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REVIEW

α-Glucosidases

V. V. Krasikov, D. V. Karelov, and L. M. Firsov*

Konstantinov St. Petersburg Nuclear Physics Institute, Gatchina, Leningrad Region, 188350 Russia;
E-mail: firsov@lf4029.spb.edu

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Abstract—This review highlights the main properties of mammalian, plant, and microbial α-glucosidases. Special attention is given to the classification of these enzymes, possible catalytic mechanisms, their tertiary structure, and the structure of major inhibitors. Experimental data on the elucidation of amino acid residues essential for catalysis are also discussed.

Key words: α-glucosidases, classification, catalysis, catalytic nucleophiles, inhibitors

α-Glucosidases (EC 3.2.1.20, α-D-glucoside glucohydrolases) are exoenzymes hydrolyzing terminal glycosidic bonds and releasing α-glucose from the non-reducing end of the substrate chain. These enzymes are widely distributed in microorganisms, plants, and animal tissues. Many α-glucosidases can hydrolyze not only oligosaccharides and synthetic α-glycosides containing α-glycosidic bonds but also α-glucans such as water-soluble starch and glycogen.

α-Glucosidases are often called transglucosidases because some of them catalyze both hydrolysis and transglucosylation, which can be described by the following scheme:

$$\text{G-O-R} + \text{H-O-H} \rightleftarrows \text{G-O-H} + \text{H-O-R},$$
$$\text{G-O-R} + \text{H-O-A} \rightleftarrows \text{G-O-A} + \text{H-O-R},$$

where G, R, and H-O-A are a glucosyl residue, aglucone, and acceptor, respectively. Both types of these reactions (hydrolysis and transglucosylation) can be considered as exchange reactions between glucosyl residues and water or acceptor protons. In these reactions, glucose is released as the α-anomer and the configuration of the substrate anomer center (C_1) persists in a transglucosylation product. This represents the major difference between α-glucosidases and glucoamylases catalyzing hydrolysis of the same substrates; however, in the latter case the reaction product is β-glucose (i.e., the reaction is accompanied by reversion of configuration at C_1 in the released glucose residue). In addition, glucoamylases do not catalyze the transglucosylation reaction.

Although α-glucosidases and glucosyltransferases formally belong to various classes, hydrolases (EC 3.2) and transferases (EC 2.4), respectively, in the present review they are considered together.

Chiba [1] classified all α-glucosidases into three groups depending on the type of preferred substrate. The first group consists of typical α-glucosidases, which are more active with phenyl-α-glucoside or sucrose as substrate rather than with maltose. The second group consists of so-called "maltases" readily hydrolyzing maltooligosaccharides (but not synthetic α-glucosides and sucrose). Enzymes of the third group exhibit maltase activity; they can also hydrolyze α-glucans (soluble starch and glycogen).

The modern classification of glycosyl hydrolases is based on the distribution of these enzymes within families characterized by close homology of amino acid sequences [2]. Since amino acid sequence and protein structure are directly related, this classification provides better emphasis on common structural features than just substrate specificity. This classification directly links evolutionary relations of glycosidases and gives useful information about mechanisms of action (via recognition of conserved blocks in the active site regions of these enzymes). So far, 81 families have been recognized. α-Glucosidases constitute two families that are called 13 and 31 [2, 3].

Although oligo-1,6-glucosidases (EC 3.2.1.10) and sucrose-α-glucosidases (EC 3.2.1.48) do not belong to any of the 81 families, they can be considered as α-glucosidases. Cyclodextrin glucan transferases (CDGT, EC 2.4.1.19) belong to family 13.

Recently Chiba proposed a slightly different classification of α-glucosidases that is also based on recognition of homologous sites of amino acid sequences of these

* To whom correspondence should be addressed.

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Table 1. Conservative blocks of amino acid sequences of α-glucosidases [4]

Enzyme	Region				
	1	2	3	4	5
Family I					
Saccharomyces cerevisae AG	106-**DLVINH**	210-**GFRI**<u>**D**</u>TAGL	276-**E**VAH	344-YIE**NHD**	
Bacillus sp. (SAM1606) AG	113-**DLVANH**	210-**GFRMD**VINA	271-**E**TGG	340-YWT**NHD**	
Candida albicans AG*	98-**DLVINH**	202-**GFRID**TAGM	263-**E**VGH	333-FIE**NHD**	
Pediococcus pentosaceus AG*	100-**DLVVNH**	197-**GFRMD**VINQ	256-**E**THG	327-FWN**NHD**	
Thermus caldophilus AG*	96-**DLVPNH**	194-**GFRVD**VLWL	245-**E**MRQ	322-VLG**NHD**	
Streptococcus mutans DG*	98-**DLVVNH**	190-**GFRMD**VIDM	236-**E**TWG	308-FWN**NHD**	
Escherichia coli TPH*	100-**DMVFNH**	196-**GLRLD**VVNL	251-**E**MSS	320-FWC**NHD**	
Bacillus subtilis TPH*	101-**DLVVNH**	198-**GFRLD**VINL	253-**E**MSS	324-FWC**NHD**	
Bacillis thermoglucosidasius OG*	98-**DLVVNH**	195-**GFRMD**VINM	256-**E**TPG	325-YLN**NHD**	
Bacillis coagulans OG*	97-**DLVVNH**	195-**GWRMD**VIGS	255-**E**AIG	327-YFE**NHD**	
Bacillis cereus OG	98-**DLVVNH**	195-**GFRMD**VINF	255-**E**MPG	324-YWN**NHD**	
Bacillus sp. OG*	97-**DLVVNH**	194-**GWRMD**VIGS	254-**E**AGG	326-YFE**NHD**	
Bee AG	119-**DFVPNH**	219-**GFRV**<u>**D**</u>ALPY	286-**E**AYT	343-VPG**NHD**	
Mosquito AG′	114-**DFVPNH**	215-**GFRID**AVPY	290-**E**GYT	351-VLG**NHD**	
Fruit fly AG′ (protein H)	115-**DFVPNH**	217-**GFRID**AVPY	297-**E**AYT	358-VLG**NHD**	
Fruit fly AG′ (protein D)	120-**DFVPNH**	222-**GFRID**AVPH	298-**E**AYS	359-VVG**NHD**	
Fruit fly AG′ (protein L)	116-**DFVPNH**	216-**GFRID**AVPH	293-**E**AYS	354-VFG**NHD**	
Family II					
Aspergillus niger AG		220-**GVWY**<u>**D**</u>**MSE**V	255-**EPG**D	316-**YVINH**D	421-**GADTCGF**
Aspergillus oryzae AG		488-**GVWYDMAE**V	523-**EPG**N	583-**YVINH**V	687-**GVDTCGF**
Emericella nidulans AG*		496-**GVWYDMSE**V	531-**EPG**N	593-**YVINH**V	697-**GVDTCGF**
Mucor javanicus AG		426-**GLWIDMNEP**			594-**GADICGF**
Candida tsukubaensis AG		522-**GIWLDMNEP**			769-**GADICGF**

Table 1. Contd.

