Mutations to alter Aspergillus awamori glucoamylase selectivity.

III. Asn20→Cys/Ala27→Cys, Ala27→Pro, Ser30→Pro,
Lys108→Arg, Lys108→Met, Gly137→Ala, 311–314 Loop,
Tyr312→Trp and Ser436→Pro

Hsuan-Liang Liu, Pedro M.Coutinho, Clark Ford¹ and
Peter J.Reilly²

Departments of Chemical Engineering and ¹Food Science and Human
Nutrition, Iowa State University, Ames, IA 50011, USA

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Introduction

Glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3)
(GA) catalyzes β-D-glucose release from the non-reducing
ends of starch and related oligo- and polysaccharide chains
by α-1,4-glucosidic bond hydrolysis (Figure 1). Aspergillus
awamori and Aspergillus niger GAs, which have identical
amino acid sequences (Svensson et al., 1983; Nunberg et al.,
1984), are widely used in industry to produce high-glucose
syrs, to be converted primarily to fructose and ethanol.
GA can also hydrolyze α-β-1,1-, α-1,2-, α-1,3- and α-1,6-
glucosidic bonds at much lower rates (Hiromi et al., 1969;
Meagher and Reilly, 1989).

GA synthesizes various α-linked di-, tri- and tetrasaccharides
in high D-glucose concentrations by reforming all the above
bonds (Pazur and Okada, 1967; Hehre et al., 1969; Pazur
et al., 1977; Nikolov et al., 1989), as the law of microscopic
reversibility mandates. This limits α-glucose yield from starch
hydrolysis to 95–96% of theoretical (Crabb and Mitchinson,
1997). In these condensation reactions, isomaltose [α-β-D-glucopyranosyl-(1→6)-D-glucose] (iG2) is thermodynamically fav-
ored since its glucosidic bond is through a primary hydroxyl
group, giving it an extra degree of freedom. Therefore, it
reaches the highest equilibrium level even though its initial
synthesis rate is much lower than that of maltose [α-D-
glucopyranosyl-(1→4)-D-glucose] (G2) (Hehre et al., 1969;
Roels and van Tilburg, 1979; Adachi et al., 1984; Beschkov
et al., 1984; Shiraishi et al., 1985; Nikolov et al., 1989), the
kinetically favored product. If GA could be engineered to
decrease its ability to synthesize iG2, higher D-glucose yields
from starch would be achieved.

In this work, we constructed three single mutations, Lys108→
Arg, Lys108→Met and Tyr312→Trp, and one insertional
mutant, Tyr311–Tyr312–Asn313–Gly314→Tyr311–Asn–Gly–
Asn–Gln–Gly314 (311–314 Loop) (Figure 2), by site-directed mutagenesis to decrease the iG2 formation
rate and to increase glucose yield. GA amino acid sequences
are aligned into six conserved regions, corresponding to the
six inter-helical loops that border the active site in the
center of the catalytic domain (Tanaka et al., 1986; Itoh et al.,
1987; Coutinho and Reilly, 1994a,b, 1997; Fierobe et al.,
1996). Both Lys108 and Tyr312 are located in the (α/ω)αβ-fold
of the catalytic domain in conserved segments that define the
active site (Aleshin et al., 1992) in the loops between α-
helices 3 and 4 and 9 and 10, respectively. The catalytic site,
where hydrolysis takes place, is defined by subsites –1 and
+1 (Davies et al., 1997).

We picked Lys108 to mutate because it is totally conserved
in all GAs except Clostridium GA, where it is missing
(Coutinho and Reilly, 1997). The Clostridium GA has relatively
high α-1,6 activity (Ohnishi et al., 1992). Lys108 is proposed
to form a water-bridged hydrogen bond with the 6-OH of the
reducing glucosyl residue of G2 at subsite +1 through a surface
water molecule (Coutinho and Reilly, 1994a). However, this
surface water does not form a hydrogen bond with either 1-
deoxyxojirimycin (Harris et al., 1993) or acarbose (Aleshin

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site-directed mutagenesis/yield

Fig. 1. Schematic diagram of hydrolysis of branched maltooligosaccharide
by GA. Reducing residues are open and non-reducing residues are closed.
Closed arrows denote sites of initial α-1,4- and α-1,6-glucosidic bond
cleavage. The reverse reaction condenses D-glucose into five different α-
linked α-glucosyl disaccharides, chiefly into the α-1,6-linked isomaltose.

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et al., 1994) owing to their shifted positions in the active site. There is no hydrogen bond in the GA–iG₂ complex through this hydroxyl group, since it is now part of the glucosidic bond. We replaced Lys108 with Arg and Met residues to study the influence of side-chain surface charges on transition-state stabilization in a well defined void formed by the aromatic residues Tyr50, Trp52, Tyr116 and Trp120 (Coutinho and Reilly, 1994a). It had not previously been mutated, so this work will be of interest in demonstrating whether highly conserved residues in conserved regions do in fact play important roles in enzyme activity, thermostability and selectivity (Coutinho and Reilly, 1994b).

