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Replacement and deletion mutations in the catalytic domain and belt region of *Aspergillus awamori* glucoamylase to enhance thermostability

Hsuan-Liang Liu, Yann Doleyres, Pedro M.Coutinho, Clark Ford[1] and Peter J.Reilly[2]

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

[2]To whom correspondence should be addressed. E-mail: reilly@iastate.edu

Three single-residue mutations, Asp71→Asn, Gln409→Pro and Gly447→Ser, two long-to-short loop replacement mutations, Gly23-Ala24-Asp25-Gly26-Ala27-Trp28-Val29-Ser30→Asn-Pro-Pro (23–30 replacement) and Asp297-Ser298-Glu299-Ala300-Val301→Ala-Gly-Ala (297–301 replacement) and one deletion mutation removing Glu439, Thr440 and Ser441 (Δ439–441), all based on amino acid sequence alignments, were made to improve *Aspergillus awamori* glucoamylase thermostability. The first and second single-residue mutations were designed to introduce a potential *N*-glycosylation site and to restrict backbone bond rotation, respectively, and therefore to decrease entropy during protein unfolding. The third single-residue mutation was made to decrease flexibility and increase *O*-glycosylation in the already highly *O*-glycosylated belt region that extends around the globular catalytic domain. The 23–30 replacement mutation was designed to eliminate a very thermolabile extended loop on the catalytic domain surface and to bring the remainder of this region closer to the rest of the catalytic domain, therefore preventing it from unfolding. The 297–301 replacement mutant GA was made to understand the function of the random coil region between α-helices 9 and 10. Δ439–441 was constructed to decrease belt flexibility. All six mutations increased glucoamylase thermostability without significantly changing enzyme kinetic properties, with the 23–30 replacement mutation increasing the activation free energy for thermoinactivation by about 4 kJ/mol, which leads to a 4°C increase in operating temperature at constant thermostability.

Keywords: *Aspergillus awamori*/belt region/catalytic domain/glycoamylase/mutations/thermostability

Introduction

Glucoamylase [α-(1,4)-D-glucan glucohydrolase, EC 3.2.1.3] (GA) is an industrially important, multidomain, *N*- and *O*-glycosylated enzyme that produces glucose from starch dextrin. A mutant GA with higher thermostability would be desirable because it could be used at higher temperatures, decreasing the required reaction time because of its higher activity.

GA undergoes irreversible thermoinactivation at moderately acidic pH and 70°C (Munch and Tritsch, 1990). Neither deamidation (Chen *et al.*, 1994a,b) nor Asp–X peptide bond hydrolysis (Chen *et al.*, 1995a) determines the rate during GA thermoinactivation at 70°C and above, confirming earlier work (Munch and Tritsch, 1990). Aggregation and precipitation occur after the rate-determining step, because GA thermoinactivation follows first-order kinetics (Munch and Tritsch, 1990). Mismatched disulfide bonds are not found at 70°C and pH 3.5 and 4.5, which means that some other mechanism must control thermoinactivation at high temperatures. This mechanism appears to be a change in the protein structure such as unfolding that destroys the integrity of the active site (Munch and Tritsch, 1990; Williamson *et al.*, 1992b; Chen *et al.*, 1994a,b).

Knowledge of amino acid sequence homology and three-dimensional structures allows the prediction of important roles of specific amino acid residues on enzyme catalytic mechanism, thermostability, substrate selectivity and structure–function relationships. The primary sequences of archaeal, eubacterial and fungal GAs were aligned by Coutinho and Reilly (1994a,b, 1997) and have served along with the GA catalytic domain three-dimensional structure (Aleshin *et al.*, 1992) as important guides for various protein engineering studies of GA (Ford, 1999; Reilly, 1999).

