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Pedro M. Coutinho
Iowa State University

Michael K. Dowd
United States Department of Agriculture

Peter J. Reilly
Iowa State University, reilly@iastate.edu

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Disciplines

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Automated Docking of α-(1,4)- and α-(1,6)-Linked Glucosyl Trisaccharides in the Glucoamylase Active Site

Pedro M. Coutinho,[†,‡] Michael K. Dowd,[§] and Peter J. Reilly*[,†]

Department of Chemical Engineering, Iowa State University, Ames, Iowa 50011, and Southern Regional Research Center, U.S. Department of Agriculture, New Orleans, Louisiana 70179

Low-energy conformers of five α-(1,4)- and α-(1,6)-linked glucosyl trisaccharides were flexibly docked into the glucoamylase active site using AutoDock 2.2. To ensure that all significant conformational space was searched, the starting trisaccharide conformers for docking were all possible combinations of the corresponding disaccharide low-energy conformers. All docked trisaccharides occupied subsites −1 and +1 in very similar modes to those of corresponding nonreducing-end disaccharides. For linear substrates, full binding at subsite +2 occurred only when the substrate reducing end was α-(1,4)-linked, with hydrogen-bonding with the hydroxymethyl group being the only polar interaction there. Given the absence of other important interactions at this subsite, multiple substrate conformations are allowed. For the one docked branched substrate, steric hindrance in the α-(1,6)-glycosidic oxygen suggests that the active-site residues have to change position for hydrolysis to occur. Subsite +1 of the glucoamylase active site allows flexibility in binding but, at least in *Aspergillus* glucoamylases, subsite +2 selectively binds substrates α-(1,4)-linked between subsites +1 and +2. Enzyme engineering to limit substrate flexibility at subsite +2 could improve glucoamylase industrial properties.

Introduction

Glucoamylase [α-(1,4)-D-glucan glucohydrolase; EC 3.2.1.3; GA] is used industrially to digest liquified starch, an important step in the production of high-fructose corn syrup. GA releases *β*-D-glucose by hydrolyzing terminal α-(1,4)-D-glucosidic bonds from the nonreducing ends of maltooligosaccharides. *Aspergillus niger* GA can cleave α-(1,6)-D-glucosidic bonds, which initiate amylopectin branches, at lower rates, and also can slowly hydrolyze α,*β*-(1,1)-, α-(1,2)-, and α-(1,3)-glucosidic bonds (Pazur and Kleppe, 1962; Meagher and Reilly, 1989). At high D-glucose concentrations, as found industrially, thermodynamics dictates the formation of differently linked condensation products by GA (Hehre et al., 1969; Nikolov et al., 1989), including the disaccharides α,*β*-trehalose, kojibiose, nigerose, maltose, and isomaltose, which have α,*β*-(1,1), α-(1,2), α-(1,3), α-(1,4), and α-(1,6) bonds, respectively, and the α-(1,4)- and α-(1,6)-linked trisaccharides maltotriose, panose, and isomaltotriose. Although the formation of α-(1,4)-linked products is kinetically favored, that of products containing α-(1,6)-glycosidic bonds is thermodynamically preferred (Nikolov et al., 1989). Understanding the molecular basis of GA interaction with its many possible substrates and products is necessary to effectively engineer GA selectivity to more closely approach the theoretical glucose yield in starch hydrolysis, responding to requirements of the starch processing industry (Crabb and Mitchinson, 1997).

Subsite mapping has established that in *Aspergillus* GAs, up to seven and six subsites are involved in malto- and isomaltooligosaccharide hydrolysis, respectively (Savel'ev et al., 1982; Meagher et al., 1989; Ermer et al., 1993). The crystal structures of complexes of the active site of *Aspergillus awamori* var. *X100* GA with the inhibitors acarbose and D-*gluco*-dihydroacarbose (Aleshin et al., 1994, 1996; Stoffer et al., 1995) revealed the locations of the first four subsites in their interaction with maltooligosaccharides, with a pH-dependent dual conformation found beyond subsite +1 (formerly designated as the second subsite but now following the recently adopted subsite terminology for glycosyl hydrolases (Davies et al., 1997)). The interactions of the substrates methyl α-maltoside and methyl α-isomaltoside in subsites −1 and +1 have been elucidated (Coutinho and Reilly, 1994a; Lemieux et al., 1996; Coutinho et al., 1997b,c), with most of the amino acid residues taking part in those interactions being conserved in fungal, yeast, and bacterial GAs (Coutinho and Reilly, 1994a,b, 1997; Henrissat et al., 1994). However, the indication that the residues at subsites +2 and +3 are significantly less conserved (Coutinho and Reilly, 1997) demands a better understanding of the interaction of these subsites with different substrates.

Automated docking with AutoDock allows the study of the interaction of proteins with flexible molecules (Goodsell and Olson, 1990; Goodsell et al., 1993, 1996). Given the inherent flexibility of carbohydrates, this technique has been successfully used to characterize protein−carbohydrate interactions. The docking of monosaccharides in enzyme active sites, like that of *β*-acetylglucosamine in lysozyme (Goodsell and Olson, 1990), and that of D-glucose, D-mannose, and D-galactose and some monosaccharide-like inhibitors in GA (Coutinho et al., 1997a), yielded binding modes close to those found by X-ray crystallography. A combination of conformational analysis with docking to model the interaction of disaccharide substrates and their analogues with GA (1) yielded binding modes of methyl

* Corresponding author. E-mail: reilly@iastate.edu. Phone: (515) 294-5968. Fax: (515) 294-2689.

[†] Department of Chemical Engineering.

[‡] Present address: Centre de Recherches sur les Macromolécules Végétales, CNRS, BP 53, F-38041 Grenoble Cedex 9, France.

[§] Southern Regional Research Center.

