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Effect of replacing helical glycine residues with alanines on reversible and irreversible stability and production of *Aspergillus awamori* glucoamylase

Hsiu-mei Chen, Yuxing Li[1], Tapobrata Panda[2], Frédéric U.Buehler, Clark Ford[3] and Peter J.Reilly[4]

Departments of Chemical Engineering, [1]Zoology and Genetics and [3]Food Science and Human Nutrition, Iowa State University, Ames IA 50011, USA

[2]Permanent address: Department of Chemical Engineering, Indian Institute of Technology, Madras, India

[4]To whom correspondence should be addressed

To decrease irreversible thermoinactivation of *Aspergillus awamori* glucoamylase, five Gly residues causing helix flexibility were replaced with Ala residues. Mutation of Gly57 did not affect thermostability. Mutation of Gly137 doubled it at pHs 3.5 and 4.5 but barely changed it at pH 5.5. The Gly139→Ala mutation did not change thermostability at pH 3.5, improved it at pH 4.5 and worsened it at pH 5.5. The Gly137/Gly139→Ala/Ala mutation gave 1.5–2-fold increased thermostabilities at pHs 3.5–5.5. Mutations of Gly251 and Gly383 decreased it at all pHs. Gly137→Ala and Gly137/Gly139→Ala/Ala glucoamylases are the most stable yet produced by mutation. Guanidine treatment at pH 4.5 decreased the reversible stabilities of Gly137→Ala, Gly139→Ala and Gly137/Gly139→Ala/Ala glucoamylases at infinite dilution while not changing those of Gly251→Ala and Gly383→Ala glucoamylases, which is, in general, opposite to what occurred with thermoinactivation. Mutation of Gly57 greatly improved the extracellular glucoamylase production by yeast, that of Gly137 barely affected it and those of Gly139 and of both Gly137 and Gly139 strongly impeded it. These observations suggest that α-helix rigidity can affect reversible and irreversible glucoamylase stability differently, that the effects of multiple mutations within one α-helix to improve stability are not always additive and that even single mutations can strongly affect extracellular enzyme production.

Keywords: α-helix/alanine/glucoamylase/glycine/guanidine/production/thermostability

Introduction

Glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3, GA), which cleaves glucose from the non-reducing ends of starch and related oligosaccharides, is commercially used to produce high-glucose syrup and ethanol. *Aspergillus awamori* and *Aspergillus niger* GAs, which are identical (Svensson *et al.*, 1983; Nunberg *et al.*, 1984), are usually employed for this, but they suffer significant irreversible thermoinactivation at 60°C, the preferred industrial temperature. Therefore, the development of a more thermostable GA would be desirable.

The *A.awamori* GA gene has been cloned and expressed in *Saccharomyces cerevisiae* (Innis *et al.*, 1985). We have used site-directed mutagenesis to eliminate the potential deamidation of GA with the mutations Asn182→Ala, Asn182→Asp, Asn182→Gln and Asn395→Gln (Chen *et al.*, 1994a,b) and to eliminate peptide hydrolysis with the mutations Asp126→Glu, Gly127→Ala, Asp257→Glu, Asp293→Gln and Asp293→Glu (Chen *et al.*, 1995). The Asn182→Ala and Asp257→Glu mutations significantly reduced the irreversible thermoinactivation rate coefficients of GA at pH 4.5 at up to 70°C. However, above 70°C neither mutation stabilized GA because protein unfolding followed by irreversible formation of incorrect structures started to become dominant. Because the major secondary structures in the catalytic domains of GAs from different species are α-helices (Aleshin *et al.*, 1992; Coutinho and Reilly, 1994b), their stabilization to prevent protein unfolding at high temperatures may enhance GA thermostabilty.

Gly residues have the highest backbone conformational flexibility of any amino acid and therefore act as α-helix breakers in proteins (Chou and Fasman, 1974), while Ala residues stabilize α-helices more than any other amino acid (Chou and Fasman, 1974; Dao-Pin *et al.*, 1990; Zhang *et al.*, 1991; Heinz *et al.*, 1992). In addition, Gly residues in the helices of proteins from mesophilic species are generally replaced by Ala residues in the corresponding proteins from thermophilic species (Menéndez-Arias and Argos, 1989). Attempts to strengthen helices to increase the thermostabilities of λ repressor (Hecht *et al.*, 1986) and glucose isomerase (Quax *et al.*, 1991) have been successful; no such attempts had previously been made with GA.