We mutated Tyr312 because it adjoins Tyr311, which participates in a hydrogen-bond network with the general catalytic base Glu400 (Harris et al., 1993) through the invariant Tyr48. The Tyr48 side chain is important for maintaining active-site geometry (Frandsen et al., 1994). The large loss in substrate affinity of Glu400→Gln GA is because of destabilization due to disruption of the hydrogen-bond network involving the Glu400 γ-carboxyl group, the Tyr48 hydroxyl group and the Tyr311 hydroxyl group (Aleshin et al., 1994). Tyr311 is also totally conserved (Coutinho and Reilly, 1997). Based on the above evidence, we chose Tyr312, located right behind Tyr311 with respect to the substrate in the enzyme–substrate complex (Aleshin et al., 1994) and not totally conserved (Coutinho and Reilly, 1997), to be mutated to Trp. This introduced a larger side chain to supply stronger support to Tyr311 in forming the tight hydrogen-bond network described above, tightening interactions at subsite +1. In addition to a changed concerted loop movement in the Loop 3–Loop 5 double-loop mutant (Fierobe et al., 1996), the higher flexibility of Tyr311 and therefore of subsite +1 caused by the Tyr312→Met mutation may have contributed significantly to its higher α₁,6-activity, as found in Hormoconis resinae GA S (Fagerström, 1991).

We introduced the 311–314 loop mutation to mimic the Rhizopus GA sequence. GAs from Rhizopus and some other subfamilies have longer amino acid sequences in the conserved region between helices 9 and 10 than do A. niger or A. awamori GAs (Coutinho and Reilly, 1994a) and therefore may have different active-site conformations. Rhizopus niveus GA has a catalytic efficiency about 20–50% that of A. niger GA for iG₂ hydrolysis (Tanaka and Takeda, 1994) with about the same catalytic efficiency for G₂ hydrolysis (Ohnishi, 1990). In addition, H. resinae GA has ratios of catalytic efficiency (kcat/ Kₐ) for α₁,4- over α₁,6-glucosidic bonds only 10% those of A. awamori/A. niger GA (Fagerström, 1991; Fagerström and Kalkkinen, 1995). Two individual loop replacements and one double loop replacement have been constructed in A. awamori GA to mimic part of the H. resinae GA sequence (Fierobe et al., 1996). The double mutation had approximately twofold higher relative catalytic efficiencies for hydrolysis of α₁,6-over α₁,4-linked substrates. Although loop replacement based on sequence homology can be a powerful tool for altering GA selectivity (Svensson et al., 1995; Fierobe et al., 1996; Stoffer et al., 1997), loop insertion or deletion had not been attempted in the GA catalytic domain.

We measured the kinetics and glucose yields of some previously constructed thermostable mutant GAs, Asn20→Cys/ Ala27→Cys (SS) (Li, 1996), Ser30→Pro (Allen, 1997), Gly137→Ala (Chen et al., 1996) and Ser436→Pro (Li et al., 1997), along with one thermosensitive mutant GA, Ala27→Pro (Li et al., 1997) to investigate a potential relationship between enzyme thermostability and selectivity (Figure 2). Asn20 is the C-terminal residue of α-helix 1 (Aleshin et al., 1992). Ala27 and Ser30 are located in a type II β-turn on an extended loop belonging to the conserved region between α-helices 1 and 2 (Coutinho and Reilly, 1994a,b, 1997). Gly137 is located in the middle of the fourth α-helix. Ser436 is located in a random coil in a packing void of unknown function in A. awamori var. X100 GA (Aleshin et al., 1994). The SS mutation was designed to create a disulfide bond on the catalytic domain surface and to stabilize GA against unfolding. The Ser30→Pro, Gly137→Ala and Ser436→Pro mutations were meant to stabilize GA by reducing the conformational entropy of unfolding and are the most stable mutant GAs in a series of previously made X→Pro and Gly→Ala substitutions (Chen et al., 1996; Allen, 1997; Li et al., 1997). Only Ala27 and Ser30 among these residues are located in conserved regions and neither of them is totally conserved (Coutinho and Reilly, 1994a,b, 1997).

Previous studies have attempted to improve GA selectivity by single-residue substitutions: Ser119→Tyr, Gly183→Lys and Ser184→His GAs all had 2.3–3.5-fold enhancement of the ratio of catalytic efficiencies of G₂ hydrolysis to iG₂ hydrolysis (Sierks and Svensson, 1994; Svensson et al., 1995). By mutating Arg305 to Lys, Frandsen et al. (1995) showed that Arg305 plays an important role in GA selectivity because it stabilizes α₁,4-linked substrates from the hydrophilic side of the substrate-binding pocket in subsites –1 and +1. This has been confirmed for all the differently-linked disaccharide substrates of GA by molecular modeling (Coutinho et al., 1997a,b). On the other hand, Asp176→Asn GA shows no significant changes in binding (Christensen et al., 1996). Asp176 is important in the rate-determining catalytic step but...
not so much in substrate binding (Sierks et al., 1990), because its side chain participates in the hydrogen-bond network involving Glu179, the general catalytic acid, and the backbone of Glu180, which forms a hydrogen bond with the 2-OH of the glucosyl ring of maltose at subsite +1 and is important in the Michaelis complex (Christensen et al., 1996).

Eleven mutations at positions not totally conserved around the GA active site were constructed by Fang et al. (1998a,b) to alter selectivity of A.wa'amori between peak glucose yield and the ratio of initial rate of iG2 significantly increased glucose yields at 55°C compared with wild-type GA. In general, there was an inverse correlation between peak glucose yield and the ratio of initial rate of iG2 formation to that of glucose formation.

Materials and methods

Methods for enzyme production and purification, protein concentration measurement, enzyme kinetic assays, maltoligosaccharide hydrolysis and glucose condensation reactions were described by Fang et al. (1998a). Specific activities were determined according to Fang et al. (1998b).

Materials

Glucose, maltose, maltotriose (G3), maltotetraose (G4), maltopentaose (G5), maltohexaose (G6) and maltoheptaose (G7) were obtained from Sigma. Isomaltose was purchased from TCI America. Maltrin M100, M180 and M250 maltodextrins, of dextrose equivalents (DE) 10, 18 and 25, respectively, and with average degrees of polymerization (DP) of 10, 6 and 4, respectively, were donated by Grain Processing Corporation. Other materials were as described by Fang et al. (1998a).