Mutant GAs have been constructed to increase thermostability by eliminating thermoinactivation sites, by increasing α-helix stability, by introducing additional disulfide linkages and by substituting Pro residues. Specifically, the *Aspergillus awamori*/*Aspergillus niger* (GAs from the two species are identical) Asn182→Ala and Asn182→Asp mutations increased GA thermostability up to 70°C, with specific activities similar to wild-type GA and the second with about 25% lower specific activity (Chen *et al.*, 1994a,b). Mutations Asp257→Gln, Asp293→Gln and Asp293→Glu gave slightly increased thermostability below 70°C at pH 4.5 with retention of about three-quarters of wild-type specific activity (Chen *et al.*, 1995a). Mutations Gly137→Ala, Gly139→Ala and Gly137→Ala/Gly139→Ala, designed to stiffen α-helix 4, significantly increased thermostability at pH 4.5 well above 70°C without loss of specific activity, although performance at other pHs varied (Chen *et al.*, 1996). Mutation Ala246→Cys, proposed to create a disulfide linkage with Cys320, increased thermostability with retention of 27 and 85% of wild-type activity with maltose and maltoheptaose substrates, respectively (Fierobe *et al.*, 1996). Double mutation Asn20→Cys/Ala27→Cys was designed to create a disulfide bond on the catalytic domain surface and successfully stabilized GA against unfolding (Li *et al.*, 1998). Mutations Ser436→Pro (Li *et al.*, 1997) and Ser30→Pro (Allen *et al.*, 1998) stabilized GA by reducing the conformational entropy of unfolding. In general, incorporating several beneficial mutations in one GA further increased thermostability and specific activity (Allen *et al.*, 1998; Li *et al.*, 1998).

This is one paper of a series (Chen *et al.*, 1994a,b, 1995a, 1996; Li *et al.*, 1997, 1998; Allen *et al.*, 1998) on site-directed mutagenesis of *A.awamori* GA to improve GA thermostability. It reports the construction and performance of three single-residue mutations, Asp71→Asn, Gln409→Pro and Gly447→Ser, two loop-shortening replacement mutations, Gly23-Ala24-Asp25-Gly26-Ala27-Trp28-Val29-Ser30→Asn-Pro-Pro (23–30 replacement) and Asp297-Ser298-Glu299-

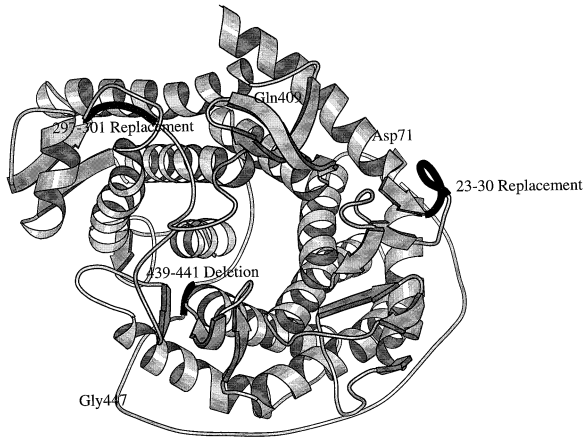

Fig. 1. View of *A.awamori* GA, showing the mutations made in the enzyme. Replacement and deletion mutations are denoted by black shading. The mutated residues Asp71, Gln409 and Gly447 are located on a short loop between α-helices 3 and 4, a short β-strand between α-helices 12 and 13 and the belt, respectively.

Ala300-Val301→Ala-Gly-Ala (297–301 replacement) and one deletion mutation removing Glu439, Thr440 and Ser441 (Δ439–441) designed by comparison with other GAs (Coutinho and Reilly, 1997). Figure 1 shows where each mutation occurs on the enzyme.

Materials and methods

Materials

YEpPM18, a yeast expression vector carrying the wild-type GA cDNA and the *Saccharomyces cerevisiae* strain C468 (α *leu2-3 leu2-112 his3-11 his3-15 mal-*) (Innis *et al.*, 1985) was a generous gift from Cetus. All restriction enzymes were purchased from Promega. Acarbose was a gift from Miles Laboratories. Peptide-*N*-glycosidase F was purchased from Oxford GlycoSystems.

Site-directed mutagenesis

Site-directed mutagenesis was performed with the Promega Altered Sites II *in vitro* mutagenesis system. An *Xba*I–*Hin*dIII fragment of pGEM-GA containing the wild-type GA cDNA (Fang and Ford, 1998) was inserted into the Promega pALTER-1 vector, making a GA cDNA-containing vector to be used as the double-stranded DNA template in the Altered Sites II system (Fang *et al.*, 1998b). The following mutagenic oligonucleotide primers were synthesized at the Iowa State University Nucleic Acid Facility: 5′-GCT CGT ACT ATC CTG AAT AAC ATC **AAT CCG CCC** GGC GCG GAC TCT GGC ATT GTC GTT GCT-3′ (23–30 replacement), 5′-CTC TTC CGA AAT GGA **A**AT ACC AGT CTC CTC T-3′ (Asp71→Asn), 5′-TAT ACC CTC AAC GAT GGT CTC AGT **GCT GGA GCT** GCG GTG GGT CGG TAC CCT GAG GAC-3′ (297–301 replacement), 5′-AG TCT GAT GGC GAG C**C**G CTT TCC GCT CGC GA-3′ (Gln409→Pro), 5′-GTC GTG CCT GCT TCT TGG GGC --- --- --- GCC AGC AGC GTG CCC GGC ACC TGT-3′ (439–441 deletion) and 5′-GCC AGC AGC GTG CCC **A**GC ACC TGT GCG GCC A-3′ (Gly447→Ser), the bold letters and the dashed lines indicating the changed and deleted nucleotides, respectively. All mutations were verified by DNA sequencing.

