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Review

Molecular Mechanism in α-Glucosidase and Glucoamylase

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The hydrolysis of glucosidic linkage catalyzed by every carbohydrate-hydrolase is a reaction in which the product retains (α→α or β→β) or inverts (α→β or β→α) the anomeric configuration of the substrate. α-Glucosidase and glucoamylase are essentially distinguished by releasing α-glucose and β-glucose, respectively, from the common substrates having α-glucosidic linkage. The distinction in the substrate specificities of the two enzymes was explained by the subsite affinities in their active sites. The amino acid sequences of the regions containing the catalytic sites were compared in α-glucosidases and glucoamylases from various sources. α-Glucosidases were suggested to be grouped into two families by their primary structures. The catalytic reaction mechanisms of carbohydrate-hydrolases were discussed in the two significant models of a nucleophilic displacement mechanism and an oxocarbenium ion intermediate mechanism.

Key words: α-glucosidase; glucoamylase; subsite affinity; Pompe's disease; D-glucal hydration

As for starch-degrading enzymes, in the 1980's the three dimensional structures were solved for α-amylases from *Aspergillus oryzae* (Taka-amylase A)[1,2] and porcine pancreas,[3] and by now from *A. niger*,[4] barley,[5] and *Pseudomonas stutzeri*.[6] On the other hand, the X-ray crystallographic analyses have been made also for *Bacillus circulans*[7] and *B. stearothermophilus* cyclodextrin glucanotransferases (CGTase),[8,9] soybean β-amylase,[10–12] *A. awamori* glucoamylase.[13] and *B. cereus* α-glucosidase[14] though this type of α-glucosidase has no starch-hydrolyzing activity.

α-Glucosidase (EC 3.2.1.20) and glucoamylase (EC 3.2.1.3) are a group of these enzymes, typical exo-type α-glucosidic *O*-linkage-hydrolases releasing D-glucose from the non-reducing end side of substrate. As shown in Fig. 1, the hydrolytic reactions occur by splitting of the bond between the anomeric carbon of the glucosyl residue and glucosidic oxygen (C_1–O). The glucosyl residue is replaced by a proton from water or an acceptor, namely an exchange reaction between the glucosyl residue and the proton in both hydrolysis and transglucosylation. α-Glucosidase and glucoamylase are not apparently distinguished from each other in view of releasing only D-glucose. However, both enzymes are essentially differentiated by the anomer type of product,[15] that is, α-glucosidase produces α-glucose and glucoamylase, β-glucose. In "Enzymes Nomenclature" (1992), some α-glucosidases, for instance, lysosomal α-glucosidase, acid maltase, and exo-1,4-α-glucosidase are erroneously described as other names of glucoamylase. Oligo-1,6-glucosidase (EC 3.2.1.10) and sucrose α-glucosidase (EC 3.2.1.48) also essentially fit into the category of α-glucosidase (EC 3.2.1.20).

In general, every hydrolysis of a glycosidic linkage by glycosidases is a reaction in which the product retains (α→α or β→β) or inverts (α→β or β→α) the anomeric configuration of the substrate. For example, even if a substrate consists only of α-glucosyl residues as starch does, β-amylase and glucoamylase produce β-anomer by "inverting," while α-amylase and α-glucosidase produce α-anomer by "retaining." "Inverting" enzymes do not catalyze the transglycosylation. Only "retaining" enzymes catalyze the transglycosylation (Fig. 1), and the reason is as follows. If the transfer reaction is caused by an inverting enzyme, the product should become the inverted anomer. However, the inverting enzyme would have to cleave the anomer-inverted product as a substrate, which is not essentially acted only by the enzyme, through the reverse reaction, because the reactions catalyzed by glycosidases are usually reversible. The phenomenon containing such a contradiction seems unlikely. It is of importance that "inverting" enzyme never catalyze the transglycosylation. When, in the reaction of an "inverting" enzyme, a by-product, different from the product that is inherently brought about by the enzyme is found, it may be generated

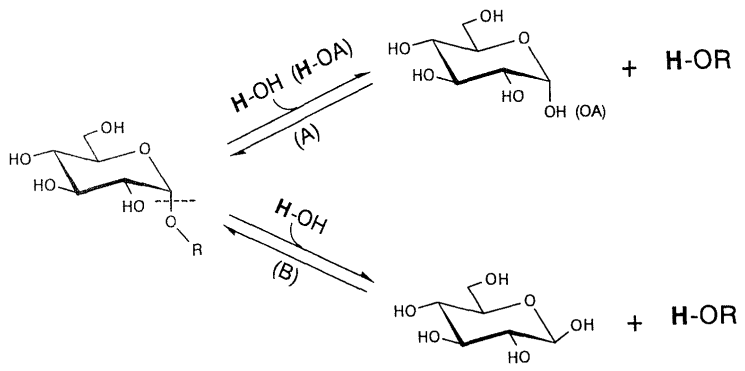

Fig. 1. Hydrolytic and Transfer Reaction Schemes of α-Glucosidase (A) and Glucoamylase (B).
R, reducing side sugar residue or aglycone; H-OA, acceptor; - - -, cleavage point.

through the reverse reaction (the condensation reaction).