Site-directed mutagenesis

Site-directed mutagenesis was performed with the Muta-Gen phagemid in vitro mutagenesis kit from Bio-Rad (Kunkel et al., 1987) on a BamHI–XhoI fragment of the wild-type GA cDNA inserted into the phagemid vector pGEMZ(+) (9). The vector was then transformed into Escherichia coli C236 (dut, ung –) to produce uracil-containing single-stranded DNA to be used as the template in site-directed mutagenesis. The following mutagenic oligonucleotide primers were synthesized at the Iowa State University Nucleic Acid Facility: 5′-GCT CTC GGT GAG CCC AGT TTC ATC GTC GAT-3′ (Lys108→Arg), 5′-GCT CTC GGT GAG CCC ATG TTC ATC GTC GAT-3′ (Lys108→Met), 5′-TAC CCT GAG GAC ACG TAC AAC GCC GGC AAC TCG CAG GGC AAC CCG TGG TTC CTG TGC-3′ (311–314 Loop) and 5′-GAG GAC AGC TAC TGG AAC GCC ACG CCG-3′ (Tyr312→Trp), the bold letters indicating the changed or added nucleotides. All mutations were verified by DNA sequencing. The mutated GA cDNAs were subcloned into YEpPM18 and then transformed into Saccharomyces cerevisiae C468 as described previously (Chen et al., 1994a). Construction of the Gly137→Ala (Chen et al., 1996), SS (Li, 1996), Ala27→Pro, Ser436→Pro (Li et al., 1997) and Ser30→Pro (Allen, 1997) GAs is described separately.

Protein electrophoresis

Soluble extracellular fractions of 5 day yeast cultures from 5 l fermentations of wild-type and Lys108→Met GAs were separated on 10% polyacrylamide gel at 60 V for 5 h along with high molecular weight markers, followed by staining with Coomassie Brilliant Blue R for 20 min and by destaining with distilled water for 24 h. The resulting gels were transferred onto a 0.2 nm nitrocellulose membrane with a Bio-Rad Trans-Blot SD semi-dry transfer cell.

Irreversible thermoinactivation

GAs (0.475 µM) were incubated in 0.05 M NaOAc buffer, pH 4.4, at 70°C for 12 min. Samples were taken at 2 min intervals, quickly chilled on ice and then stored at 4°C for 24 h before being subjected to residual activity assay at 35°C. Enzyme activity was determined with 0.117 M maltose in the same buffer. Seven samples were taken at 7 min intervals and added to 0.4 volume of 4 M Tris–HCl buffer, pH 7.0, to stop the reaction. The resulting glucose was measured by the glucose oxidase method (Rabbo and Terekhldsen, 1960).

Results

Thermoinactivation

Wild-type, Lys108→Arg, 311→314 Loop and Tyr312→Trp GAs followed first-order decay kinetics, as have all previous mutant GAs tested for thermostability (Chen et al., 1994a,b, 1995, 1996; Li, 1996; Allen, 1997; Li et al., 1997), with rate coefficients at 70°C of 0.036, 0.073, 0.075 and 0.053 min⁻¹, respectively. Except for Ala27→Pro, other mutant GAs studied here were more thermostable than wild-type GA (Chen et al., 1996; Li, 1996; Li et al., 1997; Allen, 1997).

Enzyme kinetics

After expression from yeast colonies, Lys108→Met GA produced no starch-clearing halo on an SD + His + 1% soluble starch plate. It yielded no detectable activity in the supernatant of a yeast fermentation broth after specific activity assay with 4% maltose. Lys108→Met GA gave a band with one-third the intensity of wild-type GA after submitting the concentrated supernatants to 10% SDS–PAGE. The mutated GA did not bind to an acarbose-Sepharose affinity column, since no detectable peak was observed by UV absorption at 280 nm at the retention time characteristic of GA after elution with 1.7 M Tris–HCl at pH 7.6. Therefore, this mutated GA was not studied further.

Specific activities of wild-type, Lys108→Arg, 311→314 Loop and Tyr312→Trp GAs were 15.5, 11.5, 9.07 and 11.9 IU/mg, respectively, determined with 4% (w/v) maltose in 0.05 M NaOAc buffer at 45°C, pH 4.4. All other mutant GAs studied here except Ala27→Pro had specific activities about the same as or greater than that of wild-type GA (Chen et al., 1996; Li 1996; Li et al., 1997). Specific activities of wild-type, Lys108→Arg, 311→314 Loop and Tyr312→Trp GAs were 15.5, 11.5, 9.07 and 11.9 IU/mg, respectively, determined with 4% (w/v) maltose in 0.05 M NaOAc buffer at 45°C, pH 4.4. All other mutant GAs studied here except Ala27→Pro had specific activities about the same as or greater than that of wild-type GA (Chen et al., 1996; Li 1996; Li et al., 1997). Specific activities of wild-type, Lys108→Arg, 311→314 Loop and Tyr312→Trp GAs were 15.5, 11.5, 9.07 and 11.9 IU/mg, respectively, determined with 4% (w/v) maltose in 0.05 M NaOAc buffer at 45°C, pH 4.4. All other mutant GAs studied here except Ala27→Pro had specific activities about the same as or greater than that of wild-type GA (Chen et al., 1996; Li 1996; Li et al., 1997). Values of kcat and KM for the hydrolysis of G2 to G7 in 0.05 M NaOAc buffer, pH 4.4, at 45°C are given in Table I for wild-type, SS, Lys108→Arg, 311→314 Loop and Tyr312→Trp GAs. The values of kcat increased quickly with increasing DP from G2 to G4 and more slowly thereafter, whereas those of KM followed the opposite pattern, as found previously (Hirumi et al., 1973). In general, kcat values at different DPs were roughly the same for SS GA but lower for Lys108→Arg, 311→314 Loop and Tyr312→Trp GAs than for wild-type GA.