In the present study, site-directed mutagenesis replaced five Gly residues with Ala residues on four different α-helices of *A.awamori* GA to restrict the GA conformation at high temperatures. We mutated Gly57, located near the beginning of the second α-helix of *A.awamori* GA (Figure 1a) and highly conserved as Ala in non-*Aspergillus* GAs, including a GA from the thermophilic *Humicola grisea* var. *thermoidea* (Coutinho and Reilly, 1994a). We made two single mutations and a double mutation at Gly137 and Gly139, in the middle of the fourth α-helix (Figure 1b). Mutations at Gly251 and Gly383 were in the middle of the eighth and twelfth α-helices respectively (Figure 1c and d). These four α-helices are part of an α,α-barrel of 12 helices that surround the *A.awamori* GA active site, with the second, fourth and eighth being part of the inner ring of helices and the twelfth being part of the outer ring (Aleshin *et al.*, 1992), so their stabilities can greatly affect the catalytic domain thermostability. The wild type and mutant GA genes were each expressed in *S.cerevisiae*, the secreted GAs were purified and the amounts of secreted protein, the specific activities and the irreversible thermoinactivation rate coefficients were measured. Finally, all but one GA form was denatured with different concentrations of guanidine hydrochloride (Gdn·HCl), and circular dichroism spectra were obtained at each concentration to determine the resistance to reversible denaturation that each mutation imparted.

Method

Materials

A laboratory strain of *S.cerevisiae*, C468 (α *leu2-3 leu2-112 his3-11 his3-15 mal-*) and its expression plasmid, YEpPM18,

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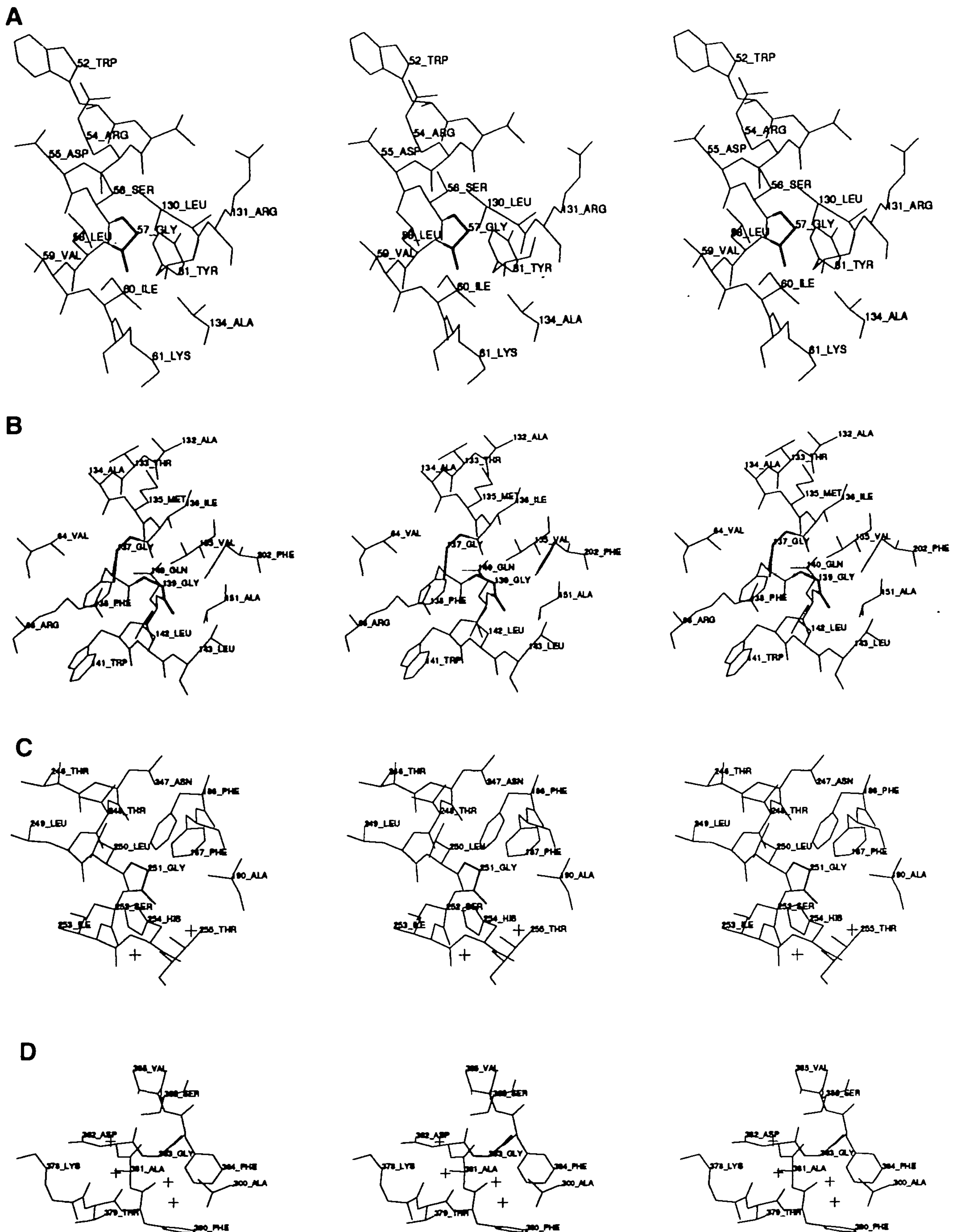

Fig. 1. 3-D structure of *A.awamori* var. *X100* GA in the vicinity of (a) Gly57, (b) Gly137 and Gly139, (c) Gly251 and (d) Gly383. All residues closer than 5 Å to these residues are displayed. Crosses signify water molecules.