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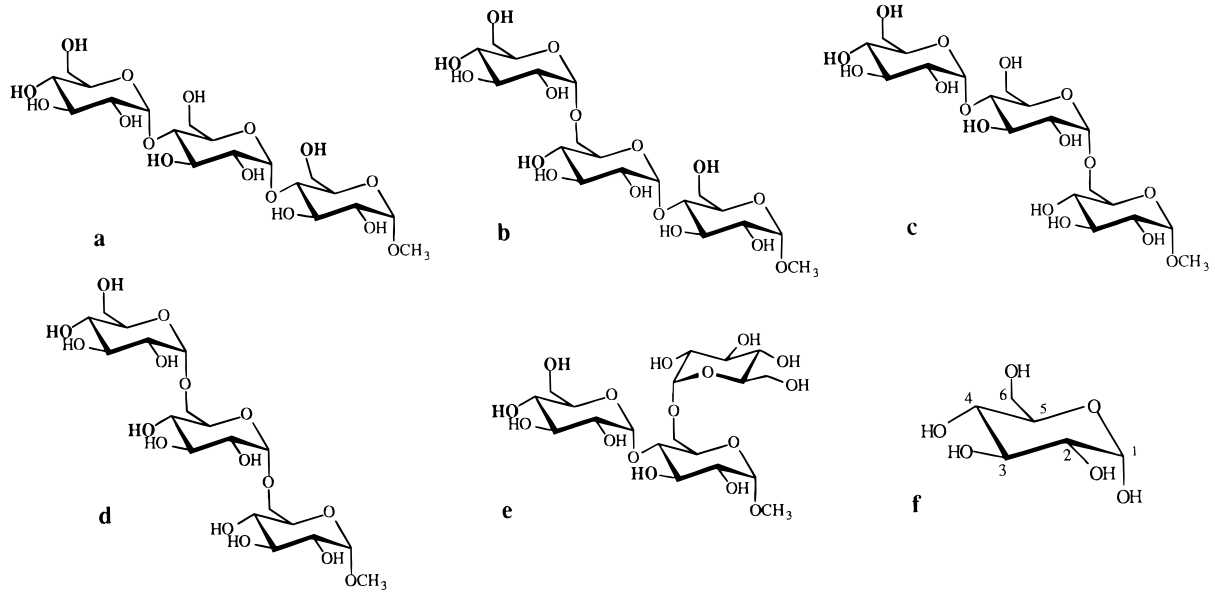


Figure 1. (a) Methyl α-maltotrioside. (b) Methyl α-panoside. (c) Methyl α-isopanoside. (d) Methyl α-isomaltotrioside. (e) Methyl α-(6^1-α-glucopyranosyl)-maltoside. (f) α-Glucose, showing numbered carbon atoms. Bold hydroxyl groups are those binding strongly to the GA active site.

α-acarviosinide (Coutinho et al., 1997a) that matched the disaccharide moiety of the inhibitor acarbose in its GA complex (Aleshin et al., 1994); (2) gave consistent binding modes of different isomaltosyl analogues, identifying the key hydroxyl group in the reducing end of isomaltose essential for correct binding in subsite +1 (Coutinho et al., 1997b); and (3) correctly identified the real substrates among all α- and *β*-linked glucosyl disaccharides, confirming that a key substrate hydroxyl group bound at subsite +1 correctly positions the glycosidic oxygen for hydrolysis and is essential for enzyme action (Coutinho et al., 1997c). The key groups identified by docking analysis matched those described in kinetic studies with maltose analogues (Bock and Pedersen, 1987; Sierks and Svensson, 1992; Sierks et al., 1992) and isomaltose (Bock et al., 1991; Lemieux et al., 1996), or were otherwise suggested for other α-linked glucopyranosyl disaccharides (Lemieux and Spohr, 1994).

Starch chains are composed of α-(1,4)-linked D-glucopyranosyl units, with branches being initiated by more flexible α-(1,6) bonds. Conformational analysis of glucose-containing disaccharides using MM3, a force field giving low-energy conformations in good agreement with the torsion angles of crystal structures (French and Dowd, 1993), confirmed that the two-bond-linked maltose is much less flexible than the three-bond-linked isomaltose (Dowd et al., 1992, 1994).

In this study, methyl α-maltotrioside [methyl α-D-glucopyranosyl-(1,4)-α-D-glucopyranosyl-(1,4)-α-D-glucoside], methyl α-panoside [methyl α-D-glucopyranosyl-(1,6)-α-D-glucopyranosyl-(1,4)-α-D-glucoside], methyl α-isopanoside [methyl α-D-glucopyranosyl-(1,4)-α-D-glucopyranosyl-(1,6)-α-D-glucoside], methyl α-isomaltotrioside [methyl α-D-glucopyranosyl-(1,6)-α-D-glucopyranosyl-(1,6)-α-D-glucoside], and methyl α-(6^1-α-D-glucopyranosyl)-maltoside (Figure 1) were docked in the *A. awamori* var. *X100* GA active site by simulated annealing with AutoDock 2.2 to estimate GA selectivity to the different trisaccharides found during starch saccharification. Initial docking conformers were constructed by making all possible combinations of low-energy conformers derived from the conformational analyses of α-maltose and methyl α-isomaltoside (Dowd et al., 1992; Coutinho et al., 1997b), and were optimized with MM3(92). An approach adapted from that recently described for docking monosaccharides and inhibitors and different glucosyl disaccharides (Coutinho et al., 1997a,b,c) was used. This study extends to trisaccharides the systematic study of the structural interaction of substrates with GA, clarifying the role of subsite +2 in substrate binding and catalysis.

Computational Methods

Different conformers of the five trisaccharides were optimized by the MM3(92) force field (Allinger et al., 1989, 1990; Lii and Allinger, 1989) (Technical Utilization Corporation, Powell, OH) with the bulk dielectric constant (ϵ) set to 4.0. These conformers, representing all possible combinations of three and seven low-energy minima described in conformational studies of α-maltose and methyl α-isomaltoside, respectively (Dowd et al., 1992; Coutinho et al., 1997b), were generated and preoptimized using PC−Model (Serena Software, Bloomington, IN). The number of conformers of each trisaccharide varied: methyl α-maltotrioside (9), methyl α-panoside (21), methyl α-isopanoside (21), methyl α-isomaltotrioside (49), and methyl α-(6^1-α-glucopyranosyl)-maltoside (21). The significant exocyclic orientations obtained in those studies were used, but the exocyclic conformation of the C-5_A—C-6_A bond at the nonreducing end was given the orientations gauche to both C-4_A and O-5_A, or gauche-gauche (*gg*), because this is the orientation found in the GA active site in all substrates and inhibitors.

The torsional angles representing ring orientations about the α-(1,4)-glucosidic linkage are defined as ϕ_{ij} = Θ(H-1_i-C-1_i-O-4_j-C-4_j) and ψ_{ij} = Θ(C-1_i-O-4_j-C-4_j-H-4_j), whereas those about the α-(1,6)-glucosidic linkage are ϕ_{ij} = Θ(H-1_i-C-1_i-O-6_j-C-6_j), ψ_{ij} = Θ(C-1_i-O-6_j-C-6_j-C-5_j), and ω_{ij} = Θ(O-6_j-C-6_j-C-5_j-H-5_j) (Figure 1), with *i* denoting the residue toward the nonreducing end and *j* denoting the one toward the reducing end, which in the five substrates studied here was capped with a methyl group. The pairs *ij* will be designated AB, BC, or A'B for the pairs of glucosyl residues docked in *A. awamori*

var. *X100* GA active site at subsites −1 and +1, subsites +1 and +2, and the first branching subsite and subsite +1, respectively.