In the case of glycosyltransferase (EC 2.4), however, this fundamental rule is not always applicable. There has been reported an interesting transferase,[16,17] inulin fructotransferase (EC 2.4.1.93). The enzyme produces α-D-fructofuranose β-D-fructofuranose 1′,1:2,3′-dianhydride (DFA III) from inulin through an intramolecular transfer reaction accompanying "inverting" of the anomer (β→α). Another different hydrolase catalyzes the hydrolysis of α-2,3′-fructofuranoside linkage of DFA III to give inulobiose of β-anomer, and also the condensation reaction (β→α) of inulobiose to DFA III.

Substrate Specificity

Various types of α-glucosidases are widely distributed in microorganisms, plants, and animal tissues. Their substrate specificities are greatly diverse. Many α-glucosidase are capable of hydrolyzing not only synthetic α-glucosides and oligosaccharides but also α-glucans such as soluble starch and glycogen. α-Glucosidases are conventionally classified into three types, based on their substrate specificities,[18] but they may reasonably be divided into two groups from recent information on the primary structures as described in the next section. Both oligosaccharides and α-glucans are hydrolyzed at a single active site.[19,20] α-Glucosidase showing the highest activity toward soluble starch among the α-glucosidases so far reported is the enzyme from sugar beet seeds.[21] Even the α-glucosidase, however, can't degrade raw starch, while barley malt α-glucosidase[22,23] has been reported to be able to hydrolyze starch granules. The substrate specificity of sugar beet α-glucosidase is presented in the Table, together with the data on *A. niger* α-glucosidase[24] and *Paecilomyces varioti* glucoamylase.[25] As can be seen from the Table, the molecular activities (k_0's) of α-glucosidases for the smallest substrate such as maltose in a series of maltooligosaccharides are not much different from the values for other maltooligosaccharides. On the other hand, in the case of glucoamylase, the k_0 values are very dependent on the polymerization degree of substrate, resulting in considerable differences in the values between maltose and other maltooligosaccharides. Glucoamylase shows an overwhelming big value in the catalytic efficiency (k_0/K_m) for soluble starch, implying that the best substrate is soluble starch. Glucoamylase is a starch-hydrolase capable of also degrading raw starch, while α-glucosidase is oligosaccharide-hydrolase.

The distinction in the substrate specificities for maltooligosaccharides between glucoamylase and α-glucosidase has been rationally explained by the subsite affinities (A_i's) in the active site based on the subsite theory of Hiromi *et al.*,[26] as shown in the histograms of the subsite affinities of Fig. 2. The finding that the second subsite has the largest affinity (A_1 values for glucoamylase, 4.82 kcal/mol, and for α-glucosidase, 5.93 kcal/mol) is in common with glucoamylase and α-glucosidase. In the subsite affinities of the first and the third subsites (A_1 and A_3), however, there is a pronounced difference. The A_3 value (1.76 kcal/mol) of glucoamylase is bigger than A_1 value (0.46 kcal/mol), but the relative A_1 and A_3 values (0.79 kcal/mol and 0.19 kcal/mol, respectively) are reversed in α-glucosidase.[19]

In the case of α-glucosidase, therefore, every maltooligosaccharides may be mainly bound in productive form, a stride over the catalytic site between the first and the second subsites. This means that the k_0 values are little dependent on the polymerization degree of substrate. However, in the glucoamylase–maltose complex, the probability of forming the non-productive binding mode, occupying all except the first and the second subsites, become more than the one of forming the productive binding mode. This appears to be the reason why maltose is slowly hydrolyzed by glucoamylase.

Primary Structure

The primary structures have been deduced so far for more than fifteen of glucoamylases. High similarity is recognized in the amino acid sequences among many glucoamylases. However, there is no similarity in the primary structures to α-amylase, β-amylase, and α-glucosidase, except that *B. ciculans* (CGTase) has only a little similarity in part of the C-terminal region binding the enzyme to starch.[27] While these enzymes have the $(\beta/\alpha)_8$ barrel-fold structure, glucoamylase has the $(\alpha/\alpha)_6$ barrel-fold structure. There is a big difference in the three-dimensional structure between glucoamylase and other amylolytic enzymes.