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containing the wild type GA gene from *A.awamori* were generous gifts from Cetus Corporation. All restriction enzymes were obtained from Promega. Acarbose was a gift from Miles Laboratories.

Site-directed mutagenesis

Site-directed mutagenesis was performed using a Muta-Gene phagemid *in vitro* mutagenesis kit from Bio-Rad (Chen *et al.*, 1994b). The synthetic oligonucleotide mutagenic primers were TCGCGACTCTGCTCTCGTCCT (Gly57→Ala), ACTGCTATGATCGCTTTCGGGCAATGG (Gly137→Ala), ACTGCTATGATCGGCTTCGCGGAATGG (Gly139→Ala), ACTGCTATGATCGCCTTCGCCCAATGGCTGCTTG (Gly137/Gly139→Ala/Ala), CACCCTCCTGGCAAGCATCCACAC (Gly251→Ala) and CTTTCGCCGATGCCTTCGTCTCTATT (Gly383→Ala), the underlined nucleotides signifying the mutated Ala codons. The phagemids containing mutated GA genes were screened, sequenced, digested by restriction enzymes, subcloned into YEpPM18 and transformed into *S.cerevisiae* as described previously (Chen *et al.*, 1994b).

Enzyme production and purification

Wild type, Gly57→Ala and Gly137/Gly139→Ala/Ala GAs were produced in 10-l batches in a 15-l working volume fermentor at pH 4.5 and 30°C over 72 h using SD-His medium (Chen *et al.*, 1994a). Gly137→Ala and Gly139→Ala GAs were produced in 5.3-l batches in a 5-l working volume fermentor, but otherwise under identical conditions. Another wild type GA sample as well as Gly251→Ala and Gly383→Ala GAs were produced in 1-l shake flasks over 5 days at 170 r.p.m. and 30°C in SD-His medium without pH control. All broth supernatants were concentrated by ultrafiltration, diafiltered against 0.5 M NaCl–0.1 M NaOAc buffer at pH 4.4 and then subjected to acarbose–Sepharose affinity chromatography to purify GA (Chen *et al.*, 1994b).

Assays

Protein concentrations were determined by the Pierce bicinchoninic acid protein assay (Smith *et al.*, 1985) with a bovine serum albumin standard. The activities of those enzymes produced by fermentor were measured with 4% maltose as substrate in 0.05 M NaOAc buffer at pH 4.5 and 50°C (Chen *et al.*, 1994b), conditions under which no inactivation occurs during the assay. With the exception of specific activity measurement, those GAs produced by shake flask were assayed under the same conditions but at 30°C. One unit (IU) is the enzyme required to produce 1 μmol/min glucose at 50°C with the above substrate and buffer. Cell densities in a 10-mm pathlength cuvette were determined at 600 nm and the resulting total optical densities were the product of the number of times the suspensions had been diluted multiplied by the optical densities of the diluted suspensions.

Irreversible thermoinactivation

Purified enzymes, 40–80 μg/ml in 0.05 M NaOAc buffer at pH 3.5, 4.5 or 5.5, were incubated at five or six different temperatures, sometimes in duplicate. Samples were removed at six or seven different times, promptly placed in an ice bath and after 24 h at 0°C they were assayed for GA activity. Each GA was irreversibly inactivated by first-order kinetics, so the thermoinactivation rate coefficients, k_d were determined by linear regression in semi-logarithmic coordinates. Standard errors were usually less than 5% of their corresponding k_d values.

Table I. Specific activities and amounts of protein remaining after purification of GAs produced by 10-l batch fermentation of *S.cerevisiae*

GA form	Protein (mg)	Specific activity (IU/mg)
Wild type	68.1	18.9
Wild type[a]	–	20.6
Gly57→Ala	108.0	19.4
Gly137→Ala[b]	59.7	22.0
Gly139→Ala[b]	29.1	22.1
Gly137/Gly139→Ala/Ala	22.3	19.0
Gly251→Ala[a]	–	17.4
Gly383→Ala[a]	–	16.2

[a]Produced in shake flasks.
[b]Produced by a 5.3-l batch fermentation; protein production is normalized to 10 l.

Reversible denaturation with guanidine hydrochloride

GA (200 μg/ml) was denatured in 0.05 M NaOAc buffer containing 0–6 M Gdn·HCl at pH 4.5 for 48 h at 30°C, followed by 2 h at 25°C (Williamson *et al.*, 1992). After denaturation, the fractions of the enzyme remaining folded were determined by measuring the change of ellipticity with circular dichroism at 220 nm with a Jasco J720 spectropolarimeter at 25°C. These were used to find the equilibrium constants between the folded and unfolded forms and from them the free energies of unfolding, ΔG_u, at different [Gdn·HCl] levels. Linear extrapolation to zero [Gdn·HCl] gave $\Delta G_{u\ H_2O}$ values for the different GA forms (Pace, 1986). We did not use the alternate method of Kellis *et al.* (1989) to calculate $\Delta\Delta G_{u\ H_2O}$ because the significantly different slopes between different enzyme forms made it less trustworthy.

