Mutational Modulation of Substrate Bond-Type Specificity and Thermostability of Glucoamylase from *Aspergillus awamori* by Replacement with Short Homologue Active Site Sequences and Thiol/Disulfide Engineering†

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Received January 31, 1996; Revised Manuscript Received March 25, 1996⊗

ABSTRACT: Rational protein engineering based on three-dimensional structure, sequence alignment, and previous mutational analysis served to increase thermostability and modulate bond-type specificity in glucoamylase from *Aspergillus awamori*. The single free cysteine, Cys320, became disulfide bonded in the Ala246→Cys mutant, thus enhancing T_{50} by 4 °C to 73 °C. Compared to wild-type, Ala246→Cys was roughly twice as active at 66 °C, but half as active at 45 °C. The alternative, elimination of the thiol group in Cys320→Ala, barely improved thermostability or altered activity. Secondly, to acquire exceptionally high specificity toward α-1,6 glucosidic linkages, characteristic of *Hormoconis resinae* glucoamylase, two short sequential mutants, Val181→Thr/Asn182→Tyr/Gly183→Ala (L3 glucoamylase) and Pro307→Ala/Thr310→Val/Tyr312→Met/Asn313→Gly (L5 glucoamylase), were made. These homologue mutants are located in the $(\alpha/\alpha)_6$-fold of the catalytic domain in segments that connect α-helices 5 and 6 and α-helices 9 and 10, respectively. The kinetics of malto- and isomaltooligosaccharides hydrolysis clearly demonstrated that combination of the mutations in L3L5 compensated adverse effects of the single replacements in L3 or L5 glucoamylases to yield wild-type or higher activity. On α-1,4-linked substrates, typically K_m increased 2-fold for L3, and k_{cat} decreased up to 15-fold for L5 glucoamylase. In contrast, on α-1,6-linked substrates L3 showed both a 2-fold increase in K_m and a 3-fold decrease in k_{cat}, while L5 GA caused a similar k_{cat} reduction, but up to 9-fold increase in K_m. L3L5 glucoamylase had remarkably low K_m for isomaltotriose through isomaltoheptaose and elevated k_{cat} on isomaltose, resulting in an approximately 2-fold improved catalytic efficiency (k_{cat}/K_m). Rational loop replacement thus proved powerful in achieving variants with enhanced properties of a highly evolved enzyme.

Glucoamylase (GA;[1] 1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) is widely applied in industrial production of high glucose syrups and catalyzes the hydrolytic release of glucose from the nonreducing ends of starch or related oligo- and polysaccharides. So far 15 GA sequences, all but one of fungal origin, have been reported; the overall sequence similarity is high in the catalytic domain of fungal GAs (Itoh et al., 1987; Henrissat et al., 1994; Stoffer, 1994). The multidomain GA (G1 form) from *Aspergillus awamori* (identical to *Aspergillus niger* GA; Svensson et al., 1983; Boel et al., 1984; Nunberg et al., 1984) consists of a catalytic domain, Ala1−Thr440, that includes two high-mannose-type *N*-glycosylated positions (Asn171 and Asn395), a Ser/Thr-rich, highly *O*-glycosylated linker from Ser441 to Ser508, and a 108-residue long C-terminal starch granule binding domain (Svensson et al., 1983, 1986, 1989; Williamson et al., 1992a; Stoffer et al., 1993). In addition to α-1,4-glucosidic bonds, *A. niger* GA cleaves α-1,6-bonds, e.g., in isomaltooligosaccharides and panose, but with about 600-fold lower catalytic efficiency (Sierks et al., 1989; Meagher et al., 1989). The catalysis of glucosidic bond hydrolysis leads to inversion of the anomeric configuration of the product (Weill et al., 1954). Kinetic analysis indicated maltooligosaccharide substrates to be accommodated at seven consecutive subsites and cleaved between subsites 1 and 2 (Hiromi, 1970; Sierks et al., 1990), as illustrated in the recent three-dimensional structures of GA from *A. awamori* var. *X100* in complex with the pseudotetrasaccharide inhibitors acarbose (Aleshin et al., 1994) and D-*gluco*-dihydroacarbose (Stoffer et al., 1995).

The catalytic domain of GA folds as an uncommon $(\alpha/\alpha)_6$-barrel (Aleshin et al., 1992). The active site, located at one side of the barrel, is formed by six α→α connecting segments that comprise short, most highly conserved sequences (Stoffer, 1994). Chemical modification in conjunction with site-directed mutagenesis and crystallography provided details on the general acid and base catalysts, Glu179 and Glu400, from the third and the sixth highly conserved regions (Sierks et al., 1990; Svensson et al., 1990; Harris et al., 1993; Frandsen et al., 1994). Other important residues in the conserved segments include Tyr48, Trp52, Arg54, Asp55 (region 1), Trp120 (region 2), Glu180 (region

† Henri-Pierre Fierobe was supported by a long-term fellowship (ALTF 524-1993) from the European Molecular Biology Organization (Heidelberg, Germany) in 1994 and by a Human Capital and Mobility fellowship (ERBCHBICT941224, E.U.) in 1995. B.B.S. was supported by a postdoctoral fellowship (94-0423/20) from the Carlsberg Foundation (Copenhagen, Denmark).

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⊗ Abstract published in *Advance ACS Abstracts,* May 15, 1996.

[1] Abbreviations: GA, glucoamylase; GdnHCl, guanidine hydrochloride; DP, degree of polymerization; G2 through G7, maltose through maltoheptaose; iG2 through iG7, isomaltose through isomaltoheptaose (α-1,6-linked throughout).

S0006-2960(96)00241-3 CCC: $12.00

3), Arg305, Asp309, and Trp317 (region 5), which were demonstrated by mutation coupled with molecular recognition and binding thermodynamic analyses to play different roles in substrate transition-state stabilization and/or ground-state binding (Clarke & Svensson, 1984b; Sierks et al., 1989; Sierks & Svensson 1993; Frandsen et al., 1994, 1995; Berland et al., 1995). Such thorough structure/function relationship investigations make an excellent basis for design of engineered variants of GAs with desired properties.

