and HBG-II exhibit non-Michaelis-Menten kinetics, which is considered as allosteric behavior for the enzymes, even though they are monomeric enzymes [75, 77]. For instance, HBG-I cleaves the linkage of maltose, sucrose, and *p*-nitrophenyl  $\alpha$ -glucoside in a mode of negative cooperativity [75], whereas HBG-II shows positive cooperativity [77]. Honey-forming HBG-III, however, follows typical Michaelis-Menten kinetics. Maltotriose is the best substrate in naturally occurring carbohydrates for HBG-III, as for HBG-I and HBG-II, but HBG-III prefers sucrose to maltose. Another enzymatic characteristic of HBG-III is the high  $k_{cat}$  and  $K_m$  values on sucrose, indicating that HBG-III is suitable for hydrolyzing the high sucrose concentrations in nectar [45].

Unlike *Drosophila*, which possess 10 AGase-coding genes its genome, European honey bee possesses only three AG-coding genes, *hbg1*, *hbg2*, and *hbg3*, in its genome. Two homologous genes of the honey bee encode the heavy chains of amino-acid transporter proteins, and the other one, AG-like gene (*AGluL*), encodes a function-unknown protein which lacks both AG and transporter features [78]. Some AGase isozymes are also found in other species of bees. A Japanese subspecies of the eastern honeybee, *Apis cerana japonica*, possesses at least two AGase isozymes in the adult body [79]. One, JBG-I, is similar to HBG-I in enzymatic properties, and the protein sequences share a 76 % amino-acid identity. JBG-I displays an even higher transglucosylation from *p*-nitrophenyl  $\alpha$ -glucoside and sucrose at low substrate concentrations. Three AGase isozymes are also reported in *Apis cerana indica* [80], and the primary structure of one has a high amino-acid identity (97 %) with HBG-III [81].

The amino-acid identities among the HBG-I, HBG-II, and HBG-III sequences are 38–44 % [80]. The three honeybee enzymes fall into GH13 subfamily 17 (GH13\_17), which is composed of AGases from insects,

including bees, mosquitos, and flies. The three-dimensional structures of the proteins in this subfamily have not yet been determined. A sequence alignment with the known structure of *B. cereus* O16G [82] suggests that the common structural features of the honeybee enzymes include the following: (1) long  $\beta \rightarrow \alpha$  loop 4; (2) short loop 6; (3) no insertion in  $\beta \rightarrow \alpha$  loop 7 (therefore, there is no structure corresponding to the B' domains in amylosucrases or sucrose phosphorylases); and (4) short  $\beta \rightarrow \alpha$  loop 8 (missing most of the sequences forming  $\alpha 8'$  and  $\alpha 8''$  in the *B. cereus* O16G structure, which corresponds to the first half of domain B' of glucan 1,6- $\alpha$ -glucosidase). All of the highly conserved amino-acid residues are conserved, including the five extra residues, Asp81, Phe168, Phe187, Gln191, and Arg413 (HBG-III numbering). In addition, one of the two Phe residues that form the aromatic clamp of trehalulose synthase [33] is conserved in loop 6, and the other on loop 5 is replaced by Tyr at the position 288. The amino-acid residue next to the catalytic nucleophile, the equivalent of Val195 in *SmdG*, is Ala. Therefore, a possible Michaelis complex of the three enzymes with sucrose might be similar to that observed in trehalulose synthase, with possible hydrogen bonding from Arg413 to O4 of the glucosyl residue and O6 of the fructosyl residue, together with a hydrophobic interaction between Tyr288 and C3 of the fructosyl moiety.

Ngwiwsara et al. [83] focused on C-terminal neighboring amino-acid residues from the nucleophile catalyst, Asp223 (HBG-III), because the two amino-acid residues corresponding to Pro226 and Tyr227 of HBG-III are characteristic signatures of GH13 enzymes [28]. The site-directed mutagenesis experiment provided strong evidence that the two residues (Pro226 and Tyr227 of HBG-III) are crucial factors in discriminating among HBG-I, -II, and -III and their enzymatic properties (Table 2). The replacement of Pro-Tyr by Asn-His (HBG-II type) resulted in drastic changes in the enzymatic properties of the parent HBG-III toward HBG-II as follows: (1) substrate preference (maltose is more suitable than sucrose for the mutant); (2) large increase in the transglucosylation ratio (68–97 % on 150 mM maltose); and (3) alternation of regioselectivity of transglucosylation, in which the mutant synthesizes an  $\alpha$ -(1  $\rightarrow$  6)-linkage rather than an  $\alpha$ -(1  $\rightarrow$  4)-linkage. Conversely, the HBG-II mutant harboring an HBG-III-like Pro-Tyr in the position preferred sucrose, having a 3.2-fold higher reaction rate ( $k_{\text{cat}}/K_m$ ), and had a decreased transglucosylation ratio, compared with parent HBG-II. The mutant HBG-II produced  $\alpha$ -(1  $\rightarrow$  4)-linked transglucosylation products from both sucrose and maltose. HBG-I-type mutants of both HBG-III and HBG-II showed high activities, particularly on maltooligosaccharides, and preferred maltose to sucrose. The transglucosylation ratio was high in both mutants and  $\alpha$ -(1  $\rightarrow$  4)-regioselectivity was

predominant. The roles of the amino acids in enzymatic functions are as follows. The His and Tyr residues at position 227 are involved in substrate preferences for maltose and sucrose, respectively. The residue at position 226 is a determinant of regioselectivity in transglucosylation, and the equivalent Pro226 and Asn226 are associated with  $\alpha$ -(1  $\rightarrow$  4)- and  $\alpha$ -(1  $\rightarrow$  6)-transglucosylation, respectively. A high transglucosylation ratio can be achieved in mutants harboring Pro226-His227, and these mutants resemble HBG-I in their high transglucosylation ratios and the amino acids (Pro233-His234) at the corresponding positions (Table 2).

## GH31 $\alpha$ -glucosidase and $\alpha$ -1,4-glucan lyase

### Overview of GH31 enzymes

GH31 is a divergent family, and it contains not only hydrolases and transglycosidases, but also lyases. In the Carbohydrate Active Enzymes (CAZy) database (<http://www.cazy.org/>) [84], AGase,  $\alpha$ -1,3-glucosidase,  $\alpha$ -xylosidase, 3- $\alpha$ -isomaltosyltransferase, 1,4- $\alpha$ -glucan 4- $\alpha$ -glucosyltransferase,  $\alpha$ -galactosidase, and GLase are present as members of GH31. Among them, 1,4- $\alpha$ -glucan 4- $\alpha$ -glucosyltransferase [85] and  $\alpha$ -galactosidase [86] have been recently discovered through bioinformatics analyses.

Although GH31 contains such significantly divergent enzymes, its three-dimensional structural information was unavailable until a decade ago. The first three-dimensional structure of a GH31 enzyme, the  $\alpha$ -xylosidase YicI from *Escherichia coli*, was determined in 2005 [87]. Thereafter, the structures of several AGases [88–93],  $\alpha$ -xylosidase [94], GLase [95], 1,4- $\alpha$ -glucan 4- $\alpha$ -glucosyltransferase [85], and  $\alpha$ -galactosidase [86] were determined, and 12 structures were available as of October 20, 2015, through the CAZy database ([http://www.cazy.org/GH31\\_structure.html](http://www.cazy.org/GH31_structure.html)).

The three-dimensional structures of GH31 enzymes are generally composed of four major domains, an N-terminal domain, a ( $\beta/\alpha$ )<sub>8</sub>-barrel catalytic domain, and two C-terminal domains. An exception is found in  $\alpha$ -xylosidase from *Cellvibrio japonicus*, *CjXyl31A*, which has an additional PA14 domain next to the N-terminal domain [94]. The active-site pocket is formed by loops of the inner  $\beta$ -barrel, as is often the case with ( $\beta/\alpha$ )<sub>8</sub>-barrel domains. The catalytic nucleophile and acid/base residues in the retaining catalytic mechanism are conserved at the end of  $\beta$ -strands 4 and 6, respectively. In addition to the ( $\beta/\alpha$ )<sub>8</sub>-barrel domain, a long bulging loop from the N-terminal domain (N-loop) generally participates in substrate binding. Interestingly, the positive subsites of *E. coli* YicI, which has a hexameric structure, are comprised of the N-terminal domains of three different monomers. In addition to the N-loop from its own

monomer, the Asp residue from an adjacent monomer, and a Trp residue from a second monomer contribute to the formation of positive subsites [96]. AGases from *Sulfolobus solfataricus* [88] and *Ruminococcus obeum* ATCC29174 [91] also form hexameric and dimeric structures, respectively; however, their active sites are formed by a single subunit. CjXyl31A has a distinctive positive-subsite architecture, in which the aforementioned PA14 domain appears to be involved [94].