Automated docking was performed with AutoDock 2.2 (Goodsell and Olson, 1990; Goodsell et al., 1993, 1996) (Scripps Research Institute, La Jolla, CA). As in previous studies, we used the GA crystal structure found in the complex with inhibitor D-*gluco*-dihydroacarbose (Stoffer et al., 1995; Aleshin et al., 1996) (Brookhaven Protein Databank entry *1gai*), with all water molecules except the putative catalytic molecule removed and nonpolar hydrogens added using Quanta 4.0 (Molecular Simulations, Inc., San Diego, CA). Atomic partial charges for GA and trisaccharides were calculated by the Gasteiger method (Gasteiger and Marsili, 1980).

Atomic interaction energy grids in a 30-Å cubic box centered at approximately subsite +1 of the GA active site were obtained by using probes corresponding to each atom type found in the substrate and tested every 0.5 Å (Goodford, 1985; Goodsell et al., 1996). An electrostatic interaction energy grid was calculated using a sigmoidal distance-dependent dielectric function to account for the solvent screening effect (Mehler and Solmajer, 1991). As before, Lennard-Jones coefficients of AutoDock 1.0 with different parameters for apolar and polar hydrogens were used, along with a distance criterion with directional attenuation to account for hydrogen bonding.

The docking of a single compound consisted of two stages, designated as docking and redocking. In docking, the nonreducing-end ring of each conformer of the substrate derived from conformational studies was superimposed on the average position of the pyranosyl rings at subsite −1 of the disaccharide substrates docked in previous studies (Coutinho et al., 1997b,c). Fifty independent Monte Carlo simulations per starting structure were performed using a variation on the short schedule (Goodsell et al., 1993), with 100 constant temperature cycles for simulated annealing. Each cycle had a maximum of 1500 steps accepted or rejected, the minimal energy structure being passed to the next cycle. The temperature was reduced by a 0.95 factor per cycle from an initial value of RT = 100 cal/mol, and the maximal rotation per step for the exocyclic torsion angles was reduced by a 0.9875 factor per cycle from the initial 15°. To reduce the number of degrees of freedom, the nonreducing-end pyranosyl ring was fixed, but flexibility of all rotatable dihedral angles was allowed. Following the docking procedure, all structures of each trisaccharide were subjected to cluster analysis with a tolerance of 1 Å for an all-atom root-mean-square (RMS) deviation from a lower-energy structure representing each cluster family.

For redocking, the global minimum structure and the low-energy structures of the significant clusters of a given trisaccharide were subjected to docking under similar conditions, but now permitting a maximal translation of 0.01 Å per step, followed again by cluster analysis. This approach allowed a complete exploration of conformational space in the first stage, but with a reduced number of degrees of freedom by fixing the nonreducing end. By permitting movement and optimizing the successful docking modes in the second stage, some economy of computer resources was obtained. As before, substrate internal energies in both docking and redocking were referenced to the MM3(92) relative energy, the energy difference between a given conformer of the substrate and the corresponding lowest-energy conformer, overcoming some of the limitations of the AutoDock force field:

$$\text{internal energy}_{(\text{docking})} = \text{relative MM3 energy} + \Delta\,[\text{AD internal energy}]_{(\text{docking})}$$

$$\text{internal energy}_{(\text{redocking})} = \text{internal energy}_{(\text{docking})} + \Delta\,[\text{AD internal energy}]_{(\text{redocking})}$$

Because both nonreducing ends of methyl α-(6^1-α-glucopyranosyl)-maltoside can potentially fit in subsite −1 of the GA active site, each end was independently docked there.

Docked Trisaccharides

All trisaccharides bound effectively in subsites −1 and +1 during docking (data not shown) with no noticeable energy penalty, an indication that the immobilization of the pyranosyl ring at subsite −1 was a good approach. The lower-energy structures representing their energetically significant clusters were then redocked, now allowing limited movement of the nonreducing end. The clusters obtained during redocking with total energies of interaction of the representative structure within 5 kcal/mol of the best interacting structure of each trisaccharide are listed in Table 1. Following analysis of the representative clusters, the structures believed to represent significant binding modes in the GA active site are depicted in Figure 2.

As in previous studies with mono- and disaccharides (Coutinho et al., 1997a,b,c), the glucosyl residues docked at subsite −1 are *gg* conformers (data not shown), following the conformation taken by the exocyclic hydroxymethyl group at the nonreducing end of substrate analogues in the crystal structures of their complexes with GA (Harris et al., 1993; Aleshin et al., 1994, 1996; Stoffer et al., 1995).

The docked trisaccharides often bind in modes similar to those of their corresponding nonreducing-end disaccharide moieties (Coutinho et al., 1997a,b). The values of ϕ_{AB}/ψ_{AB} for methyl α-maltotrioside, methyl α-isopanoside, and the productive mode of methyl α-(6^1-α-glucopyranosyl)-maltoside are not far from the values of −5°/-8° obtained in docking of methyl α-maltoside in the GA active site (Coutinho et al., 1997c), and are within 1 kcal/mol of the global minimum at −23°/−22° of α-maltose obtained by conformational mapping with MM3 (Dowd et al., 1992). Similarly, for most clusters of docked methyl α-isomaltotrioside, values of $\phi_{AB}/\psi_{AB}/\omega_{AB}$ are near −20°/146°/4°, the top productively bound methyl α-isomaltoside structure in previous GA docking studies (Coutinho et al., 1997b), and −47°/134°/47° for the conformational exploration of the transition state there with GEGOP (Lemieux et al., 1996). For methyl α-panoside, only the third cluster gives values in the same range, in what will be considered a productive binding mode. The remaining methyl α-panoside clusters correspond to unproductive modes, as does the second cluster of methyl α-(6^1-α-glucopyranosyl)-maltoside, where the α-(1,6)-linked disaccharide moiety lies in subsites −1 and +1. It is interesting to observe that, although automated docking allows only a productive