In the present study protein engineering of *A. awamori* (*A. niger*) GA was pursued to increase the thermostability and to modulate the substrate bond-type specificity. GA contains three disulfide bridges, Cys210−Cys213, Cys222−Cys449, and Cys262−Cys270 (Aleshin et al., 1992) in the catalytic domain and one, Cys509−Cys604, in the starch binding domain (Williamson et al., 1992a). The free Cys320, located near the N-terminus of α-helix 10 (Aleshin et al., 1992), was proposed to cause conformational destabilization (Munch & Tritsch, 1990). Although only one thiol group in GA seemed accessible to carboxymethylation after denaturation (B. Svensson, unpublished data), peptide mapping revealed that approximately 0.25 equiv of reagent got incorporated at Cys320, 0.25 at Cys262 + Cys270, and 0.5 at Cys469 (B. Svensson, unpublished data). This finding motivated site-directed mutation of Cys320 in an attempt to achieve stabilization through suppression of thiol/disulfide interchange. In the crystal structure of *A. awamori* var. *X100* GA (Aleshin et al., 1992) Thr246, at the N-terminus of α-helix 8, would be an excellent partner for disulfide bond formation with Cys320, if mutated to Cys. Cys320→Ala GA was made in a complementary approach, and analysis of Cys320→Ala and Ala246→Cys GAs indicated that disulfide formation led to superior stability.

Although it is not fully understood why *A. niger* GA has 600-fold higher specificity toward α-1,4- relative to α-1,6-glucosidic linkages, some insight has been gained from a combination of molecular modeling, and mutational and molecular recognition analyses. This clearly demonstrated the impact of residues in the highly conserved regions 3 and 5 on bond-type specificity (Sierks et al., 1990; Frandsen et al., 1995). Most interestingly, GA P from the fungus *Hormoconis resinae* with a very unusual only around 50-fold lower catalytic efficiency for α-1,6-glucosidic bonds (Fagerström, 1991; Fagerström & Kalkkinen, 1995) has the largest sequence variation (Figure 1) in conserved segments 2, 3, and 5 among the GA homologues (Joutsjoki & Torkkeli, 1992; Stoffer, 1994). In the present study, protein engineering of *A. awamori* GA to get loop 3 and 5 variants, designated L3, L5, and L3L5, mimicked the *H. resinae* GA (Figure 1). In L3 GA, Val181, Asn182, and Gly183 were thus replaced by Thr, Tyr, and Ala, and in L5 GA, Pro307, Thr310, Tyr312, and Asn313 by Ala, Val, Met, and Gly, respectively. L3L5 GA combines these replacements. The activity of the three GAs variants on linear malto- and isomaltooligosaccharides was for L3 and, even more pronounced, L5 lower than of *A. awamori* wild-type GA, while the double mutant L3L5 GA had improved activity on isomaltooligosaccharides.

MATERIALS AND METHODS

Enzymes and Reagents. Restriction endonucleases and T4-DNA ligase were from Promega (Madison, WI); the Vent polymerase was purchased from New England Biolabs, Inc. (Beverly, CA). The DNA sequencing kit was from Applied Biosystems (Foster City, CA). Acarbose was a generous gift of Drs. D. Schmidt and E. Truscheit, Bayer AG (Wuppertal, Germany). Acarbose-Sepharose was prepared essentially as described (Clarke & Svensson, 1984b). Maltotriose through maltoheptaose, isomaltose, isomaltotriose, the glucose oxidase kit, and 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) were from Sigma (St. Louis, MO), and maltose monohydrate was from Merck (Darmstadt, Germany). Isomaltotriose through isomaltoheptaose were generous gifts of Drs. M. Meagher and P. J. Reilly (Iowa State University). Guanidine hydrochloride (GdnHCl) was from Fluka (Buchs, Switzerland).

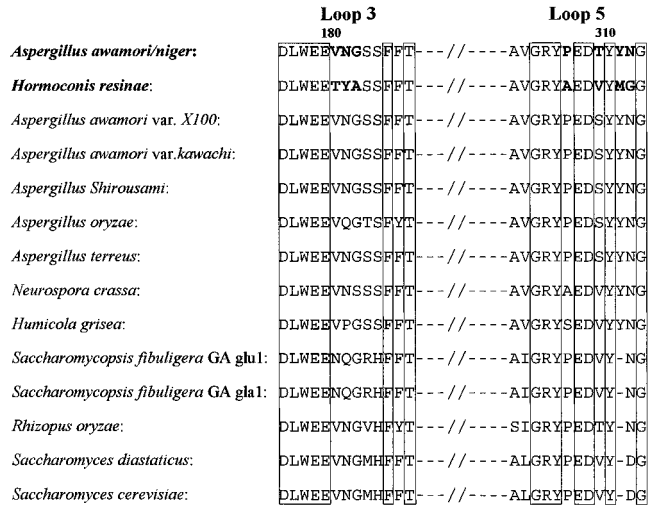

FIGURE 1: Sequence alignment of fungal GAs in loop 3 and loop 5 highly conserved regions (Stoffer, 1994). Numbers above the sequence refer to the mature GA from *A. awamori*. Residues in bold correspond to those mutated in the present study. "–" symbolizes a gap introduced for optimization of the alignment. Rectangles designate invariant residues.

Strains and Plamids. *A. awamori* wild-type and mutant genes were expressed in *Saccharomyces cerevisiae* strain C468 (α *leu2−3 leu2−112 his3−11 his3−15 mal*$^-$), obtained from Dr. J. Meade (Cetus Corporation, CA). The genes were carried by the autonomously replicating shuttle plasmid pGAC10 (Innis et al., 1985; Sierks et al., 1989). *Escherichia coli* strain DH5α (Søgaard et al., 1990) was used as host for plasmid pBS322, (Stratagene, CA) harboring the 1.57 kb *Hin*dIII−*Bam*HI cDNA fragment of *A. awamori* wild-type GA (Sierks et al., 1989), while in the case of Ala246→Cys GA, the *Dam*$^-$ strain JM 110 (Stratagene CA) was used.

Site-Directed Mutagenesis. The 1.57 kb *Hin*dIII−*Bam*HI wild-type cDNA fragment was mutated essentially as described (Higuchi et al., 1988), using a modified PCR method except that the Vent polymerase was used instead of Taq polymerase. Cys320→Ala was made using the set of mutagenic primers 5′-CCGTGGTTCCTG**GC**CACCT-TGGCTGCCGCGGAGCAGTTGTACG-3′ and 5′-CGTA-CAACTGCTCCGCGGCAGCCAAGGTG**GC**CAGGAAC-CACGG-3′ (nucleotides replaced for mutation are in bold; underlined nucleotides yield a silent mutation introducing a *Sac*II site to facilitate the control of the construct). The mutagenized fragment was amplified using an upstream forward primer 5′-GGGAAGCATCCACACCTTTGATC-CTGAGGCCGCATGC-3′ and a reverse primer complementary to the genomic DNA inverse strand 5′-TAGGGCGAAT-TCGAGCTCGGTACCCGGGGATCC-3′, downstream of the site of mutation.

Ala246→Cys was made using the mutagenic primers 5′-CCGGCAAGGACTGCAACACCCTCCTGGGATCGATCCACACCTT-3′ and 5′-AAGGTGTGGATCGATCCCAGGAGGGTGTTGCAGTCCTTGCCGG-3′ (new silent site *Cla*I is underlined). Amplification was done as above using the forward upstream primer 5′-TCGTCTTTCTTTACGATTGCTGTGCAACACCGGGCCC-3′ and the reverse downstream primer 5′-TAGGGCCAATTCGAGCTCGGTACCCGGGGATCC-3′.