The three-dimensional structure of glucoamylase was first analyzed for glucoamylase from *A. awamori* var.*X*100[13]

Table Kinetic Parameters for Hydrolysis by α-Glucosidases and Glucoamylase

Substrate	Sugar beet α-glucosidase			*A. niger* α-glucosidase			*P. varioti* glucoamylase		
	K_m (mM)	k_0 (s^{-1})	k_0/K_m ($mM^{-1}\cdot s^{-1}$)	K_m (mM)	k_0 (s^{-1})	k_0/K_m ($mM^{-1}\cdot s^{-1}$)	K_m (mM)	k_0 (s^{-1})	k_0/k_m ($mM^{-1}\cdot s^{-1}$)
Maltose	20	149	7.5	0.75	144	192	1.2	7.2	6
Maltotriose	3.7	136	36.8	0.69	181	262	0.26	27.2	105
Maltotetraose	2.3	136	59.1	1.1	193	175	0.14	43.4	310
Maltopentaose	0.64	136	213	1.9	147	77.4	0.13	41.1	316
Maltohexaose	0.42	136	324	3.2	170	53.1	0.12	41.6	347
Maltoheptaose	0.32	120	375	4.9	146	29.8	0.11	46.6	424
Maltodextrin ($\overline{DP}=17$)	0.26	54.3	209	11	127	11.5	0.03	36.5	122×10
Isomaltose	11	33.7	3.1	8.0	50.5	6.31	59	0.5	0.01
Phenyl α-glucoside	1.7	5.4	3.2	0.34	1.83	5.83	N.D.[b]	N.D.[b]	N.D.[b]
Soluble starch	0.27[a]	179	663	4.3[a]	163	37.9	1.1×10^{-3}[a]	49.1	446×10^2

[a] mMolar of nonreducing terminal residue.
[b] No detection of activity.

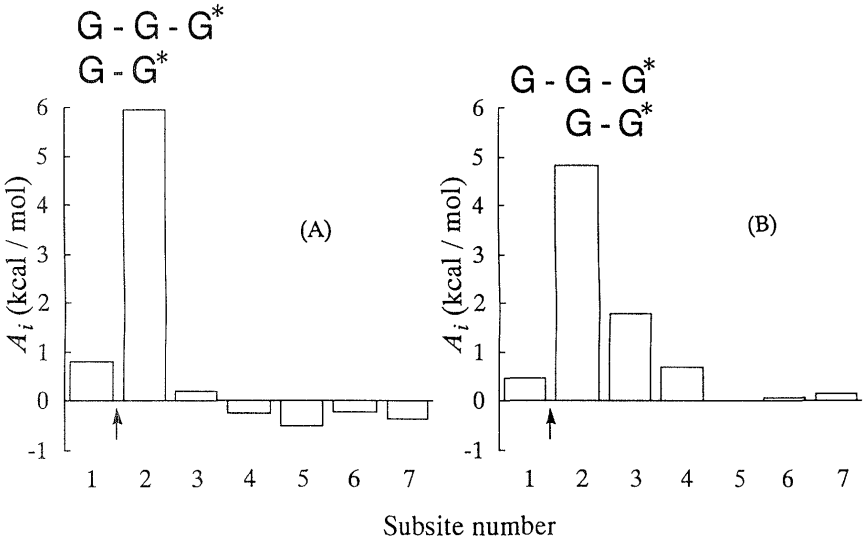

Fig. 2. Histograms of Subsite Affinities of *Aspergillus niger* α-Glucosidase (A) and *Paecilomyces varioti* Glucoamylase (B).

Arrow, the position of catalytic site; G-G*, maltose (the productive binding mode to α-glucosidase; the non-productive binding mode to glucoamylase; G-G-G*, maltotriose (the productive binding mode to both enzymes); G*, reducing glucose residue.