Table 1. Redocking of α-(1,4)- and α-(1,6)-Linked Trisaccharides in the GA Active Site

compound	cluster[a] [no. of structures]	total energy (kcal/mol)	internal energy (kcal/mol)	inter-ring dihedral angle[b]					
				ϕ_{AB}	ψ_{AB}	ω_{AB}	$\phi_{BC/A'B}$	$\psi_{BC/A'B}$	$\omega_{BC/A'B}$
methyl α-maltotrioside	1 [47/250]	−119.4	8.4	−8	−23		0	−37	
	2 [11/250]	−115.7	17.6	−10	−23		−18	−53	
methyl α-panoside	1 [41/250]	−106.9	4.7	73	140	112	17	18	
	2 [90/250]	−103.7	8.3	−70	140	108	−26	−157	
	3 [50/250]	−102.0	8.5	−41	137	44	−3	−70	
methyl α-isopanoside	**1** [50/400]	−120.4	3.5	−11	−15		−28	118	−156
	2 [64/400]	−119.7	−2.4	−12	−17		−38	131	98
	3 [16/400]	−118.9	4.5	−13	−19		23	−134	13
	4 [9/400]	−117.8	13.9	−8	−19		−85	124	−172
	5 [17/400]	−117.7	4.4	−7	−17		−58	123	−62
	6 [19/400]	−116.4	8.3	−10	−16		−38	124	−104
	7 [7/400]	−116.0	2.0	−6	−6		−20	100	−156
methyl α-isomaltotrioside	**1** [51/350]	−120.6	−5.1	−38	136	39	3	117	−106
	2 [48/350]	−119.5	10.3	−26	140	11	−14	82	8
	3 [8/350]	−118.1	8.6	−31	143	23	−2	95	−38
	4 [55/350]	−117.0	3.5	−35	140	32	−16	111	176
	5 [24/350]	−115.2	7.9	−62	143	83	27	131	−60
methyl α-(6^1-α-glucopyranosyl)-maltoside[c]	**1** [92/500]	−104.9	12.0	−6	−18		−21	−92	−83
	2 [50/500]	−100.5	14.6	−56	125	−114	−50	158	

[a] In bold are the clusters whose top structures are shown in Figure 2. [b] A = residue in subsite −1, B = residue in subsite +1, C = residue in subsite +2. In methyl α-(6^1-α-glucopyranosyl)-maltoside, A = residue in subsite −1, A′ = residue in first branching subsite, B = residue in subsite +1. [c] The clusters described for this substrate have either the maltosyl moiety or the isomaltosyl moiety at subsites −1 and +1.

class of binding modes at subsites −1 and +1 for long substrates with nonreducing-end terminal α-(1,4) linkages, there is significant variability in those substrates with α-(1,6)-linked nonreducing-end residues, with clearly unproductive modes caused by contributions from interactions taking place beyond subsite +1.

For productive cases, the binding modes beyond subsite +1 depend on the type of glycosidic bond. For linear trisaccharides, the two-bond linkage of the α-(1,4)-bound disaccharide moiety at the reducing end allows a binding mode at subsite +2 close to two slightly different conformations found there in crystallographic studies of the GA complex with D-*gluco*-dihydroacarbose (Stoffer et al., 1995; Aleshin et al., 1996). Such is the case for the second cluster of methyl α-maltotrioside and the third cluster of methyl α-panoside (Figures 2a and 2b). There we find, as in the GA complexes with acarbose and D-*gluco*-dihydroacarbose (Aleshin et al., 1994, 1996; Stoffer et al., 1995), hydrogen bonding between the exocyclical 6_C-OH and the backbones of both Gly121 and Glu179. Given that this is the only polar point of attachment and that the hydroxymethyl group containing 6_C-OH is flexible by nature, the dual conformation found there for acarbose and D-*gluco*-dihydroacarbose is not surprising. The inhibitors are, however, stabilized by the interactions with the active site of the glucosyl moiety at the reducing end in both positions that constitute subsite +3 in *A. awamori* var. *X100* GA.

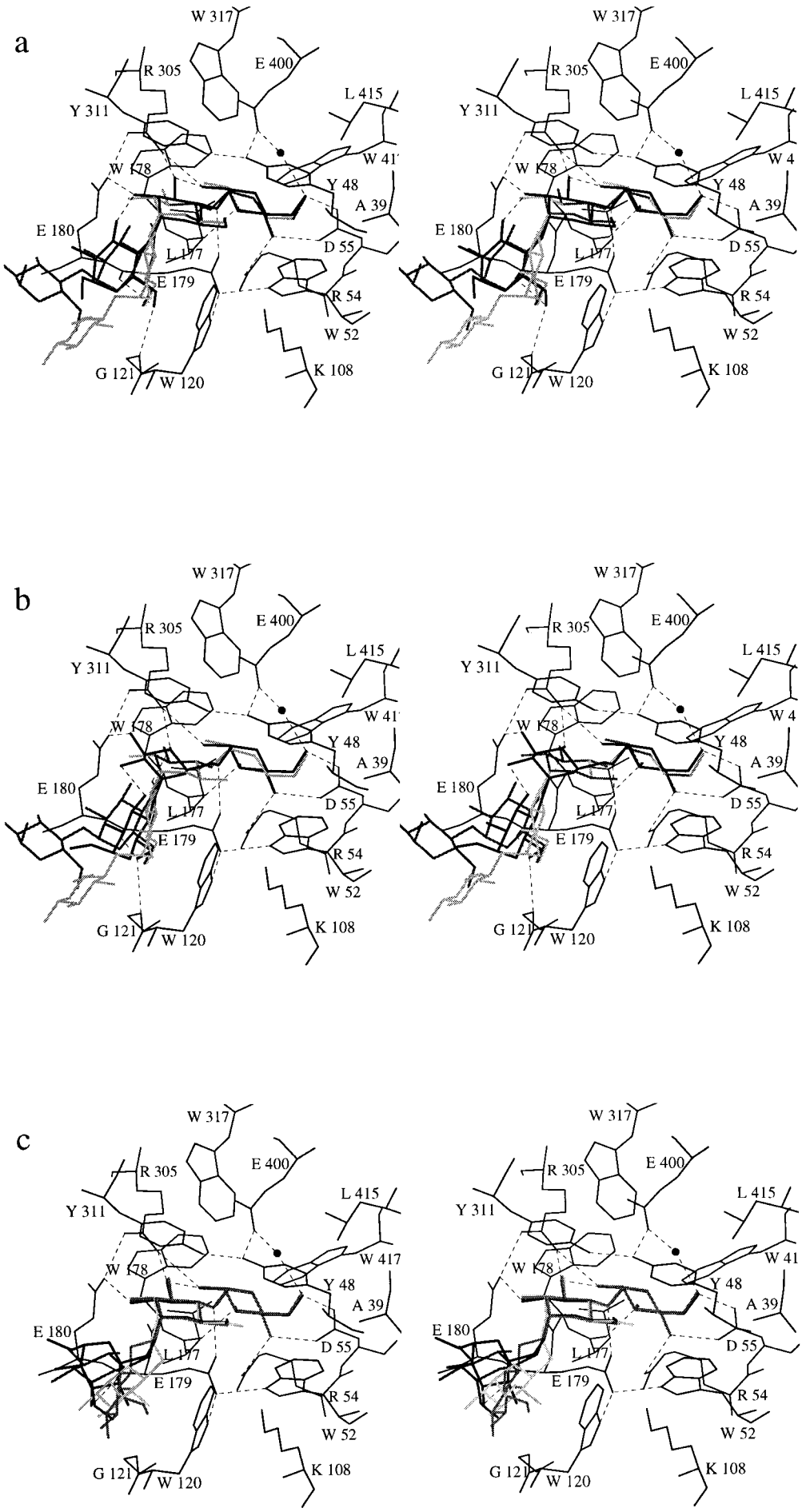
The values of ϕ_{BC}/ψ_{BC} obtained for the second docked cluster of methyl α-maltotrioside are close to the values of −25°/−62° and −21°/−67° found at pH 4.0 for the most abundant conformers of acarbose and D-*gluco*-dihydroacarbose, respectively (Stoffer et al., 1995; Aleshin et al., 1996). The least abundant conformers for both inhibitors have, however, values of −71°/−47° and −69°/−50°, respectively, for the same set of glycosidic dihedral angles, which are very close to that of −66°/−53° obtained by the only acarbose conformer in a similar GA complex at pH 6.0 (Aleshin et al., 1994). For methyl α-panoside, even though it is not possible to make a direct comparison of dihedral angles, it is very significant that the third residue of the top productive structure takes a position intermediate to that found for both conformers of D-*gluco*-dihydroacarbose at subsite +2. The GA active site can accommodate methyl α-maltotrioside and methyl α-panoside almost equally well, although a significant difference in distance exists between the two glucosyl units at the nonreducing end.