Enzyme	Region				
	1	2	3	4	5
Schwanniomyces occidentalis AG		466-**GIWADMNEV**			665-**GADVCGF**
Human lysosomal acidic AG		514-**GMWI<u>D</u>MNEP**			643-**GA<u>D</u>VCGF**
Human IM (intestine)		501-**GLWIDMNEV**			631-**GADICGF**
Rabbit IM (intestine)		501-**GLWI<u>D</u>MNEV**			631-**GADICGF**
Rabbit SE (intestine)		1390-**GLWI<u>D</u>MNEP**			1527-**GADICGF**
Pig IM (intestine)*		487-**GLWIDMNEV**			617-**GADICGF**
Pig SE (intestine)*		1396-**GLWIDMNEP**			1513-**GADICGF**
Rat IM (intestine)		510-**GLWIDMNEV**			642-**GADSCGF**
Rat SE (intestine)		1395-**GLWIDMNEP**			1539-**GADICGF**
Shrew (mouse) IM (intestine)*		487-**GLWIDMNEV**			617-**GADICGF**
Shrew (mouse) SE (intestine)*		1394-**GLWIDMNEP**			1513-**GADICGF**
Sugar-beet AG		465-**GIWI<u>D</u>MNEA**			595-**GADICGF**
Spinach AG		461-**GLWIDMNEI**			591-**GADICGF**
Barley AG		433-**GLWIDMNEI**			561-**GADICGF**
Buckwheat AG		451-**GLWIDMNEV**			581-**GADICGF**
α-Glucosidases without conservative blocks					
Sulfolobus solfataricus AG					
Thermotoga maritima AG					

Note: AG) α-glucosidase; AG′) an enzyme homologous to α-glucosidase; DG) dextran glucosidase; TPH) trehalose-6-phosphate hydrolase (α,α-phosphotrehalase); OG) oligo-1,6-glucosidase; IM) isomaltase; SE) sucrase. The main conservative amino acids are marked in bold; amino acids directly involved in catalysis are underlined. Asterisk shows enzymes added to families I and II by the authors of the present review.

enzymes [4]. He revealed four conservative regions in primary structures of α-glucosidase from yeast [5], bacilli [6, 7], and insects [8-11] (Table 1). These regions were also found in dextran glucosidase from *Streptococcus* mutants [12], α-amylases (EC 3.2.1.70) [13-20], and trehalose-6-phosphate hydrolase (EC 3.2.1.93) from *Escherichia coli* [21]. Both α-amylase and trehalose-6-phosphate hydrolase catalyzed hydrolysis of the synthetic substrate *p*-nitrophenyl-α-D-glucoside. According to Chiba's classification, these enzymes constitute family I. α-Glucosidases from mammalian tissues [22-26], plants [27-29], *Candida tsukubaensis* [30], and *Mucor javanicus* [31] lack regions 1, 3, and 4 whereas α-glucosidases from *Aspergillus niger* [32] and *A. oryzae* [33] do not contain region 1. However, all

these enzymes are characterized by the existence of the fifth region, and they constitute family II.

MAMMALIAN α-GLUCOSIDASES

Glycosylation of asparagine residues of animal glycoproteins occurs via transfer of oligosaccharide $Glc_3Man_9GlcNAc_2$ linked with dolichol phosphate to the synthesizing peptide [34, 35]. After this transfer, the oligosaccharide undergoes several sequential enzymatic conversions (processing) that starts in endoplasmic reticulum and continues in the Golgi apparatus. Initially two distinct membrane-bound α-glucosidases remove all three glucosyl residues: α-glucosidase I removes a terminal α-1,2-bound glucose residue and then α-glucosidase II removes two other α-1,3-bound glucose residues. This is accompanied by subsequent enzymatic hydrolysis of several α-1,2-bound mannose residues; the resultant oligosaccharide structure is either conserved as such (high mannose type structure) or modified into complex oligosaccharide structures during the concerted action of other α-mannosidase and various glycosyl transferases.

Mammalian α-glucosidases can be separated into two main types: acidic α-glucosidases (pH optimum varies from 4 to 5) and neutral α-glucosidases (pH optimum varies from 6 to 7) [36]. Acidic α-glucosidases are located in lysosomes of somatic cells and play an important role in glycogen metabolism. In lysosomes, glycogen can be hydrolyzed to glucose independently from phosphorolysis of glycogen in cytoplasm. Since 1963, when Hers [37] found the absence of this enzyme in patients with Pompe's disease (impairment of glycogen metabolism leading to glycogen accumulation in lysosomes of all tissues), acidic α-glucosidases from various tissues have been extensively studied in many laboratories. Acidic glucosidases hydrolyze α-1,4- and to a lesser extent α-1,6-glycosidic bonds in glucose oligo- and polysaccharides (from maltose to glycogen and starch). These enzymes also hydrolyze artificial substrates such as 4-methylumbelliferyl-α-D-glucoside, β-naphthyl-α-D-glucoside, and 6-bromonaphthyl-α-D-glucoside [35-38]. The enzymes involved into glycoprotein processing are also related to acidic glucosidases. Although neutral α-glucosidases are widely distributed in mammalian tissues and body fluids (in blood serum and urine), their biological role remains uncertain. Pig blood serum was shown to contain high glucosidase activity. This enzyme was highly purified and its properties were studied in detail [39]. At least three various neutral α-glucosidases were found in human urine [40]. They demonstrate wide substrate specificity with respect to different glucans possessing not only α-1,4- but also other types of glycosidic bonds. The comparative characteristics of mammalian acidic and neutral α-glucosidases are given in Table 2.

Table 2. Kinetic characteristics of acidic α-glucosidase from pig liver [38] and neutral α-glucosidase from pig blood serum [39]

Substrate	Acidic α-glucosidase			Neutral α-glucosidase		
	K_m, mM	k_{cat}, sec^{-1}	*v*, %	K_m, mM	V_{max}, nmole glucose/min	*v*, %
Maltose	6.7	113	(100)	2.1	392	100
Maltotriose	4.4	10.1	(82.6)	0.28	144	74
Maltotetraose	5.9	10.6	(86.6)	0.15	156	80
Maltopentaose	11.0	10.6	(86.6)	n.d.	n.d.	n.d.
Maltohexaose	4.0	11.0	(90.1)	n.d.	n.d.	n.d.
Maltoheptaose	5.6	11.3	(92.3)	n.d.	n.d.	n.d.
Kojibiose	5.0	2.22	18.2	8.33	130	33.3
Nigerose	20.0	9.1	75.5	20.0	348	89
Isomaltose	100	2.77	22.7	125	4.5	1.14
Panose	27.0	5.55	45.5	20.0	63	14.8
Phenyl-α-glucoside	8.6	1.77	14.5	1.47	215	110
Phenyl-α-maltoside	4.0	8.21	67.3	0.33	183	93
Soluble starch	8.0*	70.9	62.7	9.8*	215	110
Glycogen	10.0*	80.6	71.3	55.6*	10	5.15

Note: *v* is relative rate (%); n.d., not determined.
* K_m value is expressed as mg/ml.

PLANT α-GLUCOSIDASES

α-Glucosidases are widely distributed in plant tissues: in seeds, fruits, leaves, and roots [41]. Apparently, the main function of these enzymes consists in hydrolysis of oligosaccharides produced from starch by α-amylases, β-amylases, and dextrinases. This results in formation of glucose, an energy source for the developing plant. It is suggested that α-glucosidases can initiate the degradation of natural starch granules in barley seeds and pea chloroplasts in the absence of α-amylases [42-44]. Plant glucosidases have been purified to homogeneity from pea chloroplasts [42], onion leaves [45], barley seeds [43, 46], sugar beet [28], buckwheat [47], spinach [48], green pea [49], maize [50], and rice [51].

Most plant α-glucosidases are large proteins of molecular mass from 65 to 150 kD. Only glucosidases from pea chloroplasts [42], onion leaves [45], and banana flesh [52] are smaller proteins (40-50 kD). Sugar beet α-glucosidases I and II contain 2.7 and 8.8% neutral sugars, respectively [53]. Banana acidic α-glucosidase is a glycoprotein because it binds to ConA-Sepharose [52]. Little is known about post-translational modifications of plant α-glucosidases.