Expression and purification of GA

GA genes containing mutations were subcloned into the yeast expression vector YEpPM18 and transformed into *S.cerevisiae* strain C468 by electroporation, as described previously (Chen *et al.*, 1994b). Then mutant and wild-type GAs were produced in shaker flasks at 30°C and 170 r.p.m. for 5 days without pH control. The cultures were centrifuged to remove the yeast cells and then the culture supernatants were concentrated, dialyzed against 0.5 M NaCl–0.1 M NaOAc buffer (pH 4.4) with a 10 kDa cut-off Amicon S1 spiral ultrafiltration cartridge and applied to acarbose-Sepharose affinity chromatography for purification (Chen *et al.*, 1994b). SDS–PAGE that demonstrated enzyme homogeneity was carried out using 0.75 mm thick polyacrylamide gels (Garfin, 1990).

Specific activities and kinetic parameters

GA specific activity at 50°C and pH 4.4 was determined with 4% (w/v) maltose (Fang *et al.*, 1998b). One unit (IU) was defined as the amount of enzyme required to produce 1 μmol/min of glucose under the conditions of the assay. Values of k_{cat} and K_M on maltose were obtained at 45°C and pH 4.4 in 0.05 M NaOAc buffer (Fang *et al.*, 1998a). Enzyme concentrations were determined by the Pierce bicinchoninic acid protein assay, using bovine serum albumin as the standard.

Irreversible thermoinactivation

Wild-type and mutant GAs (0.475 μM) were incubated for 12 min in 0.05 M NaOAc buffer, pH 4.4, at six temperatures between 65 and 77.5°C. 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 50°C (Liu *et al.*, 1998). The inactivation rate coefficients (k_d) for mutant and wild-type GAs were obtained from a semilogarithmic plot of residual activity versus inactivation time. The activation free energies ($\Delta G^{\ddagger}$) for thermoinactivation of mutant and wild-type GAs were obtained from a semilogarithmic plot of k_d/T versus $1/T$.

De-N-glycosylation of Asp71→Asn mutant and wild-type GAs

Portions of 20 μg of Asp71→Asn mutant and wild-type GAs were added to 10 μl of 5× incubation buffer supplemented with 0.5% SDS and 5% β-mercaptoethanol, followed by denaturation of these two glycoproteins by heating at 100°C for 2 min. After the mixtures had cooled to room temperature, 15 μl of peptide-*N*-glycosidase F were added to the two mixtures and each was incubated for 18 h at 37°C.

MALDI mass spectra

Asp71→Asn mutant and wild-type GAs, with and without de-*N*-glycosylation, were submitted to the Protein Facility at Iowa State University to obtain MALDI mass spectra. Samples were dispersed in a large excess of a matrix material containing a chromophore for the laser light.

Results

GA specific activity and kinetics

The enzymatic properties of mutated and wild-type GAs are listed in Table I. Specific activities and catalytic efficiencies (k_{cat}/K_M) for all the mutated GAs are slightly higher than those of wild-type GAs, except for the Asp71→Asn mutated GA, which has the lowest catalytic efficiency, and the GA with mutation Δ439–443, which has the lowest specific activity. There is a positive correlation between specific activity and catalytic efficiency.