For methyl α-isopanoside and methyl α-isomaltotrioside, the longer α-(1,6)-glycosidic bond in the reducing-end disaccharide moiety causes the results to differ, leading to a variety of possible binding modes (Table 1). Different hydrogen bonding can be found for these compounds between one or more of the hydroxyl groups at the third residue and the backbones of Gly121 or Glu179. Typical cases are the top structures of methyl α-isopanoside and methyl α-isomaltotriose (Figures 2c and 2d), but there are alternative hydroxyl groups for different clusters. The three-bond linkage allows greater conformational liberty than subsite +2 in the *A. awamori* var. *X100* GA active site is designed to accommodate.

In the top productive methyl α-(6^1-α-glucopyranosyl)-maltoside (Figure 2e), the branching α-(1,6)-linked glycosyl unit has a mildly constrained conformation. Even though this trisaccharide corresponds to the branching point of amylopectin, the presence of a terminal α-(1,4)-linked methyl group instead of a glucosyl unit at subsite +2 sets no limits to the position of the α-(1,6)-linked glucosyl moiety. Significant conformational hindrance occurs at this branching point according to modeling studies (Imberty and Pérez, 1989; Buléon and Tran, 1990). In fact, extension of the α-(1,4)-linked chain would lead to steric contacts with the α-(1,6)-linked glucosyl unit (Figure 2e). This result is valid only for trisaccharides and not for longer substrates.

Atomic Interactions in the GA Active Site

The energetic contributions in intermolecular interactions between the different atoms of the top productive trisaccharide conformers and the GA active site are given in Table 2. Both polar and apolar hydrogen-atom contributions were added to those of their adjacent heavy atoms.


a
W 317
E 400
R 305
L 415
Y 311
W 178
Y 48
A 39
E 180
D 55
L 177
E 179
R 54
W 52
G 121
W 120
K 108
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c

