Mutational Analysis of the Roles in Catalysis and Substrate Recognition of Arginines 54 and 305, Aspartic Acid 309, and Tryptophan 317 Located at Subsites 1 and 2 in Glucoamylase from *Aspergillus niger*†

Torben P. Frandsen,‡ Trine Christensen,‡ Bjarne Stoffer,‡,⊥ Jan Lehmbeck,§ Claude Dupont,‡,∥ Richard B. Honzatko,▽ and Birte Svensson*,‡

Department of Chemistry, Carlsberg Laboratory, Gamle Carlsberg Vej 10, DK-2500 Copenhagen Valby, Denmark, Fungal Gene Technology, Enzyme Business, Novo Nordisk, Novo Allé, DK-2880 Bagsvaerd, Denmark, and Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50011

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ABSTRACT: The mutants Arg54 → Leu, Arg54 → Lys, Arg305 → Lys, Asp309 → Glu, and Trp317 → Phe, located at subsites 1 and 2 in glucoamylase from *Aspergillus niger*, provide insight into the importance of specific hydrogen bonds and hydrophobic interactions in substrate recognition, catalytic mechanism, and stability. As suggested from the crystal structure of a closely related glucoamylase [Aleshin, A. E., Firsov, L. M., & Honzatko, R. B. (1994) *J. Biol. Chem. 269*, 15631−15639], Arg54 in subsite 1 hydrogen bonds to the key polar group 4′-OH of maltose. The two mutants of Arg54 display losses in transition-state stabilization of 16−21 kJ mol^{-1} in the hydrolysis of different maltooligodextrins, which originate from a [(1.2−1.8) × 10^3]-fold reduction in k_{cat} and changes in K_m ranging from 25% to 300% of the wild-type values. Arg305 similarly hydrogen bonds to 2′-OH and 3-OH, located at subsites 1 and 2, respectively. Arg305 → Lys glucoamylase is not saturated at concentrations of maltose or maltoheptaose of 400- and 40-fold, respectively, the K_m of the wild-type enzyme. This mutant also has highly reduced k_{cat}. On the other hand, for the α-1,6-linked isomaltose, the Lys305 mutant surprisingly has the same K_m as the wild-type enzyme, while k_{cat} is 10^3-fold reduced. Arg305 is thus an important determinant in the distinction of the α-1,4 to α-1,6 substrate specificity. Arg305 interacts electrostatically and hydrophobically with the side chains of Asp309 and Trp317. The mutants Asp309 → Glu and Trp317 → Phe display a 12−80-fold increase in K_m for α-1,4-linked substrates, while k_{cat} is essentially unaffected; K_m for isomaltose increased 3−7-fold and k_{cat} decreased 2−4-fold compared to that for wild-type glucoamylase. Arg305, Asp309, and Trp317 are near an extraordinary secondary structure [Aleshin, A., Golubev, A., Firsov, L. M., & Honzatko, R. B. (1992) *J. Biol. Chem. 267*, 19291−19298] in which strain imposed upon Asn313 and Ser411 via hydrogen bonds forces nonbonded atoms in close contact. The Arg305 → Lys, Asp309 → Glu, or Trp317 → Phe mutants thus have reduced stability at elevated temperature and in guanidine hydrochloride. The results provide insight useful for rational engineering of bond-type specificity in glucoamylase.

The increasing number of three-dimensional structures of carbohydrate-metabolizing enzymes and carbohydrate-binding proteins improves the understanding of forces and bond types important in stabilization and specificity of protein−carbohydrate interactions. Excellent complementarity, involving hydrophobic and hydrophilic interactions, characterizes the molecular structure and the thermodynamics of protein−carbohydrate complexes (Vyas, 1991; Bundle & Young, 1992; Bourne et al., 1992; Quiocho, 1993; Sigurskjold et al., 1994). Two different and complementary approaches have been used to describe in detail the energetics of ligand binding to *Aspergillus niger* glucoamylase (GA)[1] (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3). One is site-directed mutagenesis that addresses the significance of specific side chains. Another is molecular recognition in which substrate analogs are used to identify atoms or groups of atoms important in binding and assess their contribution to the binding energy. Structural elements in glucoamylase and its substrates are thus assigned particular roles in substrate recognition and catalysis (Bock & Pedersen, 1987; Sierks et al., 1989, 1990, 1993; Sierks & Svensson, 1992, 1993; Frandsen et al., 1994a,b; Svensson et al., 1994). Recently the crystal structure of the acarbose complex of a closely related glucoamylase has become available (Aleshin et al., 1994), which allows the assignment of a fraction of the total binding energy contribution to specific protein−carbohydrate interactions.