Plant α-glucosidases are often isolated from a given object as multiple forms with different molecular masses, isoelectric points, and pH optima. However, it remains unclear whether these multiple forms are products of various genes or they stem from posttranslational modification. For example, cloning experiments revealed that the presence of one gene encoding spinach glucosidase and four enzymatic forms with various substrate specificities [54] appear during posttranslational modification [29]. Most plant α-glucosidases are characterized by pH optimum at 4-5, but some of these enzymes have pH optimum at 6-7 [42, 52]. So, by analogy with mammalian α-glucosidases [36], the plant enzymes can also be separated into groups of acidic and neutral glucosidases. Plant α-glucosidases do not require metal ions as cofactors; these enzymes are characterized by wide substrate specificity (Table 3). They are most active with respect to α-1,4 glycosidic bonds in short oligosaccharides, but they exhibit individual specificity in hydrolysis of disaccharides with other bond types and longer maltooligosaccharides. This is an important difference between α-glucosidases and glucoamylases; the latter are characterized by increased affinity for longer chain maltooligosaccharides [55]. Most plant α-glucosidases can degrade soluble starch and the rates of hydrolysis of starch and maltooligosaccharides are comparable; some enzymes can attack natural starch granules [42, 43]. Since plant α-glucosidases catalyze substrate hydrolysis without change of anomer center configuration, the transglycosylation reaction was determined for some (but not all) enzymes. For example, the buckwheat seed enzyme synthesized kojibiose (α-1,2), nigerose (α-1,3), maltose (α-1,4), and trisaccharides from soluble starch [56]. However, role of transglycosylation *in vivo* remains unclear.

MICROBIAL α-GLUCOSIDASES

Various types of α-glucosidases with different (and wide) substrate specificity have been found in microor-

Table 3. Kinetic parameters of α-glucosidases from buckwheat [56], sugar beet [56], and barley [41]

Substrate	Buckwheat			Sugar beet			Barley		
	k_{cat}, sec^{-1}	K_m, mM	k_{cat}/K_m, $sec^{-1}\cdot mM$	k_{cat}, sec^{-1}	K_m, mM	k_{cat}/K_m, $sec^{-1}\cdot mM$	k_{cat}, sec^{-1}	K_m, mM	k_{cat}/K_m, $sec^{-1}\cdot mM$
Maltose	123	6.3	19.5	149	20.0	7.5	33.9	1.05	32.3
Maltotriose	136	4.0	34.0	68	3.7	18.4	14.0	0.65	21.5
Maltotetraose	131	2.8	46.8	68	2.3	29.4	12.4	1.4	8.8
Maltopentaose	150	1.8	83.3	68	0.6	113	13.5	1.7	7.9
Maltohexaose	162	1.3	125	68	0.4	170	11.5	4.6	2.5
Maltoheptaose	134	0.8	160	60	0.3	200	12.4	7.1	1.7
Kojibiose	21	3.2	6.6	16	1.3	12.3	20.3	11.0	1.8
Nigerose	124	12.0	10.3	195	17.0	11.5	2.6	14.0	0.2
Isomaltose	2.5	74.0	0.03	33.7	11.0	3.1	36.8	9.0	4.1
Phenyl-α-glucoside	4.9	40.0	0.12	5.4	1.7	3.2	n.d.	n.d.	n.d.
Soluble starch	103	1.1	97.2	179	0.3	597	n.d.	n.d.	n.d.

Note: n.d., not determined.

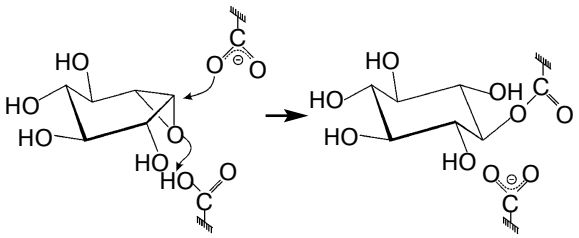
Table 4. Kinetic characteristics of α-glucosidase from *A. niger* [58]

Substrate	k_{cat}, sec^{-1}	K_m, mM	k_{cat}/K_m, sec^{-1}·mM
Maltose	54.8	0.76	73.1
Maltotriose	69.3	0.69	100
Maltotetraose	74.1	1.1	67.4
Maltopentaose	56.4	1.9	29.7
Kojibiose	12.4	4.6	2.7
Nigerose	33.8	12.0	2.8
Isomaltose	19.3	8.0	2.4
Phenyl-α-glucoside	0.7	0.36	1.9
Soluble starch	62.3	4.3	14.5

ganisms. The enzyme from *Aspergillus niger* has been intensively studied for many years [57-59]. This enzyme possesses wide substrate specificity; it can hydrolyze such substrates as maltose, kojibiose, nigerose, isomaltose, phenyl-α-glucoside, phenyl-α-maltoside, oligosaccharides, maltodextrin, and soluble starch (Table 4). Similar properties are exhibited by α-glucosidases from *A. oryzae* [60], *Bacillus subtilis* [61], and *B. cereus* [62]. The α-glucosidases from hyperthermophilic archaeobacteria (*Pyrococcus furiosus*, *P. woesei*, *Thermococcus profundus*, *T. litoralis*, *Thermotoga maritima*) are now attracting much attention [63-70]. The enzyme from *P. furiosus* [66] is active at temperatures up to 115°C. A glucosidase from *T. maritima* [70] hydrolyzes *p*-nitrophenyl-α-D-glucoside, maltose, and maltotriose but it does not hydrolyze starch, amylose, and amylopectin. The temperature optimum of this enzyme is at 75°C.

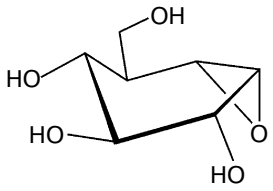
ELUCIDATION OF AMINO ACID RESIDUES OF THE ACTIVE SITE AND EVALUATION OF PROTEIN–CARBOHYDRATE INTERACTIONS

The increasing number of known nucleotide sequences of genes encoding α-glucosidases suggests the role of certain conserved amino acid residues in catalysis (Table 1). These suggestions can be verified by direct chemical modification of certain amino acid residue or by site-directed mutagenesis. Ionizable groups essential for catalysis were found in α-glucosidases from sugar beet [71] and *A. niger* [72]. In the latter case, kinetic pH dependences of V_{max} and K_m [72] gave $pK_{e1} = 3.2$ and $pK_{e2} = 6.4$ values for a free protein. Reduction of dielectric permeability increased the p*K* values. The heat of ionization values ΔH were 0.4 and ~0 kcal/mole. Water-soluble carbodiimide completely inactivated the enzyme, and substrate (maltose) reduced the inactivation rate. These data suggest that the first group is carboxylate (-COO^-), whereas the second is a carboxy group (-COOH). For this enzyme a catalytic amino acid residue was recognized by modification with conduritol B epoxide (CBE):



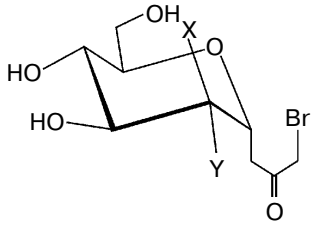
(1)

A competitive inhibitor (Tris) reduced the inactivation rate. Incorporation of one mole CBE to one mole enzyme completely abolished its catalytic activity. It is suggested that the active site -COO^- attacks CBE C_1 (Scheme 1); this method can be used to identify a nucleophile at the active site. Similar results were also obtained for sugar beet α-glucosidase [73], intestine sucrase-isomaltase [74], and human acidic α-glucosidase [75]. Use of CBE and other epoxides as probes for a catalytic residue sometime produces erroneous results because of high epoxide reactivity and lack of a C-5-hydroxymethyl residue in CBE which allows alternative binding of the inhibitor in the active site and reactions with acidic noncatalytic residues [76]. Perhaps, (R, 6S)-cyclophellitol, possessing a hydroxymethyl group at C_5 [77], can provide more specific inhibitor binding at the active site (Scheme 2):



(2)

Recently, Withers [78] introduced a method for determination of an amino acid involved in general acid catalysis at the active site. C-Mannoside-1′-bromo-3′-(α-D-mannopyranosyl)-2′-propanol (Scheme 3a) is an irreversible inhibitor of yeast α-glucosidase:



(3)

α-C-mannoside: X = OH, Y = H (a),
α-C-glucoside: X = H, Y = OH (b).

