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Glucoamylase mutants with decreased K_m-values for C-6 substituted isomaltosides

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Abstract

Glucoamylase can catalyze the hydrolysis of methyl 6*R*-*C*-alkyl α-isomaltosides. A Trp120→Phe mutant altered in the +2 binding subsite had a K_m of 0.089 mM for methyl 6*R*-*C*-methyl-α-isomaltoside compared to a K_m of 0.71 mM for the wild-type enzyme. This reflects an eight-fold lower K_m for this substrate; however, the k_{cat} for the mutant was decreased 200-fold compared with the wild-type glucoamylase. With increasing size of the substituent to 6*R*-*C*-ethyl and -isopropyl, Trp120→Phe and wild-type glucoamylase have similar K_m values, while k_{cat} for the mutant increases 10- and 100-fold, respectively, approaching wild-type values. Perturbation of the structural integrity around the general acid catalyst Glu179, through elimination of the Trp120 NE1 hydrogen-bond to Glu179 OE2 in Trp120→Phe glucoamylase, seems to be counteracted by the larger C-6 substituents. The apparent complementarity between enzyme and substrate analogs emphasizes the favorable impact of hydrophobic forces in protein-carbohydrate interactions. Wild-type glucoamylase and Val181→Ala/Asn182→Ala/Gly183→Lys/Ser184→His, a quadruple mutant located beyond subsite +3, essentially maintain k_{cat} in substrates with the three different 6*R*-*C* substituents, while K_m increases from 0.45 to 47.0 mM for methyl-6*R*-*C*-isopropyl α-isomaltoside. Compared with the wild-type enzyme, the quadruple mutant has 1.5–3.7-fold improved specificity (k_{cat}/K_m) for the parent and 6*R*-*C*-alkyl isomaltosides, but 10-fold lower activity for the α-(1→4)-linked maltose caused mainly by a low k_{cat}. This mutation at distant subsites thus influences hydrolysis of disaccharides, corroborating the presence of long-range effects on the catalytic site.

Keywords: Substrate specificity; Methyl-6*R*-*C*-methyl-, -6*R*-*C*-ethyl-, and -6*R*-*C*-isopropyl-α-isomaltosides; Protein–carbohydrate interactions; Binding loop mutant

1. Introduction

Glucoamylase (GA) (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) an inverting, exo-acting carbohydrase, has 500-fold higher specificity (k_{cat}/K_m) for maltose compared with isomaltose [1–4]. Efficient hydrolysis of both substrates depends on strong hydrogen bonds between GA and specific OH-groups of the α-(1→4)- and α-(1→6)-linked glucosyl residues accommodated at subsites −1 and +1, respectively, as demonstrated using deoxy-maltosides [5–7] and deoxy-isomaltosides as substrates [8,9]. Deoxygenation of OH-4′, -6′, or -3 in maltose and OH-4′, -6′, or -4 in isomaltose thus reduced the transition-state

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stabilization energy, $\Delta\Delta G^{\ddagger}$, by 18–24 kJ mol^{-1} [6–9]. Such high $\Delta\Delta G^{\ddagger}$ values agree with loss of a charged hydrogen-bond [10] to Arg54, Asp55, Glu180, or Arg305, seen from the crystal structure of *Aspergillus awamori* var. *X100* GA in complex with the α-(1→4)-linked analogs and potent inhibitors acarbose and D-*gluco*-dihydroacarbose [11–13]. A modelled glucoamylase–isomaltose complex is compatible with the energetics determined by molecular recognition of deoxy-isomaltosides [8,9].