Values of KM were higher than wild-type GA for Lys108→Arg GA, lower for 311→314 Loop GA and about the same for SS and Tyr312→Trp GAs. These results led to lower catalytic efficiencies than wild-type GA for Lys108→Arg GA and approximately the same values for the other mutated GAs. The resulting transition-state energies, Δ(ΔG), used to estimate
the binding strength of the enzyme–substrate complex in the transition state, were positive for substrates of all DPs for Lys108→Arg GA and were both positive and negative, but always small, for the other mutated GAs.

Table I also shows kinetic values for Ig2 hydrolysis by the same GAs. All GAs had drastically lower kcat and drastically higher KM values than with G2, as found previously (McGether et al., 1989). SS, 311–314 Loop and Tyr312→Trp GAs had lower values of kcat on Ig2, than did wild-type GA, whereas Lys108→Arg GA had a much higher value. All four mutant GAs had lower KM values. This led to higher catalytic efficiencies on Ig2 for Lys108→Arg GA and lower values for the other three mutant GAs, especially 311–314 Loop GA, compared with wild-type GA. The ratios of the catalytic efficiency for G2 to that for Ig2 were higher than those for wild-type GA for 311–314 Loop and Tyr312→Trp GAs, about the same for SS GA and much lower for Lys108→Arg GA.

Maltodextrin hydrolysis

Figures 3, 4 and 5 show the results of 30% (w/v) DE 10 maltodextrin hydrolyses at 35, 45 and 55°C, respectively, with 1.98 mM GA. Data (not shown) for DE 18 and DE 25 maltodextrins are similar. SS and 311–314 Loop GAs had the highest peak glucose yields and Lys108→Arg GA had the lowest yields. Glucose concentrations decreased after reaching maximum values because of conversion to oligosaccharides, especially at higher temperatures. Initial glucose formation rates are given in Table II. They were generally lowest for 311–314 Loop GA because its specific activity with 4% (w/v) maltose substrate was only 60% that of wild-type GA. Thermostable mutant GAs generally had higher initial glucose formation rates than wild-type GA, whereas thermosensitive GAs had lower rates. Initial glucose formation rates often increased slightly with increasing substrate DE. Activation energies of initial rates from 35 to 55°C were determined by plotting ln(initial rate) vs 1/T and were averaged over the three maltodextrin substrates, since there was no significant difference between them. They were 66, 69, 84, 85 and 90 kJ/mol for Lys108→Arg, SS, Ser436→Pro, Ala27→Pro, Gly137→Ala, Tyr312→Trp, Ser30→Pro, wild-type and 311–314 Loop GAs, respectively, averaging 80 kJ/mol. Activation energies for only the first and last GAs differed from each other by more than the 95% level of confidence, suggesting that these two GAs may have undergone more significant active-site conformational changes than the other GAs.

Glucose condensation reactions

Formation of Ig2 from condensation of 30% (w/v) glucose at 35, 45 and 55°C with 1.98 mM GA is shown in Figure 6. Initial rates were obtained by fitting iG2 concentration vs time, using adjustable parameters, a being initial rate and t being incubation time, and are given in Table II. SS and 311–314 Loop GAs had the lowest initial rates at all three reaction temperatures and Lys108→Arg and Ala27→Pro GAs had the highest initial rates. Activation energies were 40, 55, 61, 62, 66, 69, 84, 85 and 90 kJ/mol for Ser436→Pro, Ala27→Pro,
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Fig. 3. Glucose formation during the incubation at 35°C of 30% (w/v) DE 10 maltodextrin with 1.98 µM GA in 0.05 M NaOAc buffer, pH 4.4, GAs: (a) wild-type (○, ——), Lys108→Arg (●, ——), 311–314 Loop (▲, ⋯⋯), Tyr312→Trp (△, —); (b) wild-type (○, ——), SS (□, ——), Ala27→Pro (■, ——), Ser30→Pro (♦, ——), Gly137→Ala (▲, ⋯⋯) and Ser436→Pro (●, ——).

Lys108→Arg, Gly137→Ala, SS, Ser30→Pro, 311–317 Loop, Tyr312→Trp and wild-type GAs, respectively. The activation energies of the second to fifth GAs differed from those of the first GA and last three GAs by more than the 95% confidence level. Except for the latter two GAs, activation energies for iG2 formation were less than those for G2 hydrolysis, with significant differences between the two activation energies for Ala27→Pro, Ser30→Pro, Gly137→Ala and Ser436→Pro GAs.

Selectivity for α-1,6-linked product synthesis versus α-1,4-linked substrate hydrolysis

The ratio of the initial rate of iG2 formation by condensation of 30% glucose to that of glucose formation from 30% DE 10, 18 and 25 maltodextrin hydrolysis was taken to estimate the selectivity for α-1,6-linked product synthesis over α-1,4-linked substrate hydrolysis (Table II). Lys108→Arg and SS GAs had the highest and lowest ratios, respectively, among wild-type and mutant GAs at all reaction temperatures. The 311–314 Loop GA also had low ratios, as did Gly137→Ala GA at 45 and 55°C. These results can be compared with the ratios of catalytic efficiencies of G2 to those of iG2, where 311–314 Loop GA had the highest value, Lys108→Arg GA had the lowest and SS and Tyr312→Trp GAs had values similar to wild-type GA.

