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Substrate recognition by amyloglucosidase: evaluation of conformationally biased isomaltosides

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ABSTRACT

Amyloglucosidase catalyzes the hydrolysis of methyl β-maltoside (**1**) 30–50 times more rapidly than methyl α-isomaltoside (**2**). It is established that OH-6′, OH-4′ and OH-3 are intimately involved in the hydrolysis of the maltoside whereas it is OH-6′, OH-4′, and OH-4 which are involved in key polar interactions with the enzyme in the case of isomaltoside. Conformational analyses based on HSEA calculations indicate that the dispositions in space of OH-3 of maltose relative to OH-4′ and OH-6′ in the preferred conformation for the maltoside (**1**) is energetically more readily achieved by methyl 6*R*-*C*-methyl-α-isomaltoside (**3**), than for its 6-*S*-isomer (**4**). A kinetic evaluation of the hydrolysis in fact has shown that the *R*-compound is more strongly bound by the enzyme ($K_m = 0.9$ mM) than the parent isomaltoside ($K_m = 24.5$ mM), whereas the *S*-compound has the weakest enzyme binding ($K_m = 90$ mM). Since the k_{cat} values were all within the range 0.85 ± 0.20 s^{-1}, it is evident that the relative rates of hydrolysis are related to the relative ease for the compounds to achieve an interaction of a hydroxyl group in the aglycon of an α-D-glucopyranoside with the enzyme for the formation of the enzyme–substrate complex. The relative rates of hydrolysis of the α-glucosides of the 1,3-dihydroxy-*trans*-decalins, **5** and **6**, provide further support for this highly desirable but not necessary recognition for the orientation of the reducing glucose unit in the active site.

INTRODUCTION

It has long been appreciated that the industrially important enzyme, amyloglucosidase (EC 3.2.1.3, AMG) is sensitive to the structure of the aglycon of α-D-glucopyranosides[1–3]. The enzyme catalyzes a rapid release of D-glucose from the nonreducing end of starch, glycogen and a variety of oligosaccharides[4,5]. Although simple alkyl α-D-glucopyranosides are hydrolyzed extremely slowly, if at all, the α-(1 → 4) linkage of maltose is hydrolyzed 30–50 times faster than the α-(1 → 6) bond of isomaltose[6,7]. A series of kinetic studies, using as substrates deoxy,

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deoxy-fluoro, chloro-deoxy, and *O*-methyl derivatives of methyl β-maltoside, have shown that the 3,4′,6′- and 4,4′,6′-hydroxyl groups of maltosides and isomaltosides, respectively, are essential structural requirements for rapid hydrolysis[8–12]. Modification of any one of these groups decreases the rate by about one thousand fold. Therefore, efficient catalysis involves some type of interaction of these three hydroxyl groups with the enzyme. The importance of these polar hydrogen bonds is similar to that of the key polar groups for substrate recognition as was discovered for the binding of oligosaccharides by lectins and antibodies[13,14].

Conformational analyses based on HSEA calculations[15] suggest that the preferred conformations of methyl β-maltoside[11,16] (**1**) and its α-isomer[17] in water are in high abundance with internuclear distances of about 7.1 and 7.3 Å separating O-3 from O-4′ and O-6′, respectively. In contrast to maltose, which has a well-defined conformational preference, isomaltose is conformationally ill-defined because of the freedom of rotation about the C-5 to C-6 bond of the reducing unit. Therefore, it seemed likely that its ease of hydrolysis by AMG, as compared to simple alkyl α-D-glucopyranosides, would be related to an ability to achieve a conformation that would bring its OH-4 in a spatial relationship to OH-4′ and OH-6′ close to that for OH-3 of maltose without much demand in conformational energy. HSEA calculations supported this conjecture[18]. Furthermore, it had been demonstrated that the introduction of a *C*-methyl group at the 6-position of the reducing unit of β-D-Gal-(1 → 4)-β-D-GlcNAc-(1 → 6)-D-Gal importantly restricted the freedom of rotation about the β-(1 → 6) bond[19]. In fact, one isomer was more strongly bound by a monoclonal antibody than either the other isomer or the parent compound[20]. Furthermore, unequivocal evidence was provided that the role of the C-6 methyl group was to render more facile the achievement of the conformer that is recognized by the antibody[20]. Therefore, the diastereoisomeric C-6 methyl derivatives of methyl α-isomaltoside, compounds **3** and **4**, were synthesized in order to assess the effect that introducing the *C*-methyl group would have on the rate of hydrolysis by AMG.