Most recently, Miyazaki et al. [86] found the first GH31  $\alpha$ -galactosidases in *Pedobacter heparinus* and *Pedobacter saltans*. Almost all of the  $\alpha$ -galactosidases are distributed in GH27 and GH36, which are members of clan GH-D, together with GH31 in the CAZy database. Subsite -1 of *P. saltans*  $\alpha$ -galactosidase (*PsGal31A*) has a structure distinct from that of GH31 AGases/ $\alpha$ -xylosidases and GH27/GH36  $\alpha$ -galactosidases. Meanwhile, the prominent spatial pattern of GH27/GH36  $\alpha$ -galactosidases was found in the active site of *PsGal31A*. Thus, Trp486 on  $\beta \rightarrow \alpha$  loop 8 of *PsGal31A* is spatially superimposed with a Trp residue on  $\beta \rightarrow \alpha$  loop 1 in GH27/GH36  $\alpha$ -galactosidases. The Trp residue, conserved in GH27 and 36, seems to exclude an equatorial 4OH group [97–100]. The spatially conserved Trp486 of *PsGal31A* can thus confer the  $\alpha$ -galactosidase activity on the enzyme. The discovery of *PsGal31A* indicated a strong evolutionary linkage in clan GH-D, which has not only conserved protein folding but also substrate recognition.

### GH31 $\alpha$ -glucosidase

GH31 AGases can be found in important biological processes. For example, GAA is responsible for the hydrolysis of the  $\alpha$ -(1  $\rightarrow$  4)- and  $\alpha$ -(1  $\rightarrow$  6)-glucosidic linkages of glycogen in the lysosome. A genetic deficiency in this lysosomal enzyme leads to the accumulation of glycogen inside the lysosome, resulting in lysosomal expansion in many tissues with cardiac and skeletal muscle being the most severely affected. This serious disorder is called Pompe disease or glycogen storage disease type II. To understand Pompe disease, a large number of studies on GAA have been performed. Thus far, approximately 500 genetic mutations responsible for the disease have been reported (The Pompe Disease Mutation Database, <http://cluster15.erasmusmc.nl/klgn/pompe/mutations.html> [101]). From a structural standpoint, it is interesting that the proteolytic events of GAA are required to form the mature enzyme and obtain the optimal activity toward glycogen [102]. The precursor polypeptide is translated as a 110-kDa peptide that undergoes a series of proteolytic processing events, which convert it into a 70-kDa polypeptide. The proteolytic processing increases the enzyme activity by 7–tenfold.

GH31 AGase plays an important role in the quality control of nascent glycoproteins in the endoplasmic reticulum. A de novo glycoprotein undergoes quality control, which is carried out by the calnexin (CNX)/calreticulin (CRT) cycle [103]. The glycoprotein, having a terminal  $\alpha$ -(1  $\rightarrow$  3)-glucoside in the *N*-linked sugar chain, can enter the CNX/CRT cycle, and the properly folded glycoprotein leaves this cycle. The departure signal from the CNX/CRT cycle is the removal of the terminal  $\alpha$ -(1  $\rightarrow$  3)-glucoside, which is hydrolyzed by endoplasmic reticulum-resident glucosidase II [104]. Glucosidase II functions as a heterodimer composed of  $\alpha$ - and  $\beta$ -subunits. The  $\alpha$ -subunit, corresponding to a catalytic subunit, shows a significant similarity to GH31 AGases. The  $\beta$ -subunit is believed to function as a lectin, which recognizes the oligomannose branches of the *N*-glycan [105, 106], and is required for solubility, stability, activity, and the localization of glucosidase II [107–109].

In general, most GH31 AGases are associated with the complete digestion of starch into glucose and, thus, are widespread in organisms that depend on plant starch as an energy source. For instance, sucrase–isomaltase (SI) and maltase–glucoamylase (MGAM) in the mammalian small intestine are involved in the degradation of dietary starch. Orally ingested starch is initially broken down into maltooligosaccharides, such as maltose, maltotriose, and short maltooligosaccharides having  $\alpha$ -(1  $\rightarrow$  6)-branches by means of the salivary and pancreatic  $\alpha$ -amylases. SI and MGAM are responsible for hydrolyzing the resultant oligosaccharides into glucose. SI and MGAM individually possess two catalytic subunits: an N-terminal subunit (NtSI and NtMGAM) and a C-terminal subunit (CtSI and CtMGAM). Each catalytic subunit has diverged from a common ancestor and is a member of GH31, but shows discriminative substrate selectivity, as well as commonly possessed  $\alpha$ -(1  $\rightarrow$  4)-specificity [110]. NtMGAM and CtMGAM are mainly responsible for the hydrolysis of  $\alpha$ -(1  $\rightarrow$  4)-linkages but exhibit different preferences for the substrate's degree of polymerization (DP): CtMGAM prefers to hydrolyze substrates with higher DP values than NtMGAM [92, 111]. NtSI is associated with the hydrolysis of  $\alpha$ -(1  $\rightarrow$  6)-glucosidic linkages [90]. CtSI can hydrolyze  $\alpha$ -(1 $\leftrightarrow$ 2) $\beta$  linkages in sucrose [112, 113].

Understanding how their catalytic properties, including substrate specificity, relate to their structures is under intense study (Table 3). Because the inhibition of these enzymes leads to a delay in glucose production, they may have therapeutic roles in type II diabetes. Currently, the three-dimensional structures of these subunits, except CtSI, are available [89, 90, 92]. These structures are protein complexes with inhibitors, including an acarbose and a kotalanol. Every structure is composed of four major domains, the N-terminal domain, the ( $\beta$ / $\alpha$ )<sub>8</sub>-barrel catalytic

**Table 3** Amino-acid residues associated with substrate and catalytic reaction specificities in GH31 enzymes

	Amino acid	Position	Remarks
NtMGAM	Tyr299	$\beta \rightarrow \alpha$ loop 1 <sup>a</sup>	A substitution with Trp provides $\alpha$ -(1 $\rightarrow$ 6)-specificity
CtMGAM	Tyr1251	$\beta \rightarrow \alpha$ loop 1 <sup>a</sup>	A substitution with Trp provides $\alpha$ -(1 $\rightarrow$ 6)-specificity
NtSI	Trp327	$\beta \rightarrow \alpha$ loop 1 <sup>a</sup>	The residue can be responsible for $\alpha$ -(1 $\rightarrow$ 6)-specificity
<i>R. obeum</i> AGase	Trp169	$\beta \rightarrow \alpha$ loop 1 <sup>a</sup>	A substitution with Tyr caused a reduction in $\alpha$ -(1 $\rightarrow$ 6)-specificity
<i>S. occidentalis</i> AGase	Trp324	$\beta \rightarrow \alpha$ loop 1 <sup>a</sup>	A substitution with Tyr causes severe reductions in $\alpha$ -(1 $\rightarrow$ 6)-specificity in hydrolysis and transglycosylation
<i>Bacillus</i> sp. AGase	Tyr268	$\beta \rightarrow \alpha$ loop 1 <sup>a</sup>	A substitution with Trp provides $\alpha$ -(1 $\rightarrow$ 6)-specificity
	Glu545	$\beta \rightarrow \alpha$ loop 8 <sup>a</sup>	A substitution with Gly provides $\alpha$ -(1 $\rightarrow$ 6)-specificity
Sugar beet AGase	Phe236	N-loop <sup>b</sup>	The residues participate in the formation of subsites +2/+3 and responsible for guiding the substrate to distal subsites
	Asn237	N-loop <sup>b</sup>	
	Leu240	N-loop <sup>b</sup>	The residue participates in the formation of subsite +4
	Ser497	Subdomain b2 <sup>a</sup>	The residue participates in the formation of subsite +4
	Lys493	Subdomain b2 <sup>a</sup>	The residues participate in the formation of subsites +5, +6 and +7
	Gly499		
	Arg500		
<i>G. lemaneiformis</i> GLase	Val501		
	Pro502		
	Asn459	$\beta \rightarrow \alpha$ loop 3 <sup>a</sup>	The residue can be associated with function of Asp553 as base catalyst
	Arg649	$\beta \rightarrow \alpha$ loop 5 <sup>a</sup>	The residue can be associated with function of Asp553 as base catalyst
	Val556	$\beta \rightarrow \alpha$ loop 4 <sup>a</sup>	The residue can be associated with an apolar environment around Arg649. An equivalent residue in <i>S. solfataricus</i> AGase, Glu323, to Val confers a slight lyase activity