consisting of 471 amino acid residues. In the catalytic domains, Glu-179 and Glu-400 are deduced to be the catalytic amino acid residues directly involved in the hydrolytic reaction. The Glu residues corresponding to the two amino acid residues in the three-dimensional structure of *A. awamori* glucoamylase are considered to be the catalytic amino acid residues also in other glucoamylase.[28,29] These catalytic amino acid residues are conserved in all glucoamylases[29] except for *Saccharomyces* glucoamylase (Fig. 3), but a Glu residue corresponding to Glu-400 of *A. awamori* glucoamylase is not present in the conserved region of glucoamylase from *S. cerevisiae* and *S. diastaticus*.[28,29] However, the possibility has been suggested that a misreading in the gene analysis may be responsible for the lack of a catalytic Glu residue,[32] because a common conservative region (509-GELSEQF) containing the catalytic Glu residue is newly aligned by shifting the open reading frame on the hypothetical insertion of a nucleotide (T) into the gene sequence between 1490-T and 1491-C. In addition, the yeast *Schwanniomyces occidentalis* glucoamylase has been pointed out to be not related to any other glucoamylases.[33] The glucoamylase, however, may be an α-glucosidase, since there is high similarity in the primary structure with α-glucosidases, *i.e.*, human acid α-glucosidase,[34] human intestinal isomaltase,[35] rabbit or rat intestinal sucrase and isomaltase,[36,37] and *A. niger* α-glucosidase.[38]

As for α-glucosidases, more than twenty kinds of complete amino acid sequences have been reported. *Saccharomyces carlsbergensis* (*S. cerevisiae*) α-glucosidase,[39] the amino acid sequence of which was for the first time deduced to be an α-glucosidase, showed high similarity in the amino acid sequence to *B. cereus* α-glucosidase.[40] They make a family, but have no similarity with α-glucosidases from mammals,[34-37] fungus,[38,41] and plants.[42-44] Four conservative regions (1, 2, 3, and 4) including the active site are known in the protein sequences of α-glucan-hydrolyzing enzymes represented by α-amylases.[45] Figure 4 shows the conserved regions in α-amylases, its related enzymes[45,46] and α-glucosidases. The four regions are conserved also in α-glucosidases from *S. carlsbergensis*, *B. cereus*, a *Bacillus*

Glucoamylase source	Catalytic region 1	Catalytic region 2
Aspergillus awamori (*X100*)	176-DLWE[a]EV	396-GSMSE[a]QF
Aspergillus niger	176-DLWE EV	396-GSMSE QY
Neurospora crassa	175-DLWE EV	395-GSLAE QF
Hermoconis resinae	176-DLWE ET	399-GSLSE QF
Rhizopus oryzae	311-DLWE EV	540-GSLSE EF
Saccharomycopsis fibuligera	207-DLWE EN	452-GSLNE QL
Saccharomyces cerevisiae	261-DLWE EV	472-GYLIL EF
		(509-GELSE QF)[b]
Clostridium sp.	431-ERWE EI	628-GIISE QV

Fig. 3. Conserved Amino Acid Sequences in Glucoamylases Containing Glutamic Acid Residue Deduced as Catalytic Amino Acid Residue of *Aspergillus awamori* Glucoamylase.

[a] Underlined E, the catalytic glutamyl residue.
[b] Newly aligned by hypothetically inserting a nucleotide (T) into the next position of 1490-T.

sp.,[47] and insects,[48-51] though they have no similarity in the overall protein sequence with α-amylases. Interestingly, the primary structures of *Saccharomyces* and *Bacillus* enzymes are highly similar[52] with also those of dextrans glucosidase (an exodextranase in EC 3.2.1.70) from *Streptococcus mutans*[53] and trehalose-6-phosphate hydrolase (EC 3.2.1.93) from *Escherichia coli*,[54] both of which show a hydrolytic activity on synthetic substrates such as *p*-nitrophenyl α-glucosidide. α-Glucosidases from mammals,[34-37] plants,[42-44] *Candida tsukubaensis*,[55] and *Mucor javanicus*[41] are lacking the regions 1, 3, and 4, and α-glucosidases from *A. niger*[38] and *A. oryzae*[56] are devoid of only the region 1. These findings imply that α-glucosidases can reasonably be classified into the two families I and II from the viewpoint of primary structure. It seems likely that the two families have independently evolved by convergent evolution from different ancestral proteins.