Its interaction with enzyme can be described by the following scheme:

$$EH + R\text{-}Br \longleftrightarrow EH * R\text{-}Br \longrightarrow E\text{-}R + HBr \quad (4)$$

α-C-Mannoside causes rapid irreversible enzyme inactivation, which follows pseudo first order kinetics. Although the authors failed to synthesize α-C-glucoside (Scheme 3b), they identified Glu276 as general acid catalyst. Among glucosidase family 13 members, this residue is completely conserved; it corresponds to Glu233 of pig pancreatic α-amylase and Glu257 of *B. circulans* 251 CDGT.

Chiba demonstrated [79] that bee α-glucosidase I is inactivated by diethylpyrocarbonate, which modifies one histidine residue. It seems unlikely that this histidine residue is directly involved in substrate cleavage. It is possible that this histidine is involved in substrate recognition. Such function of active site histidine residues as exhibiting of affinity for OH-groups of the substrate was found for His210, His122, and His296 of Taka-amylase A [80, 81].

Both conservative regions of human lysosomal acidic α-glucosidase (Table 1) have been well investigated using site-directed mutagenesis [75]. In mammalian and plant α-glucosidases, Asp513 is completely conservative; however, the mutant enzyme with substitution Asp513→Glu exhibits catalytic activity (13% of the wild-type enzyme), so this residue does not play a critical role in the catalysis. Mutations Asp518→Asn(or Glu or Gly) were accompanied by complete enzyme inactivation. It is suggested that this Asp is the catalytic nucleophile. Mutational analysis in the second conservative region Asp645→Glu(His) suggests the involvement of Asp645 in substrate binding [82].

The contribution of each hydroxy group of maltose to the energy of binding of both the basal and transition states of the enzyme–substrate complex was investigated using barley α-glucosidase [83]. The authors synthesized corresponding monodeoxyanalogs of maltose, and k_{cat} and K_m values were determined. Ratios of catalytic constants determined for a given analog and maltose were used for calculation of $\Delta\Delta G^{\neq}$ (contribution of hydrogen bond energy of the corresponding hydroxy group in the energy of transition state stabilization). The Michaelis constant ratio can be used to evaluate the contribution of each hydroxy group in stabilization of the basal state. Figure 1 shows that hydrogen bonds with hydroxy groups at C-6, C-4, and to a lesser extent at C-3 and C-2 of nonreducing maltose residue are especially important for catalysis. For reducing residues, only the hydroxy group at C-3 is essential; the others provide insignificant stabilization, and they are probably exposed to the solution.

THREE-DIMENSIONAL STRUCTURE OF GLUCOSIDASES

Three-dimensional structures have been obtained for α-amylases from *A. oryzae* (Taka-amylase A) [80, 81], pig pancreas [84], *A. niger* [85], barley [86], and *Pseudomonas stutzeri* [87]. All these structures have similar topological features: domain organization of tertiary structure and

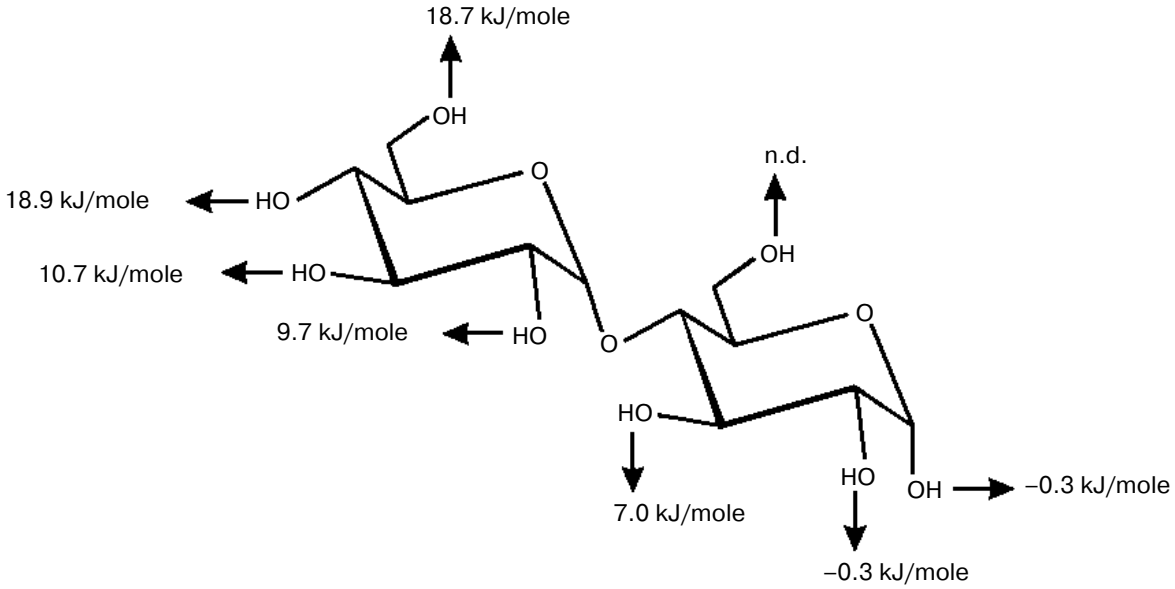


Fig. 1. Changes in free energy of activation of maltose hydrolysis during substitution of hydroxy groups for hydrogen atoms (n.d., not determined).