Discussion

GA has often been mutated to determine the effect of important residues around the active site on catalytic mechanism and selectivity (Siersks et al., 1989, 1990, 1993; Sierks and Svensson, 1992, 1994, 1996; Bakir et al., 1993; Olsen et al., 1993; Frandsen et al., 1995, 1996; Svensson et al., 1995; Christensen et al., 1996; Fierobe et al., 1996; Stoffer et al., 1997), but all previous papers described GA selectivity based on changes in $k_{cat}$ and $K_M$ for substrate hydrolysis. Initial rates of glucose and iG2 formation and peak glucose yields from mutated GAs were not measured until Fang et al. (1998a,b) studied high-concentration glucose condensation and maltodextrin hydrolysis reactions. In the present work, we used their strategy on newly designed mutant GAs and on those derived from earlier thermostability studies.

SS GA had initial rates of iG2 formation 25–50% those of wild-type GA, even though initial glucose formation rates increased. Among the GAs tested here, SS GA is the only one whose mutation introduced a covalent bond. This may have made the enzyme even more rigid and restricted its natural flexing action, favoring the α-1,4 over the α-1,6 substrate binding conformation.

Introduction of unfavorable contacts between the Pro pyrrolidine ring and neighboring groups, such as the N atoms of Val29 and Ser30, by the Ala27→Pro mutation, resulted in decreased GA thermostability (Li et al., 1997). The mutation yields incompatible φ and ψ angles and a less well packed
GA may have favored formation and hydrolysis of α-1,6-linked substrates, reducing glucose yield.

Ser30→Pro GA produced comparatively fewer α-1,6 bonds at increased temperatures, suggesting that the introduction of a Pro residue at the second position of a type II β-turn in this region causes an entropy change not only to reduce conformational unfolding, but also to favor the α-1,4 binding mode at the active site.

Lys108→Arg GA had higher initial rate ratios for iG2 formation over glucose formation than any other GA at all temperatures and with all maltodextrin substrates (Table II), leading to up to 4% decreases in peak glucose yields compared with wild-type GA (Figure 7). It has only 75% of the specific activity of wild-type GA. Our aim in replacing Lys108 by Arg was to make a stronger hydrogen-bonding bridge with the 6-OH of the reducing residue of G2 through a water molecule (Coutinho and Reilly, 1994a) to stabilize the GA–G2 complex, since Arg has more surface charge at pH 4.4 than Lys owing to its strongly protonated δ-guanido group. The Lys108→Arg mutation instead increased the binding affinity of the α-1,6-linked iG2 compared with wild-type GA. Lys108 is involved in the enzyme–substrate complex in the conserved segment between helices 3 and 4 containing three invariant residues, Trp120, Gln124 and Asp126 (Coutinho and Reilly, 1994a). The Trp120 loop region plays an important role in directing conformational changes controlling the postulated rate-limiting product release step from maltooligosaccharide hydrolysis (Natarajan and Sierks, 1996). Trp120 is the key residue in stabilizing the transition-state structure (Sierks et al., 1989). The Lys108→Arg mutation may push the Trp120 loop to the catalytic center, restricting glucose release and causing it to become a competitive substrate. This would lead to iG2 synthesis instead of maltooligosaccharide hydrolysis. On the other hand, the surface charge in the GA–G2 complex introduced by this mutation may cause destabilization by rearrangement of the unbalanced charge in the transition state. The same pressure on Trp120 may also perturb binding of the non-reducing end of maltose at subsite +1 while limiting movement of the non-reducing end of the more flexible and solvent-exposed iG2 (Coutinho et al., 1997a,b), facilitating its hydrolysis.

Complexes of GA with different inhibitors suggest that the 6-OH group of the residue at subsite +1 projects into a void large enough to accommodate an additional glucosyl residue (Aleshin et al., 1994), especially when it is linked through an α-1,6-branch from the former residue (A.Aleshin and R.B.Honzatko, personal communication, 1995). This has been recently modeled by substrate docking (Coutinho et al., 1998).

Since Lys108 is in an extended conformation within this void, the Lys108→Arg mutation would impact significantly on the binding of this branched glucosyl ring without seriously altering the electrostatic charge of GA. This hypothesis strongly agrees with experimental results from the present work showing that Lys108→Arg GA favors α-1,6 bond hydrolysis and formation much more than does wild-type GA. Also, at high glucose concentrations glucose molecules are more likely to present at both subsites −1 and +1, allowing Glu179 to serve as a catalytic base in abstracting a proton from a hydroxyl group of the glucose residue in subsite +1. The activated hydroxyl group would then displace the 1-OH of β-d-glucose at subsite −1, with Glu400 acting as the catalytic acid in this synthetic reaction. Generally, wild-type GA cannot specifically recognize the orientations of the different OH groups in the glucosyl residue at subsite +1, maltose binding better because of a hydrophobic interaction with the hydroxymethyl group found there (Coutinho et al., 1997b). However, with the 6-OH group projecting into the void described above and with the electrostatic effect caused by the Lys108→Arg GA mutation, it is more likely for this mutant GA to form an α-1,6-branched product like iG2 rather than other disaccharides.