GA thermostability

The irreversible thermoinactivation of wild-type and mutated GAs follows first-order kinetics. Figure 2 shows the effect of

Table I. GA specific activities at 50°C and pH 4.4 using 4% maltose substrate and GA kinetic parameters on maltose at 45°C and pH 4.4, both in 0.05 M NaOAc buffer

GA form	Specific activity (IU/mg GA)	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} mM^{-1})
Wild-type	20.6 ± 0.2[a]	8.67 ± 0.17	0.72 ± 0.03	12.0
Asp71→Asn	19.6 ± 0.6	7.59 ± 0.11	0.78 ± 0.04	9.7
Gln409→Pro	21.9 ± 0.8	8.71 ± 0.22	0.68 ± 0.02	12.8
Gly447→Ser	21.5 ± 0.9	9.13 ± 0.19	0.71 ± 0.04	12.9
23–30 replacement	23.4 ± 0.7	9.32 ± 0.29	0.70 ± 0.05	13.3
297–301 replacement	22.2 ± 0.8	9.38 ± 0.20	0.76 ± 0.06	12.3
Δ439–441	18.4 ± 0.5	8.13 ± 0.13	0.69 ± 0.05	11.8

[a]Standard error.

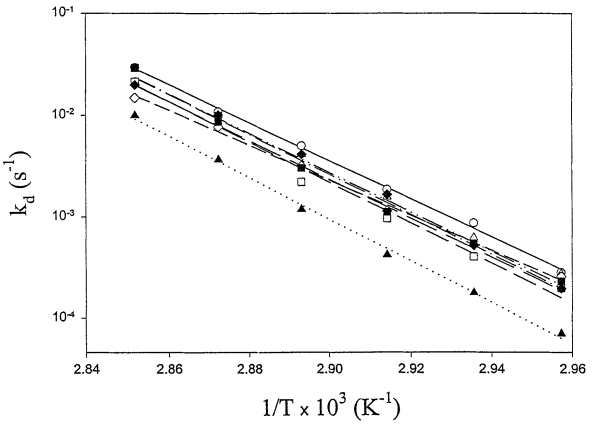

Fig. 2. Effect of temperature on thermostability of *A.awamori* GA at pH 4.4. Symbols: wild-type (○,—), Asp71→Asn (△, – ·· –), Gln409→Pro (□, — —), Gly447→Ser (◇, – –), 23–30 replacement (▲, ...), 297–301 replacement (◆, – –), Δ439–441 (■, – · –).

Table II. Changes in the activation free energy for thermoinactivation ($\Delta\Delta G^{\ddagger}$) at 65 and 75°C for mutated GAs relative to wild-type GA

GA form	$\Delta\Delta G^{\ddagger}$ (kJ/mol)	
	65°C	75°C
Asp71→Asn	0.5	0.3
Gln409→Pro	1.8	1.2
Gly447→Ser	0.7	1.5
23–30 replacement	4.4	3.5
297–301 replacement	1.2	0.7
Δ439–441	1.0	0.7

temperature on k_d for wild-type and mutated GAs, and Table II shows the changes of the activation free energies for thermoinactivation ($\Delta\Delta G^{\ddagger}$) for mutated GAs relative to wild-type GA at 65 and 75°C. All the mutated GAs have positive $\Delta\Delta G^{\ddagger}$ values, meaning that they all are more thermostable than wild-type GA at both temperatures. The 23–30 replacement mutation gave the highest positive $\Delta\Delta G^{\ddagger}$ values of all the mutated GAs at both 65 and 75°C and therefore yielded the most thermostable mutated GA. All the mutated GAs except that with mutation Gly447→Ser have lower $\Delta\Delta G^{\ddagger}$ values at 75 than at 65°C, indicating that their thermostabilities are less increased over that of wild-type GA by mutation at the higher temperature.

De-N-glycosylation and MALDI mass spectra

MALDI mass spectra of untreated and de-*N*-glycosylated Asp71→Asn mutant and wild-type GAs showed that the molecular weights of the two forms decreased by 2.2 and 2.3 kDa, respectively, upon *N*-deglycosylation. This suggests that Asp71→Asn mutant GA is as *N*-glycosylated as wild-type GA. Therefore, this mutation does not cause *N*-glycosylation of Asn71 as was possible. These results also confirm that the two *N*-glycosylation sites of wild-type GA have ~13 sugar residues, as found by X-ray crystallography (Aleshin *et al.*, 1994).