Val181→Thr/Asn182→Tyr/Gly183→Ala (L3) was obtained using the primers 5′-TATGATCTCTGGGAAGAAACTTACGCGTCGTCTTTCTTTACG-3′ and 5′-CGTAAAGAAAGACGACGCGTAAGTTCTTCCCAGAGATCATA-3′ (new site *Mlu*I is underlined). The forward upstream primer 5′-CTCGGTGAACCCAAGTTCAATGTCGAC-3′ and the reverse downstream primer 5′-CAGGGCTGGAAGGTGGAGTCGTCGCATGC-3′ were used for amplification.

Pro307→Ala/Thr310→Val/Tyr312→Met/Asn313→Gly (L5) was performed using the mutagenic primers 5′-GTGGGTCGGTACGCTGAGGACGTCTACATGGGCGGCAACCCG-3′ and 5′-CGGGTTGCCGCCCATGTAGACGTCCTCAGCGTACCGACCCAC-3′ (new site *Aat*II is underlined). The forward upstream primer 5′-GGGAAGCATCCACACCTTTGATCCTGAGGCCGCATGC-3′ and the reverse downstream primer 5′-CACTGGTATAGAGCATCGTACAACTGCTCCGCGGC-3′ were used for the amplification.

The combined L3L5 mutant was obtained by ligating the *Hin*dIII−*Sph*I 0.885 kb fragment, containing the loop 3 mutations, with the 0.666 kb *Sph*I−*Bam*HI fragment containing the loop 5 mutations, and cloning the entire *Hin*dIII−*Bam*HI fragment in pBS322.

E. coli clones were screened for the newly introduced restriction sites and subsequently sequenced using the Taq dyedeoxy terminator cycle sequencing kit. The mutated selected plasmid was then digested by *Hin*dIII and *Bam*HI and the fragment cloned into pGAC10. *S. cerevisiae* strain C468 was transformed using the LiCl method (Ito et al., 1983), and clones were selected on minimal salt medium containing histidine (40 μg/mL).

Yeast Growth and GA Purification. Cells were grown in minimal salt medium (6.7 g/L yeast nitrogen base without amino acids, 10 g/L succinic acid, 5 g/L NaOH, 0.111 M glucose, and 0.1 g/L histidine, pH 5.1) at 30 °C, in a 15 L fermentor, for 48 h. When the glucose was consumed, a second portion of glucose was added to a final concentration of 0.111 M, and the fermentation continued for another 2−3 days. The secretion level at the end of the fermentation was 10 mg/L for wild-type and 0.5−3.5 mg/L for GA variants. The culture was centrifuged (4000*g*, 30 min) and the supernatant concentrated (Pellicon cell, Amicon, 20 kDa cutoff). A final volume of around 300 mL was applied on a 2.5 × 10 cm DEAE-Sepharose column (Pharmacia, Sweden) in 25 mM sodium phosphate, pH 7.0, and GA was subsequently eluted by a linear gradient from 0 to 0.4 M NaCl (2 × 500 mL) in the same buffer. Fractions containing activity toward maltose were pooled, and GA was purified by affinity chromatography on individual acarbose-Sepharose columns (1.1 × 5 cm) as previously described (Clarke & Svensson, 1984b) and followed by dialysis against 50 mM sodium acetate, pH 4.5. To avoid cross-contamination, new columns (acarbose-Sepharose and DEAE-Sepharose) were used for each mutant. Homogeneity was checked by SDS−PAGE (12.5%; Phast-System Pharmacia). The protein concentration was estimated by amino acid analysis or spectrophotometrically using $\epsilon_{280} = 1.37 \times 10^5$ M^{-1} cm^{-1} for the GA G1 form (Clarke & Svensson, 1984a). The G1 form is used throughout the present study.

Analysis of Thiol Groups. GA (typically 10 nmol) was incubated in 0.2 mM 5,5′-dithiobis(2-nitrobenzoic acid), 6 M GdnHCl, and 50 mM Tris, pH 8 (final volume: 1 mL). After 10 min, the absorbance was measured at 412 nm (Riddles et al., 1983), and the concentration of thiol was calculated using a value 13 700 M^{-1} cm^{-1} for the liberated 2-nitro-5-thiobenzoate anion. The extinction coefficient was confirmed by establishing a standard curve for cysteine (0−30 μM) under the same conditions.

Enzyme Kinetics. Initial rates of hydrolysis were determined at 45 °C in 50 mM sodium acetate, pH 4.5, using up to 15 concentrations, from $^1/_{10}K_m$ to $8K_m$, of maltooligosaccharides, isomaltose, and isomaltotriose. For L5 GA hydrolysis of isomaltotriose the maximum substrate concentration was $4K_m$. Since limited amounts of isomaltotetraose through isomaltoheptaose were available, only eight concentrations from $^1/_{10}K_m$ to $(4-5)K_m$ were used. Glucose released was quantified by the glucose oxidase method monitored in microtiter plates with a CERES UV900 (BIOTEK) reader (Fox & Robyt, 1991; Palcic et al., 1993; Frandsen et al., 1994). The enzymatic hydrolysis of the various substrates were followed for 5−30 min. k_{cat} and K_m were determined by direct fit to the Michaelis−Menten equation.

Thermostability. GA (0.6 μM) in 0.1 M sodium acetate, pH 4.5, was incubated at varying temperatures (20−85 °C) for 5 min and then immediately frozen on an alcohol bath at −80 °C. After thawing, residual activity was measured toward 70 mM maltose essentially as above. T_{50} is defined as the temperature corresponding to a loss of 50% of the initial activity.

GdnHCl Denaturation. Using a procedure previously described in detail (Frandsen et al., 1995), GA (0.25 μM) was incubated for 1 h at room temperature at varying concentrations (0−7.7 M) of GdnHCl in 50 mM sodium acetate, pH 4.5. Protein unfolding was determined by the decrease in fluorescence (excitation at 280 nm and emission at 320 nm) using a Perkin-Elmer LS50 luminescence spectrometer. The fraction of unfolded enzyme, f_u,was calculated using the equation $f_u = (F_N - F_{obs})/(F_N - F_U)$, where F_N and F_U are the fluorescence intensities recorded for the folded and the unfolded protein, respectively, and F_{obs} is the fluorescence observed at a given concentration of GdnHCl. The standard free energy was not calculated, since the unfolding of GA was not reversible (Williamson et al., 1992b).