The observations that Lys108→Met GA (i) formed no halos on starch plates after expression from yeast colonies, (ii) had no detectable activity in concentrated fermentation culture supernatants, (iii) formed a GA band with one-third the intensity of wild-type GA on 10% SDS–PAGE gel and (iv) could not be purified by acarbose-Sepharose affinity chromatography suggest that this mutated GA is expressed in an inactive form because a positively charged residue at position 108 may be essential to activate Glu179. Met108, having no side-chain surface charge and being non-polar and relatively unreactive, cannot form hydrogen bonds with substrates or activate Glu179. The mutated GA cannot be separated by an acarbose-Sepharose column because no salt link can be formed between the amino linkage of acarbose and the catalytic acid Glu179.

These two mutations demonstrate that changing Lys108 strongly affects GA. Removing its surface charge yields a GA that does not bind substrates and therefore totally loses activity, while increasing its surface charge favors α-1,6 bond synthesis over α-1,4 bond hydrolysis. Lys108 is not only important for helping to retain active-site conformation for substrate binding but can also have a specific role in hydrolysis.
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Gly137 is located in the middle of the α-helix 4, which is part of the inner ring of the αβ-barrel around the active site. The Gly137→Ala mutation increased GA thermostability by strengthening this α-helix and preventing the catalytic domain from unfolding (Chen et al., 1996). Even though Gly137 is not directly involved in catalysis or substrate binding, the strong stabilization of this helix by Ala residue substitution has made the conformational adjustment around the catalytic cavity disfavor α-1,6-linked substrate formation, especially at higher temperatures. This results in higher glucose yields for this mutant GA than for wild-type GA.

The mutation giving 311–314 Loop GA decreased specific activity by about 40%, with ratios of initial rates of iG₂ formation to that of glucose formation about 50–70% of those of wild-type GA. Introducing a long sequence into the Gly137→Ala active-site environment increases the binding affinity for both α-1,4- and α-1,6-linked substrates because the conformational change may reduce the binding energies required to form the enzyme–substrate complex. This allows the substrate to progress into subsite −1 more easily while undergoing a slow unimolecular enzyme–substrate complex migration step. A ‘dead-end pocket’ model of the GA active site was proposed based on X-ray structural studies, suggesting that water displacement from subsite −1, where a cluster of seven water molecules exists in the absence of substrate, is a crucial step in the GA catalytic mechanism (Aleshin et al., 1994). The conformational change by the 311–314 Loop mutation may also help to accommodate the simultaneous transport of water molecules as the substrate enters in or product diffuses from subsite −1. Product release after hydrolysis, the proposed rate-limiting step for maltooligosaccharide hydrolysis (Kitahata et al., 1981; Natarajan and Sierks, 1996; Sierks and Svensson, 1996; Sierks et al., 1997), may be relatively easier and faster than from wild-type GA because of the bigger loop introduced around the active site and this may make iG₂ synthesis less likely. The 311–314 Loop GA is less thermostable than most other GAs, so it is of interest to combine this mutation with another conferring thermostability, assuming that mutational effects will be additive (Matsumura et al., 1986; Wells, 1990).

Table II. Initial rates of glucose and iG₂ formation in the hydrolysis of 30% (w/v) maltodextrins and condensation of 30% (w/v) glucose, respectively and their relative ratios for wild-type and mutant GAs at 35, 45 and 55°C

<table>
<thead>
<tr>
<th>GA form</th>
<th>Initial rates (mol/mol GA·s)</th>
<th>Initial rate iG₂(initial rate glucose)×10³</th>
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<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>iG₂×10³</td>
</tr>
<tr>
<td></td>
<td>DE 10 DE 18 DE 25</td>
<td>DE 10 DE 18 DE 25</td>
</tr>
<tr>
<td>35°C</td>
<td></td>
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<tr>
<td>Wild-type</td>
<td>32.8 ± 1.4</td>
<td>15.2 ± 0.5</td>
</tr>
<tr>
<td>SS</td>
<td>48.1 ± 2.2</td>
<td>7.07 ± 0.33</td>
</tr>
<tr>
<td>Ala27→Pro</td>
<td>41.4 ± 1.9</td>
<td>43.7 ± 1.9</td>
</tr>
<tr>
<td>Ser30→Pro</td>
<td>39.7 ± 1.8</td>
<td>22.9 ± 0.8</td>
</tr>
<tr>
<td>Lys108→Arg</td>
<td>32.6 ± 1.6</td>
<td>50.9 ± 1.9</td>
</tr>
<tr>
<td>Gly137→Ala</td>
<td>44.9 ± 2.3</td>
<td>40.2 ± 3.3</td>
</tr>
<tr>
<td>311–314 Loop</td>
<td>20.9 ± 1.1</td>
<td>6.71 ± 0.24</td>
</tr>
<tr>
<td>Tyr312→Trp</td>
<td>26.9 ± 1.4</td>
<td>15.5 ± 0.6</td>
</tr>
<tr>
<td>Ser346→Pro</td>
<td>45.1 ± 2.6</td>
<td>47.5 ± 1.8</td>
</tr>
<tr>
<td>45°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>101 ± 5</td>
<td>39.6 ± 2.9</td>
</tr>
<tr>
<td>SS</td>
<td>129 ± 6</td>
<td>16.4 ± 1.0</td>
</tr>
<tr>
<td>Ala27→Pro</td>
<td>99.5 ± 5.3</td>
<td>79.2 ± 4.2</td>
</tr>
<tr>
<td>Ser30→Pro</td>
<td>98.8 ± 5.4</td>
<td>51.0 ± 2.2</td>
</tr>
<tr>
<td>Lys108→Arg</td>
<td>77.1 ± 4.3</td>
<td>85.5 ± 4.7</td>
</tr>
<tr>
<td>Gly137→Ala</td>
<td>102 ± 6</td>
<td>30.7 ± 1.8</td>
</tr>
<tr>
<td>311–314 Loop</td>
<td>61.2 ± 3.8</td>
<td>14.6 ± 1.1</td>
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<tr>
<td>Tyr312→Trp</td>
<td>78.4 ± 4.5</td>
<td>34.3 ± 1.8</td>
</tr>
<tr>
<td>Ser346→Pro</td>
<td>87.9 ± 5.3</td>
<td>68.3 ± 3.6</td>
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<tr>
<td>55°C</td>
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<tr>
<td>Wild-type</td>
<td>240 ± 15</td>
<td>129 ± 7</td>
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<tr>
<td>SS</td>
<td>268 ± 17</td>
<td>32.4 ± 2.7</td>
</tr>
<tr>
<td>Ala27→Pro</td>
<td>258 ± 18</td>
<td>163 ± 9</td>
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<tr>
<td>Ser30→Pro</td>
<td>284 ± 20</td>
<td>119 ± 6</td>
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<tr>
<td>Gly137→Ala</td>
<td>152 ± 11</td>
<td>216 ± 14</td>
</tr>
<tr>
<td>311–314 Loop</td>
<td>191 ± 14</td>
<td>49.5 ± 2.7</td>
</tr>
<tr>
<td>Tyr312→Trp</td>
<td>169 ± 13</td>
<td>117 ± 7</td>
</tr>
<tr>
<td>Ser346→Pro</td>
<td>301 ± 22</td>
<td>124 ± 8</td>
</tr>
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</table>