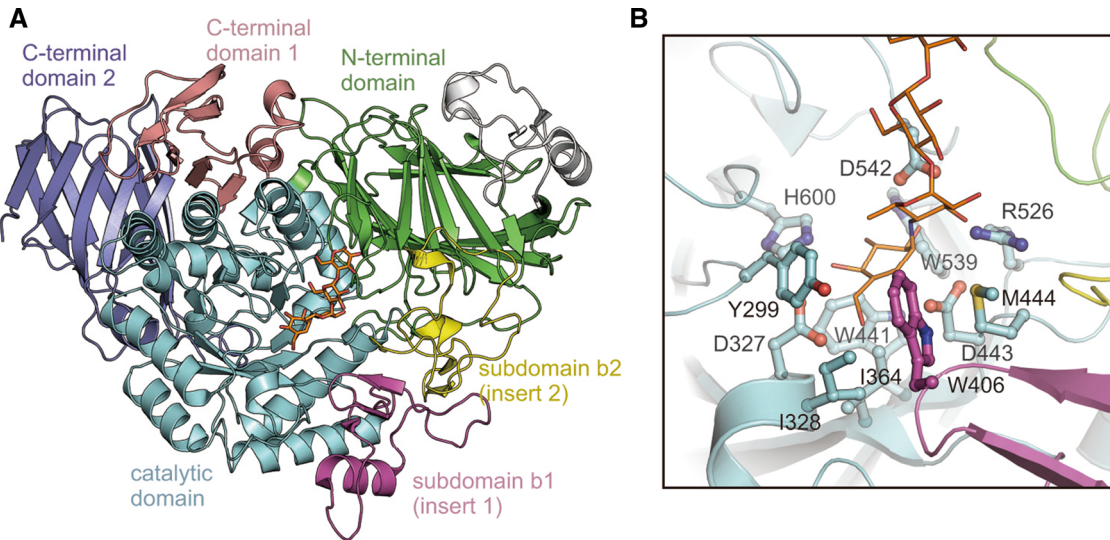
<sup>a</sup> Of the catalytic domain

<sup>b</sup> Of the N-terminal domain

domain, and two C-terminal domains (Fig. 4a). The catalytic domain has two insertions, insert 1 and 2, after  $\beta$ -strand 3 and 4, respectively. The overall structures of these subunits are almost identical, but insert 1 is different. The insertion 1 of CtMGAM has an extra helical segment consisting of 21 amino-acid residues, and is dissimilar to the others [92] (Fig. 4c). Subsite -1 is in an active-site pocket formed by the loops of the  $\beta$ -barrel in the catalytic domain. Residues associated with the formation of subsite -1 are strongly conserved among these subunits. In NtMGAM, Tyr299, Asp327, Ile328, Ile364, Trp406, Trp441, Asp443, Met444, Arg526, Trp539, Asp542, and His600 are located within a 4 Å distance of a valienamine unit, which lies at subsite -1, of acarbose (Fig. 4b). Asp443 and Asp542 provide a catalytic nucleophile and general acid/base, respectively. The side chains of Asp327, Arg526, and His600 form a hydrogen bond with the hydroxy groups of the valienamine. Among these residues, an aromatic residue on  $\beta \rightarrow \alpha$  loop 1 of the catalytic domain, corresponding to Tyr299 in NtMGAM, is unusually varied (Fig. 4c). Both subunits of MGAM have a Tyr residue (Tyr299 of NtMGAM and Tyr1251 of CtMGAM), whereas NtSI possesses Trp327. This Trp residue is regarded as an important residue conferring the  $\alpha$ -(1  $\rightarrow$  6)-

**Fig. 4** Three-dimensional structure of a GH31 AGase and a multiple sequence alignment of GH31 AGases. **a** Overall structure of NtMGAM (PDB, 2QMJ). Different colors are used for each domain: N-terminal domain green; catalytic domain cyan; subdomain b1 magenta; subdomain b2 yellow; C-terminal domain 1 pink; and C-terminal domain 2 slate. **b** The active site of NtMGAM. Residues located within a 4-Å distance of a valienamine unit are shown by sticks. **c** Multiple sequence alignment of catalytic domains of GH31 AGases referred in this literature. SOG and BspGH31 are abbreviation of *S. occidentalis* (GenBank ID: BAE20170.1) and *Bacillus* sp. AGases (GenBank ID: BAQ19546.1), respectively. Other four characters, 3lpp, 2qmj, 3top, 3weo, 2g3n, and 3n04, are pdb id codes and stand for NtSI, NtMGAM, CtMGAM, sugar beet AGase, *S. solfataricus* AGase, and *R. obeum* AGase, respectively. The residues at subsite -1 are marked by the inverted triangle. The residues involved in  $\alpha$ -(1  $\rightarrow$  4) and  $\alpha$ -(1  $\rightarrow$  6) specificities are indicated by star signs. Subdomain b1 and b2 are indicated by magenta and yellow boxes, respectively. The extra segment existed in CtMGAM is underlined

specificity of NtSI [90]. Indeed, the substitution of Tyr299 of NtMGAM and Tyr1251 of CtMGAM with Trp residues increases the  $k_{\text{cat}}/K_{\text{m}}$  value for the hydrolysis of isomaltose [92]. The crystal structure of an  $\alpha$ -(1  $\rightarrow$  6)-specific AGase from *R. obeum* explained the binding situation in isomaltose [91]. The bulky side chain of an equivalent Trp residue, Trp169, is juxtaposed to the flexible  $\alpha$ -(1  $\rightarrow$  6)-