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* Address correspondence to this author at: Department of Chemistry, Carlsberg Laboratory, DK-2500 Copenhagen Valby, Denmark. Telephone: + 45 33 27 53 45. Facsimile + 45 33 27 47 08.

‡ Carlsberg Laboratory.

§ Novo Nordisk.

▽ Iowa State University.

⊥ Present address: University of Copenhagen, Department of Chemistry, Laboratory IV, Universitetsparken 5, DK-2100 Copenhagen Ø, Denmark.

∥ Present address: Université du Québec, Institut Armand-Frappier, Department de Microbiologie Appliquée, Laval, Québec, Canada H7N 4Z3.

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[1] Abbreviations: bp, base pair; GA, glucoamylase; GdnHCl, guanidine hydrochloride.

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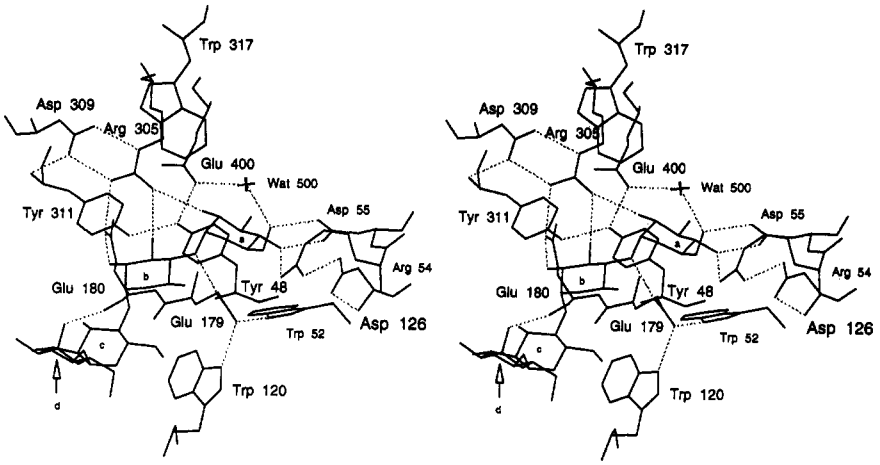

FIGURE 1: Stereoview of the active site of glucoamylase from *A. awamori* var *X100* with bound D-*gluco*-dihydroacarbose (Stoffer et al., 1995). Atoms within hydrogen bond donor−acceptor distance of 3.2 Å are represented by dashed lines. The rings of D-*gluco*-dihydroacarbose are labeled a−d from the nonreducing end.

GA catalyzes the hydrolysis of glucose from nonreducing ends of starch and related oligo- and polysaccharides with inversion of the anomeric configuration. GA tolerates a broad range of aglycon moieties, cleaving α-1,6 glucosidic bonds and aryl glucosides with 500- and 50-fold less activity, respectively, than for maltose (Hiromi et al., 1966; Sierks et al., 1989; Ermer et al., 1993). The active site crevice has seven kinetically identified consecutive subsites. Hydrolysis occurs between subsites 1 and 2 (Hiromi et al., 1983; Sierks et al., 1990; Fagerström, 1991), and Glu179 and Glu400 are shown by chemical modification, mutation, and crystallography to act as general acid and base catalyst, respectively (Sierks et al., 1990; Svensson et al., 1990; Harris et al., 1993; Frandsen et al., 1994a). Tyr48 OH is hydrogen bond donor to γ-COO^- of Glu400, and mutation demonstrated Tyr48 to be critical for catalysis; nonbonding electrons of Tyr48 OH perhaps contribute to stabilization of an oxocarbonium ion−substrate intermediate (Frandsen et al., 1994a).