*Standard error.

The Gly137 is located in the middle of α-helix 4, which is part of the inner ring of the αβ-barrel around the active site. The Gly137→Ala mutation increased GA thermostability by strengthening this α-helix and preventing the catalytic domain from unfolding (Chen et al., 1996). Even though Gly137 is not directly involved in catalysis or substrate binding, the strong stabilization of this helix by Ala residue substitution has made the conformational adjustment around the catalytic cavity disfavor α-1,6-linked substrate formation, especially at higher temperatures. This results in higher glucose yields for this mutant GA than for wild-type GA.

The mutation giving 311–314 Loop GA decreased specific activity by about 40%, with ratios of initial rates of iG₂ formation to that of glucose formation about 50–70% of those of wild-type GA. Introducing a long sequence into the Gly137→Ala active-site environment increases the binding affinity for both α-1,4- and α-1,6-linked substrates because the conformational change may reduce the binding energies required to form the enzyme–substrate complex. This allows the substrate to progress into subsite −1 more easily while undergoing a slow unimolecular enzyme–substrate complex migration step. A ‘dead-end pocket’ model of the GA active site was proposed based on X-ray structural studies, suggesting that water displacement from subsite −1, where a cluster of seven water molecules exists in the absence of substrate, is a crucial step in the GA catalytic mechanism (Aleshin et al., 1994). The conformational change by the 311–314 Loop mutation may also help to accommodate the simultaneous transport of water molecules as the substrate enters in or product diffuses from subsite −1. Product release after hydrolysis, the proposed rate-limiting step for maltooligosaccharide hydrolysis (Kitahata et al., 1981; Natarajan and Sierks, 1996; Sierks and Svensson, 1996; Sierks et al., 1997), may be relatively easier and faster than from wild-type GA because of the bigger loop introduced around the active site and this may make iG₂ synthesis less likely. The 311–314 Loop GA is less thermostable than most other GAs, so it is of interest to combine this mutation with another conferring thermostability, assuming that mutational effects will be additive (Matsumura et al., 1986; Wells, 1990).

Tyr312→Trp GA was made to study the importance of the Tyr311–Glu400–Tyr48 hydrogen-bond network on substrate affinity without destroying it. The indole side chain of Trp is larger and stiffer than the Tyr side chain while both side chains are aromatic, so a Tyr312→Trp mutation is a good choice to make this hydrogen-bond network narrower and to form a stronger affinity with substrate. Tyr312→Trp GA had slightly higher ratios of initial rates of iG₂ formation to that of glucose formation (Table II), with peak glucose yields about 0.5% lower, 0.4% higher and 2% higher than for wild-type GA at 35, 45 and 55°C, respectively (Figure 7).

Ser346→Pro had a lower initial rate ratio for iG₂ formation over glucose formation than wild-type GA and had an increased glucose yield only at 55°C. The space-filling effect engendered by inserting the Pro rigid backbone into a random coil in a
packing void of unknown function causes more hydrophobic interaction at higher temperatures, thus reducing the conformational entropy of unfolding. This entropy decrease helps GA to make a conformational adjustment favoring an $\alpha$-1,4-linked substrate, the same as it did for Ser30$\rightarrow$Pro mutant GA.

Based on the results from the above thermostable and thermosensitive mutant GAs, enzyme selectivity can be dramatically altered not only by mutations around the active site that directly involve catalytic or substrate-binding mechanisms, but also by mutations made away from the active site that may cause global entropy and conformational changes. A very dynamic view of GA selectivity is arising, not only from the mutated GAs studied here, but also from double-loop active-site mutations of Fierobe et al. (1996). These results, coupled with the identification of GA folding units and their role in activity and thermostability (Coutinho and Reilly, 1997) may
lead to a new approach in designing more industrially effective GAs.