As presented in Fig. 4, the Asp residue in the region 2 is confirmed to be an essential amino acid residue directly involved in the catalytic reaction by the chemical modification with conduritol B epoxide.[51,57-59] Probably the Asp residue functions in common as a catalytic residue in every α-glucosidase. However, another catalytic amino acid residue has not become thoroughly apparent. In the case of α-glucosidases in faimily I, the catalytic amino acid

Enzyme	Region 1	Region 2	Region 3	Region 4	Region 5
– α-Amylase family –					
Aspergillus oryzae AMY	117-DVVANH	202-GLRIDTVKH	230-EVLD	292-FVENHD	
Bacillus stearothermophilus NPL	242-DAVFNH	324-GWRLDVANH	357-EIWH	419-LLGSHD	
Pseudomonas amyloderamosa IAM	291-DVVYNH	370-GFRFDLASV	454-EWSV	502-FIDVHD	
Klebsiella aerogenes PUL	600-DVVYNH	671-GFRFDLMGY	704-EGWD	827-YVSKHD	
Bacillus stearothermophilus CGT	131-DFAPNH	221-GIRMDAVKH	253-EWFL	319-FIDNHD	
Escherichia coli BE	335-DWVPNH	401-ALRVDAVAS	426-EFGG	521-LPLSHD	
– α-Glucosidase family I –					
Saccharomyces cerevisiae AG	106-DLVINH	210-GFRIDTAGL	276-EVAH	344-YIENHD	
Bacillus cereus AG	98-DVVVNH	195-GFRMDVINF	255-EMPG	324-YWNNHD	
Bacillus sp. (SAM 1606) AG	113-DLVANH	210-GFRMDVINA	271-ETGG	340-YWTNHD	
Honeybee (I) AG[a]	DLVPNH	GFRIDAVPH	EAFS	VMGNHD	
Honeybee (III) AG	119-DFVPNH	219-GFRVDALPY	286-EAYT	343-VPGNHD	
Mosquito AG'	114-DFVPNH	215-GFRIDAVPY	290-EGYT	351-VLGNHD	
Fruit fly (protein H) AG'	115-DFVPNH	217-GFRIDAVPY	297-EAYT	358-VLGNHD	
Fruit fly (protein D) AG'	120-DFVPNH	222-GFRIDAVPH	298-EAYS	359-VVGNHD	
Fruit fly (protein L) AG'	116-DFVPNH	216-GFRIDAVPH	293-EAYS	354-VFGNHD	
– α-Glucosidase family I-like –					
Streptococcus mutans DG	98-DLVVNH	190-GFRMDVIDM	236-ETWG	308-FWNNHD	
Escherichia coli TPH	100-DMVFNH	196-GLRLDVVNL	251-EMSS	320-FWCNHD	
– α-Glucosidase family II –					
Aspergillus niger (P2) AG		220-GVWYDMSEV	255-EPGD	316-YVINHD	421-GADTCGF
Aspergillus oryzae AG		488-GVWYDMAEV	523-EPGN	583-YVINHV	687-GVDTCGF
Mucor javanicus AG		426-GLWIDMNEP			594-GADICGF
Candida tsukubaensis AG		522-GIWLDMNEP			769-GADICGF
Schwanniomyces occidentalis AG		466-GIWADMNEV			665-GADVCGF
Human lysosomal acid AG		514-GMWIDMNEP			643-GADVCGF
Human intestinal (isomaltase) AG		501-GLWIDMNEV			631-GADICGF
Rabbit intestinal (isomaltase) AG		501-GLWIDMNEV			631-GADICGF
Rabbit intestinal (sucrase) AG		1390-GLWIDMNEP			1527-GADICGF
Rat intestinal (isomaltase) AG		510-GLWIDMNEV			642-GATSCGF
Rat intestinal (sucrase) AG		1395-GLWIDMNEP			1539-GADICGF
Sugar beet AG		465-GIWIDMNEA			595-GADICGF
Spinach AG		461-GLWIDMNEI			591-GADICGF
Barley AG		433-GLWIDMNEI			561-GADICGF
Buckwheat AG[a]		451-GLWIDMNEV			581-GADICGF

Fig. 4. Conserved Amino Acid Sequences in α-Amylase, Related Enzymes and α-Glucosidases Containing Regions Deduced as Catalytic Sites.

[a] S. Chiba *et al.*, unpublished data. AMY, α-amylase; NPL, neopullulanase; IAM, isoamylase; PUL, pullulanase; CGT, cyclomaltodextrin glucanotransferase; BE, 1,4-α-glucan branching enzyme; AG, α-glucosidase; AG', α-glucosidase-like; DG, dextran glucosidase; THP, trehalose-6-phosphate hydrolase (α,α-phosphotrehalase).