Substitution at C-6 in isomaltose (Fig. 1) hinders rotation about the C-5–C-6 bond. The 6*R*-C-alkyl enantiomer is conformationally biased to have optimal distribution of the OH-4′, -6′, and -4 groups for recognition by GA [14]. Wild-type GA thus has an ca. 30-fold lower K_m for methyl 6*R*-*C*-methyl-α-isomaltoside than for methyl α-isomaltoside [15]. The binding energetics of the conformationally biased OH-4 and -6-deoxy-methyl *6R*-*C*-methyl-α-isomaltosides for wild-type and selected active site GA mutants, Glu180→Gln and Asp309→Glu, furthermore demonstrated that the key polar interactions at subsite −1 in the GA-isomaltoside transition-state complex are optimized through a change from the predominant solution *gauche–gauche* to an enzyme-bound *trans–gauche* conformer, which is induced by GA substrate interaction at subsite +1 [8]. To increase insight into structural features of both GA and isomaltose that are important for catalysis, a kinetic study of the hydrolysis of methyl 6*R*-*C* alkyl α-isomaltosides by wild-type, Trp120→Phe

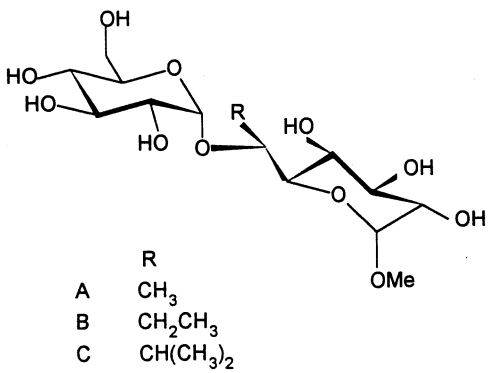

Fig. 1. Molecular structures of conformationally biased α-(1→6)-linked substrates: methyl 6*R*-*C*-methyl-α-isomaltoside (A), methyl 6*R*-*C*-ethyl-α-isomaltoside (B), and methyl 6*R*-*C*-isopropyl-α-isomaltoside (C).

[16], and Val181→Ala/Asn182→Ala/Gly183→Lys/Ser184→His mutant GAs has been carried out. These mutations at the conserved substrate binding α→α loops 2 and 3 of the catalytic $(\alpha/\alpha)_6$-barrel affect subsite +2 and subsites beyond +3, respectively [12]. They highlight the versatility of GA catalysis and emphasize the significance of hydrophobic interactions in protein–carbohydrate complexation. Surprisingly, the quadruple mutant at a distance from the catalytic site reduced the rate of hydrolysis for maltose, but not isomaltose, which stresses that knowledge on long-range effects is both necessary and useful in protein engineering.

2. Results and discussion

Isomaltosides substituted at C-6 are conformationally biased and maintain the substrate key polar groups, OH-4′, -6′, and -4, in optimal register for recognition and transition-state stabilization by GA [8,14,15]. Steady state kinetics showed that while the rate of GA catalyzed hydrolysis was essentially the same for the parent isomaltoside and the three 6*R*-*C*-methyl, -ethyl-, and -isopropyl analogs (Fig. 1), K_m increases with increasing size of the alkyl group, being 0.71, 3.30, and 47.0 mM compared with 20 mM for the parent substrate (Table 1). Compared with α-methyl isomaltoside, the methyl and ethyl substituents at C-6 thus enhance recognition. The transition-state stabilization as a consequence is improved by 8.2 and 5.6 kJ mol^{-1} (Table 1), arguably due to a conformational bias where the preferred conformer for binding is the dominant species in solution [15]. Although the K_m for methyl 6*R*-*C*-isopropyl-α-isomaltoside increased 70-fold compared with the 6*R*-*C*-methyl analog, it was only doubled compared with the unsubstituted isomaltoside (Table 1). Apparently GA has the capacity to accommodate and hydrolyze isomaltosides carrying larger substituents at C-6. The binding mechanism, however, may differ from that of the parent substrate, since the conformational adjustment to a productive enzyme complex formation is easier for the substituted isomaltosides [8]. Presteady-state kinetic stud-

Table 1
Kinetic parameters for wild-type and mutants of *Aspergillus niger* glucoamylase for the hydrolysis of conformationally biased isomaltosides[a]