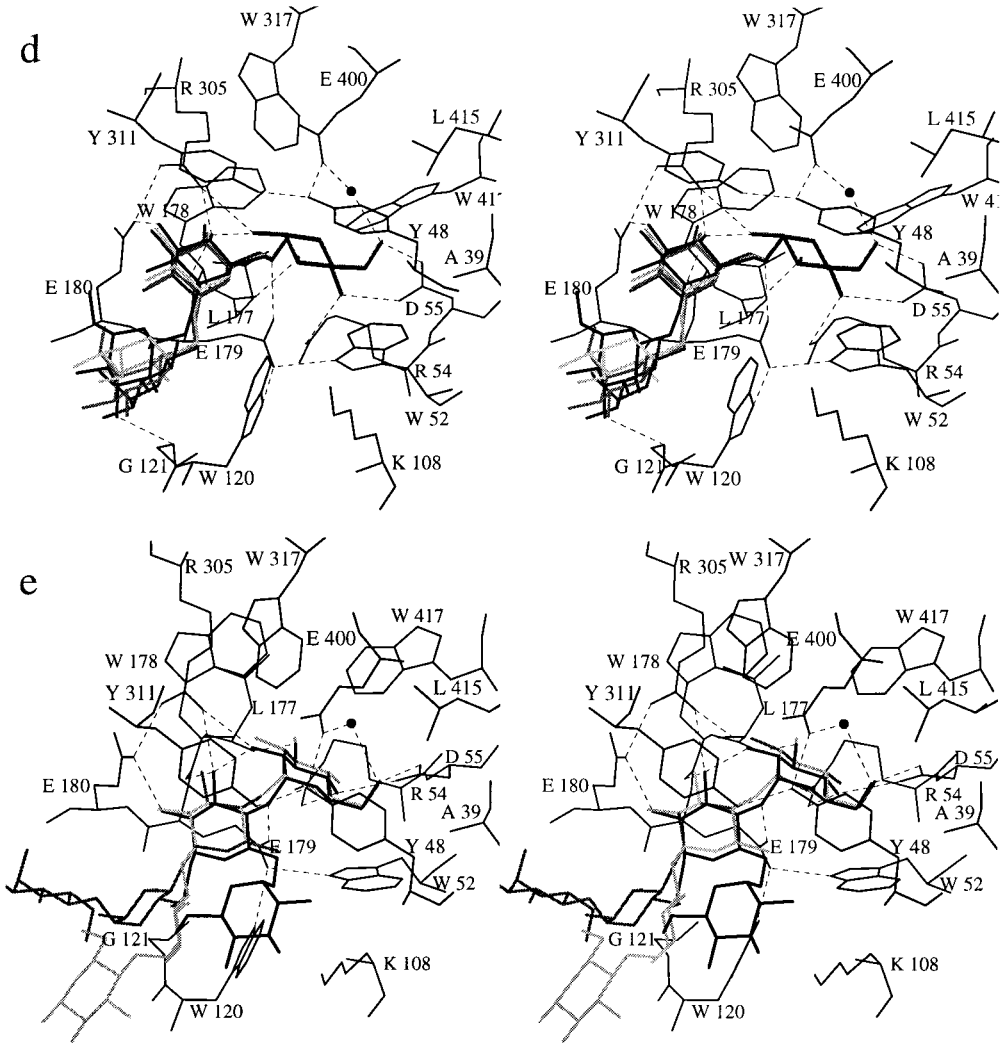

Figure 2. Stereoscopic plots of trisaccharides docked in the GA active site. Residues surrounding the subsites −1 and +1 and the catalytic water are shown, with hydrogen bonding indicated by dashed lines. Unless otherwise indicated, all docked structures (in black) are compared with the more abundant and less abundant conformers of D-*gluco*-dihydroacarbose (dark and light gray). (a) Methyl α-maltotrioside. (b) Methyl α-panoside. (c) Top four optimal methyl α-isopanoside structures ordered by energy of interaction (black to light gray). (d) Top four optimal methyl α-isomaltotrioside structures ordered by energy of interaction (black to light gray). (e) Methyl α-(6^1-α-D-glucopyranosyl)-maltoside. Plots were prepared with Molscript (Kraulis, 1990).

In subsites −1 and +1, the interactions are very similar to those described earlier for docked methyl α-maltoside and methyl α-isomaltoside (Coutinho et al., 1997b,c). Polar interactions are stronger at the critical OH-4_A, OH-6_A, and OH-3_B groups for the α-(1,4)-linked nonreducing-end disaccharide moieties of methyl α-maltotrioside, methyl α-isopanoside, and methyl α-(6^1-α-glucopyranosyl)-maltoside, and for the corresponding OH-4_A, OH-6_A, and OH-4_B groups for the nonreducing-end α-(1,6)-linked methyl α-panoside and methyl α-isomaltotrioside. The importance of these critical groups in binding and catalysis has been demonstrated in kinetic studies with substrate analogues (Bock and Pedersen, 1987; Sierks and Svensson, 1992; Sierks et al., 1992; Lemieux et al., 1996). The individual role of the different hydroxyl groups as been recently described in docking studies with isomaltose analogues (Coutinho et al., 1997b).

Relatively strong hydrophobic interactions exist with C-6_A for all trisaccharides and with C-6_B for the nonreducing-end α-(1,4)-linked trisaccharides. A strong interaction at C-6_A partially explains the stereochemical distortions of the substrate nonreducing end likely to occur during catalysis (Aleshin et al., 1996), whereas a hydrophobic environment for the C-6_B position had been described for the GA digestion of maltose analogues (Bock and Pedersen, 1988). Together with the low flexibility of the α-(1,4)-glycosidic bond, no equivalent contribution to the interaction at C-6_B exists for other α-linked glucosyl disaccharides (Coutinho et al., 1997c), which could to some extent explain the selectivity of GA in digesting disaccharides. As described in previous docking studies, strain occurs at the oxygen involved in the glycosidic bond to be cleaved, which probably is the basis for catalysis (Coutinho et al., 1997b,c). Interestingly, a stronger unfavorable interaction occurs at glycosidic oxygen O-6_B involved in branching in methyl α-(6^1-α-glucopyranosyl)-maltoside, which is forced by the large glucosyl group to a contact distance of only 3.2 Å from atom CH-2 in Trp120. Because this sterically hindred glycosidic oxygen is only 0.4 Å away from the position taken by OH-6_B in docked methyl α-maltoside (Coutinho et al., 1997c), which exhibits no steric contacts, it suggests that for the hydrolysis of branched substrates to occur, some GA active-site residues may have to change position to accommodate the branch and to allow the substrate to bind properly in the catalytic site.

Table 2. Atomic Contributions to Intermolecular Energy (kcal/mol) in the Interaction of Optimal α-(1,4)- and α-(1,6)-Linked Glucosyl Trisaccharide Structures with the GA Active Site[a−c]

atom	methyl α-maltotrioside	methyl α-panoside	methyl α-isopanoside	methyl α-isomaltotrioside	methyl α-(6^1-α-glucopyranosyl) maltoside
$C-1_A$	−5.1	−5.2	−5.2	−5.1	−5.2
$C-2_A$	−5.2	−5.2	−5.2	−5.2	−5.2
$C-3_A$	−4.8	−5.1	−4.9	−4.9	−4.9
$C-4_A$	−5.1	−5.0	−5.1	−5.0	−5.0
$C-5_A$	−4.9	−4.9	−4.9	−4.9	−4.9
$C-6_A$	**−8.6**	**−8.9**	**−8.6**	**−8.9**	**−8.9**
$O-2_A$	−5.4	−4.2	−5.2	−4.3	−4.3
$O-3_A$	−8.7	−5.5	−8.3	−5.8	−5.9
$O-4_A$	**−9.9**	**−8.8**	**−9.7**	**−9.1**	**−8.3**
$O-5_A$	−1.6	−1.6	−1.6	−1.5	−1.6
$O-6_A$	**−7.9**	**−9.6**	**−8.9**	**−9.8**	**−8.6**
$C-1_B$	−2.1	−1.7	−2.2	−1.7	−2.0
$C-2_B$	−1.5	−0.9	−2.2	−2.4	−3.7
$C-3_B$	−4.3	−3.7	−4.2	−3.7	−4.4
$C-4_B$	−4.2	−1.6	−4.3	−1.7	−4.1
$C-5_B$	−3.7	−3.3	−3.8	−3.4	−3.8
$C-6_B$	**−5.7**	−5.0	**−5.8**	−5.0	**−6.1**
$O-2_B$	−3.3	−0.8	−3.6	−0.6	−3.2
$O-3_B$	**−6.7**	−4.2	**−5.9**	−2.4	**−7.1**
$O-4_B$	0.1	**−5.7**	−0.2	**−5.9**	0.2
$O-5_B$	−0.8	−0.8	−0.9	−0.8	−0.8
$O-6_B$	−2.2	−0.2	−2.7	0.0	5.4
$C-1_{C\ (or\ A')}$[d]	−0.9	−1.0	−1.5	−3.1	−3.2
$C-2_{C\ (or\ A')}$	−0.5	−0.7	−2.5	−2.6	−3.7
$C-3_{C\ (or\ A')}$	−1.1	−0.6	−1.8	−1.7	−2.5
$C-4_{C\ (or\ A')}$	−1.6	−2.2	−2.4	−3.4	−1.0
$C-5_{C\ (or\ A')}$	−2.2	−2.2	−1.1	−1.3	−1.8
$C-6_{C\ (or\ A')}$	**−6.1**	**−5.3**	−2.6	−3.4	−0.9
$O-2_{C\ (or\ A')}$	−0.1	0.0	−1.6	−1.1	−1.9
$O-3_{C\ (or\ A')}$	−0.4	−0.2	−2.0	−1.9	−2.3
$O-4_{C\ (or\ A')}$	−0.8	−0.6	−3.4	−1.6	−0.9
$O-5_{C\ (or\ A')}$	−0.7	−1.1	−0.5	−0.8	−0.7
$O-6_{C\ (or\ A')}$	**−9.6**	**−4.4**	−0.7	−0.8	−0.6
$C-1_{D\ (or\ C)}$[e]	−6.4	−0.2	−0.3	−1.4	−3.9
$O-1_{D\ (or\ C)}$	−0.7	−0.1	−0.1	−0.3	−1.2
$GA-L_A$	−67.1	−64.0	−67.5	−64.5	−62.8
$GA-L_B$	−35.2	−28.0	−35.9	−27.7	−29.6
$GA-L_C$[d]	−24.1	−18.2	−20.1	−21.5	−5.0
$GA-L_D$[e]	**−7.1**	−0.3	−0.4	−1.7	
$GA-L_{A'}$[d]					−19.5
GA-L	−133.4	−110.5	−123.9	−115.5	−116.9
$L_{internal}$	17.6	8.5	3.5	−5.1	12.0
total	−115.7	−102.0	−120.4	−120.6	−104.9
k_{cat} (s^{-1})[f]	42.1	11.9		1.32−1.55	
K_M (mM)[f]	0.51	13.2		10.5	
k_{cat}/K_M ($mM^{-1}\ s^{-1}$)[f]	82.5	0.90		0.13−0.15	
$t_{1/2}$ (min)[g]	5				24