residue may be the Glu or Asp residue in the regions 3 and 4, as well as in α-amylases. It is conceivable that, in the case of α-glucosidases in family II, such a catalytic amino acid residue is present in a different region. Human acid α-glucosidase has been pointed out to be important in the metabolism of glycogen in lysosomes.[60] The deficiency of the enzyme activity causes a glycogen-storage disease type II (Pompe's disease).[61–63] Recently it has been reported that the loss of the acid α-glucosidase activity may be due to the replacement of the Asp-645 residue by a Glu or His residue in the region 5 (Fig. 4) by the genetic analyses for the lysosomal acid α-glucosidases of patients with Pompe's disease.[62,63] The amino acid sequence in the region 5 is highly conserved in the α-glucosidase family II. Therefore, the Asp residue may be considered to be directly involved in the hydrolytic reaction as a catalytic residue together with the Asp residue in the region 2. As shown in Fig. 4, only in the case of rat intestinal α-glucosidase (isomaltase),[37] the Asp residue is deduced to be exceptionally replaced by a Thr residue, suggesting that the Asp residue may not always be involved in the catalytic reaction. However, there is a possibility of misreading in the cDNA sequence analysis, as well as in the case of *S. cerevisiae* glucoamylase,[30] because the amino acid sequence of -GATSCGF- in the region 5 of rat isomaltase is easily alterable into the one of -GADICGF- by inserting a nucleotide (G) in the next place of 1270-G and deleting 1276- or 1277-T (1265-GGC·GCG·ACA·TCT·TGT·GGA·TTT- to -GGC·GCG·GAC·ATC·TGT·GGA·TTT-).

Catalytic Reaction Mechanism

As mentioned previously, glucoamylase and α-glucosidase produce β-glucose and α-glucose, respectively, from the

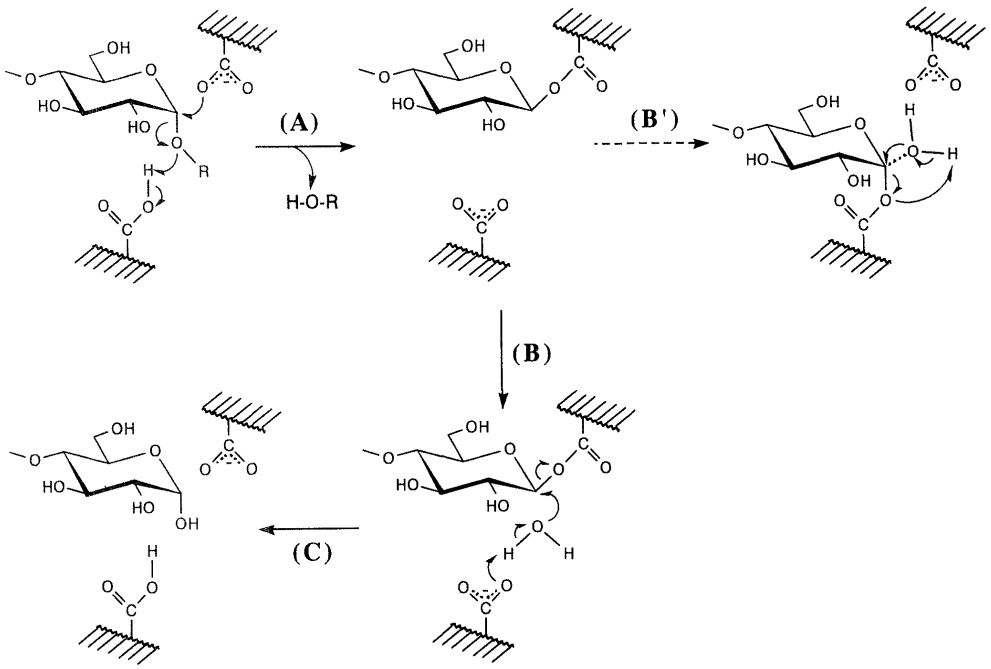

Fig. 5. Nucleophilic Double Displacement Mechanism in Hydrolytic Reaction of α-Glucosidic Linkage.

common substrate in the hydrolytic reaction. This implies that their reaction mechanisms may be essentially different from each other. Various models have been proposed for the catalytic reaction mechanisms of carbohydrate-hydrolase in the transition state, but reasonable models remain to be established. There seem to be the two significant models of a nucleophilic displacement mechanism and an oxocarbenium ion mechanism in the models so far proposed. In the former, as shown in Fig. 5, the two catalytic ionizable groups of a carboxyl (it can't be completely ruled out a possibility of imidazole, in the enzyme[64] having an optimum pH in a neutral region), –COOH, and a carboxylate, $-COO^-$, cleave the glucosidic linkage cooperatively by direct electrophilic and nucleophilic attacks against the glucosyl oxygen and anomeric carbon atoms, respectively, resulting in a covalent β-glucosyl-enzyme complex by a single displacement involving anomer inversion. Subsequently, the β-carboxyl-acetal bond is replaced with the hydroxyl ion of water to stop the hydrolysis, retaining the anomer of product by double displacement. The double displacement mechanism is adequate for explaining the reaction, which retains the anomeric configuration of the substrate (Fig. 5).