Substrate	Wild-type				Trp120 → Phe				L3A[b]			
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	$\Delta\Delta G^{\ddagger}$[c] (kJ mol^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	$\Delta\Delta G^{\ddagger}$ (kJ mol^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	$\Delta\Delta G^{\ddagger}$ (kJ mol^{-1})
Maltose	10.7	1.21	8.84		0.18	0.91	0.20[d]		1.68	1.83	0.92	
Isomaltose	0.41	19.8	0.021		0.041	19.6	0.0021		0.63	24.8	0.025	
Methyl-α-isomaltoside	0.88	20.0	0.044		0.024	8.7	0.0028		0.77	8.7	0.089	
Methyl 6*R*-*C*-methyl-α-isomaltoside	0.68	0.71	0.96	−8.2	0.0034	0.089	0.038	−6.9	0.65	0.45	1.44	−7.4
Methyl 6*R*-*C*-ethyl-α-isomaltoside	1.2	3.3	0.36	−5.6	0.032	2.3	0.014	−4.3	2.00	1.5	1.34	−7.2
Methyl 6*R*-*C*-isopropyl-α-isomaltoside	0.80	47.0	0.017	2.5	0.31	51	0.0061	−2.1	0.95	39.5	0.024	3.5

[a] 45 °C; 0.05 M sodium acetate, pH 4.5.
[b] Val181 → Ala/Asn182 → Ala/Gly183 → Lys/Ser184 → His.
[c] $\Delta\Delta G^{\ddagger} = -RT \ln[(k_{cat}/K_m)_a/(k_{cat}/K_m)_b]$, where a and b refer to analog and parent substrate, respectively.
[d] 50 °C [16].

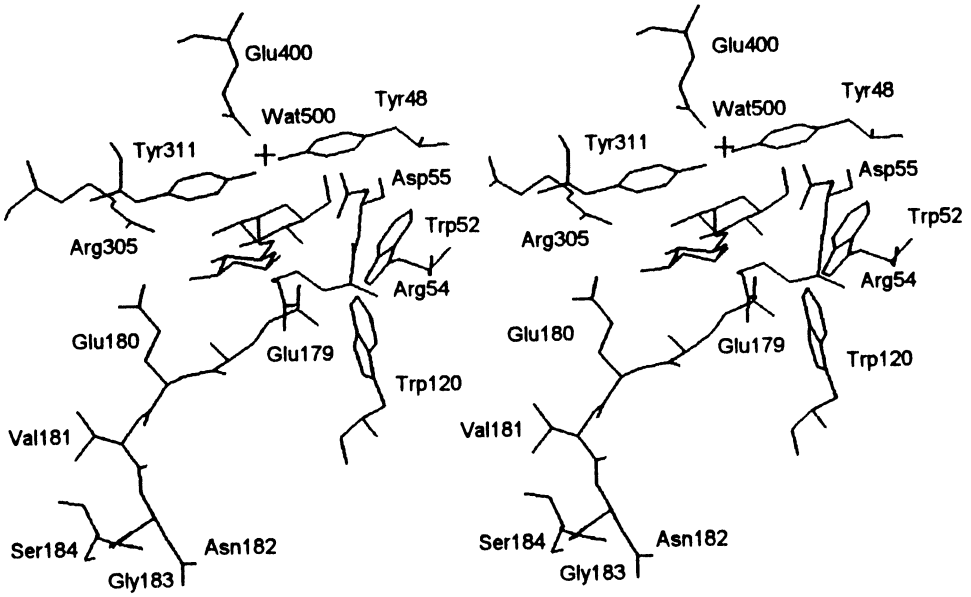

Fig. 2. Stereo view of the active site of glucoamylase from *Aspergillus awamori* var. *X100* with isomaltose modelled into the active site.

ies suggested that binding of isomaltose includes an additional step compared with maltose [17].