RESULTS AND DISCUSSION

The conformational analyses[18] revealed that the *C*-6-*R*-methyl compound (**3**) has the highest population of the possible conformations in which the spatial arrangement of the key polar groups, as illustrated by the conformational formulas (Fig. 1), are in close correspondence to that of maltose (**1**). As further indicated in Table I, the population of the key conformer of methyl α-isomaltoside (**2**) was expected to be appreciably lower. Achievement of the key conformer was estimated to be most difficult for the 6-*S*-derivative (**4**). It could be expected, therefore, that the relative rates of hydrolysis would be in the order **3** > **2** > **4**.

The kinetic parameters for the hydrolysis of compounds **2**–**4** by *Aspergillus niger* amyloglucosidase are summarized in Table I. These were performed by quantitating the glucose formed as a reaction product using glucose oxidase and peroxidase

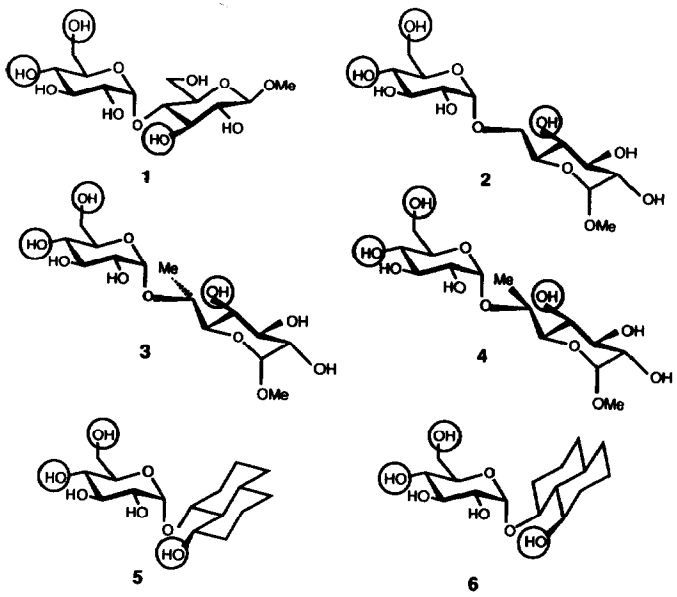

Fig. 1. The OH-4′, OH-6′, and OH-3 or OH-4 groups (circled) of methyl α-maltoside (**1**), and methyl α-isomaltoside (**2**) which are expected to form the key polar grouping for amyloglucosidase hydrolysis. Conformational formulas display how compounds **2**–**6** may achieve conformations that can provide the polar interaction with amyloglucosidase that is key to the rapid hydrolysis of **1**.

enzymes. The parameter k_{cat} will contain terms for all steps after the formation of the first enzyme–substrate (ES) complex. It is seen that the rates of decomposition of the ES complexes to the products of hydrolysis vary less than two-fold for these three substrates, since the k_{cat} values are in the range of 0.7 to 1.1 s^{-1}. However, major effects on the reversible formation of the complex are reflected by the K_m values which range from a low of 0.7 mM for the best substrate, the methyl 6-*R*-methyl-α-isomaltoside (**3**), to 90 mM for the poorest 6-*S*-diastereoisomer (**4**). The specificity constants k_{cat}/K_m values follow a similar trend, ranging from 0.012 mM^{-1} s^{-1} for the 6-*S*-analogue to 0.92 mM^{-1} s^{-1} for the 6-*R* analogue. As was to

TABLE I

Kinetic parameters for the hydrolysis of conformationally biased α-D-glucopyranosides by *Aspergillus niger* amyloglucosidase

Substrate	Population of key conformer	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})
1 [a]	High	7.8	1.3	6.0
2	Intermediate	1.04	24.5	0.042
3	High	0.68	0.71	0.96
4	Low	1.1	90.0	0.012
5	High	0.039	0.9	0.043
6	Low	0.14	≥ 15	0.0092

[a] Maltose.

be expected on the basis of the relative demands in conformational energy for achievement of the key conformer, the k_{cat}/K_m value for methyl α-isomaltoside (**2**) was intermediate at 0.042 $mM^{-1}\ s^{-1}$.