However, if this mechanism is applied to "inverting" enzymes, triple displacements including the formation of an additional covalent α-glucosyl-enzymes intermediate (α-carboxyl-acetal covalent intermediate, Fig. 5(B′) between the proton donor carboxyl group and a glucosyl residue should be taken into account in the reaction accompanying anomeric inversion.[65] Such displacements seem unlikely to occur in the hydrolytic reaction. It is so difficult to prove directly the existence of a glucosyl–enzymes intermediate in the reaction process, but the formation of β-carboxyl-acetal covalent intermediate was suggested in the reaction of porcine pancreatic α-amylase on ^{1}H-NMR spectra.[66] The catalytic mechanism of *A. oryzae* α-amylase (Taka-amylase A) has been explained by the double displacement mechanism from the effects of the substituent and solvent deuterium isotope on the kinetic parameters.[67] The peptide tagged with a β-glucosyl residue at the catalytic aspartic acid residue has been isolated from the pepsin digest of the enzymes-substrate complex formed in the transfer reaction of *Streptococcus sorbinus* α-glucosyl-transferase, though the enzyme is not a hydrolase.[68]

On the other hand, in the oxocarbenium ion intermediate mechanism, the two catalytic groups of carboxyl and carboxylate participate cooperatively in the hydrolytic reaction as shown in Fig. 6. The carboxylate group is considered to promote oxocarbenium ion formation and to stabilize the occurring intermediate, while the carboxyl group attacks the oxygen of glucosidic linkage. The subsequent nucleophilic displacement of the hydroxyl ion of water to oxocarbenium ion is allowed to finish the reaction (S_N1 mechanism). The oxocarbenium ion intermediate model has been applied to interpret the catalytic mechanism of many carbohydrate-degrading enzymes, including lysozymes, amylases, and glucosidases. It is, however, too difficult also to prove the existence of an oxocarbenium ion as the reaction intermediate. Although is not direct evidence, the α-secondary kinetic isotope effects have been reported to be observed in the hydrolytic reactions by lysozyme,[69,70] glucoamylase,[71,72] and α-glucosidase.[73] These findings strongly support the suggestion that an oxocarbenium ion intermediate mechanism is suitable for these enzymes reactions. This mechanism is applicable to both "retaining" and "inverting" enzymes.[65] As shown in Fig. 6, when the hydroxyl ion of water attacks the anomeric carbon from the "above" and "below" sides of the plane of the oxocarbenium ion, a β-anomer and an α-anomer should be produced, respectively. Generally speaking, the hydrolytic reaction of carbohydrate-hydrolase is interpreted by the general acid-base catalytic mechanism. In the case of *A. awamori* glucoamylase (identical to *A. niger* glucoamylase), a carboxylate group of Glu-400 and a carboxyl group of Glu-179 are demonstrated to be situated in the active site as the putative catalytic base and acid,

respectively, so that the substrate is interposed between the two groups. A water molecule is found near a carboxyl group of Glu-400 by the analysis of three dimensional structure.[13,29,74] The Glu-179 and Glu-400 residues in *A. awamori* glucoamylase are speculated to correspond to the Glu 434 and Glu-632 residues, respectively, in *Clostridium* sp. glucoamylase.[75] "The inverting" mechanism of the product anomer for these glucoamylases is proposed to occur *via* an oxocarbenium ion intermediate[76] (S_N1 mechanism) in *A. awamori* glucoamylase (Fig. 6), a single displacement[75] (S_N2 mechanism) in *Clostridium* sp. glucoamylase, with a nucleophilic hydroxyl group of water associated with a carboxylate of G-400 (G-632 in *Clostridium* sp. enzyme). The oxocarbenium ion intermediate mechanism is consistent[77] with the observations of α-secondary kinetic isotope effects,[71,72,78] but the latter displacement mechanism is not. In the reaction mechanism, a proton of the water acting as nucleophile is pulled out by the carboxylate of Glu-400, resulting in the formation of a carboxyl group. However, the proton should be finally transferred to the dissociated carboxyl group of Glu-179 for the further reaction, and seems unlikely to fill the space directly in the carboxylate group of Glu-179. Additionally the distance between the water and the carboxyl group of Glu-179 is so far for such a direct transfer of the proton. How does the proton from water move to the carboxylate group of Glu-179? In the "inverting" mechanism of the product anomer, the migration process of the proton into the dissociated proton donor has remained ambiguous, also in both the single displacement mechanism and the oxocarbenium ion intermediate mechanism of any enzyme.