[a] $GA-L_i$: intermolecular energy per subsite; GA-L: total intermolecular energy per ligand; $L_{internal}$: internal energy of the substrate; A: subsite −1; B: subsite +1; C: subsite +2; D: subsite +3; A′: first branching subsite. [b] Bold: Important hydroxyl groups for binding and catalysis and important carbons for hydrophobic interactions. [c] Underline: Glycosidic oxygen. [d] Methyl α-maltotrioside, methyl α-panoside, methyl α-isopanoside, and methyl α-isomaltotrioside have a glucosyl residue in C; methyl α-(6^1-α-glucopyranosyl)-maltoside has a glucosyl group in A′. [e] Methyl α-maltotrioside, methyl α-panoside, methyl α-isopanoside, and methyl α-isomaltotrioside have a methyl residue in D; methyl α-(6^1-α-glucopyranosyl)-maltoside has a methyl group in C. [f] Kinetic data at 45 °C for hydrolysis of maltotriose, panose, and isomaltotriose by *A. niger* GA (Meagher and Reilly, 1989). [g] Kinetic data at 27 °C for similar concentrations of methyl *β*-maltotrioside and methyl *β*-(6^1-α-glucopyranosyl)-maltoside by *A. niger* GA (Bock, 1987).

At subsite +2, strong polar interactions are found at the $OH-6_C$ of productively bound methyl α-maltotrioside and methyl α-panoside conformers, because it hydrogen-bonds to both the backbone amide nitrogen of Gly121 and the backbone carbonyl oxygen of Glu179. No significant polar interactions occur for the top optimal structures of the remaining linear trisaccharides. In addition, there is a strongly favorable hydrophobic interaction with CH_2OH-6_C in both methyl α-maltotrioside and methyl α-panoside. Methyl α-maltotrioside has a stronger overall interaction than methyl α-panoside at this hydroxymethyl group, probably caused by the tighter binding of the former at subsite +1. At subsite +2, the α-linked methyl group of methyl α-(6^1-α-glucopyranosyl)-maltoside interacts rather strongly with GA. Moreover, the reducing-end α-linked methyl group of methyl α-maltotrioside finds a strong hydrophobic pocket at subsite +3 on the side corresponding to the most abundant conformers of acarbose and D-*gluco*-dihydroacarbose in the GA active site at pH 4.0 (Stoffer et al., 1995; Aleshin et al., 1996).

Tight binding at subsites −1 and +1 occurs only when a glucosyl substrate containing an α-(1,4)-linkage is found at the catalytic site, as with the $GA-L_i$ values in Table 2. Moreover, the tightest binding is observed for methyl α-maltotrioside, the only trisaccharide containing two α-(1,4)-linkages. The easier accommodation of this substrate to the active site explains the low values

of K_M for maltotriose hydrolysis by *A. niger* GA (Meagher and Reilly, 1989). Steric hindrance at subsite +1, namely the close contact between O-6_B with Trp120, is probably the cause of the lower rate observed in the hydrolysis of methyl β-(6^1-α-glucopyranosyl)-maltoside by *A. niger* GA compared with that of methyl β-maltotrioside (Bock, 1987). Similar steric hindrance is found due to the O-6_B-linked acetyl group in phenyl 6-*O*-acetyl-α-maltoside, which causes a eightfold increase in K_M while maintaining a similar k_{cat} compared with phenyl α-maltoside hydrolysis by *Rhizopus delemar* GA (Hiromi et al., 1973). Unfortunately, mutations at Trp120 that could remove the steric contact and so more effectively hydrolyze branched substrates lead to a strong perturbation of catalysis in *A. awamori* GA (Sierks et al., 1989), due to the loss of a critical hydrogen bond between Trp120 and Glu179. Even though a productive binding mode has been found for methyl α-panoside, the possibility of low-energy unproductive binding modes might explain the same magnitude of K_M obtained for panose and isomaltotriose (Meagher and Reilly, 1989). On the other hand, the different clusters found for methyl α-isomaltotrioside, even after allowing variation at subsite +2, are all productive at the catalytic site. The extra hydrogen bond found in subsite +2 for methyl α-panoside might further stabilize the transition state in panose hydrolysis and permit a k_{cat} an order of magnitude higher than for isomaltotriose, which lacks strong interactions there. A better picture of binding than Table 2 would compensate for the low energy values for the interaction of methyl α-panoside at subsite +2 by including solvent effects, whereas for methyl α-isomaltotrioside, unfavorable entropic effects coming from the multiplicity of its possible forms should balance the same values. However, to further emphasize the importance of the productive binding mode described for methyl α-panoside, the presence of an extra α-linked glycosyl unit at O-6_C in oligosaccharides, whose nonreducing end is otherwise identical with that of panose, such as 6^3,6^4-di-*O*-glucopyranosyl maltotetraose and 6^3,6^5,6^6-tri-*O*-glucopyranosyl maltohexaose, renders the digestion of that extra group by GA very difficult (Okada et al., 1994), most likely by making hydrogen bonding at subsite +2 impossible. The specific interaction of subsite +2 with methyl α-panoside, not possible with methyl α-isomaltotrioside, suggests that in the characterization GA specificity, the study of panose hydrolysis might be more relevant than that of isomaltooligosaccharides of DP 4 or higher.