As is typical of both aromatic and charged side chains (Vyas, 1991; Quoicho, 1993), Arg54, Arg305, Asp309, and Trp317 in GA participate in carbohydrate binding (Aleshin et al., 1992, 1994). These four invariant residues (Coutinho & Reilly, 1994; Henrissat et al., 1994) are located in the first or the fifth of the six α → α connecting segments that form the active site cavity in the catalytic $(\alpha/\alpha)_6$-barrel domain of GA (Aleshin et al., 1992). Arg54 hydrogen bonds in subsite 1 to the 4-OH of ring a of the inhibitor D-*gluco*-dihydroacarbose and Arg305 in subsites 1 and 2 to the 2- and 3-OH of rings a and b, respectively (see Figure 1; Stoffer et al., 1995). Arg305 is also engaged in electrostatic and hydrophobic interactions with the side chains of Asp309 and Trp317. Molecular recognition of different monodeoxymaltosides by single-residue GA mutants is a useful strategy for mapping the energetics of individual enzyme−substrate transition-state interactions. Comparable to increases in activation energy due to mutational elimination of specific glucoamylase−substrate hydrogen bonds, $\Delta(\Delta G^{\ddagger})$ values of 11 and 18 kJ mol^{-1} arise by substitution with hydrogen of the 4′- or 3-OH, respectively, of maltose which are so-called key polar groups required for GA catalysis (Bock & Pedersen, 1987; Sierks & Svensson, 1992; Sierks et al., 1992; Svensson et al., 1994). To elucidate the significance of the different interactions in GA for substrate bond-type specificity and catalysis, the mutants Arg54 → Leu/Lys, Arg305 → Lys, Asp309 → Glu, and Trp317 → Phe were made and their enzymatic and stability properties are discussed in relation to relevant three-dimensional models of GA−oligosaccharide inhibitor complexes (Aleshin et al., 1992, 1994; Stoffer et al., 1995) and to GA-catalyzed hydrolysis of maltose analogs (Sierks & Svensson, 1992; Sierks et al., 1992; Svensson et al., 1994). A most unusual effect was obtained by replacement of Arg305, which was concluded to be crucial in α-1,4 as opposed to α-1,6 glucosidic bond substrate specificity.

MATERIALS AND METHODS

Materials. Recombinant (AnMT 833) and commercial (AMG 200L) GA were obtained as culture filtrates from Novo Nordisk (Bagsvaerd, Denmark) and purified as described (Svensson et al., 1982; Stoffer et al., 1993). Acarbose-Sepharose was prepared as described earlier (Clarke & Svensson, 1984b). Isomaltose, maltotriose through maltoheptaose, and the glucose oxidase kit were from Sigma (St. Louis, MO); the glucose dehydrogenase kit and maltose monohydrate were from Merck (Darmstadt, Germany). Guanidine hydrochloride (GdnHCl) was from Fluka (Buchs, Switzerland). Water was drawn from a Milli-Q system (Millipore, Bedford, MA). Other chemicals used were of analytical grade.

Construction of Mutant Genes. The *A. niger* GA gene (Boel et al., 1984) was mutated essentially as described (Frandsen et al., 1994a) using a modified PCR method (Nelson et al., 1989). For the two mutations at position 54, the sets of primers were R54K, 5′-TTCTACACCTGGAC-**TAAG**GACTCTGGTCTC-3′ and 5′-GAGACCAGAGTC-**CTT**AGTCCAGGTGTAGAA-3′, and R54L, 5′-TTCTA-CACCTGGACTC**TA**GACTCTGGTCTC-3′ and 5′-GAG-ACCAGAGTC**TA**GAGTCCAGGTGTAGAA-3′ (nucleotides replaced for mutation are in bold). The mutagenized fragment was amplified using a reverse hybrid primer (A, 5′-CAAGGGCCCGGTGTT-3′) complementary to the genomic DNA inverse strand downstream of the site of mutation and a forward primer (B, 5′-GAGCCTAAGCT-TCATCCCCAGCATCATTAC-3′) upstream of the site of mutation.