Results

GA production

Similar growth curves and glucose utilization rates were observed in fermentor cultures to produce wild type, Gly57→Ala and Gly137/Gly139→Ala/Ala. In all three cases, the cell optical densities at 70 h exceeded 18. No activity loss was observed for any of these GAs upon acarbose–Sepharose affinity chromatography. The amounts of purified GAs recovered from fermentation broths decreased in the order of Gly57→Ala > wild type > Gly137→Ala > Gly139→Ala > Gly137/Gly139→Ala/Ala (Table I).

GA specific activity

The specific activities at 50°C and pH 4.5 of purified mutated GAs are close to that of wild type GA (Table I).

GA thermostability

The irreversible thermoinactivation of all GAs was measured at 70°C and pHs 3.5, 4.5 and 5.5, with the resulting k_d values shown in Figure 2. The two wild type GAs, one produced in a fermentor and the other by shake flask, have similar thermostabilities at pH 4.5. Gly57→Ala GA has approximately the same thermostability as fermentor-grown wild type GA at all three pHs. Gly137→Ala GA is twice as stable as wild type GA at pHs 3.5 and 4.5 but is equally stable at pH 5.5. It is the only GA studied having its optimal pH for thermostability displaced below pH 4.5 and being more stable at pH 3.5 than at pH 5.5. Gly139→Ala GA is as stable as wild type GA at pH 3.5, more stable at pH 4.5 and less stable at pH 5.5. Gly137/Gly139→Ala/Ala GA is somewhat more stable than wild type GA at pH 3.5 and is twice as stable at pH 4.5 and 5.5. Serious decreases in thermostability occur at pHs 3.5

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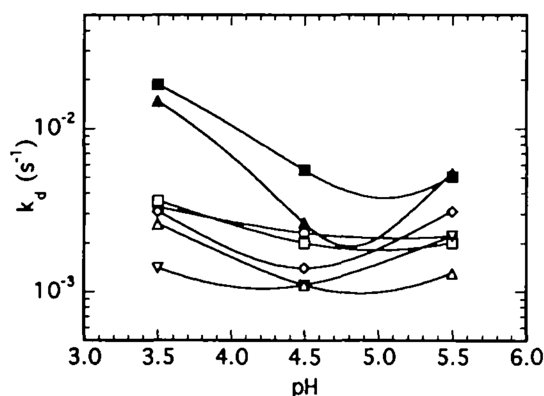

Fig. 2. Effect of pH on first-order thermoinactivation rate coefficients of (○,●) wild type, (□) Gly57→Ala, (▽) Gly137→Ala, (◇) Gly139→Ala, (△) Gly137/Gly139→Ala/Ala, (▲) Gly251→Ala and (■) Gly383→Ala GAs. Closed symbols indicate GAs produced by shake flask culture.

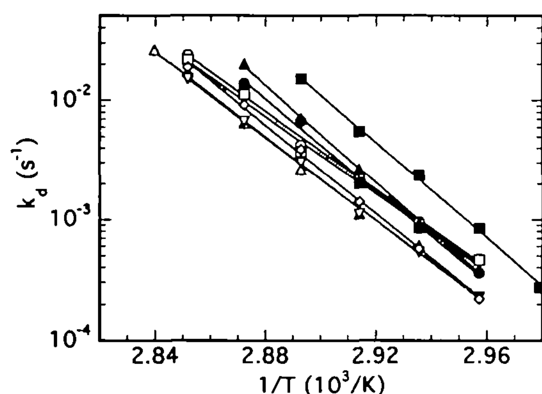

Fig. 3. Effect of temperature on first-order thermoinactivation rate coefficients of wild type and mutant GAs. Symbols as in Figure 2.

and 5.5 for Gly251→Ala GA and at all three pHs for Gly383→Ala GA.

The effect of temperature on the k_d values of all six GAs at pH 4.5 is shown in Figure 3. Wild type GAs produced by the fermentor and shake flask are equally stable near 65°C, but the former is slightly more stable at higher temperatures. The Gly57→Ala GA stability is unchanged from that of wild type GA at different temperatures as it is at different pHs. GAs with the single mutations Gly137→Ala and Gly139→Ala and the double mutation Gly137/Gly139→Ala/Ala are all more stable than wild type GA at pH 4.5, the first and third being equally stable through all temperatures and both being more stable than Gly139→Ala GA. Finally, the Gly251→Ala mutation destabilizes GA at pH 4.5 at higher temperatures, while the Gly383→Ala mutation severely destabilizes GA throughout the temperature range studied.