**C**

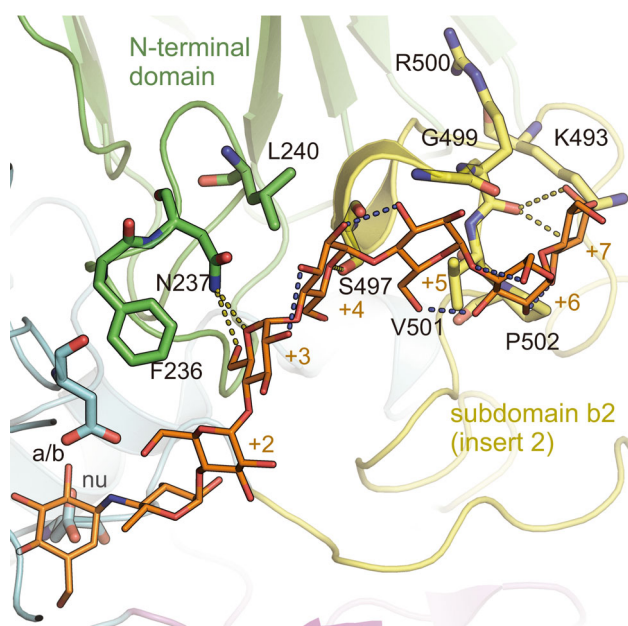
	β1	β2	β3	β4 nu
31pp	LPAPFAWNLGFLSRWYKSLDVKVEWRRNREAGTFFQVQVTDIDYMEDKDFTYDQVAFN--GLPQFVQDLFDHGGKYVITLDFATISIGRRAN--GT			
2qmj	RPALPSYWALGFHLSTRYEGTLDNMRVVERNRAAQLPYDVOHADIDYMERDERRDFTYDSDVDFK--GFPEFVNLHNGGKLVITLDFATIS--NNSS--SK			
3top	RPVMMPYWSLGFQLGRYQNDSEIASLYDEMVAACIPYVVOYSDIDYMERQLDFTLS-PKFA--GFALINRMKADGMRVILLDFATISGNE--TQ			
3weo	RPAPMPYWALGFHQRWYQNDVNEIETVVDKYAEARILEVMMTDIDYMAAFKDFLDPVHFPLDKMQQFVTKLHNGRQYVPLDFGINTN--K			
SOG	LPAPFQYWSLGFHQRWYQDIEKLSVVENFKFNIPLETIWSIDIDYMSYKDFTYDPHRFPLDEYRKFLELHKNCHYVPLDAAITVVPNPNNATDN			
2g3n	KPFLPPYWALGFYMSRYVYPODKVVELVDIMOKEGFRVAGVFLDIHMYDSYKLFTHWHPYRFP--EPKKLIDELHKNVKLITLDFGIRVD--Q			
2g3n	RSYIPPKFAFGFGOSRWYTIKEDFRAVAKGYRENHPTIDMIYDIDYMODKDFTVNEKNFP--DFEFVKEMKDQELRLIPLDAAVYKVE--K			
3n04	RAPTPRWALGFHQRWYVYKTEQEVRLAKTFKEIPLDAIHLDIHMYDGYRVFTFDRSRFP--KPEKVVLELKEQGVHIVSIVDFGVKQD--P			
BspGH31				
	<b>insert 1</b>			
31pp	TYATYERGCNTQHWINESDGSPTIICEVWP	GLITVYPDETINNCIDWVANECSIFHQ-----EVQYDGLWIDMNEFSS		
2qmj	PYGFYDRGSDMKIWNSSDGVYPLICEVWP	GQITVFPDYTNCAVAMTKFELFHN-----QVEFDGIWIDMNEFSS		
3top	PYPAFTIRCVEDDVFIKYPNDGDIWVCKVWPDFPDVVVNGSLDWDSDQVELYRAYVAFPDFFRNSTAKVWKRETEELYNPNQNERSLKFDGIWIDMNEFSS	GPVYYPDFLLAARSFVVDTEKRRFD-----ILPIDGIWIDMNEFSS		
3weo	SVGTFIRGMSVFIKRD--GNPYLICEVWP	GYVYYPDFLLAENIQEYMNKVIKDWLSE-----LTPFDGIWIDMNEFSS		
SOG	EYQPFHYNETDVFILKRD--GSLYICEVWP	GTITVYPDFFRDTRWVAGLISEWLS-----QGVDGIWIDMNEFSD		
2g3n	NYSPLFSG--MGKFCIEES--GELFVCKMWP	GDITVFPDFMLNREARKVFGDKYRFLID-----QGIEGFIWIDMNEFAI		
2g3n	GVEYEECVKNNYFKRED--CSDFVAVWVP	GRSAFPDETINKEVREWVQKHAYYAN-----MGIEGIWIDMNEFSV		
3n04	EYHLYKEGIQNDYFCVYLE--GEVFFEDVWP			
BspGH31				
	<b>insert 2</b>			
31pp	ITQE--STKG-----CNVNK-----LNYPP			
2qmj	FVDE--SVSG-----CSTNN-----LNNPP			
3top	FVNC--AVSPG-----CRDAS-----LNNPP			
3weo	FITSAP-----TPGST-----LDNPP			
SOG	FCVESC-----GTGKYFENPAYPPFTVGSKATSYVPGFDVSNASEWKSISATAKTSSTSSVSSSSSTIDSMTNLAPGKGNINYP			
2g3n	FISR--AIEIRDVLSS--LPV-----WAMQ-			
3n04	FYSSEGLAEAKEFAGEFAKDTEGKIHP-----			
BspGH31	FNETK-----			
		β5	β6	a/b
31pp	FTPDILDK--LMYSKT--ICVDAVQN----WGKQYDI--SLYGYSAIATECAVQKVFHPNKRSEFILLTRSTFAGSGRFAAHMLGDNTASWEQMEWSTITGMLE			
2qmj	FTRPILDG--YIFCKT--LCVDAVQH----WGKQYDI--HNLGYSAVATAEAAKTVFHPNKRSEFILLTRSTFAGSGKFAAHMLGDNTATWDLRWSIPGVLE			
3top	YMPHLESRDRLSSKT--LCMESQIILPDESLVQHVMV--HNLGWSQTRPTIEAVQEVIT--GQRGVITRSTFSPSGRWAGHMLGDNTAAWDLKKSITGMME			
3weo	YKINNSGRRVPIKNT--IPATAVHY----GNVTEVNA--HNLGYFLESQATREALVRTS--NERPFLLRSTFAGSGRYTAHWIGDNTAARWDLQYSIPTMLN			
SOG	YAIYNMQGSDLAATHA--VSPNATHA--DGTVEYDI--HNLGYLQENATYHALLVFPNKRPFMISRSTFPGAGKWTGHWGDNADWAYAFSIPQAFS			
2g3n	--QFRDR--L--VTI--FPDNVHY--LRKRVKHEK--RNAPLYEAMATFKGFRFSH--RNEIFILSRAGYAGIQRYAFITWIGDNTSPWDLKLQQLVLLG			
2g3n	--AKMKDI--VNSPEDYKRFYH--VNEKKIRHDK--HNLFGYNTRAAGAFERIDPEKRFMFPSRYIGMHRVGGIWDGDKSWSHILLNLKMLPS			
3n04	-----AKMKDI--VNSPEDYKRFYH--VNEKKIRHDK--HNLFGYNTRAAGAFERIDPEKRFMFPSRYIGMHRVGGIWDGDKSWSHILLNLKMLPS			
BspGH31	-----TMDVNVVHE--NDQPRTRREL--HNYGVMWGKATYEGMKKQLGNKRFLLTRAGFAGVQRYSAVMTGDRNSFWEHLELSLPLMCMN			
	β7	β8		
31pp	FSLFGIPLVGADICGFV-----AETTEELCRRWMLGAFYPPFSRNHNSDGEYHODFAHFGQNSLLVKSSROYLTITRYILLPFLYTLFYKAHVFCETVAR			
2qmj	FNLFGIPLVPGDICTGFA-----LDTPEELCRRWMLGAFYPPFSRNHNGQYKDDPASFGADSLLLNNSRHLYLNIRYITLLPYLYTLFFRAHSRQDTVAR			
3top	FSLFGISYTGADICTGFF-----QDAEYECVRRWMLGAFYPPFSRNHNTITRRQDPVSWD--VAFVNIISRTVLTQTRYITLLPYLYTLMHKAHTEGVTVVR			
3weo	FGLFGVPMIGADICTGFA-----ESTTEELCRRWMLGAFYPPFSRDHSARDTHOELYLW--ESVAASARTVLLGLRYQLLPYLYTLMYDANLRGPIAR			
SOG	MGIAGLPPFGADYCGFN-----GNSDSELCRRWMLGSEFFPYRNHNYLGAIDQEPYVW--ESVAEATRTSMATRYILLPYLYTLLHESITGLPIIR			
2g3n	LSISGVFVCGDITGGFQGRNFAEIDNSMOLLVKYVALALFFPYRSHKATGDIDTEVFLP--DYKKEVKEIVELRYKFLPYLYSIALEASEKHPYIIR			
2g3n	LNMCGMYTGADLCGFG-----DDITRDLLRFLALGVITPLMRDAAEATREQECYQFE--NIEDFRSVINARVRLVYLYSEYMKALNDODMYFK			
3n04	LGVSCLVFFVGFVCGFA-----HDSNGQLLTRWTVQVAFYPPFSRNHSVIESVROEPWAFG--EVEYQIIRYIQLRYQMLPFLYSIFAEANETGVPIIR			
BspGH31				

glucosidic linkage with three bonds and appears to constrain its movement. The importance of Trp169 to  $\alpha$ -(1  $\rightarrow$  6)-specificity was shown by a site-directed mutagenesis study, in which the substitution of Trp169 with Tyr substantially reduced the hydrolysis activity toward isomaltose and converted the  $\alpha$ -(1  $\rightarrow$  6)-specific AGase into an  $\alpha$ -(1  $\rightarrow$  4)-specific enzyme [91].