Structural Analysis. The complex between GA from *A. awamori* var. *X100* and D-*gluco*-dihydroacarbose at pH 4 (Stoffer et al., 1995) and the program package from Biosym were used for structure analysis and structure energy minimization. After removal of the disordered residues and correction of the bond order of the carbonyl group of *N*-acetylglucosamine, hydrogen atoms were added to the structure, selecting a pH value of 4.0 and the standard force field *cvff*. The complex was then minimized in DISCOVER for 1000 iterations using the steepest descendance algorithm. After the initial minimization, the mutations L3 (Val181→Thr, Asn182→Tyr, and Gly183→Ala) and L5 (Pro307→Ala, Thr310→Val, Tyr312→Met, and Asn313→Gly) were introduced in the complex, and D-*gluco*-dihydroacarbose was

FIGURE 2: Stereoview of the α-helix elements of GA from *A. awamori* var. *X100* in complex with D-*gluco*-dihydroacarbose (Stoffer et al., 1995). Side chains of Cys320 and Thr246 are in bold. The dashed line represents the distance between OG1 of Thr246 and SG of Cys320.

Table 1: Determination of Thiol Groups in Wild-Type, Cys320→Ala, and Ala246→Cys GAs from *A. awamori*

protein	[protein] (μM)	[SH] (μM)	[SH]/[protein]
wild-type	9.7	10.7	1.10
	19.4[a]	19.8	1.02
C320A	11.5	0.1	0.01
	11.5[a]	0.1	0.01
A246C	10.2	3.9	0.38
	10.2[a]	4.0	0.39

[a] Duplicate experiment.

replaced by a model of isomaltose (Stoffer, 1994) followed by another 2000 iterations in DISCOVER using the steepest descendance algorithm.

RESULTS

Cys320→Ala and Ala246→Cys GAs

Design of the Ala246→Cys Mutant. Thr246 at the N-terminus of α-helix 8 in GA from *A. awamori* var. *X100* (Aleshin et al., 1992), 94% identical to *A. awamori* GA, has OG1 at a distance of 3.20 Å from SG of Cys320, located near the N-terminus of α-helix10 (Figure 2). Upon rotation around the Cα-Cβ bond of Cys320 from −66.14° to −123.24°, the distance from Thr246 oxygen atom (OG1) and Cys320 sulfur atom (SG1) is reduced to 2.00 Å (the torsion angle Cβ,SG1 (Cys320), OG1,Cβ (Thr246) changes from 40.80° to 92.03°), thus yielding an ideal geometry for disulfide bond formation. Ala246 in *A. awamori* GA, therefore, was mutated to Cys aiming at introduction of the extra disulfide bond Cys246−Cys320.

Thiol Group Analysis. Successful formation of the fifth disulfide bridge in Ala246→Cys GA, involving Cys246 and Cys320, was deduced from the low thiol content found under denaturating conditions. Wild-type, having free Cys320 (Aleshin et al., 1992), and Cys320→Ala GAs served as positive and negative controls, respectively (Table 1). Since Ala246→Cys GA contained 0.4 thiol group per molecule, the Cys246−Cys320 disulfide formation was assumed to occur in an average yield of 80%. According to the crystal structure (Aleshin et al., 1992), all half-cystines of GA are far from Cys246 or Cys320. The disulfide formation was spontaneous and attempts to complete the reaction by oxygen bubbling under alkaline conditions caused irreversible denaturation.

Enzymatic Properties. Both GA mutants showed modest or no change in activity dependent on the substrate. Cys320→Ala GA thus elicited marginal decrease in k_{cat} and increase in K_m for hydrolysis of maltose and gave wild-type values for maltoheptaose and isomaltose hydrolysis (Table 2). The disulfide mutant Ala246→Cys GA (disulfide bonded at 80%) was fully active on maltoheptaose, while similar small losses for the disaccharide substrates in catalytic rate (50%) and affinity (2-fold) were observed.

Conformational Stability. The two mutations only slightly affected the conformational stability in GdnHCl (Figure 3). At low concentrations of GdnHCl, Ala246→Cys and Cys320→Ala, in that order, thus unfolded more easily than wild-type GA. The thermostability (Figure 4), however, was clearly improved for both Cys320→Ala and Ala246→Cys GAs, as illustrated by T_{50} values of 70.5 °C for Cys320→Ala and 73 °C for Ala246→Cys GAs. In comparison, T_{50} is 69 °C of wild-type GA secreted by *S. cerevisiae*, while recombinant *A. niger* wild-type GA has T_{50} of 71 °C, and wild-type GA purified from a commercial *A. niger* GA T_{50} of 67 °C (Svensson et al., 1986; Stoffer et al., 1993; Frandsen et al., 1995). This improved thermostability of the disulfide mutants is also reflected by the time course of maltose hydrolysis at 66 °C (Figure 5). Although the initial velocities are almost identical at this temperature, the hydrolysis by Ala246→Cys continues linearly for much longer than Cys320→Ala and wild-type GAs.

Loop Replacement Mutants

Thermostability. The *A. awamori* GA variants, L3, L5, and L3L5, had excellent stability at elevated temperatures, T_{50} for all being 69 °C and essentially identical to T_{50} of the wild-type GA (curves not shown).

Kinetic Parameters on Maltooligosaccharides. The activity of the three loop-variant GAs was tested using the substrates maltose through maltoheptaose (Table 3). In the case of L3 GA, k_{cat} values were 50−85% of k_{cat} of the parent wild-type, the clearly decreased values being for maltose and maltohexaose. K_m increased up to 2-fold compared to wild-type for all substrates (Table 3). The replacements in L3 GA, Val181→Thr/Asn182→Tyr/ Gly183→Ala, thus had very little effect on the activity toward maltooligosaccharides.

In contrast, the substitutions in L5 GA, Pro307→Ala/ Thr310→Val/Tyr312→Met/Asn313→Gly, induced drastic and unexpected changes (Table 3). For maltose K_m increased 3.5-fold compared to wild-type GA, whereas, for longer maltooligosaccharides, K_m approached wild-type values even more closely than in the case of L3 GA. L5, however, has k_{cat} of 36% for maltose but only 5−8% for maltotriose

Table 2: Kinetic Parameters[a] for Hydrolysis of Maltose, Isomaltose, and Maltoheptaose by Wild-Type, Cys320→Ala, and Ala246→Cys GAs from *A. awamori*

	maltose			maltoheptaose			isomaltose		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	k_{cat} (s^{-1})	K_m(mM)	k_{cat}/K_m (s^{-1} mM^{-1})
WT	11.3 ± 0.26[b]	1.8 ± 0.14	6.2 ± 0.50	41.1 ± 0.79	0.12 ± 0.008	348 ± 24.5	0.33 ± 0.013	36 ± 4	0.0091 ± 0.001
C320A	7.9 ± 0.38	2.6 ± 0.35	3.2 ± 0.46	46.2 ± 1.54	0.13 ± 0.016	358 ± 46.0	0.31 ± 0.015	42 ± 6	0.007 ± 0.001
A246C	4.9 ± 0.20	2.9 ± 0.29	1.7 ± 0.19	34.9 ± 0.79	0.12 ± 0.01	296 ± 26.0	0.19 ± 0.025	76 ± 23	0.0025 ± 0.0008

[a] Determined at 45 °C, 50 mM sodium acetate, pH 4.5. [b] Standard deviation.