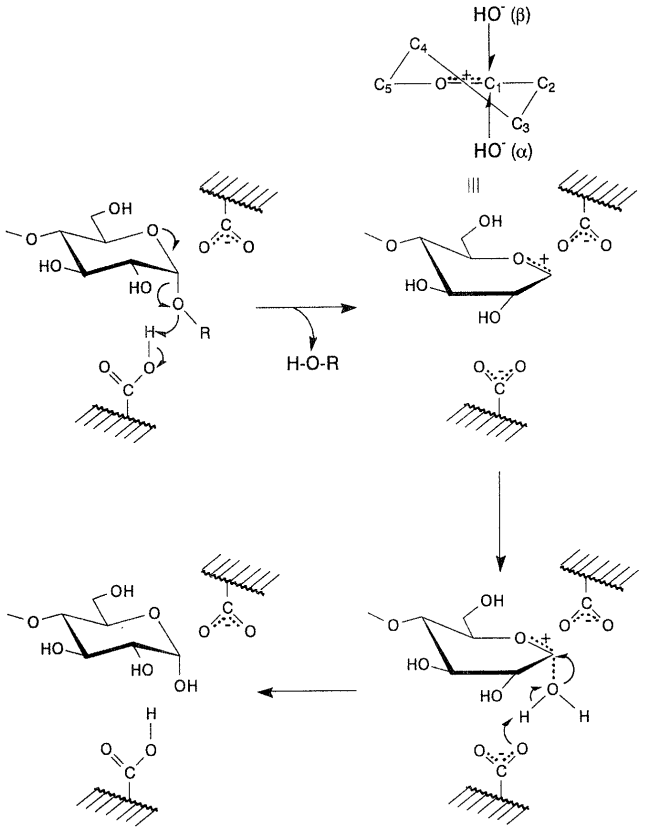

Fig. 6. Carbenium Ion Intermediate Mechanism in Hydrolytic Reaction of α-Glucosidic Linkage.

α-Glucosidase and glucoamylase catalyze the hydration of the double bond of D-glucal to produce 2-deoxy-D-glucose (Fig. 7). Both α-glucosidase and glucoamylase have been found to protonate the C_2 position of D-glucal from different directions, "above" and "below", but catalyze nevertheless the hydrolysis of the same α-glucosidic linkage of the substrate.[65] As shown in Fig. 7, when the hydration was done in ^2H_2O (D_2O), the C_2 position of D-glucal was deuterated (protonated in hydration) from "above" the plane in an axial orientation by α-glucosidase, and from "below" the plane in an equatorial orientation by glucoamylase on ^{1}H-NMR spectra.[79] There has been no evidence supporting the idea that the same catalytic groups as in the hydrolytic reaction are involved in the hydration (deuteration) of D-glucal. However, if the C_2 position of D-glucal is protonated by the proton donor of a catalytic group functioning in the hydrolysis, why is it that the directions of protonation are different from each other in α-glucosidase and glucoamylase? The findings seem to provide interesting and important informations needed to establish a reasonable reaction mechanism in the hydrolysis by these enzymes.

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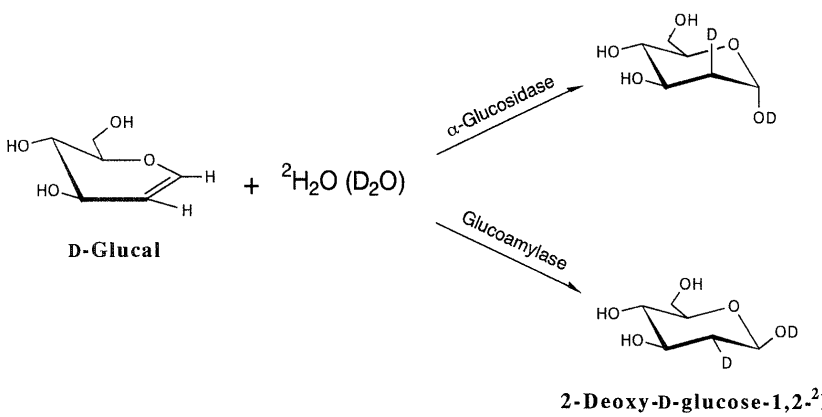

Fig. 7. Deuteration Reaction to D-Glucal with ^2H_2O (D_2O) by α-Glucosidase and Glucoamylase.

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