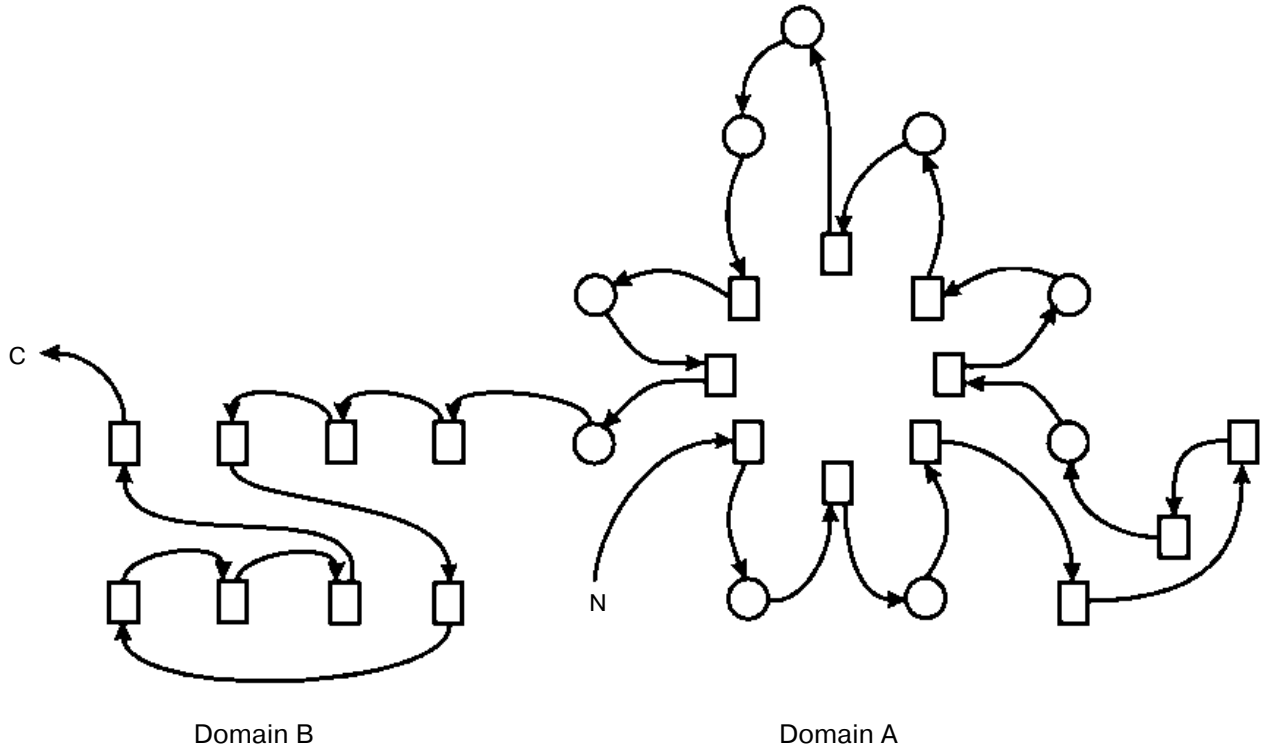


Fig. 2. Topological diagram demonstrating organization of α-helices (circles) and β-strands (rectangles) along polypeptide chain of Taka-amylase A. The structure of $(\beta/\alpha)_8$ barrel of domain A is on the right side. Antiparallel structure of domain B (β-sandwich type) is shown on the left.

organization of one of the domains as super-secondary structure of eight sequential α-helices and β-strands known as $(\beta/\alpha)_8$ barrel. In all these enzymes, the active site is a slit formed at the C-terminus of the β-sheet of the $(\beta/\alpha)_8$ barrel.

Figure 2 shows a topologic diagram of the polypeptide chain arrangement of Taka-amylase A [82].

Three-dimensional structures of CDGT from *B. circulans* [88-90] and *B. stearothermophilus* [91, 92] are homologous to those of α-amylases and also contain $(\beta/\alpha)_8$ barrel as the main topological element. Structure of the inactive CDGT mutant (Glu257→Ala) in the complex with β-cyclodextrin (CD) derivative, S-(α-D-glucopyranosyl)-6-thio-β-CD, was determined at resolution 2.4 Å [93]. The complex was fixed after the cyclization reaction (but before product diffusion into solution). Analysis revealed that β-CD bound to CDGT is deviated from a symmetrical conformation stronger than crystal β-CD dodecahydrate [94]. Electron density and temperature factor are not the same along the ring. The most pronounced deviation from β-CD symmetry was observed in subsites 1 and 1′ in the region of the cleaving bond (according to this nomenclature the exoglucose unit is bound to the fourth β-CD residue). The highest electron density was determined in subsite 2′, which is the most probable fixation point of α-glucan on the enzyme. Here side chains of several aromatic amino acids involved into the binding of glucose residues are located.

Recently, the structure of oligo-1,6-α-glucosidase from *B. cereus* has been recognized [95]. This type of exo α-glucosidases lacks starch-hydrolyzing activity. The enzyme consists of three domains. The N-terminal domain is characterized by the structure of $(\beta/\alpha)_8$ barrel with additional α-helices. The structure of the subdomain includes α-helix, triple-stranded antiparallel β-sheet, and long loops. The C-terminal domain consists of eight antiparallel β-strands. Three catalytic amino acid residues, Asp199, Glu255, and Asp329, are located in a deep slit formed by the subdomain and helix cluster of the $(\beta/\alpha)_8$ barrel. In 1999 preliminary results on the structure of 6-phospho-α-glucosidase from *B. subtilis* have been reported [96]. This enzyme is involved in catabolism of α-glucosides formed via the phosphoenolpyruvate-dependent maltose transferase system of *B. subtilis*.

CATALYTIC MECHANISM OF α-GLUCOSIDASES

α-Glucosidases catalyze two types of reactions: transfer of a glucosyl residue from the non-reducing end of an oligosaccharide onto water (hydrolysis) or transfer

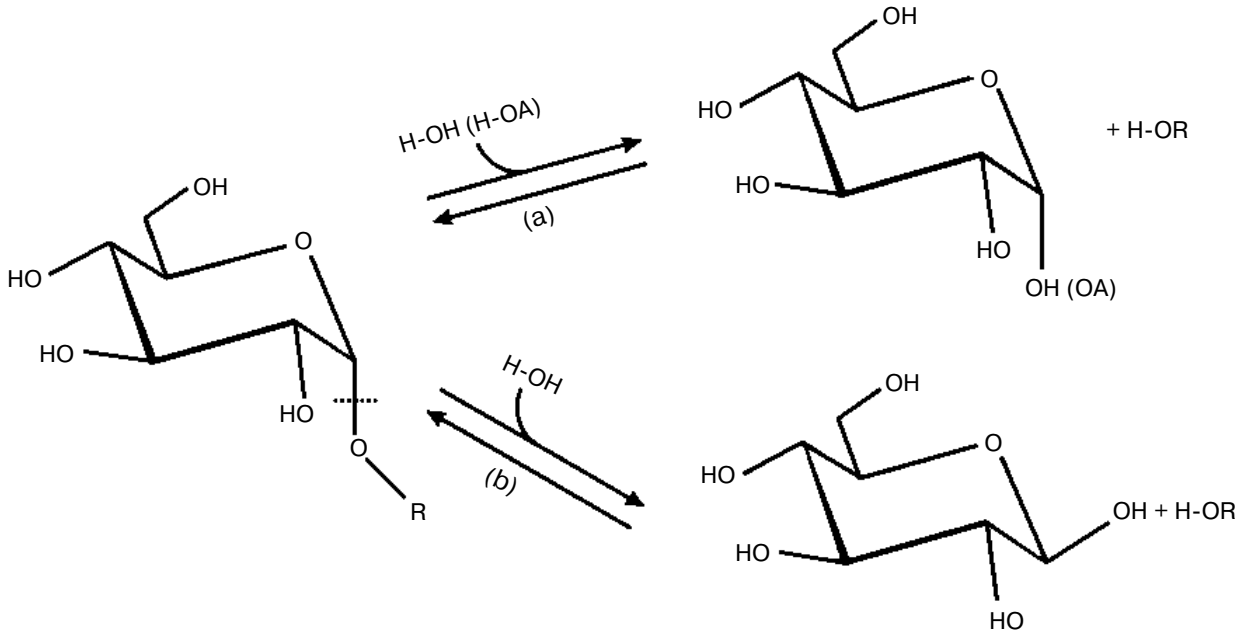


Fig. 3. Schemes of hydrolytic and transglucosylation reactions catalyzed by α-glucosidase (a) and glucoamylase (b).

of this residue onto an acceptor molecule (transglucosylation). These reactions can be formally considered as nucleophilic substitution at saturated carbon atom C_1 of the anomer center; they can proceed via either retention or inversion of the anomeric configuration of the reaction product. So, two types of glucosyl transferring enzymes (retaining and inverting) are recognized. α-Glucosidases and glucoamylases belong to the first and second type of glucosyl transferring enzymes, respectively (Fig. 3). Only retaining enzymes catalyze the transglucosylation reaction. If an inverting enzyme were to catalyze such a reaction, the reaction product would be the inverted anomer and in the reversed reaction this would be a reversed anomer substrate. Thus, inverting enzymes catalyze oligosaccharide synthesis only via reversal of the condensation reaction: maltose ↔ 2 glucose.