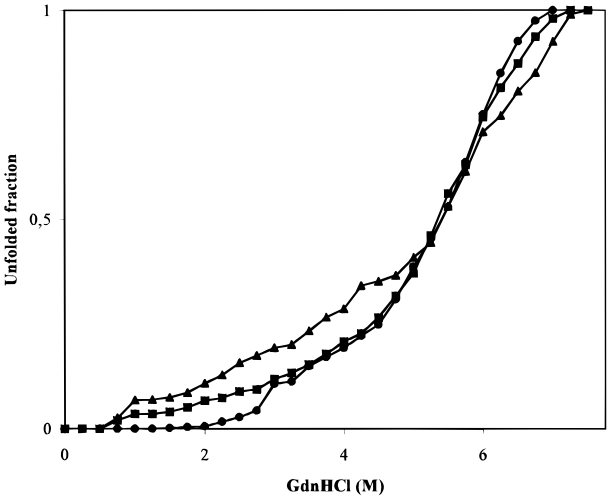

FIGURE 3: Denaturation in presence of GdnHCl for wild-type (●), Cys320→Ala (■), and Ala246→Cys (▲) GAs (see Materials and Methods). The fraction of unfolded protein is plotted as a function of [GdnHCl].

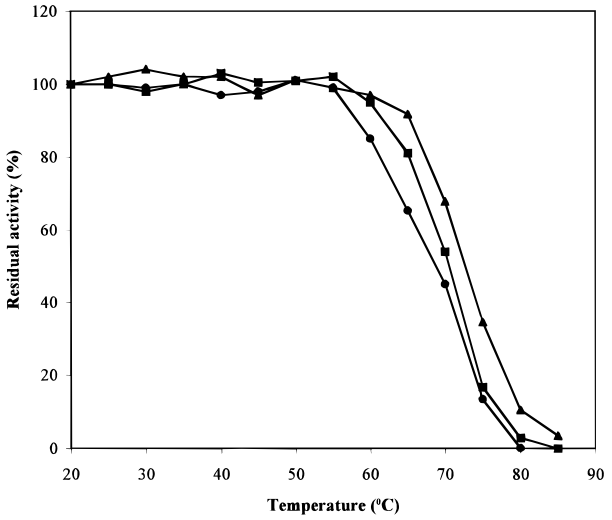

FIGURE 4: Temperature dependence of the stability (see Materials and Methods) of wild-type (●), Cys320→Ala (■), and Ala246→Cys (▲) GAs.

through maltoheptaose of wild-type GA. This is the first time that a mutation of GA resulted in higher k_{cat} for maltose than for the normally superior maltooligosaccharide substrates.

The combination of the loop replacements in L3L5 GA resulted in higher activity toward maltooligooligosaccharides which was superior to that of L3 GA and almost as high as wild-type GA, K_m values were essentially unchanged, while k_{cat} varied with the substrate length in the 50−90% range (Table 3).

Kinetic Parameters on Linear α-1,6-Linked Oligosaccharides. The substrate series isomaltose through isomaltoheptaose was used to examine the effect of GA active site loop mutations on the specificity towards α-1,6-linkages. For wild-type GA, k_{cat} increased 4-fold from isomaltose to isomaltotriose and then steadily another 2-fold up to isomaltoheptaose, while K_m was 3-fold lower for isomaltotriose and longer substrates than for isomaltose (Table 4). These variations of the kinetic parameters with substrate length are in excellent agreement with data reported for *A. niger* GA purified from a commercial enzyme (Meagher et al., 1989).

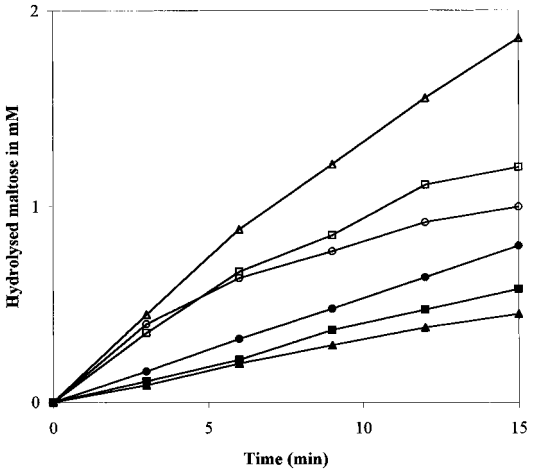

FIGURE 5: Activity at 45 °C (●, ■, ▲) and 66 °C (○, □, △) of wild-type (●, ○), Cys320→Ala (■, □), and Ala246→Cys (▲, △) GAs. 70 mM maltose in 50 mM sodium acetate, pH 4.5, was used as substrate. The enzyme concentration for wild-type and both mutant GAs was 0.081 μM. The glucose liberated was quantified as described in Materials and Methods.

Remarkably, k_{cat} of L3 GA toward isomaltose is 70% and only 25−40% for the longer isomaltooligosaccharides compared to k_{cat} values of wild-type GA. The affinities of L3 GA remained constant, corresponding to $K_m = 22.8 \pm 2.5$ mM regardless of the substrate length. K_m values slightly improved for isomaltose but increased 2-fold compared to wild-type GA for the longer substrates (Table 4).

L5 GA displayed very different alterations in catalytic properties toward isomaltooligosaccharides. While k_{cat} was 30−40% of that of wild-type for all tested substrates, K_m was doubled for isomaltose, 10-fold increased for isomaltotriose, and 4-fold increased for longer α-1,6-linked substrates relative to wild-type GA (Table 4). On maltooligosaccharides L5 GA in contrast gave wild-type level K_m values (Table 3).