As an example of how this work can yield insights into the functioning of GAs mutated to improve industrial performance, hydrogen bonding to Gly121 appears to play a very important role in substrate binding and catalysis. The steric impossibility of forming a hydrogen bond with the backbone amine nitrogen of residue 121 in the recently designed Gly121→Ala GA blocks substrate interaction with subsites +2 to +6 so that maltooligosaccharides are only slightly more effectively hydrolyzed than maltose (Fang et al., 1997). On the other hand, Gly121→Tyr GA (Natarajan and Sierks, 1996), where such a hydrogen bond also remains impossible, is likely to effectively compensate for this handicap by stacking the glucose residue of maltooligosaccharides at subsite +2 on the surface of the Tyr residue. This mutation blocks the side of subsite +2 occupied by the most abundant conformers of acarbose and D-*gluco*-dihydroacarbose in their *A. awamori* var. *X100* GA complexes but not the other maltooligosaccharide chains that are now oriented only in the direction of a surface patch conserved in *Aspergillus*-related GAs (Coutinho and Reilly, 1997; Coutinho and Reilly, unpublished results) that could define subsites +4 and higher. This orientation appears to be favorable, as Gly121→Tyr GA is slightly more effective in hydrolyzing maltooligosaccharides than wild-type GA is (Natarajan and Sierks, 1996). Unlike Gly121→Ala GA, which has reduced activity against isomaltose (Fang et al., 1997), Gly121→Tyr GA is more active in hydrolyzing isomaltose than is wild-type GA (Natarajan and Sierks, 1996). This feature could come from an interaction between the Tyr120 hydroxyl group and the isomaltose reducing-end residue at subsite −1, suggesting that the introduction of other bulky residues at position 121 that do not affect subsite +1 could lead to good characteristics in the industrial use of GA.

It is interesting that strong interactions occur between the hydroxymethyl groups of every glucosyl unit in methyl α-maltotrioside and the active site, with hydrogen bonding at OH-6_A and OH-6_C and with relatively strong hydrophobic interactions with every C-6. Unfortunately, almost no specific data on the GA hydrolysis of maltotriose analogues are available, although analogues lacking each hydroxymethyl group have been produced for that purpose (Takeo et al., 1991). We know that 6-deoxy-6-halogenomaltotrioses are not GA substrates (Omichi and Matsushima, 1978), which parallels the inability of GA to hydrolyze 6^2-deoxy-6-halogenomaltosides (Bock and Pedersen, 1988). Studies with methyl 4^3-*O*-methyl-β-maltotrioside (Bock and Skrydstrup, 1992) were unfortunately likely compromised by GA contamination with α-amylase. To our knowledge, no data exist for GA interaction with analogues of the remaining trisaccharides.

The energy values in Table 2 are indicative only of interactions in the active site and should not be taken quantitatively, as many approximations were made in this study. Nevertheless, the approach of combining a library of trisaccharide conformers based on results from conformational mapping of disaccharides with docking by AutoDock can identify important clusters of results. However, the large number of degrees of freedom and the wide conformational space available rendered convergence more difficult for simulated annealing of trisaccharides than for that of disaccharides. It is possible that, given the large number of parameters used in AutoDock, better interaction energies could be found by refining the approach.

Summary and Conclusions

The interaction of five α-(1,4)- and α-(1,6)-linked glucosyl trisaccharides with the GA active site has been simulated by Monte Carlo techniques to further investigate the molecular basis of GA specificity. An adaptation of the docking protocol developed in recent studies (Coutinho et al., 1997a,b,c) was used, combined with all possible MM3-optimized trisaccharides derived from a library of low-energy conformers of maltose and methyl α-isomaltoside (Dowd et al., 1992; Coutinho et al., 1997b). This approach determined the productive binding modes of the trisaccharides in the subsites −1 and +1 where catalysis takes place, and found the modes by which some linear trisaccharides effectively bind at subsite +2 and by which branched saccharides are likely to interact with a first branching subsite.

Productive binding in subsites −1 and +1 is similar to that obtained by the corresponding disaccharides in previous docking studies (Coutinho et al., 1997b,c), with identification of all the critical hydroxyl groups serving as the anchors required for substrate binding and catalysis. This process has now been extended to subsite +2 for methyl α-maltotrioside and methyl α-panoside, where OH-6_C appears to play an important role. An equivalent hydroxyl group involved in binding at subsite +2 was described for the binding of maltotetraose-like inhibitors (Aleshin et al., 1994, 1996; Stoffer et al., 1995). This study shows that the nonreducing-end α-(1,6)-linked methyl α-panoside can bind at subsite +2 in a mode surprisingly similar to that of α-(1,4)-linked linear substrates. The GA active site, and particularly subsite +1 as shown before (Coutinho et al., 1997c), is very adaptable even in its interaction with some longer substrates.

The study results indicate that stress occurs upon binding of branched substrates, which suggests that some rearrangement of GA active-site residues is necessary for hydrolysis to take place.

The use of a library of conformers, representing all important conformational states of the substrate, as initial structures for docking ensures the exploration of significant conformational space and demonstrates again the ability of AutoDock to explore and explain important features of substrate flexibility in carbohydrate−protein interactions. Complementary to the crystallographic studies of GA complexes with maltooligosaccharide analogues (Aleshin et al., 1994, 1996; Stoffer et al., 1995), the extension to differently linked trisaccharides of the systematic exploration of the interaction of GA with many mono- and disaccharide substrates and their analogues (Coutinho et al., 1997a,b,c) demonstrates both the versatility of the *Aspergillus* GA subsite +1 and the role of its subsite +2 in promoting the hydrolysis of substrates α-(1,4)-linked between subsites +1 and +2. Understanding the molecular basis of *Aspergillus* GA interaction with different substrates supports current efforts to engineer its selectivity to further optimize saccharification in starch processing (Crabb and Mitchinson, 1997).

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