That GA binds methyl 6*R*-*C*-ethyl-α-isomaltoside with a modest increase in K_m suggests that the substituent fits into a cavity of the GA surface. In the crystal structure of GA–acarbose, a pseudomaltotetrasaccharide inhibitor, the first two rings are sequestered from solvent at binding subsites -1 and $+1$, but a solvent cavity appears in the vicinity of atom C6B [12], into which the introduced 6*R*-*C* substituents of bound isomaltose analogs is likely to project. The isopropyl substituent, however, is too large. High ligand–protein complementarity for GA complexes is in agreement with the entropically and enthalpically favorable binding of the inhibitors 1-deoxynojirimycin and acarbose [18]. Also, *O*-methylated isomaltosides are hydrolyzed extremely slowly, except when the *O*-methyl group protrudes into solvent at the periphery of the binding site [8,9].

The Trp120→Phe GA mutant at subsite $+2$ (Fig. 2) unexpectedly has an eight-fold lower K_m of 0.089 mM for methyl 6*R*-*C*-methyl-α-isomaltoside than the wild-type GA (Table 1). For the 6*R*-*C*-ethyl- and 6*R*-*C*-isopropyl counterparts, K_m, however, increased to 2.3 and 51 mM, respectively, comparable with the wild-type values. Mutation showed that Trp120 is very important for transition-state stabilization in the hydrolysis of maltose and longer maltooligosaccharides [16,19], whereas the activity (k_{cat}/K_m) is above the wild-type level for the shortest possible substrate analog, α-D-glucopyranosyl flouride [20]. Compared with the wild-type enzyme, k_{cat} is 200-fold lower for Trp120→Phe for methyl 6*R*-*C*-methyl-α-isomaltoside, but gradually increases for the 6*R*-*C*-ethyl- and 6*R*-*C*-isopropyl analogs to $k_{cat} = 0.31\ s^{-1}$ approaching the value of $0.80\ s^{-1}$ determined for the wild-type GA (Table 1).

Trp120 both stacks with the hydrophobic face of the glucosyl residue at the binding subsite $+2$ [11–13] and forms an aromatic cluster, together with Tyr50, Trp52, and Tyr116 surrounding the hydrophobic side chain of Lys108 [11,21]. These contacts and the stabilization of the general acid catalyst Glu179 by hydrogen-bonding Trp120 NE1 to Glu179 OE2, will be perturbed in Trp120→Phe GA [11]. The pH–activity profile for maltose hydrolysis by Trp120→Phe GA (Fig. 3) indicates that the pK_a of Glu179 in free enzyme is elevated to 6.5, compared with 5.9 in the wild-type (Fig. 3(a)). Both mutant and wild-type GAs have a pK_a of ca. 6.0 in the substrate-complexes (Fig. 3(b)). The Trp120→Phe mutant binds substrate more tightly than wild-type GA in agreement with

pre-steady-state binding measurements that show the dissociation of bound substrate is much slower than for wild-type [19]. In combination with a strong interaction between the substrate methyl group at C-6 and the hydrophobic cavity at the active site in Trp120→Phe GA (Fig. 2) this might account for the unusually low K_m compared with wild-type GA. While the efficient binding of substrate analog to Trp120→Phe GA has a negative affect on transition-state stabilization and the rate of catalysis (Table 1), activity is recovered with the weaker binding analogs. In addition, larger substituents at C-6 may enhance stabilization of Glu179, and hence compensate for the loss of the Trp120 NE1 to Glu179 OE2 hydrogen-bond.