In 1953, Koshland suggested [97] that glycosyl hydrolases operating with retention of anomeric configuration of the reaction product catalyze the reaction by a double displacement mechanism in which two sequential stages can be distinguished.

1. After the initial substrate binding in the active site, a nucleophilic group of the enzyme (ionized carboxy group of aspartate, glutamate, or histidine imidazole) attacks anomeric carbon atom C_1 from the opposite side of the leaving group. This results in formation of covalent glucosyl–enzyme intermediate with anomer inversion at C_1. This stage is characterized by proton transfer from the corresponding donor at the active site (from carboxy group) to glycoside oxygen (i.e., general acid catalysis takes place).

2. At the second stage, a hydroxyl (in the case of the hydrolysis reaction) or sugar residue (transglycosylation reaction) attacks the formed ether bond; this is accompanied by configuration inversion at C_1, and the resultant product has the same anomeric configuration as the initial substrate. At this stage, important role is played by a base, which attracts a water proton (or sugar residue hydroxyl during transglycosylation). This increases nucleophilic properties of the attacking oxygen (general base catalysis). The carboxylate of aspartate or glutamate may play the role of such base. At this stage, the enzyme returns to the initial protonated state (Fig. 4). This scheme adequately describes the catalytic mechanism, and the problem remaining consists of elucidation of some important details of this scheme.

Experimental detection of β-glucosyl–enzyme intermediate formation in the active site of α-glucosidase. This is a rather complex problem because the accumulation of significant quantities of such product could be achieved only in the case when the stage of intermediate hydrolysis is rate limiting. This happens quite rarely. For example, in the case of lysozyme (the first retaining glucosidase studied within the framework of Koshland's model), substrates limiting the reaction rate at this stage were not found. Covalent β-glucosyl–enzyme intermediate was identified in cryoenzymatic experiment with pig pancreatic α-amylase using ^{13}C-NMR spectroscopy [98]. Glucosyl–enzyme intermediates were isolated for dextran sucrases catalyzing sucrose inversion to insoluble polymers in dental caries. Such intermediates were also obtained for the enzyme from *Leuconostoc mesenteroides* [99] and *S. sobrinus* [100, 101].

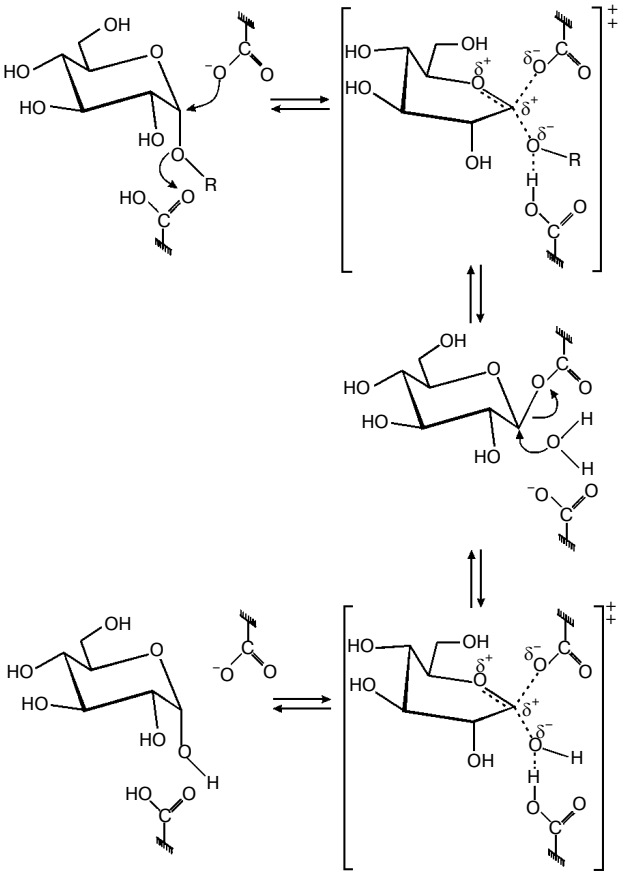
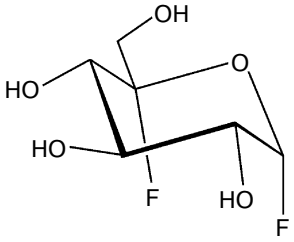


Fig. 4. Nucleophilic double displacement mechanism during hydrolysis reaction of an α-glucoside bond.

Withers's group developed another methodology for accumulation of these intermediates. They successfully used 2-deoxy-2-fluoroglycosyl fluorides for identification of active site nucleophiles of retaining glycosidases (β-glycosidase only) [102]. A good leaving group (F^-) accelerates only the first stage of the enzymatic reaction, whereas the fluoride at C-2 slows both stages, and this results in accumulation of 2-deoxy-2-fluoro-α-glucosyl–enzyme intermediate [103, 104].

Catalytic nucleophiles were identified in several β-glucosidases after the reaction with substrate followed by proteolytic degradation of the enzyme and isolation of labeled peptide by electrospray mass-spectrometry [105-107]. The other substrate, 5-fluoro-glucosylfluoride, was used for identification of catalytic nucleophiles of α-glucosidases [108]:



(5)

The C-5 epimer (for which the hydroxymethylene residue has the L-idoconfiguration) is an effective inhibitor of yeast α-glucosidase.

For both 5-fluoro-compounds, the inductive effect of fluoride at C-5 near the developing positive change on the endocyclic oxygen in the transition state will slow both stages of the enzymatic reaction. This is because the good leaving group (F^-) at the anomeric center will accelerate only the first stage, and this results in the accumulation of glucosyl–enzyme intermediate. This methodology was used to identify Asp214 of *Saccharomyces cerevisiae* α-glucosidase as the catalytic nucleophile [109].

How stages shown in Fig. 4 are realized? The mechanism of bimolecular nucleophilic substitution (S_N2 mechanism) and the mechanism of carbocation formation (S_N1 mechanism) are the two main mechanisms responsible for realization of these processes. Choice between these two models is rather complex. The S_N2 mechanism implies synchronous attack of a carboxyl proton on glycoside oxygen (general acid catalysis) with simultaneous attack on C_1 with formation of intermediate product, β-glucosyl carboxylate. The second half of this reaction consists in attack on the intermediate from the opposite side by a water molecule with general base catalysis of this process by carboxylate. Each of these half-reactions follows the S_N2 mechanism.

Figure 4 illustrates the mechanism of carbocation formation in the transition state. This scheme suggests the same sequence of events as in the S_N2 mechanism, but one or both transition states are carbocations. It is suggested that a carboxylate group stabilizes the developing carbocation charge whereas a carboxy group attacks oxygen of the glucoside bond.