To test whether changes of pH affected the thermostability behavior at temperatures other than 70°C, k_d values were measured for Gly137→Ala and Gly139→Ala GAs at temperatures between 65 and 77.5°C and at pHs 3.5 and 5.5. Curves at those pHs are nearly parallel to curves at pH 4.5 of the same mutant GAs shown in Figure 3, suggesting that changing the pH does not change the thermoinactivation mechanism for these GA forms.

Activation enthalpies ($\Delta H^{\ddagger}$) and entropies ($\Delta S^{\ddagger}$) for thermoinactivation, calculated by the transition state theory through plots of ln k_d/T versus $1/T$, are shown in Table II for wild type and mutant GAs at pH 4.5 and, in addition, for Gly137→Ala and Gly139→Ala mutant GAs at pHs 3.5 and 5.5. The values of the free energies ($\Delta G^{\ddagger}$) at 65°C derived from them are a good measure of the relative thermostabilities of the different GAs at that temperature and the designated pHs.

GA denaturation with Gdn·HCl

Figure 4 and Table III show that Gly137→Ala, Gly139→Ala, Gly137/Gly139→Ala/Ala and Gly383→Ala GAs have higher values of $[Gdn{\cdot}HCl]_{50\%}$, where the enzyme is half unfolded and $\Delta G_u = 0$, than does wild type GA, which indicates that they are more stable at high values of [Gdn·HCl]. Gly251→Ala, on the other hand, has a slightly lower value of $[Gdn{\cdot}HCl]_{50\%}$ than does wild type GA. The linear nature of the ΔG_u versus [Gdn·HCl] curves of the different GA forms (Figure 4) indicates that unfolding of the catalytic and starch-binding domains occurred more or less to the same extent at the same values of [Gdn·HCl]. When values of ΔG_u were extrapolated to zero [Gdn·HCl] to give $\Delta G_{u\ H_2O}$, Gly137→Ala, Gly139→Ala and Gly139/Gly137→Ala/Ala GAs all had lower values than wild type GA, suggesting that they were less stable to reversible denaturation under optimal conditions than was wild type GA (Table III). On the other hand, Gly251→Ala and Gly383→Ala GAs had almost the same values of $\Delta G_{u\ H_2O}$ as wild type GA. These differences occurred because the slopes of the linear ΔG_u versus [Gdn·HCl] curves were much lower for the first three GA forms than for the others. The values of $\Delta G_{u\ H_2O}$ can be converted to the fractions of different GA forms that are unfolded at 25°C at infinite dilution (Table III). These range from approximately 10^{-5} for wild type and Gly251→Ala GAs to 3×10^{-3} for Gly139→Ala GA.

Discussion

GA production

As in this work, Chen *et al.* (1994a,b, 1995) and Flory *et al.* (1994) observed changes in extracellular GA production from mutations of non-glycosylated sites of *A.awamori* GA expressed in *S.cerevisiae*, suggesting that single mutations can affect the net protein production. However, no general correlation between the extracellular GA production and either the thermostability or specific activity or between the latter two factors is apparent from data gained here and earlier.

In the two mutated GAs, Gly139→Ala and Gly137/Gly139 →Ala/Ala, where major decreases in extracellular enzyme production were observed, the mutations may strengthen the fourth α-helix and make folding of the newly synthesized mutant polypeptide chain more time-consuming than folding of the helix in wild type GA. If this occurs, some of the unfolded mutant chain may not be processed correctly, but instead may be degraded intracellularly before glycosylation, resulting in a low net production. In addition, retardation of the biosynthesis of the mutant polypeptide chain may also cause its slow and low production. Conversely, the higher production of Gly57→Ala GA may be due to a higher biosynthesis rate of the polypeptide chain, faster correct folding of its newly synthesized polypeptide chain or better intracellular stability than wild type GA.

GA specific activity

The crystal structure of the closely related *A.awamori* var. *X100* GA (Aleshin *et al.*, 1992) shows that the C_α of Gly57 is completely buried in other hydrophobic amino acids (Figure 1a). The C_α of Gly137 is only slightly exposed to solvent, with the residue located near Val64 and covered by

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Table II. Activation parameters for the thermoinactivation of wild type and mutant GAs

GA form	pH	$\Delta H^{\ddagger}$ (kJ/mol)	$\Delta S^{\ddagger}$ (J/mol·K)	$\Delta G^{\ddagger a}$ (kJ/mol)
Wild type	4.5	311 ± 10[b]	610 ± 29	105
Wild type[c]	4.5	365 ± 15	766 ± 43	106
Gly57→Ala	4.5	308 ± 13	601 ± 37	105
Gly137→Ala	3.5	326 ± 19	653 ± 56	106
Gly137→Ala	4.5	330 ± 6	661 ± 17	107
Gly137→Ala	5.5	319 ± 12	635 ± 36	105
Gly139→Ala	3.5	336 ± 8	686 ± 23	104
Gly139→Ala	4.5	344 ± 7	701 ± 21	107
Gly139→Ala	5.5	313 ± 6	617 ± 17	104
Gly137/Gly139→Ala/Ala	4.5	330 ± 14	660 ± 41	107
Gly251→Ala[c]	4.5	388 ± 8	834 ± 24	105
Gly383→Ala[c]	4.5	379 ± 9	816 ± 25	103

[a]At 65°C.
[b]Standard error.
[c]Produced in shake flasks.