As opposed to the single loop mutants, the double loop variant, L3L5 GA, possessed superior catalytic efficiency (k_{cat}/K_m) on the isomaltooligosaccharides (Table 4). The same trend was seen also in the higher activity, close to wild-type level, of L3L5 GA for maltooligosaccharides (Table 3). Two distinct effects caused L3L5 to be more efficient than wild-type GA toward α-1,6-linked substrates. First, on isomaltose k_{cat} essentially was doubled; secondly, on higher

Table 3: Kinetic Parameters[a] for Hydrolysis of Maltooligosaccharides by Wild-Type, L3, L5, and L3L5 GAs from *A. awamori*

	WT			L3		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
G2	11.3 ± 0.26[b]	1.82 ± 0.14	6.2 ± 0.50	5.65 ± 0.16	3.58 ± 0.29	1.6 ± 0.14
G3	30.9 ± 0.7	0.249 ± 0.021	124 ± 10.8	21.4 ± 0.5	0.44 ± 0.03	48 ± 3.4
G4	38.3 ± 1.5	0.119 ± 0.015	322 ± 42.5	27.2 ± 1.39	0.22 ± 0.01	127 ± 8. 8
G5	38.4 ± 1.1	0.098 ± 0.009	393 ± 37.8	33.8 ± 1.21	0.23 ± 0.028	144 ± 18.0
G6	53.8 ± 1.5	0.108 ± 0.010	499 ± 48.3	24.7 ± 0.32	0.133 ± 0.006	186 ± 8.7
G7	41.0 ± 0.79	0.118 ± 0.008	348 ± 24.5	31.8 ± 1.13	0.171 ± 0.022	186 ± 24.8
	L5			L3L5		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
G2	4.1 ± 0.099	6.4 ± 0.42	0.64 ± 0.045	5.69 ± 0.069	1.61 ± 0.083	3.5 ± 0.19
G3	1.70 ± 0.04	0.33 ± 0.02	5.2 ± 0.34	20.6 ± 0.43	0.264 ± 0.017	78 ± 5.6
G4	2.56 ± 0.04	0.182 ± 0.007	14.1 ± 0.59	32.4 ± 0.71	0.177 ± 0.013	183 ± 14.0
G5	2.99 ± 0.03	0.142 ± 0.004	21.1 ± 0.63	34.6 ± 0.89	0.119 ± 0.009	291 ± 23.2
G6	2.94 ± 0.03	0.116 ± 0.005	25.4 ± 1.1	34.1 ± 0.70	0.099 ± 0.007	391 ± 27.8
G7	2.66 ± 0.03	0.125 ± 0.005	21.7 ± 0.90	31.7 ± 0.51	0.101 ± 0.005	314 ± 16.3

[a] Determined at 45 °C in 50 mM sodium acetate, pH 4.5. [b] Standard deviation.

Table 4: Kinetic Parameters[a] for Hydrolysis of Isomaltooligosaccharides by Wild-Type, L3, L5, and L3L5 GAs from *A. awamori*

	WT			L3		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
iG2	0.33 ± 0.013[b]	36.3 ± 3.9	0.0091 ± 0.001	0.232 ± 0.007	22.9 ± 1.9	0.010 ±.0.001
iG3	1.29 ± 0.015	11.5 ± 0.48	0.112 ± 0.0048	0.35 ± 0.016	20. 9 ± 2.9	0.017 ± 0.003
iG4	1.93 ± 0.048	13.5 ± 0.84	0.143 ± 0.0096	0.63 ± 0.043	23.4 ± 3.1	0.027 ± 0.004
iG5	2.06 ± 0.075	11.7 ± 1.27	0.177 ± 0.0203	0.68 ± 0.024	23.4 ± 2.0	0.029 ± 0.003
iG6	2.44 ± 0.077	10.6 ± 1.01	0.230 ± 0.0231	0.90 ± 0.070	20.9 ± 3.9	0.043 ± 0.009
iG7	2.51 ± 0.070	11.4 ± 0.95	0.220 ± 0.0193	1.07 ± 0.051	25.5 ± 2.8	0.042 ± 0.005
	L5			L3L5		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
iG2	0.108 ± 0.012	67.36 ± 18.6	0.0016 ±0.0005	0.59 ± 0.018	27.59 ± 2.7	0.0267 ± 0.003
iG3	0.481 ± 0.064	108 ± 25.06	0.0045 ±0.0012	1.16 ± 0.038	5.44 ± 0.82	0.229 ± 0.035
iG4	0.57 ± 0.035	44 ± 4.43	0.013 ±0.0015	1.29 ± 0.036	5.39 ± 0.48	0.24 ± 0.022
iG5	0.722 ± 0.034	52.54 ± 4.46	0.014 ±0.0014	1.44 ± 0.015	4.86 ± 0.19	0.296 ± 0.012
iG6	0.76 ± 0.04	42.8 ± 3.50	0.018 ±0.0018	1.61 ± 0.039	4.25 ± 0.41	0.378 ± 0.038
iG7	0.99 ± 0.079	49.8 ± 7.35	0.020 ±0 0034	1.58 ± 0.022	5.03 ± 0.27	0.313 ± 0.017

[a] Determined at 45 °C in 50 mM sodium acetate, pH 4.5. [b] Standard deviation.

isomaltooligosaccharides, although k_{cat} was not quite at the wild-type level, the substrate affinity was enhanced corresponding to around 2-fold decrease in the K_m values (Table 4).

DISCUSSION

Designed Mutants with Improved Stability. The three-dimensional structure of GA from *A. awamori* var. *X100* (Aleshin et al., 1992) clearly shows that the thiol group of Cys320 in *A. awamori* GA, conserved in only two GAs (Stoffer, 1994), by disulfide bond formation to a cysteine introduced at position 246 may stabilize the contact between the parallel α-helices 8 and 10 of the inner "cylinder" of the $(\alpha/\alpha)_6$-fold pattern (Aleshin et al., 1992). This approach as well as mutation of Cys320 to either Ser, Ala, or Ile, present at the equivalent position in other GAs (Stoffer, 1994), represent obvious strategies for enhancing the stability of *A. awamori* GA. The disulfide Cys246−Cys320 thus formed in Ala246→Cys GA was considered responsible for improvement of stability and catalytic efficiency at elevated temperatures. A slightly higher sensitivity to GdnHCl compared to wild-type and Cys320→Ala GAs was probably due to the minor content of free thiol groups in Ala246→Cys GA. In contrast, elimination of the single free thiol group, as in Cys320→Ala GA, barely altered stability or activity properties, in agreement with the structural model (Aleshin et al., 1992). This supports the proposition that disulfide interchange in *A. niger* GA, suggested by the equimolar carboxymethylation of wild-type GA G1 in 6 M GdnHCl distributed among Cys262, Cys270, Cys320, and Cys469 (B. Svensson, unpublished data), was not associated with the unfolding. Attempts to further analyze the unfolding process by using differential scanning calorimetry failed due to irreversible denaturation.