It is clear that both variants represent extreme cases, and it is possible that each of the half-reactions occurs via a transition state possessing features of both S_N1 and S_N2 mechanisms. In other words, the degree of glycoside bond cleavage may significantly vary in the transition state.

There are at least three sets of experimental data supporting the involvement of a carbocation in the catalysis.

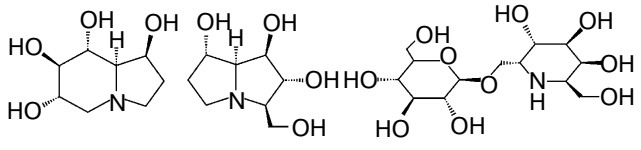
First, if carbocation formation is the rate-limiting stage, it is possible to measure an α-deuterium (or tritium) secondary kinetic isotope effect; in this case the expected k_H/k_D value is > 1.05. The solvent isotope effect is sensitive to the hybridization state at the anomeric center during the transition from basal to transition state if this transition is the rate-limiting step. Although such experiments cannot discriminate S_N1 and S_N2 mechanisms, they are useful tools for evaluation of the development of a carbocation in the transition state [110].

There are several reports on the measurement of kinetic isotope effects in α-glucosidases [111-113]; in the

first case, the effect (k_H/k_D value) did not differ from unity. Lack of a measurable isotope effect means that carbocation formation is not the rate-limiting step. For other retaining glycosidases (lysozyme [114, 115], *E. coli* β-galactosidase [116], *A. wentii* glucosidase A [117], rabbit small intestine sucrase-isomaltase [113]), the α-deuterium secondary kinetic isotope effect is 1.1-1.25. This indicates significant carbocation character of the transition state. If intermediate deglycosylation is the rate-limiting step, the value of the isotope effect $k_H/k_D > 1$ suggests covalent nature of the glucosyl–enzyme intermediate because in this case the transition of the intermediate to the transition state is accompanied by reduction of hybridization degree sp^3-sp^2. In the case of an ion-like intermediate, as suggested for lysozyme (structure of ion couple glycosyl carbocation/enzyme carboxylate), the transition state would be characterized by an increase in hybridization state and, consequently, the α-deuterium kinetic isotope would be reversed.

Second, the involvement of the carbocation in the catalysis is supported by the above-mentioned data on irreversible enzyme inhibition by such compounds as 2-deoxy-2-fluoro-α-D-glucosylfluoride or 5-fluoro-α-D-glucosylfluoride. In these compounds, the inductive effect of fluoride at C-2 or C-5 of the glucose ring destabilizes the transition state glucosyl cation and promotes formation of a stable glucosyl–enzyme intermediate.

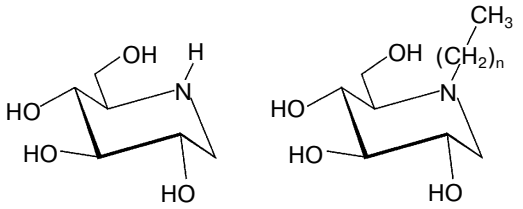
Third, ligands imitating characteristic features of a carbocation (negative charge and/or semi-chair conformation) act as inhibitors. δ-Gluconolacton possessing a semi-chair conformation is a competitive inhibitor of bovine liver α-glucosidase with K_i value of $5 \cdot 10^{-4}$ M (the K_m for maltose is $1.2 \cdot 10^{-3}$ M) [111]. Inhibitors carrying positive charge are much more potent inhibitors; even Tris inhibits glucosidase activity. Nojirimycin inhibits lysosomal acidic α-glucosidase from human liver with K_i about 10^{-6} M [118]. Two α-glucosidases from endoplasmic reticulum were tested for inhibition by the natural antibiotic castanospermum and by a synthetic disaccharide with a methylene spacer for imitation of the cleaving bond [119]:



Castanospermine Australine Disaccharide analog

(6)

It was shown that castanospermine and the disaccharide more potently inhibit glucosidase I and glucosidase II, respectively [120]. Deoxynojirimycin and its N-alkyl analogs are potent inhibitors of glucosidase I [121]:



Deoxynojirimycin N-Alkyl-deoxynojirimycin

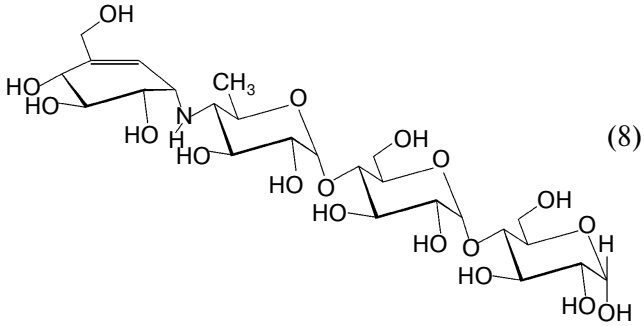
(7)

The K_i values for deoxynojirimycin and N-methyl derivative are 1.0 and 0.07 μM, respectively.

Australine [122], a polyhydroxylated pyrrolizidine alkaloid isolated from seeds of the Australian tree *Castanospermum australe*, is a potent competitive inhibitor of α-glucosidase from *A. niger* (IC_{50} = 5.8 μM), but it does not inhibit β-glucosidase, α- and β-mannosidases, and α- and β-galactosidases. Australine also inhibits the processing system of glucosidase I buts has weak effect on glucosidase II.

Analyzing structures of australine, castanospermine, and the related alkaloid swainsonine [122], it was concluded that the six-member ring structure typical for indolizidine alkaloids (castanospermine, swainsonine) and also for deoxynojirimycin is not an essential component for glucosidase inhibition. It is suggested that the presence of nitrogen in the ring and the configuration of hydroxyl groups versus nitrogen are the main preconditions required for manifestation of inhibition. Yeast α-glucosidase is effectively inhibited by a five-member ring inhibitor 1,4-dideoxy-1,4-imino-D-arabinitol with K_i value of 0.18 μM [123], deoxynojirimycin is less potent (K_i = 33 μM) [114], whereas castanospermine did not inhibit the enzyme at all.

Acarbose, a pseudotetrasaccharide from *Actinoplanes* species, is one of the most potent inhibitors of α-glucosidases [124, 125].



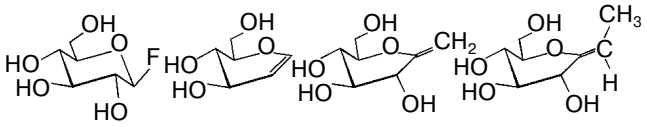
(8)

Its structure apparently resembles the transition state of a substrate. In fact, the first ring at the non-reducing end of the acarbose chain acquires a flattened conformation due to the presence of a double bond, and the imine nitrogen (pK 5) is positively charged under conditions of

the enzymatic reaction. The K_i values were determined for CDGT ($2 \cdot 10^{-7}$ M) [126] and glucoamylase (10^{-10} M) [127]. To determine whether acarbose is a transition state analog [126], amino acids constituting the active site of CDGT [128] were mutated and for each of eight mutants k_{cat}, K_m, and K_i values (for acarbose) were determined. The plot of the dependence log (K_m/k_{cat}) versus log K_i (acarbose) demonstrated high correlation with $r = 0.98$ and slope of 2.2. This suggests that acarbose exhibits properties of a transition state analog. Acarbose is now under clinical trial as a drug for the treatment of diabetes mellitus type I and II. It inhibits acidic α-glucosidases, glucoamylases, sucrases, maltases, and isomaltases in the small intestine [129].