It is well established[21] that the preferred conformation about a glycosidic bond invariably has the anomeric hydrogen in opposition to an aglyconic hydrogen, as is displayed in the structural formula **5** for one of the glucosides of 1,3-dihydroxy-*trans*-decalin. Thus, it is evident that the free hydroxyl group at the 3-position of the *trans*-decalin aglycon can occupy a spatial position relative to OH-4′ and OH-6′ similar to that of OH-3 in **1** (refs 12 and 22). On the other hand, the introduction of the α-D-glucosyl unit to form the diastereoisomeric glucoside (**6**) must be expected to provide a glucoside for which the assumption of the key conformation displayed in the formula **6** is very difficult. The high energy of this conformer appears reflected by the high K_m value and low specificity constant k_{cat}/K_m (Table I). Whether or not the achievement of this conformation was in part hindered by nonbonded interaction with the enzyme as well as unfavorable intramolecular interactions to achieve the key conformation depicted in the formula remains unknown. In the case of isomer **5**, the key conformer is surely[21] much more readily achieved than that for **6**. Therefore, it was not surprising that **5** was hydrolyzed with a K_m value close to compounds **1** and **3** and a specificity constant (k_{cat}/K_m) five times greater than for **6** (Table I). That the rate of hydrolysis of **6** is four times greater than that for **5** indicates that differential forces of interaction influence the decomposition of the complex (H_2O–enzyme–glucoside) to the product of hydrolysis. These observations clearly indicate that the complex with AMG can entertain an extremely wide range of structural differences in the aglycon which suggests that the aglycon largely remains in contact with water. The role of a hydrogen bond between the enzyme and hydroxyl group of the aglycon must be related to easing the achievement of a conformation favorable to hydrolysis as well as to the binding to the binding-site. It is noteworthy in this regard, that the polar interaction may help bring about a rotation about the glycosidic bond that weakens the *exo*-anomeric effect and, thereby, activates the anomeric center to nucleophilic attack by water[23,24].

In view of the similarity of the k_{cat} values for the conformationally biased isomaltosides, there can be little doubt that, once formed, the various complexes in each case are hydrolyzed with equal ease. Therefore, it appears that changes in nonbonded interactions with the protein as a result of the differences in structure are not encountered. Studies are in progress to assess this possibility using the general approach developed for oligosaccharide binding studies[25], with all seven possible monodeoxy- and O-CH_3 methyl α- isomaltosides.

EXPERIMENTAL

Materials.—Glucoamylase G1 was isolated from *Aspergillus niger* by affinity chromatography on an acarbose-Sepharose column[26]. Isomaltose, maltose, and a

glucose diagnostic kit including glucose oxidase–peroxidase capsules, *o*-dianisidine, and glucose standard solutions were from Sigma. The synthesis of compounds **2–4** and **5** and **6** have been described previously (refs 18 and 12, 22, respectively). Compound **6** contains 10% of compound **5**, but the kinetics suggest there is negligible contribution of this material.

Methods.—The kinetics of the hydrolysis of the substrate analogues were measured by monitoring glucose production by coupling to glucose oxidase and peroxidase reactions. The reaction volume has been reduced and the coupled assay adapted for use of a microplate reader to measure absorbances[27]. Incubations were carried out in 1.5-mL microcentrifuge tubes in a heating block thermostated at 45°C. Reaction volumes were 100 μL in 50 mM sodium acetate buffer, pH 4.5, and reaction was initiated by the addition of 1 to 20 μg of amyloglucosidase in 10 μL of assay buffer. Samples were mixed quickly on a vortex mixer then centrifuged briefly in a microfuge. Aliquots (15 μL) were removed at 2 to 20 min intervals and transferred to microtiter wells containing a quench solution of 300 μL 1 M Tris · HCl buffer, pH 7.6 with 1.5 units of glucose oxidase, 0.3 units of horseradish peroxidase, and 24 μg of *o*-dianisidine. The quenched solutions were covered with foil to protect them from light and color development was allowed to proceed for 1 h at ambient temperature, or until there was no further increase in absorbance. Absorbances were read at 450 nm in a Perkin–Elmer Microplate reader. Various amounts of D-glucose were added to each plate to obtain a standard curve. Linear regression analysis of glucose production as a function of time was carried out with the linear program in Enzfitter. Six to eight substrate concentrations were employed ranging from approximately 0.2 to 6 times K_m. The kinetic parameters V_{max} and K_m were obtained by fitting the initial rate data vs substrate concentration to the Michaelis–Menten equation using the Enzfitter program. In general, the errors for the V_{max} values were no more than 8%, and no greater than 10% for K_m. Values of k_{cat} were determined by dividing V_{max} values by enzyme concentration using a molecular weight of 82 700 and $\epsilon_M = 1.37 \times 10^5$ M^{-1} cm^{-1} (refs 28 and 29), for amyloglucosidase.

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