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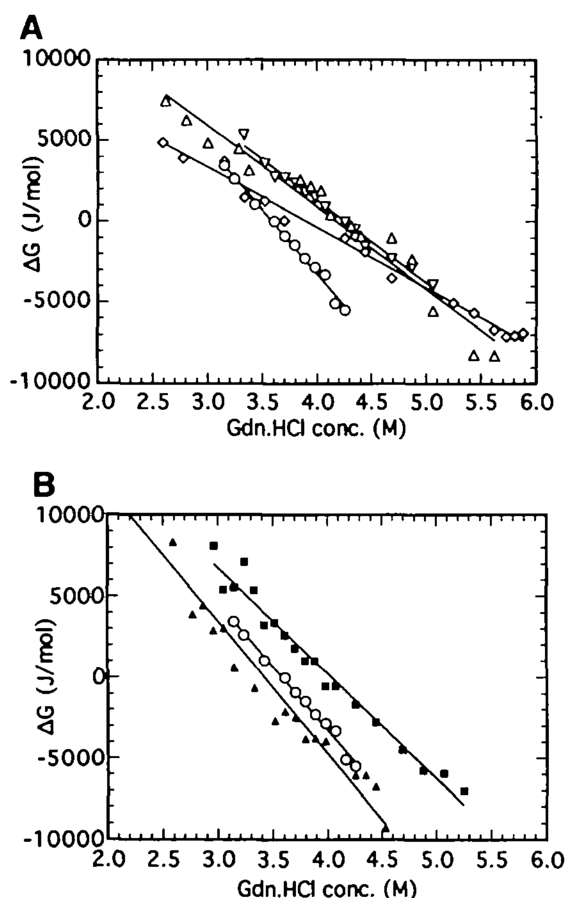

Fig. 4. Effect of [Gdn·HCl] on reversible denaturation of wild type and mutant GAs at pH 4.5 and 25°C. Symbols as in Figure 2.

hydrophilic Arg68 and Gln140 residues, while the C_α of Gly139 is completely buried, surrounded by both hydrophobic and hydrophilic residues (Figure 1b). Gly251 is also completely buried and is part of a hydrophobic microdomain consisting also of Asn247, Thr255, Cys320, Ala324 and Ser418, with the added methyl group caused by Gly→Ala mutation contacting the neighboring Phe182 residue. The Gly383 residue is on the surface with the added methyl group in the replacement Ala residue pointed out into the solution. The retention of specific activity by all six mutated GAs suggests that in all cases the enzyme can accommodate both the increased hydrophobicity and increased steric effect caused by the replacement of a hydrogen atom with a methyl group and that these changes do not affect the GA catalytic conformation.

GA thermostability

The essentially equal thermostabilities at different temperatures and pH 4.5 of the two wild type GAs, one produced by a fermentor and the other by a shake flask, indicate that the mode of enzyme production affects the GA thermostability little. This suggests that we can compare the thermostabilities of Gly57→Ala, Gly137→Ala, Gly139→Ala and Gly137/Gly139→Ala/Ala GAs, produced by a fermentor, with those of Gly251→Ala and Gly383→Ala GAs, produced by a shake flask.

Very different changes in thermostabilities occurred with the six different Gly→Ala mutations. The virtually identical thermostabilities of wild type and Gly57→Ala GAs at different pHs and temperatures indicates that strengthening the second α-helix has no effect on the GA stability.

Unlike mutations designed to eliminate the deamidation and peptide hydrolysis of *A.awamori* GA (Chen *et al.*, 1994a,b, 1995) and the other mutations designed to strengthen the α-helices reported here, the Gly137→Ala and Gly137/Gly139→Ala/Ala mutations stabilize GA over a wide range of temperatures and pHs, while the Gly139→Ala mutation stabilizes the enzyme at pH 4.5 and lower temperatures. This suggests that they strengthen the structure of the fourth α-helix of *A.awamori* GA and consequently prevent unfolding of the whole catalytic domain. The first two are the most thermostable mutants of *A.awamori* GA over all the conditions reported to date. Since (i) the Gly137/Gly139→Ala/Ala double mutation stabilizes GA less than the Gly137→Ala mutation at pH 3.5, while Gly139→Ala GA is as stable as wild type GA, (ii) the Gly137/Gly139→Ala/Ala double mutation stabilizes GA no more than the Gly137→Ala single mutation at pH 4.5, while the Gly139→Ala GA is less stable than they are but more stable than wild type GA and (iii) Gly137/Gly139→Ala/Ala GA is more stable than wild type GA at pH 5.5, while GA137→Ala GA is no more stable than wild type GA and Gly139→Ala GA is less stable, it appears that the increases in the thermostability caused by two mutations in a single α-helix are not necessarily additive.