In addition,  $\alpha$ -(1  $\rightarrow$  4)-specific AGases that have a Trp residue at the corresponding position exist [114]. A mutational analysis of a *Schwanniomyces occidentalis* AGase (SOG) has helped to elucidate the relationship between specificity and the residue at this site. SOG preferentially hydrolyzes  $\alpha$ -(1  $\rightarrow$  4)-glucosidic linkages, and the  $k_{\text{cat}}/K_{\text{m}}$  value for the hydrolysis of maltose is 14-fold greater than that of isomaltose, even though it has Trp324 at the corresponding site [115]. Mutating Trp324 to Tyr causes a substantial reduction in the  $k_{\text{cat}}/K_{\text{m}}$  value for the hydrolysis of isomaltose, and the Trp residue is necessary for the hydrolysis of  $\alpha$ -(1  $\rightarrow$  6)-glucosidic linkages even in the  $\alpha$ -(1  $\rightarrow$  4)-preferring enzyme [114]. This result demonstrates that the Trp residue on the  $\beta \rightarrow \alpha$  loop 1 is not associated with the substrate selectivity but rather with the recognition of  $\alpha$ -(1  $\rightarrow$  6)-glucosidic linkages. The mutational analysis also indicated that the residue on the  $\beta \rightarrow \alpha$  loop 1 is linked to transglycosylation properties [114]. Transglycosylation occurs when the glycosyl-enzyme intermediate is decomposed by an alcohol molecule, such as sugar, instead of a water molecule. The transglycosylation of GH31 AGases is important for the production of beneficial oligosaccharides on an industrial scale, and isomaltooligosaccharides and nigerooligosaccharides are produced by the transglycosylation of GH31 *Aspergillus niger* and *Acremonium* sp. AGases, respectively. The reaction specificity of wild-type SOG is similar to that of the *A. niger* AGase. The enzyme predominantly produces panose [ $\alpha$ -Glc-(1  $\rightarrow$  6)- $\alpha$ -Glc-(1  $\rightarrow$  4)-Glc] from maltose by the transfer of a glucose moiety to maltose, forming an  $\alpha$ -(1  $\rightarrow$  6)-glucosidic linkage. However, W324Y-SOG yields maltotriose from maltose as the major product by forming an  $\alpha$ -(1  $\rightarrow$  4)-glucosidic linkage, but it also produces a small amount of panose, since the mutation decreases the  $\alpha$ -(1  $\rightarrow$  6)-specificity not only in hydrolysis but also in transglycosylation. In addition to maltotriose, W324Y-SOG produces significant amounts of centose,  $\alpha$ -Glc-(1  $\rightarrow$  4)-[ $\alpha$ -Glc-(1  $\rightarrow$  2)]-Glc, catalyzing the formation of  $\alpha$ -glucosidic linkages between 2OH groups of the glucose residue at the reducing end of maltose and the C1 of the glucosyl donor. The production of centose is undetectable in the wild-type reaction. The mutation of Trp to Tyr can create the space for this branched oligosaccharide to be accommodated, because it involves the reduction in the volume of the side chain.

Research on an AGase from *Bacillus* sp. AHU 2001 corroborated that  $\beta \rightarrow \alpha$  loop 8 is associated with  $\alpha$ -(1  $\rightarrow$  6)-recognition, as well as that of  $\beta \rightarrow \alpha$  loop 1 [116]. This AGase, having Tyr268 on the equivalent loop, displays broad substrate specificity. The enzyme can hydrolyze the glucosidic linkages of  $\alpha$ -(1  $\rightarrow$  4),  $\alpha$ -(1  $\rightarrow$  3),  $\alpha$ -(1  $\rightarrow$  2), and  $\alpha$ -(1 $\leftrightarrow$ 1)- $\beta$ , but its specificity to isomaltose is quite low. Binding affinities of isomaltose in both the ground and transition states to the enzyme appear to be poor. The  $k_{\text{cat}}/K_{\text{m}}$  value for the hydrolysis of isomaltose is 0.26 % that of maltose, and the  $K_{\text{m}}$  value for isomaltose is comparatively greater than those for other substrates. The substitution of Tyr268 for Trp provides the ability to hydrolyze isomaltose with a decreasing  $K_{\text{m}}$  and increasing  $k_{\text{cat}}/K_{\text{m}}$ . However, it causes a reduction in the hydrolysis activity toward maltooligosaccharides. Meanwhile, a mutation of Glu545 on  $\beta \rightarrow \alpha$  loop 8 to Gly enhances the  $\alpha$ -(1  $\rightarrow$  6)-selectivity without the large reduction in the hydrolysis ability. The residue does not seem to directly interact with the substrate. The mutation on  $\beta \rightarrow \alpha$  loop 8 next to loop 1 may lead to a slight conformational change and affect the orientation of residues, such as Tyr268, that directly contact the substrate.

In addition to the studies of the regioselectivity in GH31 AGase, the specificity toward substrates with different DP values has been examined. As with the two subunits of MGAM, GH31 AGases exhibit various chain-length specificities [117, 118]. Most GH31 AGases display substrate specificity toward shorter maltooligosaccharides, such as maltose and maltotriose, whereas several AGases, especially those from plants, have the capability to act on long-chain substrates, such as amylose and soluble starch. This specificity is notably prominent in sugar beet AGase (SBG). The  $k_{\text{cat}}/K_{\text{m}}$  value for the hydrolysis of soluble starch is 90 times higher than that of maltose [119]. SBG has no extra domain, such as a starch binding domain, and its overall structure is similar to every subunit of SI and MGAM [94]. The acarviosyl unit, which occupies subsites  $-1$  and  $+1$ , of acarbose binds to SBG in a manner similar to that of NtMGAM and CtMGAM. The valienamine is located at subsite  $-1$  in a manner similar to that described above, and the 4,6-dideoxy-4-amino-glucose moiety contacts subsite  $+1$  through hydrogen bonds between its O2 and O3 atoms and a conserved Asp residue, corresponding to Asp232 in SBG, on the N-loop. The binding modes of glucose residues at subsites  $+2$  and  $+3$  vary between SBG and both of the MGAM subunits. NtMGAM, which prefers short-chain oligosaccharides, has no clear  $+2$  and  $+3$  subsites. The subunits have a few interactions with the two glucose rings of the reducing end of acarbose [89]. Subsite  $+2$  of CtMGAM, which has a preferred substrate specificity to longer maltooligosaccharides, is affected by the



**Fig. 5** Binding aspect of acarviosyl-maltohexaose to sugar beet AGase. The individual domains are colored as described in the legend to Fig. 4. The residues discussed in the text are shown as stick models and numbered. Hydrogen bonds between the protein and acarviosyl-maltohexaose are indicated by broken yellow lines, and the intramolecular hydrogen bonds in acarviosyl-maltohexaose are represented by broken blue lines

bulky side chain of Trp1369 in the above-mentioned 21 amino-acid segment, and subsite +3 mainly consists of Phe1560 on  $\beta \rightarrow \alpha$  loop 7 [92]. In SBG, the N-loop is closely associated with the substrate binding at subsites +2 and +3 [93]. Phe236 lies at subsites +2 and +3, and the glucose moiety at the reducing end is constrained by hydrogen bonds to the N atom of Ala234 and the N $\delta$ 2 atom of Asn237. A complex structure with an acarviosyl-maltohexaose provides further insights into the relation between the N-loop and the long-chain substrate [120, 121] (Fig. 5). Leu240 forms subsite +4, and Phe236 and Asn237 seem to have another function, guiding the reducing end of the substrate to insertion 2, in addition to the formation of subsites +2 and +3 (hereafter, designated as subdomain b2 in accordance with the definition of Tagami et al. [93]). In subdomain b2, a region from Lys493 to Pro502, which includes a  $3_{10}$  helix, participates in substrate binding. Several hydrogen bonds, between the O $\gamma$  atom of Ser497, and O5 and O6 of the glucose residue at subsite +4, and between the O atoms of Gly499 and Arg500, and O2 and axially oriented O1 of the glucose residue at subsite +7, contribute to substrate binding. The side chains of Lys493, Val501, and Pro502 lie within a 4-Å distance of the glucose residues at subsites +6 and +7, and seem to contribute to substrate binding at distal subsites through van der Waals contacts, but mutations of these