The increased magnitude of the transition-state energy associated with a loss of a hydrogen bond between a substrate and an uncharged group is about 2.1–6.3 kJ/mol, while that caused by loss of a hydrogen bond between a substrate and a charged group is about 14.6–18.8 kJ/mol (Fersht et al., 1985). Lys108→Arg GA had the highest transition-state energy for all the α-1,4-linked substrates (0.9–3.1 kJ/mol), with that for iG2 being negative (~3.0 kJ/mol), meaning that the higher surface charge introduced by this mutation destabilizes GA–maltoligosaccharide complexes owing to the redistribution of the unbalanced charge of this complex, while it stabilizes the GA–iG2 complex because of the tighter hydrogen bonding in the transition state. However, no hydrogen bond is formed or destroyed by this mutation. The small Δ(AG) values for all the other mutant GAs (~0.4 to 1.9 kJ/mol) indicate that their mutations cause only minor effects on substrate binding in the transition state.

With one exception, Tyr312→Trp GA, only those mutated GAs with very low (Lys108→Arg GA) and very high (311–314 Loop GA) activation energies for iG2 formation were located around the active site. This suggests that the activation energy can be affected differently by the mutations located near or distant from the active site. Most of the mutations in this work decreased the activation energy for the condensation reaction to a greater degree than for the hydrolysis reaction, with the largest differences between the two being caused by mutations substituting Pro and Ala residues.

The hypothesis that decreasing the ability of GA to synthesize iG2 would increase the glucose yield was proved by Fang et al. (1998a,b). In this study, we have also proved this hypothesis at 35, 45 and 55°C with a different group of mutated GAs. Figure 7 demonstrates an inverse relationship between peak glucose yields and relative initial rate ratios for iG2 formation over glucose formation. Mutant GAs that favor iG2 formation have peak glucose yields that are usually higher with DE 18 and DE 25 maltodextrins than with DE 10 maltodextrin (Figure 7), opposite to that observed with native GA (Lee et al., 1976) and often with mutant GAs with low ratios of initial rate of iG2 formation to that of glucose formation. Because of the single-chain hydrolysis mechanism of GA, it takes more time for longer glucosyl chains to be degraded into glucose than shorter ones. Even though there are 2.5 times as many chains to be degraded in DE 25 maltodextrin as there are in DE 10 maltodextrin, there are one-fifth to one-sixth fewer glucosidic bonds in the glucosyl chains to be digested in DE 25 maltodextrin than in DE 10 maltodextrin of the same mass concentration. The initial rates for DE 25 maltodextrin hydrolysis are often substantially higher than for DE 10 maltodextrin, but with no significant difference between them at high substrate concentration even though the catalytic efficiency is higher for longer than for shorter DP substrates at low substrate concentrations. The higher initial glucose concentration in DE 25 maltodextrin (7.4% compared with 0.6 and 0.9% in DE 10 and DE 18 maltodextrins, respectively) allows the peak glucose yield to be reached about 18–24 h faster and higher for DE 25 than for DE 10 and 18 maltodextrins. This is especially true for those mutant GAs that favor iG2 formation because the iG2 formation is already very significant in the 18–24 h interval before glucose yield reaches its peak, thus decreasing the maximal peak attainable.

Table II shows that different reaction temperatures result in different selectivities for mutant and wild-type GAs. For instance, the ratio of initial rate of glucose formation to that of iG2 formation for Ser436→Pro GA is much higher that that of wild-type GA at 35°C, whereas it is lower at 55°C. Also, glucose yields tended to decrease with increasing temperature for wild-type and most mutant GAs. This result agrees with the catalytic efficiency ratios calculated by Coutinho (1996) from literature data on A. awamori/A. niger GA, in which ratios of rates of iG2 synthesis to those of G2 hydrolysis increased with increasing temperature whereas the ratios of rates for maltoligosaccharide hydrolysis to those of G2 hydrolysis decreased.

Three thermostable mutant GAs studied here, SS, Gly137→Ala (at 45 and 55°C) and Ser436→Pro (at 55°C) GAs, had better selectivity for α-1,4 bonds than wild-type GA, while one, Ser436→Pro GA (at 35 and 45°C), had more selectivity for α-1,6 bonds and one, Ser30→Pro GA, had about the same selectivity. On the other hand, one thermosensitive mutant GA, 311–314 Loop GA, had better selectivity for α-1,4 bonds, while two, Ala27→Pro and Lys108→Arg GAs, had better selectivity for α-1,6 bonds and one, Tyr312→Trp GA, had about the same selectivity as wild-type GA. Even though we found no general correlation between thermostability and substrate specificity, thermostable mutants in the most hydrophobic folding unit (Coutinho and Reilly, 1997) tend to have a positive role in reducing the α-1,6-activity of GA, the reverse being observed for thermosensitive mutants.

In the most hydrophobic folding unit, the region between the C-terminus of α-helix 1 and the following extended loop between α-helices 1 and 2 appears to be critical for irreversible thermoactivation based on mutational analysis (Li, 1996, Li et al., 1997; Allen, 1997). Analysis of the GA models also shows that this extended loop is located on the surface of catalytic domain and is somewhat thermolabile. It is of great potential in the future to investigate the contribution of this region to thermostability and selectivity by more single, combined or deletion mutation studies.

In conclusion, we have successfully reduced iG2 formation in the glucose condensation reaction and therefore increased glucose by the SS and 311–314 Loop mutations and in part with the Gly137→Ala mutation. We also have proved by the Lys108→Met and Lys108→Arg mutations that conserved residue Lys108 is important in both catalytic mechanism and substrate binding. The goal to form a narrower Glu400–Tyr48–Trp 311–314 Loop GA, hydrolysis by GA, with glucose yields being higher than for DE 25 maltodextrin hydrolysis by GA, with glucose yields being higher and relative iG2 formation rates being lower at lower temperatures.

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References