Introduction of disulfides guided by three-dimensional structures has previously successfully improved the stability of, e.g., dihydrofolate reductase (Villafranca et al., 1983, 1987), the N-terminal domain of λ repressor (Sauer et al., 1986), T4 lysozyme (Matsumura et al., 1989), subtilisin (Mitchinson & Wells, 1989), *N*-(5′-phosphoribosyl)anthranilate isomerase (Eder & Wilmanns, 1992), ribonuclease H (Kanaya et al., 1991), *Bacillus circulans* xylanase (Wakarchuk et al., 1994), and ribonuclease (barnase) from *Bacillus amyloquefaciens* (Clarke et al., 1995). Generally, the increase in stability as measured by calorimetry, irreversible thermal denaturation, resistance to denaturing agents, and stability and activity at elevated temperatures was larger than that of Ala246→Cys GA. In one extreme case, engineering of three disulfide bonds in T4 lysozyme increased T_m by 23.4 °C (Matsumura et al., 1989). All these examples, however, involve small proteins or domains (less than 25 kDa) without disulfide bridges. GA, in contrast, is a

multidomain glycoprotein having a molecular mass of 82 kDa containing three disulfides in one domain and one in another (Svensson et al., 1983; Aleshin et al., 1992; Williamson et al., 1992a). In this light, the modest increase in thermostability by engineering of the Cys246−Cys320 disulfide in Ala246→Cys GA is considered successful.

Homologue Engineering of Substrate Specificity by Active Site Loop Replacements. The remarkably high conservation of fungal GAs at short sequences belonging to α→α connecting segments in the active site area of the $(\alpha/\alpha)_6$-barrel proposes that correlation exists between variations in substrate specificities and structure. The ideal test involves homologue engineering of *A. awamori* GA based on *H. resinae* GA, the latter representing the only available sequence of a GA with reasonably high activity toward α-1,6-linkages (Fagerström, 1991). Sequence comparison reveals that three of the six α→α connecting segments, namely, from α-helices 3 to 4 (region 2), 5 to 6 (region 3), and 9 to 10 (region 5), in *H. resinae* GA are distinctly different from the other GAs (Figure 1). In the present work emphasis has been on replacements in regions 3 and 5.

The substitution in L3L5 GA from *A. awamori* of two short sequences by the corresponding *H. resinae* residues improved the catalytic efficiency (k_{cat}/K_m) toward isomaltooligosaccharides. Some changes in maltooligosaccharides hydrolysis were also observed, but the largest was a 50% decrease in k_{cat} for maltose while affinity remained almost identical compared to wild-type. The single loop replacements, in contrast, reduced the activity compared to both wild-type and L3L5 GAs. L5 GA had low activity on both α-1,4- and α-1,6-linked oligosaccharides, while L3 GA mainly lost activity on isomaltooligosaccharides. The strongest effect for both single-loop variants was in the action on isomaltotriose and longer substrates. k_{cat}/K_m values for L3 GA were thus only 16−20% of the wild-type, albeit slightly higher toward isomaltose. Similar effects were observed with L5 GA suggesting that the protein part affected is associated with the function of the outermost binding subsites, i.e., beyond subsites 1 and 2 of the active site area. While calculation of individual subsite binding affinities according to the method of Hiromi et al. (1983) was not meaningful for L5 (k_{int} and the affinity at subsite 1 cannot be determined) and also questionable for L3, the subsite maps of L3L5 GA (data not shown) were similar to those of wild-type GA, for both α-1,4- and α-1,6-linked substrates.

Several residues in loops 3 and 5 were previously subjected to mutational analysis. Replacement of Val181-Asn182-Gly183-Ser184 by Ala-Ala-Lys-His thus caused a 7-fold decrease and a minor increase in k_{cat} for maltose and isomaltose hydrolysis, respectively, with virtually no effect on K_m (Svensson et al., 1995).

Arg305 from loop 5 has been identified as a crucial determinant in binding of α-1,4- but not of α-1,6-linked substrates (Frandsen et al., 1995). In the D-*gluco*-dihydroacarbose complex of GA from *A. awamori* var. *X100* (Figure 6A; Stoffer et al., 1995), Arg305 NH1 is thus hydrogen bonded to OH3 of ring b ($d = 2.72$ Å) and less strongly to OH2 of ring a ($d = 3.0$ Å). The hydrogen bond network includes Arg305 NH2 that interacts with OD1 of Asp309, and a possible interconnection to loop 3 exists through hydrogen bonding of Arg305 NH2 to Glu180 OE2. Tyr311, moreover, stacks to ring b. Mutation of Asp309 reduced affinity and k_{cat}, especially toward maltooligosaccharides, while replacement of Trp317 essentially induced decrease in all substrate affinities (Frandsen et al., 1995). Considering such wide variation in effect of mutations in either the L5 GA target sequence, or residues interacting with this sequence the region in question is clearly critical for the specificity of GA. Arguably, however, successful prediction of enzymatic properties of L5 and L3L5 or other related GA variants will be extremely difficult.

In an attempt to interprete the kinetic properties of the present loop mutants in structural terms, a model of an isomaltose complex based on the D-*gluco*-dihydroacarbose GA complex (Figure 6A; Stoffer et al., 1995) was made for both wild-type (Figure 6B) and L3L5 (Figure 6C) GAs followed by energy minimization. Obviously, this only provides a hypothetical structural basis for understanding the effects of the replacements. Moreover, it will not be possible to identify structural features that explain the enhanced enzymatic properties of the double loop L3L5 GA mutant, as opposed to L3 and L5 GAs. The two modeled complexes (Figure 6B,C) illustrate that, despite substitution of four side chains in loop 5, including a Pro to Ala replacement, and of three in loop 3, the backbone in the segments involved may adopt a conformation close to that of wild-type. Furthermore, the interactions of GA with isomaltose are maintained (Figure 6B,C). The lack of drastic changes in the architecture of L3L5 GA is in accordance with only minor variations in kinetic parameters for L3L5 compared to wild-type GA (Tables 3 and 4). We speculate that small modifications in hydrogen bond lengths, especially involving Glu180, Arg305, and/or Asp309, might be responsible for the slight improvement of the kinetic parameters on isomaltooligosaccharides achieved in L3L5 GA.

Clearly, more residues than the seven replaced in L3L5 GA play important roles for the substrate bond-type specificity of GA. Thus, even though the activity towards α-1,6-linked substrates is enhanced compared to that of *A. awamori* wild-type GA, values of the L3L5 GA relative specificity, $[(k_{cat}/K_m)_{Gn}/(k_{cat}/K_m)_{iGn}]$, for maltooligosaccharides over isomaltooligosaccharides remain larger than those of *H. resinae* GA (Table 5). The relative specificity of the *A. awamori* wild-type, its three loop variants, and *H. resinae* GAs are most variable for the shorter substrates. Thus, for disaccharides, the relative specificity of L3L5 GA is reduced 5-fold compared to *A. niger* wild-type GA and 4 times as high, respectively, as that of *H. resinae* GA (Table 5). With the trisaccharides these differences are slightly diminished, whereas a 3-fold decrease of L3L5 GA compared to the *A. niger* enzyme and a 10-fold increase compared to *H. resinae* GA is obtained with the tetrasaccharide substrates. L3 GA interestingly from the trisaccharide and beyond essentially doubled the relative specificity compared to wild-type GA, whereas on the disaccharide it decreased 4-fold. Surprisingly variations of L5 GA were more modest (Table 5).