Manifestation of potent inhibition apparently requires hydrogen bonding between the imine nitrogen and a catalytic acid. Using a set of β-glucosidases, Legler [130] demonstrated that the transition of N_1-alkyl-D-glucosylamines to N_1-butyl- (or dodecyl)-D-gluconamidines is accompanied by ~10-fold increase of inhibitory effect; the inhibitor geometry changes from tetrahedral C_1-geometry to planar sp^2 amidine geometry. According to the authors' viewpoint, totally protonated amidines cannot accept protons from the catalytic acid.

Hehre [131], Withers [132], and Lehmann [133] studied interactions of glycosidases (and, in particular, α-glucosidases) with substrates possessing a double bond at C_1 (glycals, glyco-heptenitols, glyco-octenitols) or "wrong" anomeric configuration (β-D-glucosylfluoride as substrate for α-glucosidase):



β-D-Glucosyl-fluoride — Glucal — Gluco-heptenitol — Gluco-octenitol (9)

α-Glucosidases form productive complexes with these substrates (possibly due to the absence of large substituents with "wrong" orientation at C_1) and hydrolyze them. However, none of the α-glucosidases reacts with the smallest "wrong" glucoside, methyl-β-D-glucopyranoside, possibly due to conformational restraints of the equatorial substituent at C_1 seized by the active site-constituting amino acid residues.

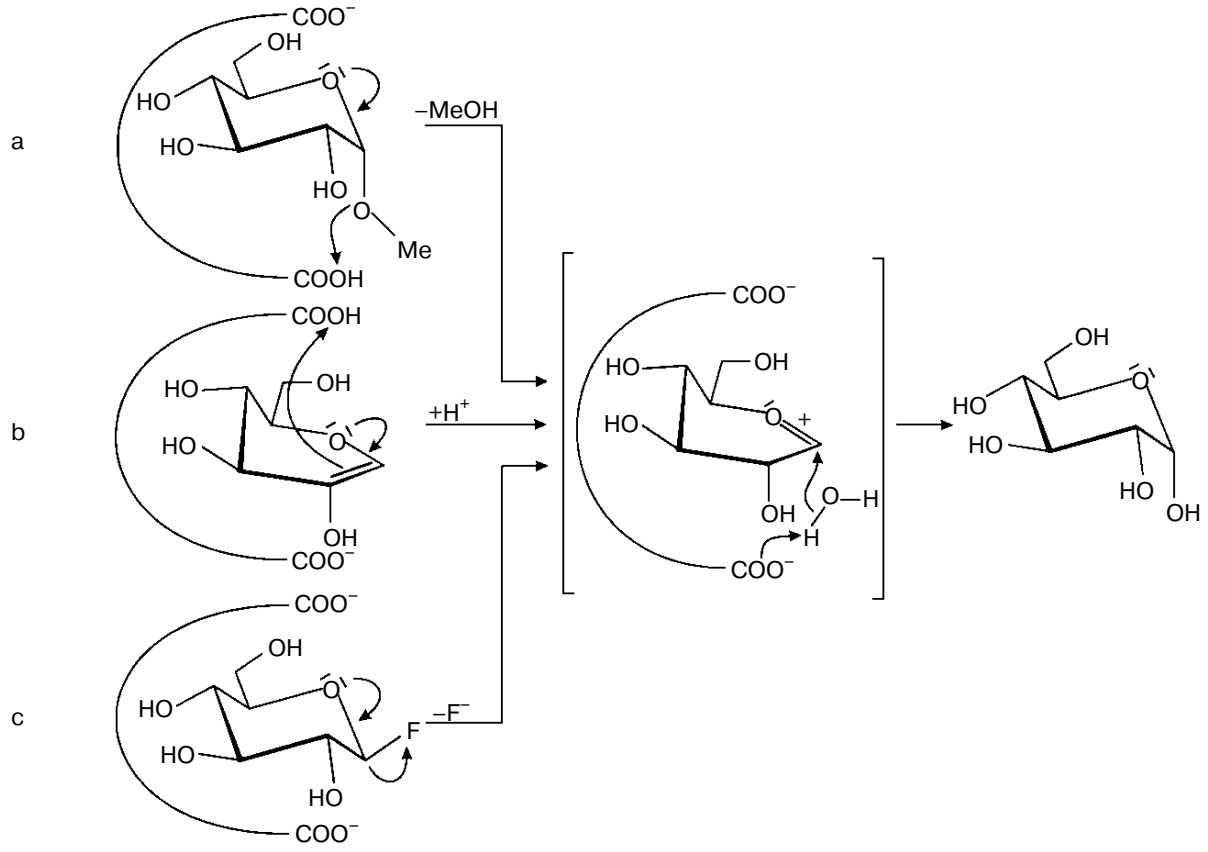


Fig. 5. Proposed mechanisms of α-glucosidase hydrolysis of substrates with various anomeric configurations. Plastic and conserved reaction phases are indicated on the left and right sides, respectively.

α-Glucosidases rapidly hydrolyze α-glucosylfluoride with α-glucose formation; they can also hydrolyze β-glucosylfluoride with α-glucose formation but the rate of reaction is significantly slower than in the first case. Hydrolysis of prochiral substrates (glucal, enitols) also yields α-anomeric products (2-deoxy-α-glucose, α-glucoheptulose, α-glucooctulose). To explain these effects, Hehre [134] proposed the minimal scheme shown in Fig. 5.

In each case, the undivided electron pair of the substrate ring oxygen is involved in cleavage of the glucosidic linkage at C_1 with formation of a carbocation structure as the general transition state. The principal point of this scheme consists in reversed protonation of catalytic carboxyls in cases (a) and (b). This is supported by NMR experiments that elucidated the proton location at C_2 (axial or equatorial versus the planar ring) during glucal hydratation in D_2O [135]. Thus, Hehre introduced an important notion on the catalytic mechanism for α-glucosidases (and other glycosidases). According to Hehre, two phases of hydrolysis, plastic and conserved, are recognized. In the plastic phase, catalytic carboxyls may switch their functional role depending on substrate structure: a nucleophile becomes a general acid and vice versa. For example, a glucal double bond near a nucleophile increases its pK and therefore promotes its protonation. During the conserved phase, hydratation is strictly controlled by protein structure irrespectively to substrate configuration; for α-glucosidases, it always proceeds in the axial position with α-anomeric product formation.

It should be noted that mechanisms of retaining β-glucosidases have been investigated much more fully than those of α-glucosidases [136]. This can be explained by the availability of a representative set of synthetic substrates possessing β-anomeric configuration. Studies of α-glucosidases hydrolyzing substituted phenyl glucosides would require the following experiments.

A) Determination of Bronsted's dependences (log k_{cat} versus pK_a of aglycon phenol, log k_{cat}/K_m versus pK_a of aglycon phenol). In the case of "good" leaving groups (characterized by low pK_a values), deglycosylation of glucosyl–enzyme intermediate would be the rate-limiting step of the catalysis. On the contrary, in the case of "poor" leaving groups (high pK_a), glycosylation will be the rate-limiting step. These conclusions can be experimentally verified by adding a nucleophilic reactant, e.g., dithiothreitol. In the case of a "good" leaving group, addition of an exogenous nucleophile will increase the reaction rate, whereas in the case of a "poor" leaving group no effect would be expected [137].

B) Measurement of secondary kinetic isotope effects for these substrates would allow the evaluation (at least qualitatively) of the transition state structure of the rate-limiting step: degree of carbocation development (S_N1 mechanism contribution when the effect is >1.1) and participation (pre-association) of enzyme nucleophile in the transition state (S_N2 mechanism contribution).

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