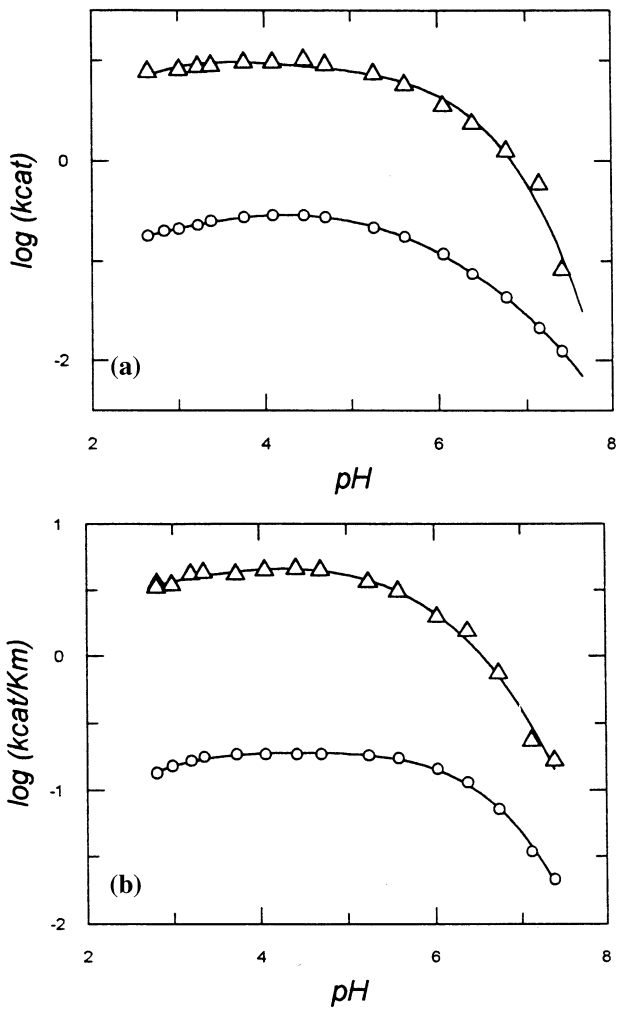

Fig. 3. pH–activity profiles for (a) maltose-complexed ($\log k_{cat}$) and (b) uncomplexed ($\log k_{cat}/K_m$) wild-type (△) and Trp120→Phe (○) GA at 45 °C. pK_a values were estimated using Dixon plots [31].

The present kinetic study of the isomaltoside analogs reemphasizes that Trp120 plays a key role in GA activity [16,22,23].

The tetrapeptide Val181-Ser184 (Fig. 2) is located in the third highly conserved active site α→α segment of the $(\alpha/\alpha)_6$-barrel adjacent to Glu179, the general acid catalyst, and Glu180, which is crucial in binding of both α-(1→4)- and α-(1→6)-linked substrates at subsite +1 [7,8,11,12,24]. Previously mutation, homologue loop replacement, modelling and molecular recognition using substrate analogs revealed that residues in the conserved α→α loops 3 and 5 determine the substrate bond-type specificity [2,4,8,21,24,25]. The present quadruple mutant Val181→Ala/Asn182→Ala/Gly183→Lys/Ser184→His, guided by a sequence motif that is associated with the strict α-(1→4)-bond specificity as found for α-amylases, provides strong support for the importance in the relative bond-type specificity for α-(1→4)- versus α-(1→6)-substrates (Table 1; Fig. 2). The mutation thus barely affects activity on isomaltose, whereas k_{cat} for maltose hydrolysis decreased seven-fold in spite of the distance from the binding subsites −1 and +1 of this mutation (Table 1; [25]). Even though the 6*R*-*C*-substituted isomaltosides and maltose have similar conformations [14,15], the quadruple mutant GA displays 1.5- and 4-fold higher k_{cat}/K_m for the methyl 6*R*-*C*-methyl- and -ethyl-α-isomaltoside, respectively, than wild-type enzyme.

The action of wild-type and the two active-site mutants on the conformationally biased isomaltosides shows that GA efficiently binds and catalyzes hydrolysis of a wide range of analogs of natural oligosaccharide substrates. This property is related to the capacity of GA to accommodate and hydrolyze α-(1→4) as well as α-(1→6) bonds. Moreover, the specificity and activity of GA are readily modulated by mutation in active site loop sequences, which need not contain residues involved in substrate binding or catalysis [25,26]. The present loop mutation demonstrates a selective decrease in activity for α-(1→4)-linked substrates, while activity towards α-(1→6)-bonds remains at wild-type level.