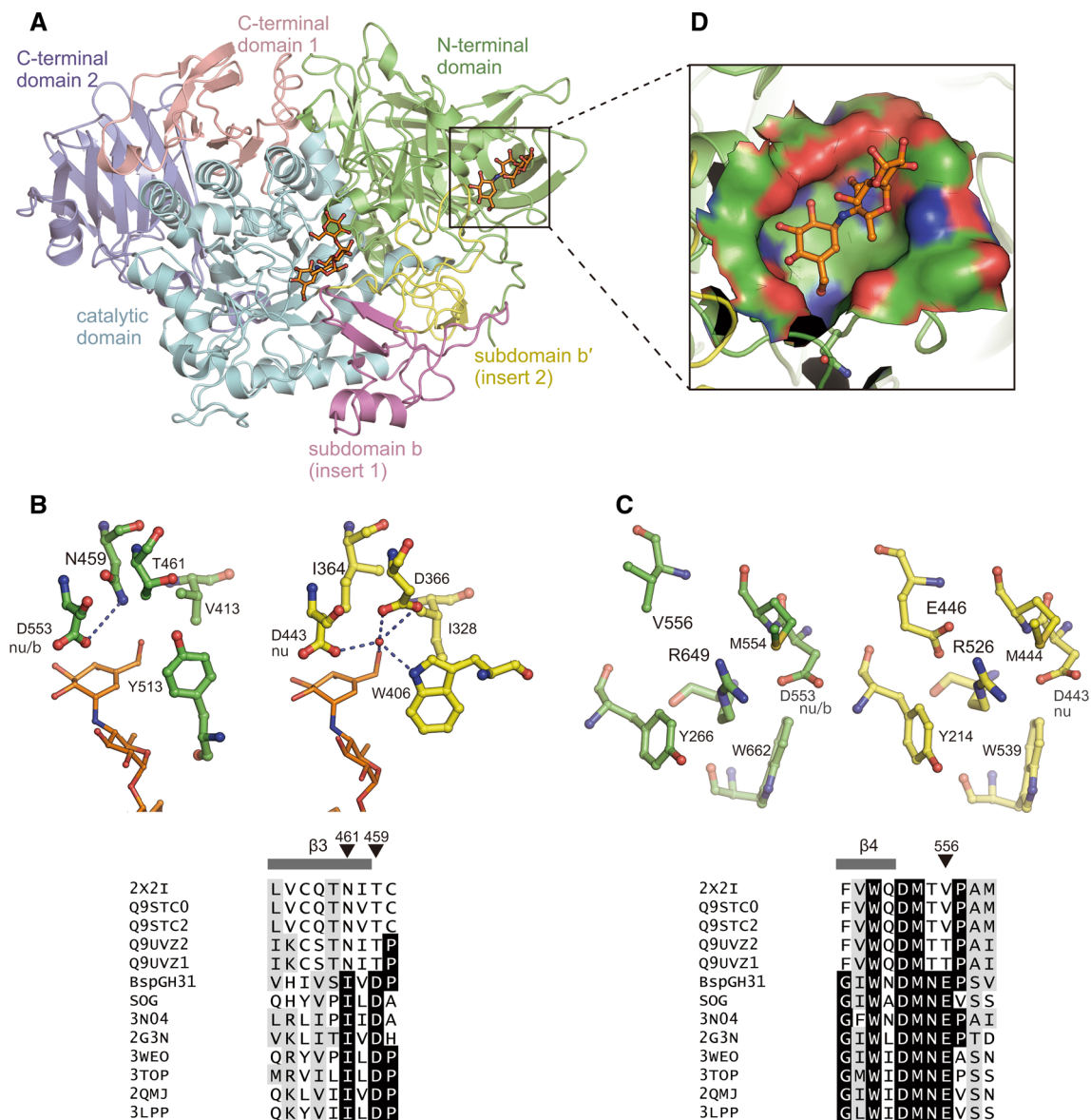
residues to Ala have little effect on the long-chain specificity. The side chains of these residues are, therefore, not as important as they may seem. The complex structure containing acarviosyl-maltohexaose offers an intriguing insight into long-chain specificity from a different perspective. The glucose chain far from the active site retains its native helical conformation, which is an important factor for the long-chain specificity of SBG. Five glucose residues from the reducing end form intermolecular hydrogen bonds, and the torsion angles of their glucosidic linkages are similar to those of cycloamylose DP26 [122]. The structures of the distal subsites suitable for this substrate's conformation suggest that SBG exploits the self-stabilizing properties of  $\alpha$ -(1  $\rightarrow$  4)-glucans to obtain the stable ES complex with the long-chain substrates. The stable ES complex may contribute to the stability of the transition state and lead to the reduction in the specific constant  $k_{\text{cat}}/K_m$  for the hydrolysis of long-chain substrates.

### $\alpha$ -1,4-Glucan lyase

The most notable feature of GH31 is that the family contains lyases as well as hydrolases [123]. GLase, which was discovered in red algae and fungi by Yu et al., cleaves the  $\alpha$ -(1  $\rightarrow$  4)-glucosidic linkage at the non-reducing end of starch, glycogen, and maltooligosaccharides, and releases 1,5AnFru [11, 13–15]. Its catalytic mechanism is clearly distinct from that of the well-known polysaccharide lyases (PLs), which cleave uronic acid-containing polysaccharides. The PLs cleave the glycosidic linkage by  $\beta$ -elimination at the O4–C4 bond, and result in the formation of a reducing end and an unsaturated hexenuronic acid residue [124, 125] (Fig. 1c). Unlike those of PLs, substrates of GLase are neutral  $\alpha$ -(1  $\rightarrow$  4)-glucans, and the enzyme cleaves their C1'–O4 bond, and forms the enol type 1,5AnFru, which has a double bond between C1 and C2 [11] (Fig. 1b).

It is generally accepted that the catalytic reaction of GLase is divided into glycosylation and deglycosylation steps, as with the other GH31-retaining hydrolases [126, 127]. The glycosylation step involves proton donation to the leaving group oxygen and the cleavage of the  $\alpha$ -glucosidic linkage. Simultaneously, the catalytic nucleophile attacks the anomeric carbon and forms a covalent glycosyl–enzyme intermediate. During this step, the oxocarbenium ion character is developed at the transition state. The intermediate is decomposed by an E2 mechanism with strong E1 characteristics during the deglycosylation step.

The structure of *Gracilariopsis lemaneiformis* GLase is similar to those of GH31 hydrolases, containing four major domains [95] (Fig. 6a). The Asp residues, which function as the catalytic nucleophile and acid/base in the retaining hydrolases, are conserved on the loops after  $\beta$ -strands 4 and



**Fig. 6** Three-dimensional structure of a GH31 GLase and differences in the active-site structure between a GLase and an AGase. **a** Overall structure of *G. lemaneiformis* GLase (PDB, 2X2I). The individual domains are colored as described in the legend to Fig. 4. **b** The differences in hydrogen bonds around a catalytic residue between a GLase (left, shown by green) and NtMGAM (right, shown by yellow). Hydrogen bonds are indicated by broken lines. A multiple sequence alignment indicates the variation in the corresponding residues.

Q9STC0, Q9STC2, Q9UVZ2, and Q9UVZ1 are UniProtKB accession numbers, and stand for *G. lemaneiformis* GLase 2, *G. lemaneiformis* GLase 4, *Morchella costata* GLase, and *M. vulgaris* GLase, respectively. Other abbreviations are defined in the legend to Fig. 4. **c** An apolar environment around Arg649 in GLase (left, green) and the environment around a correspond Arg526 in NtMGAM (right, yellow). **d** a pocket-shaped secondary substrate-binding site in the N-terminal domain

6, as Asp553 and Asp665. The amino-acid residues associated with the substrate recognition through a hydrogen bond at subsite -1 are conserved among GLases and AGases. The side chains of Asp412, Arg649, and His731 form hydrogen bonds with the hydroxy groups of the valienamine unit, and these residues are equivalent to Asp327, Arg526, and His600 in NtMGAM. Phe373, located on  $\beta \rightarrow \alpha$  loop 1, corresponds to the aromatic residue

associated with  $\alpha$ -(1  $\rightarrow$  4)- and  $\alpha$ -(1  $\rightarrow$  6)-specificity in GH31 AGases and is thought to be associated with the  $\alpha$ -(1  $\rightarrow$  4)-specificity of GLase.