Among the active site α→α loops, conspicuous sequence differences between the *A. awamori* and *H. resinae* GAs are also found near the critical Trp120 from the conserved region 2 (Clarke & Svensson, 1984b; Sierks et al., 1989). Noticeably, Ser119 in *A. awamori* GA corresponds to Pro in *H. resinae* GA. Most GAs have Ser and Ala, Pro being present in only *Saccharomyces diastaticus* and *H. resinae* GAs. The invariant Trp120 in *A. awamori* GA, was earlier shown to play a critical role in transition-state stabilization (Sierks et al., 1989) and may thus differ in spatial orientation in the latter GA. Although OG of Ser119 hydrogen bonds to 3OH of the fourth ring in D-*gluco*-dihydroacarbose (Stoffer et al.,

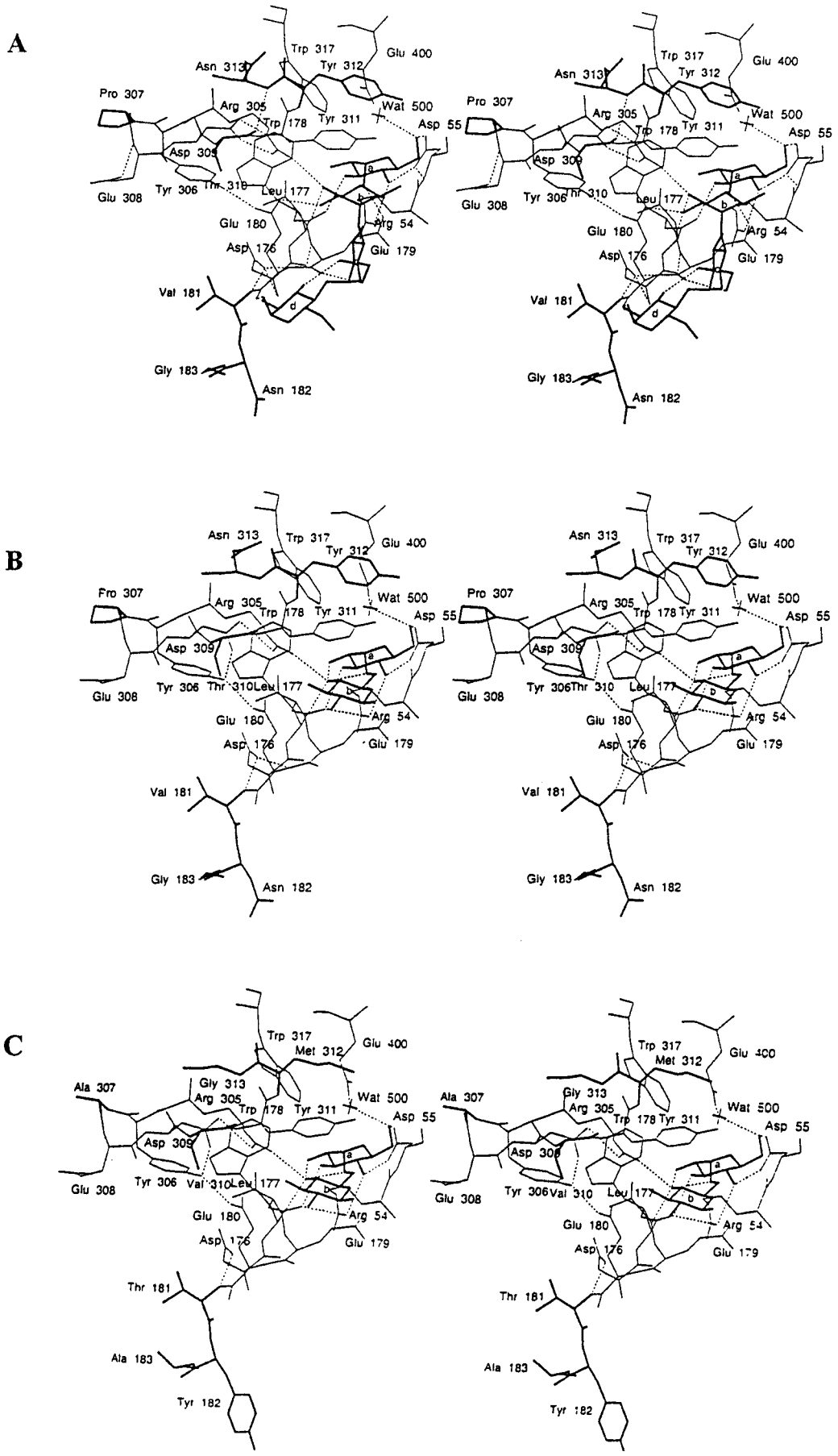

FIGURE 6: Stereoview of the active site of (A) *A. awamori* var. *X100* GA in complex with D-*gluco*-dihydroacarbose (Stoffer et al., 1995), (B) *A. awamori* var. *X100* GA in complex with isomaltose (modeled, see Materials and Methods), and (C) L3L5 GA in complex with isomaltose (modeled, see Materials and Methods). Isomaltose, D-*gluco*-dihydroacarbose, and residues substituted in the present study are in bold. Dashed lines represent hydrogen bonds.

Table 5: Relative Specificity[a] of *A. awamori* Wild-Type, L3, L5, and L3L5 GAs, and *H. resinae* GA

DP	*A. awamori* wild-type	L3	L5	L3L5	*H. resinae* GA[b]
2	681	160	400	131	27
3	1107	2823	1156	341	109
4	2252	4704	1085	763	79
5	2220	4966	1507	986	43
6	2169	4326	1411	1034	54
7	1582	4429	1085	1003	40

[a] $(k_{cat}/K_m)_{Gn}/(k_{cat}/K_m)_{iGn}$. [b] Data from Fagerström (1991).

1995), Ser119→Tyr GA did promote α-1,6-condensation reactions (Svensson et al., 1995) and has wild-type level activity toward maltose and slightly suppressed activity on isomaltose (Sierks & Svensson, 1994). Site-directed mutagenesis of Ser119 to Pro in *A. awamori* GA alone and in combination with mutations as in L3 and L5 GA are proposed to lead to a variant having even lower relative specificity than L3L5 GA.

ACKNOWLEDGMENT

We are grateful to Sidsel Ehlers for excellent technical assistance and to Dr. Ib Svendsen, Bodil Corneliussen, and Lone Sørensen for amino acid and N-terminal sequence analysis. Drs. Nathalie Juge and Ikuo Matsui are thanked for helpful discussions.

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BI960241C