Hecht *et al.* (1986) replaced the two Gly residues in the third α-helix of the *N*-terminal domain of the λ repressor with Ala residues, yielding two single and one double mutant more

Table III. Parameters for reversible denaturation of wild type and mutant GAs with Gdn·HCl

GA form	$[\text{Gdn·HCl}]_{50\%}$ (M)	Slope (kJ/mol·M)	$\Delta G_{u\ H_2O}$ (kJ/mol)	Fraction denatured at 25°C in water
Wild type	3.59	-7.83 ± 0.26^a	28.1 ± 1.0^a	1.19×10^{-5}
Gly137→Ala	4.25	-5.06 ± 0.17	21.5 ± 0.7	1.71×10^{-4}
Gly139→Ala	3.90	-3.71 ± 0.10	14.5 ± 0.5	2.88×10^{-3}
Gly137/Gly139→Ala/Ala	4.17	-5.06 ± 0.29	21.1 ± 1.2	2.01×10^{-4}
Gly251→Ala[b]	3.42	-8.17 ± 0.40	28.0 ± 1.4	1.24×10^{-5}
Gly383→Ala[b]	4.04	-6.50 ± 0.26	26.3 ± 1.0	2.47×10^{-5}

[a]Standard error.
[b]Produced in shake flasks.

stable against reversible denaturation than the wild type protein. In this case the double mutant was more thermostable than either single mutant, unlike the bulk of our results.

In general, the Gly251→Ala and Gly383→Ala mutations decreased the GA thermostability. In the former case, this could have been caused by the close contact of the added methyl group with the Phe 182 residue. No similar explanation is possible in the latter case, where the added methyl group points into the solution. With neither mutation do the ϕ and ψ angles enter forbidden regions, their values being -55 and $-44°$ respectively for Gly251 and -61 and $-39°$ respectively for Gly383. Both are in region 3 in a Ramachandran plot, which is allowed for Ala residues (Némethy *et al.*, 1966; Flory *et al.*, 1994).

Replacing completely or largely buried Gly residues with Ala residues did not change the GA thermostability at Gly57, in general increased it at Gly137 and Gly139 and decreased it at Gly251. Replacement of the solvent-exposed Gly383 residue caused a large decrease in the thermostability. Heinz *et al.* (1992) suggested that the substitution with Ala residues of fully solvent-exposed residues within α-helices increased the protein thermostability, while replacements of buried or partially buried residues decreased the stability. However, the bulk of our results do not agree with this suggestion.

As pointed out earlier (Chen *et al.*, 1995), the values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are positively correlated, as are their standard errors. Disproportionately greater increases of the latter, signifying an increase in the number of transitional-inactivated conformations, than of the former, signifying a higher energy requirement to unfold to reach the transitional-inactivated state, lead to lower values of $\Delta G^{\ddagger}$, signifying lower thermostabilities of the enzyme form at the chosen temperature, in this case 65°C. To illustrate this, the values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for samples of wild type GA produced by a fermentor are significantly lower than those for samples of wild type GA produced by a shake flask because at higher temperatures the thermostabilities of the latter are slightly lower (Figure 3). However, their $\Delta G^{\ddagger}$ values are virtually identical because their thermostabilities at 65°C are almost the same.

GA denaturation with Gdn·HCl

Denaturation experiments with Gdn·HCl showed that the stabilities to reversible denaturation at low temperature in water and to irreversible thermoinactivation in general did not agree. Gly137→Ala, Gly139→Ala and Gly137/Gly139→Ala/Ala GAs were more stable than wild type GA to thermoinactivation at pH 4.5, but were less stable to reversible denaturation. Gly137→Ala GA was more stable than Gly139→Ala and Gly137/Gly139→Ala/Ala GAs to irreversible thermoinactivation at pH 4.5, while Gly139→Ala GA was less stable than the others to reversible denaturation. As with irreversible thermoinactivation, the stability of the double mutation to reversible denaturation was not the sum of the changed stabilities of the two single mutations. Gly251→Ala GA and Gly383→Ala GAs were stable as wild type GA to reversible denaturation but were less stable to irreversible thermoinactivation. The different relative susceptibilities of the various GA forms to the two types of inactivation are almost surely due to the very different mechanisms by which they occur. Reversible denaturation is a physical phenomenon, whereas irreversible thermoinactivation is caused largely by chemical changes that prevent correct refolding.