Among the residues at subsite -1, Val413, Asn459, Thr461, and Tyr513 vary from those of the AGases (Fig. 6b, c). The variety seems to be associated with differences in the catalytic mechanism between AGase and GLase. The elimination reaction of GLase requires a base



catalyst responsible for H<sub>2</sub> abstraction. The crystal structure analysis indicates that Asp553, which acts as nucleophile in the glycosylation step, is suitably positioned to act as a base catalyst. The base catalyst function is likely associated with the hydrogen bond between of Asp553 and Asn459 (Fig. 6b). The comparable Asn residue is substituted with Ile (Ile364 in NtMGAM) or Ser in AGases, and the hydrogen bond is absent in known AGase structures. Instead, the nucleophilic catalysts of AGases form hydrogen bonds with Asp (Asp366 in NtMGAM) and Trp (Trp406 in NtMGAM) through water molecules. These amino-acid residues are conserved in AGases and should be important for AGase activity. GLase possesses Tyr513 at the same site as the Trp residue and has no corresponding hydrogen bond. Rozeboom and co-workers further refer to an effect of Arg649 in  $\beta \rightarrow \alpha$  loop 5 on the ability of Asp553 to act as the base catalyst [95]. They note that an apolar environment, which is formed by the side chains provided by Tyr266, Met554, Val556, and Trp662, lowers the  $pK_a$  of the guanidinium group of Arg649, and the lowered group affects the ionization state of Asp553 (Fig. 6c). The importance of the apolar environment for lowering the  $pK_a$  of the guanidinium group was described by Guillén Schlippe and Hedstrom [128]. The lower  $pK_a$  may withdraw the proton extracted from the sugar by Asp553, and Asp553 can retain function as the base catalyst. Interestingly, AGases conserve the Glu residue instead of Val556. This acidic side chain may disturb the apolar environment and eliminate the ability of the catalytic nucleophile to act as the base catalyst. The importance of the amino-acid residue at position 556 (Val/Thr in GLases versus Glu in AGases) for the lyase activity was confirmed by site-directed mutagenesis studies. The mutant AGase, which possesses Ala instead of Glu484, from *Schizosaccharomyces pombe* retains its D-glucal hydration activity (the reverse reaction of GLase), even though it loses most of the hydrolysis activity [129]. The mutation of Glu323 in *S. solfataricus* AGase to Val confers a slight but significant lyase activity, whereas it causes a lack of hydrolase activity [95].

The crystal structure of GLase facilitated our understanding of its unusual catalytic mechanism and revealed the presence of a unique secondary sugar-binding site in its N-terminal domain [96] (Fig. 6d). The ligand molecule is in the pocket-shape binding site and trapped by large numbers of hydrogen bonds. The subunits of MGAM, NtSI, and SBG have no such pocket-shaped structures in the corresponding area, and the binding site is unique in GLase. The existence of the secondary sugar binding site can generate the efficient purification of GLases using  $\beta$ -cyclodextrin-sepharose 6B column chromatography [13].

## Discrimination of GH13 and GH31 $\alpha$ -glucosidases

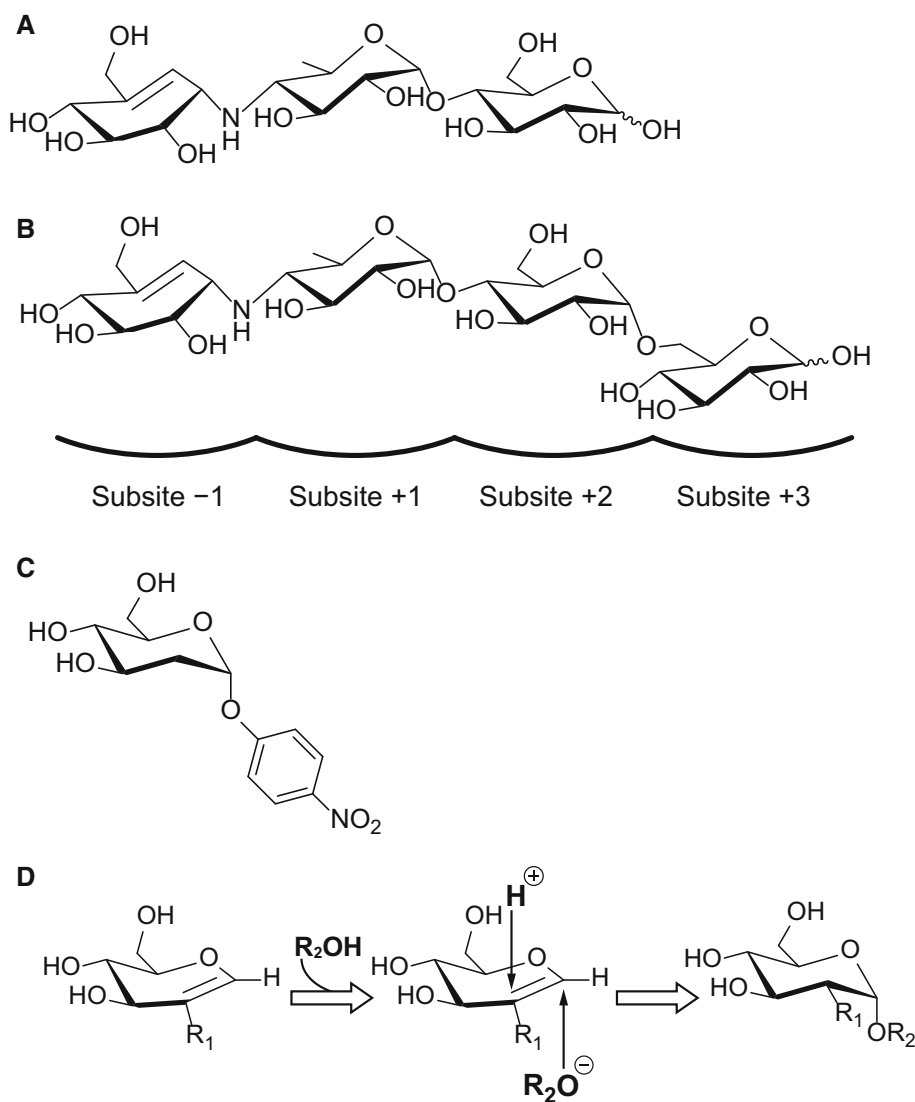
### Recognition of inhibitors

We are interested in the differences between the reactions of GH13 and 31 AGases, which occur through the recognition of inhibitors and unique substrates. As described in the introductory section, AGase inhibitors are potential candidate antidiabetic agents. Park and his colleagues have synthesized two kinds of acarbose derivatives, acarviosyl-glucose, and isoacarbose (Fig. 7a, b), using maltogenic amylase-catalyzed hydrolysis and the transglycosylation of acarbose, respectively [130, 131]. Both pseudosaccharides reduce the activities of  $\alpha$ -amylase and cyclomaltodextrin glucanotransferase through a mixed-type inhibition, and they display stronger inhibitory actions to pancreatic  $\alpha$ -amylase than acarbose [132]. However, the effect on AGases is ambiguous, because the degree of inhibition varies in enzymes, including those from baker's yeast (GH13) and rat small intestines (GH31). This variation is due to structural differences between GH13 and 31 AGases. Acarviosyl-glucose and isoacarbose are competitive inhibitors to both enzyme groups but have distinct inhibitory magnitudes [133]. In GH31 enzymes from *A. niger*, *S. pombe*, rice, and buckwheat, the inhibitors have identical  $K_i$  values at the  $\mu$ M level. For the GH13 enzymes from *B. subtilis*, brewer's yeast, and honeybee (isozyme III), the inhibition of acarviosyl-glucose ( $K_i$  values with  $\mu$ M to sub- $\mu$ M levels) is much higher than that of isoacarbose [ $K_i$  value at a sub-M level ( $\mu$ M level for honeybee isozyme III)]. This phenomenon is explained by the +3 subsites of the two AGase groups, which differentially recognize the  $\alpha$ -(1  $\rightarrow$  6)-glucosyl reducing end of isoacarbose (Fig. 7b). GH13 has difficulty binding this  $\alpha$ -(1  $\rightarrow$  6)-glucosyl residue to subsite +3, possibly owing to a steric hindrance in the formation of a competitive EI-complex, whereas subsite +3 of GH31 can accept an  $\alpha$ -(1  $\rightarrow$  6)-glucosyl moiety to form the sterically unhindered EI-complex. Isoacarbose clearly discriminates between GH13 and 31 AGases. Two acarbose derivatives inhibit the AGases in the small intestine and insect, indicating that these pseudosaccharides may be promising candidates for antidiabetic agents and insecticides. Furthermore, isoacarbose can be used to measure the sole activity of GH13 AGase in biotic samples containing the two enzyme groups.