Discussion

Of the three potential *N*-glycosylation sites in *Aspergillus* GAs, Asn171 and Asn395 but not Asn182 are *N*-glycosylated in *A.niger* (Svensson *et al.*, 1983), *A.awamori* var. *X100* (Aleshin *et al.*, 1992) and *A.awamori* GA, the latter expressed in *S.cerevisiae* (Chen *et al.*, 1994b). GAs from the first two sources have β-C1-linked *N*-acetyl-D-glucosamine residues initiating five- and eight-residue carbohydrate chains on Asn171 and Asn395, respectively (Aleshin *et al.*, 1994). Mutation Asn171→Ser GA has very similar catalytic efficiencies for malto- and isomaltooligosaccharide hydrolysis and very similar thermostability to wild-type *A.niger* GA (Stoffer *et al.*, 1997), while mutation Asn395→Gln dramatically decreases secretion as well as thermostability without changing specific activity (Chen *et al.*, 1994b), meaning that different *N*-glycosylation sites in the catalytic domain may play different roles in GA expression and unfolding.

In the present study, Asp71 between α-helices 2 and 3 in the *A.awamori* GA catalytic domain was replaced by Asn because GAs from *Neurospora crassa*, *Hormoconis resinae*, *Humicola grisea*, *Rhizopus oryzae* and *Saccharomycopsis fibuligera* all have Asn residues potentially involved in *N*-glycosylation at this position (Coutinho and Reilly, 1994b, 1997). This mutation did not yield *N*-glycosylation, but did slightly increase thermostability compared with wild-type GA while slightly decreasing specific activity.

A second single mutation was the replacement of Gln409 in *A.awamori* GA by Pro because GAs from *N.crassa*, *H.resinae*, *H.grisea*, *Arxula adeninivorans*, *Methanococcus jannaschii* and *Clostridium* sp. have Pro at this position (Coutinho and Reilly, 1997). Pro residues restrict backbone bond rotation because of their pyrrolidine rings and therefore they are believed to decrease entropy during protein unfolding by reducing the numbers of unfolded conformations that can be

sampled by the protein (Matthews *et al.*, 1987). Suzuki *et al.* (1989, 1991) proposed that increased numbers of Pro residues often correlate with increased protein thermostability. In general, the criteria used for the successful replacement of amino acid residues by Pro are that their values of ϕ, the dihedral angle of the Cα–N bond, be $-80° < \phi < -60°$ (Balaji *et al.*, 1989) and that they be located in turns or random coils, the favored locations of Pro residues (Nicholson *et al.*, 1992; Ueda *et al.*, 1993).

The effect of the Gln409→Pro mutation on GA thermoinactivation shows that the backbone rigidity, greater space filling and more hydrophobic interactions introduced by substituting Pro can help to prevent GA from unfolding under elevated temperatures.

Mutation Gly447→Ser GA was constructed to decrease flexibility and increase *O*-glycosylation in the already highly *O*-glycosylated belt region that extends around the catalytic domain between residues 441 and 470. The absence of a side chain in Gly gives the peptide backbone much greater conformational flexibility; therefore, our purpose here was to investigate whether replacing Gly447 tended to limit possible conformations and therefore to stabilize GA. The replacement with Ser was also based on homology because GAs from *Aspergillus terreus*, *Aspergillus oryzae* and *H.grisea* all have a Ser residue in position 447 (Coutinho and Reilly, 1997).

The increased thermostability caused by this mutation supports previous findings (Evans *et al.*, 1990; Libby *et al.*, 1994) that the belt region plays a role in GA thermostability by stabilizing a critical hydrophobic folding subunit (Coutinho and Reilly, 1997) and that *O*-linked carbohydrates appear to increase the rigidity and decrease the thermal vibration of the GA polypeptide chain (Williamson *et al.*, 1992a,b). In addition, this mutated GA has slightly higher specific activity and catalytic efficiency than wild-type GA, suggesting that this mutation does not significantly alter the structure of the enzyme around its active site or its interaction with the substrate. This result was expected because the belt region is located on the surface of the catalytic domain and does not play an important role in the GA catalytic mechanism.

Deletion mutations have been made to analyze the role of the belt, linker and starch-binding domain of *A.awamori* GA (Evans *et al.*, 1990; Libby *et al.*, 1994; Chen *et al.*, 1995b). In this study, we constructed two long-to-short loop replacement mutations and one deletion mutation in the same GA to improve its thermostability. A short-to-long loop replacement mutation had already been performed (Liu *et al.*, 1998), leading to a more selective but less active GA.