Conclusion

We have successfully used site-directed mutagenesis to enhance the thermostability of *A.awamori* GA expressed in *S.cerevisiae* by strengthening its α-helical structure. Single substitutions of Gly137 and Gly139 with Ala as well as the double mutation of both residues stabilized GA against irreversible thermoinactivation following protein unfolding, but single substitutions of Gly57, Gly251 and Gly383 with Ala did not. The production of the Gly139→Ala and Gly137/Gly139→Ala/Ala mutants was severely impeded, while that of the Gly57→Ala mutant was greatly improved over the wild type GA. The irreversible formations of an incorrect structure after reversible enzyme unfolding, deamidation (Chen *et al.*, 1994a,b) and peptide hydrolysis (Chen *et al.*, 1995) can all cause irreversible thermoinactivation of GA at pH 4.5, with the contribution of the first being more important than those of the latter two above 70°C. Although there are other factors contributing to GA inactivation such as glycosylation (Williamson *et al.*, 1992; Chen *et al.*, 1994b; Libby *et al.* 1994) and surface charges (Munch and Tritsch, 1990), these promising results contribute to our understanding of the thermostabilization of GA and other enzymes. Furthermore, because it is reasonable to expect that the thermostabilization resulting from independent mutations is additive (Baase *et al.*, 1986; Matsumura *et al.*, 1986; Wells, 1990; Heinz *et al.*, 1992), the combination of a number of amino acid substitutions may prove useful for substantially improving the thermostability of GA.

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References

Aleshin,A., Golubev,A., Firsov,L.M. and Honzatko,R.B. (1992) *J. Biol. Chem.*, **267**, 19291–19298.

Baase,W.A., Muchmore,D.C. and Becktel,W.J. (1986) *Biophys. J.*, **49**, 109a.

Chen,H.-M., Bakir,U., Reilly,P.J. and Ford,C. (1994a) *Biotechnol. Bioengng*, **43**, 101–105.

Chen,H.-M., Ford,C. and Reilly,P.J. (1994b) *Biochem. J.*, **301**, 275–281.

Chen,H.-M., Ford,C. and Reilly,P.J. (1995) *Protein Engng*, **8**, 575–582.

Chou,P.Y. and Fasman,G.D. (1974) *Biochemistry*, **13**, 222–245.

Coutinho,P.M. and Reilly,P.J. (1994a) *Protein Engng*, **7**, 393–400.

Coutinho,P.M. and Reilly,P.J. (1994b) *Protein Engng*, **7**, 749–760.

Dao-Pin,S., Baase,W.A. and Matthews,B.W. (1990) *Proteins: Struct. Funct. Genet.*, **7**, 198–204.

Flory,N., Gorman,M., Coutinho,P.M., Ford,C. and Reilly,P.J. (1994) *Protein Engng*, **7**, 1005–1012.

Hecht,M.H., Sturtevant,J.M. and Sauer,R.T. (1986) *Proteins: Struct. Funct. Genet.*, **1**, 43–46.

Heinz,D.W., Baase,W.A. and Matthews,B.W. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 3751–3755

Innis,M.A., Holland,M.J., McCabe,P.C., Cole,G.E., Wittman,V.P., Tal,R., Watt,K.W.K., Gelfand,D.H., Holland,J.P. and Meade,J.H. (1985) *Science*, **228**, 21–26.

Kellis,J.T., Jr, Nyberg,K. and Fersht,A.R. (1989) *Biochemistry*, **28**, 4914–4922.

Libby,C.B., Cornett,C.A.G., Ford,C. and Reilly,P.J. (1994) *Protein Engng*, **7**, 1009–1114.

Matsumura,M., Yasumura,S. and Aiba,S. (1986) *Nature*, **323**, 356–358.

Menéndez-Arias,L. and Argos,P. (1989) *J. Mol. Biol.*, **206**, 397–406.

Munch,O. and Tritsch,D. (1990) *Biochim. Biophys. Acta*, **1041**, 111–116.

Némethy,G., Leach,S.J. and Scheraga,H.A. (1966) *J. Phys. Chem.*, **70**, 998–1004.

Nunberg,J.H., Meade,J.H., Cole,G., Lawyer,F.C., McCabe,P., Schweikert,V., Tal,R., Wittman,V.P., Flatgaard,J.E. and Innis,M.A. (1984) *Mol. Cell Biol.*, **4**, 2306–2315.

Pace,C.N. (1986) *Methods Enzymol.*, **131**, 266–280.

Quax,W.J., Mrabet,N.T., Luiten,R.G.M., Schuurhuizen,P.W., Stanssens,P. and Lasters,I. (1991) *Bio/Technology*, **9**, 738–742.

Smith,P.K., Krohn,R.I., Hermanson,G.T., Mallia,A.K., Gartner,F.H., Provenzano,M.D., Fujimoto,E.K., Goeke,N.M., Olson,B.J. and Klenk,D.C. (1985) *Anal. Biochem.*, **150**, 76–85.

Svensson,B., Larsen,K., Svendsen,I. and Boel,E. (1983) *Carlsberg Res. Commun.*, **48**, 529–544.

Wells,J.A. (1990) *Biochemistry*, **29**, 8509–8517.

Williamson,G., Belshaw,N.J., Noel,T.R., Ring,S.G. and Williamson,M.P. (1992) *Eur. J. Biochem.*, **207**, 661–670.

Zhang,X.-J., Baase,W.A. and Matthews,B.W. (1991) *Biochemistry*, **30**, 2012–2017.

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