### Recognition of D-glucal and anhydrofructose

An  $\alpha$ -2-deoxyglucoside derivative with a *p*-nitrophenyl moiety (*p*-nitrophenyl 2-deoxy- $\alpha$ -D-arabino-hexopyranoside; Fig. 7c) was subjected to hydrolysis by GH13 and 31 AGases [134]. Interestingly, GH31 hydrolyzes this  $\alpha$ -2-

**Fig. 7** Saccharides differently recognized by GH13 and GH31 AGases. Acarviosyl-glucose (a) and isoacarbose (b) binding to subsites of GH13 and GH31 AGases; c *p*-nitrophenyl  $\alpha$ -2-deoxyglucoside (*p*-nitrophenyl 2-deoxy- $\alpha$ -D-arabino-hexopyranoside); d structures of D-glucal ( $R_1 = H$ ) and anhydrofructose ( $R_1 = OH$ ), as well as reaction schemes of AGase-catalyzed hydration ( $R_2 = H$ ) and 1-alkoxy-2-hydro addition ( $R_2 = \text{alkyl or sugar}$ ) by *trans*-additions



deoxyglucoside, while GH13 cannot, indicating that both enzyme groups differentially recognized the 2OH group of the glycon glucose residue. An identical result based on 2OH recognition was also obtained from the hydration of D-glucal and the 1,2-enol form of anhydrofructose, which have different structures concerning the 2OH group: existence and non-existence of 2OH for 1,2-enol anhydrofructose and for D-glucal, respectively (Fig. 7d). These double-bond-containing monosaccharides mimic the reaction intermediates (oxocarbenium ion intermediate) of AGase, and in particular, D-glucal and its derivatives have been studied relative to glycosylases for a long time [135]. Chiba et al. found that GH31 AGases catalyze the hydration of D-glucal, while GH13 cannot [136]. D-Glucal might inhibit the hydrolytic reaction of GH13 AGase, because it has a similar conformation to the reaction intermediate, as described above. However, D-glucal does not exhibit any

inhibitory effect; thus, it is neither a substrate nor an inhibitor of GH13 AGase, implying it is incapable of approaching the catalytic site [137]. For 1,2-enol anhydrofructose, both GH13 and GH31 hydrate this 2OH-containing substrate. Therefore, the 2OH group is essential and non-essential for the reactions of the GH13 and GH31 enzymes, respectively. These results agree with the data on the  $\alpha$ -2-deoxyglucoside substrate [134].

During hydration, the  $\text{OH}^-$  of water attacks the C1 of D-glucal to form  $\alpha$ -2-deoxyglucoside; thus, we expect that the reaction in the presence of alcohol can generate alkyl (or aryl)  $\alpha$ -2-deoxyglucoside by the addition of the alkoxy group to C1 (1-alkoxy-2-hydro addition; Fig. 7d). Alkyl glycosides showed antibiotic activity [138], leading us to synthesize  $\alpha$ -2-deoxyglucoside. We selected the *A. niger* AGase for this purpose [139], because the enzyme displays a significantly high alcohol-resistance, maintaining 90 and

75 % residual activity in 50 and 70 % methanol, respectively. This great stability may be due to the high 25 % sugar chain content [140, 141]. However, the deglycosylated enzyme still exhibits the same alcohol-resistance, and therefore, the protein portion might contribute to this interesting property. *A. niger* AGase synthesizes 14 kinds of alkyl and aryl  $\alpha$ -2-deoxyglucosides (containing novel types) showing extremely high yields, with an approximately 90 % production of derivatives involving methyl, ethyl, 1-propyl, or 1-pentyl groups [139]. The reduction in the water concentration by the addition of alcohol to reaction mixtures contributes to the suppression of hydration and the enhancement of product yields. As observed above, the alcohol can be utilized for 1-alkoxy-2-hydro addition, suggesting that the sugar (or sugar alcohol) also becomes a substrate. This reaction requires a non-alcoholic solvent to diminish the side-reactions, alcohol-addition, and hydration. Therefore, we selected 70 % acetone, in which the *A. niger* AGase maintains about 40 % residual activity [142]. The enzyme generates seven kinds of  $\alpha$ -2-deoxyglucoside derivatives of monosaccharides, disaccharides, and monosaccharide-type sugar alcohols, and high yields are observed in glucose (70 %) and xylitol (87 %) conjugates. The advantage of 1-alkoxy-2-hydro addition to D-glucal is the one-step reaction that synthesizes a single-species of  $\alpha$ -2-deoxyglucoside without forming any by-product.

### Novel metabolic anhydrofructose pathway that includes $\alpha$ -glucosidase

As mentioned above, GH13 and GH31 AGases hydrate the 1,2-enol forms of anhydrofructose to form glucose, which has a conformation similar to that of the reaction intermediate. However, the *N*-linked sugar chain-trimming glucosidase II (GH31 AGase) cannot catalyze the hydration [143], probably owing to the distinct substrate specificity that recognizes 1,2-enol anhydrofructose. The hydration time-course performed by GH13 and GH31 AGases exhibits a plateau, although enough anhydrofructose is present [137]. This phenomenon might be caused by a keto-enol tautomerism [11, 12] to decrease the intermediate-mimic 1,2-enol form (Fig. 1b). Thus, we attempted to increase the concentration of 1,2-enol anhydrofructose by conducting a GLase-associated reaction with starch, and the resulting 1,2-enol was directly subjected to AGase hydration to form glucose. This combined reaction using the two enzymes is as follows: starch  $\rightarrow$  1,2-enol anhydrofructose  $\rightarrow$  glucose. For this system, we selected a GH13 AGase having no hydrolytic activity on starch. The plateau in the time-course of glucose production disappears, and glucose accumulation is observed, indicating that 1,2-enol is a

substrate of the AGase. Furthermore, the  $\alpha$ -anomer of glucose is generated; thus, GH13 AGase catalyzes the hydration through a *trans*-addition (Fig. 7d). For the GH31 AGase, we cannot utilize the above system, because the enzyme enables the production of detectable levels of glucose from starch. To elucidate the occurrence of *trans*-addition by the GH31 enzyme, 1-alkoxy-2-hydro addition was achieved using the GH31 *A. niger* AGase in the presence of ethanol. Ethyl  $\alpha$ -glucoside was synthesized from anhydrofructose, indicating that GH31 also catalyzes *trans*-addition [137]. This AGase produces a small amount of anhydrofructose during the hydrolytic reaction [144].

As described, AGases catalyze the hydration of anhydrofructose and the production of glucose. This mechanism may contribute to the formation of glucose from exogenously added anhydrofructose during cultivation of microorganisms, since most AGases are secreted enzymes. The resultant glucose is immediately incorporated into the cells of microorganisms. Yu et al. [145] observed the intriguing phenomena that *A. niger* can grow using anhydrofructose as the sole carbon source, while baker's yeast is unable to thrive even in 0.47-M anhydrofructose. These findings are of interest, because *A. niger* excretes a large quantity of AGase in the medium [140], but the yeast enzyme is a non-secreting protein localized inside the cell [146, 147]. In nature, AGase-secreting microorganisms may obtain energy from extracellular anhydrofructose by way of glucose, suggesting the presence of a novel metabolic pathway to salvage unutilized anhydrofructose [137].

### Conclusion

Starch and sucrose are the basic abundant sources of carbohydrates for living organisms; therefore,  $\alpha$ -glucosidase-active enzymes are ubiquitous in nature. A wide range of catalytic specificities are found in AGases and related enzymes, including GLases. They show distinctive catalytic properties in substrate specificity, such as  $\alpha$ -(1  $\rightarrow$  4)- and  $\alpha$ -(1  $\rightarrow$  6)-glucosidic linkages, and chain length specificities, such as preferences toward maltose and longer maltooligosaccharides. Enzymes catalyzing transglycosylation and elimination are also included here, although  $\alpha$ -glucosidase itself is defined by its hydrolytic activity. Most AGases and their related enzymes, which catalyze the variety of reactions that retain the  $\alpha$ -configuration of substrates, fall into two families, GH13 and GH31, because of their sequence similarities, in spite of their diverse catalytic specificities. Even between the two families GH13 and GH31, the similar ( $\beta/\alpha$ )<sub>8</sub> barrel fold is shared as a catalytic domain, with one catalytic residue closely situated in their structures, and a remote but significant sequence homology

is observed [148]. This review includes the recent advances in determining the structural features responsible for the diverse enzyme activities in both families. Some typical amino-acid residues that drastically change the enzyme activity through mutation, i.e., structural elements associated with function, are listed in Tables 2 and 3.

The drastic expansion of sequence information has been achieved by the revolutionary development of sequencing technology. Along with this, rigorous functional gene annotations are of growing importance, because predicting gene functions is essential in understand living organisms. Unfortunately, the functional analyses of the translational products are difficult to perform. Currently, the number of determined three-dimensional protein structures is still limited and structural predictions are not completely reliable. Hence, sequence-based information is still very useful, and sequence elements associated with function need to be determined through the functional analyses of proteins in combination with three-dimensional structural analyses of selected proteins. Gene mining and screening are also required to find new and novel activities.

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