Previously made mutations suggest that the extended loop on the catalytic domain surface between α-helices 1 and 2 is critical for irreversible thermoinactivation and substrate selectivity of GA (Allen *et al.*, 1998; Li *et al.*, 1998; Liu *et al.*, 1998, 1999). It belongs to the most hydrophobic folding unit, which appears to influence strongly GA thermostability (Coutinho and Reilly, 1997). To investigate further the role of the loop and the region around it, a replacement mutation involving residues 23–30 was constructed to mimic the short amino acid sequence from *R.oryzae* GA. Previous studies led to two thermostable mutations, Asn20→Cys/Ala27→Cys (Li *et al.*, 1998) and Ser30→Pro (Allen *et al.*, 1998) and one thermosensitive mutation, Ala27→Pro (Li *et al.*, 1997), in this region. This extended loop exhibits a larger flexibility than its adjacent regions, shown by *B*-factors in *A.awamori X100* GA crystallographic studies (Aleshin *et al.*, 1992, 1994), and takes a different position in the *S.fibuligera* GA structure (Sevčik *et al.*, 1997). The 23–30 replacement mutation was designed to eliminate the long loop, tightening this region together with the rest of the catalytic domain and therefore preventing it from unfolding.

The 23–30 replacement mutation increased specific activity slightly compared with wild-type GA and led to the most thermostable mutant GA constructed in this study, giving an ~4°C increase in operating temperature at constant thermostability. The current study further highlights the importance of this loop region on GA thermostability.

Residues Asp297 to Val301 occur in the random coil region of unknown function between α-helices 9 and 10. To understand the function of this region, we replaced residues 297–301 with three residues, Ala, Gly and Ala, that mimic the amino acid sequence of *S.fibuligera* GA (Coutinho and Reilly, 1994a). The specific activity of this GA is only 20% that of *A.niger* GA on soluble starch (Solovicová *et al.*, 1996). Hence this region is perhaps important for GA specific activity and thermostability.

The 297–301 replacement mutation slightly increased specific activity and thermostability compared with wild-type GA, meaning that GA thermostability was increased by bringing two α-helices closer together to increase unfolding entropy. The GA catalytic domain is composed of 13 α-helices (52% of all its amino acid residues), with 12 of the helices folding into an unusual α/α barrel. Many of the remaining amino acids form β-sheets to link these α-helices (Aleshin *et al.*, 1992). The random coils between different α-helices may also play an important role in GA thermostability. For instance, mutation Ser436→Pro, changing a residue located in a random coil of unknown function, strongly decreases the entropy of unfolding by occupying more space and therefore enhancing hydrophobic interactions in this void (Li *et al.*, 1997).

Deletion mutation Δ439–441 was made to decrease belt flexibility and *O*-glycosylation. This particular deletion was made because *Corticium rolfsii* GA does not have these three amino acids. A change in stability of this mutant GA should tell us more about the role played by the belt region in GA thermostability. Mutations in the belt region are interesting because they should not affect the center of the catalytic domain and thus should not modify specific activity. The belt region is important because this highly *O*-glycosylated domain may be involved in GA secretion, since the catalytic domain cannot be expressed extracellularly without this belt region (Libby *et al.*, 1994). In addition, its presence may prevent the catalytic domain from unfolding at higher temperatures.

Mutation Δ439–441 increases GA thermostability more at lower than at higher temperatures. Three different effects may be involved. Reducing belt flexibility should decrease enzyme unfolding at all temperatures. However, the deletion of the three amino acids brings Gly438 adjacent to Ala442, possibly causing Gly–Ala bond hydrolysis as seen previously (Chen *et al.*, 1995a). Furthermore, the loss of two *O*-glycosylation sites caused by the deletion may partially counteract increased belt rigidity. Overall, both the Δ439–441 and Gly447→Ser mutations strengthen the structure of the belt region of *A.awamori* GA and decrease unfolding of the catalytic domain.

In summary, we have successfully increased GA thermostability by three single-point, two replacement and one deletion mutation. The 23–30 replacement mutation is the most thermostable mutation yet obtained in GA, along with the Ser30→Pro/

Gly137→Ala and Asn20→Cys/Ala27 →Cys/Ser30→Pro/Gly-137→Ala mutations (Allen *et al.*, 1998), which also contain mutated residues in this part of GA. Therefore, the extended loop region on the catalytic domain surface between α-helices 1 and 2 plays a very important role in determining GA thermostability as well as substrate selectivity. The highly *O*-glycosylated belt region on the catalytic domain surface between residues 440 and 471 also has an important role in the mechanism governing the irreversible thermoinactivation of GA; this result strongly agrees with previously suggested functions of the *O*-glycosylation region, which include protecting GA from thermal denaturation. Amino acid sequence homology provides direct and important information in predicting the functions of some important amino acid residues.

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