3. Experimental

Enzymes and substrates.—Wild-type [27] and mutant GAs [16,19] were purified from culture filtrates (Novo-Nordisk, Bagsvaerd, Denmark) using individual columns of acarbose–Sepharose [23] followed by ion-exchange chromatography [28]. Construction of Trp120→Phe has been reported previously [16], while Val181→Ala/Asn182→Ala/Gly183→Lys/Ser184→His GA was constructed using the following primer 5′-GCA GAT CTC TGG GAA GAA GCC GCT AAG CAC TCT TTC TTT ACG-3′. Transformation and expression in *Aspergillus niger* was performed essentially as described [27]. The G1 form [29,30] is used in the present study and enzyme concentrations were determined spectrophotometrically at 280 nm using $\varepsilon = 1.37 \times 10^5$ M^{-1} cm^{-1} for wild-type and the quadruple mutant GAs [22] and $\varepsilon = 1.31 \times 10^5$ M^{-1} for Trp120→Phe GA. The synthesis of α-methyl-isomaltoside [9] and methyl 6*R*-*C*-methyl-α-isomaltoside [14,15] have been described, while that of the closely related analogues, methyl 6*R*-*C*-ethyl- and methyl 6*R*-*C*-isopropyl-α-isomaltoside will be reported elsewhere (R.U. Lemieux et al., in preparation).

Assays.—The activity of wild-type and mutant GAs was determined at 45 °C in 50 mM sodium acetate, pH 4.5, essentially as described [15,27]. The hydrolysis of methyl-α-isomaltoside, methyl 6*R*-*C*-methyl-α-isomaltoside, and methyl 6*R*-*C*-ethyl-α-isomaltoside was followed at 8–10 substrate concentrations [S_0] in the range $0.1 \times K_m$–$4 \times K_m$, but for methyl 6*R*-*C*-isopropyl-α-isomaltoside only up to $1.5 \times K_m$, due to the poor affinity and low quantities of the analog. The enzyme concentration (E_0) was ca. 5×10^{-4} mM for wild-type and the quadruple mutant GAs, and ranged from 5×10^{-3} to 3×10^{-2} mM for Trp120→Phe GA. From the reaction mixture (100 μL) aliquots (15 μL) were transferred at appropriate time intervals to microtitre plate wells containing 1 M Tris, pH 7.6 (200 μL) to stop the reaction, as well as 5 U mL^{-1} glucose oxidase (*A. niger*), 1 U mL^{-1} peroxidase (horseradish), and 0.21 mg mL^{-1} *o*-dianisidine for glucose analysis. In the case of Trp120→Phe GA catalyzed hydrolysis of methyl 6*R*-*C*-methyl-α-isomaltoside, the reaction was performed in a volume of 1000 μL to ensure sensitivity in the glucose analysis, even when low substrate concentrations were used, as dictated by a low K_m. The absorbance was read at 450 nm after 1 h incubation at rt, and quantitated using D-glucose as the standard. V_{max} and K_m were obtained by fitting initial rates as a function of S_0 to the Michaelis–Menten equation and k_{cat} was calculated as $V_{max}/[E_0]$. The errors for the V_{max} values are within 12% and for k_{cat} within 6%. The pH activity dependence was determined using 50 mM citrate/phosphate buffers at 16 different pH values ranging from 2.6 to 7.4. To estimate pK_a of the catalytic groups in the GA-substrate complex and the free GA, respectively, maltose was used as substrate at concentrations ca. $10 \times K_m$ and $0.1 \times K_m$,. pK_a-values were estimated using Dixon plots [31]. Enzyme concentrations were $1.1–3.8 \times 10^{-3}$ mM and $0.055–0.19 \times 10^{-3}$ mM for Trp120→Phe and wild-type GA, respectively.

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