For mutation at position 305, the sets of primers were R305K, 5′-CGGTGGGT**AAG**TACCCTGAGGACACG-

TACTACAAC-3′ and 5′-GTTGTAGTACGTGTCCTCA-GGGTACTTACCCACCG-3′ (nucleotides replaced for mutation are in bold). As above, a reverse hybrid primer (C, 5′-CGCTGCCGGATCCAGTGGGGGTAGCCGTCG-3′) and a forward primer (D, 5′-TCTGGGAAGAAGTTAACCG-GTCGTCTTTCT-3′) were used for amplification.

Mutations of Asp309 and Trp317 were done using the primers D309E, 5′-GGTCGGTA*T*CCTGAGGA**G**ACGTAC-TAC-3′, and W317F, 5′-CGGCAACCCGT**T**CTTCCT*C*T-GCACCTTGGC-3′ (nucleotides in bold were replaced for mutation and in italics for silent mutations, facilitating control of the construct by restriction enzyme analysis). A reverse hybrid primer (E, 5′-GTATGATCAGGATGCTAGAC-CCCATCCTTTAACTATAGCG-3′) was used downstream of the GA C-terminus, composed of a 3′ 21-nucleotide sequence complementary to the genomic DNA inverse strand and a 5′ 19-nucleotide unique sequence, a forward primer (F, 5′-CCGTTCACAAGCTGAAGAGC-3′) was used upstream of the GA N-terminus, and a reverse primer (G, 5′-GTATGATCAGGATGCTAGAC-3′) of identical sequence to the unique 5′ 20-nucleotide half of primer A was used.

PCR, cloning, sequencing, transformation, and expression of *A. niger* were done essentially as described (Frandsen et al., 1994a).

Purification. Recombinant wild-type and mutant GAs were isolated from culture filtrates (Frandsen et al., 1994a) using individual acarbose-Sepharose columns (Clarke & Svensson, 1984b) followed by purification on HiLoad Q-Sepharose (Stoffer et al., 1993). Homogeneity was evaluated by Tricine-SDS−PAGE, amino acid, and N-terminal sequence analyses and hydrolysis of 4-nitrophenyl α-D-glucopyranoside (Stoffer et al., 1993). The concentration of mutant protein in the culture liquids was estimated to be 0.50 (15:1), 0.06 (10:1), 0.11 (14:1), 0.70 (4:1), and 0.15 (4:1) g L^{-1} for Arg54 → Leu, Arg54 → Lys, Arg305 → Lys, Asp309 → Glu, and Trp317 → Phe GAs, with the G1:G2 ratios shown in brackets.

Analytical Techniques. Protein concentrations were determined by amino acid analysis and/or spectrophotometrically at 280 nm using $\epsilon = 1.37 \times 10^5$ M^{-1} cm^{-1} for GA G1, $\epsilon = 1.09 \times 10^5$ M^{-1} cm^{-1} for GA G2 (Clarke & Svensson, 1984a), and for Trp317 → Phe GA G1 $\epsilon = 1.31 \times 10^5$ M^{-1} cm^{-1} as calculated from lack of one tryptophan compared to wild-type GA (Svensson et al., 1983).

Enzyme Assays. Initial rates of hydrolysis were determined for maltose through maltoheptaose, isomaltose, and 4-nitrophenyl α-D-glucopyranoside at 45 °C in 0.05 M sodium acetate at pH 4.5 using up to 12 substrate concentrations ranging from $^1/_8K_m$ to $8K_m$. Released glucose was measured by the glucose oxidase method with monitoring in microtiter plates (Fox & Robyt, 1991; Palcic et al., 1993; Frandsen et al., 1994a). The kinetic measurements were performed using the G1 form of mutant and wild-type GAs and enzyme concentrations: 0.567−26.9 μM for Arg54 → Leu, Arg54 → Lys, and Arg305 → Lys GAs; 0.0045−2.78 μM for Asp309 → Glu and Trp317 → Phe GAs; 0.00117−0.83 μM for wild-type GA. k_{cat} and K_m were obtained by fitting values of initial rates, v, as a function of v/[S] to the Eadie−Hofstee equation using the program ENZFITTER (Leatherbarrow, 1987). Activity on 4-nitrophenyl α-D-glucopyranoside was measured using total volumes of 300 μL and initiating hydrolysis by addition of enzyme to a final concentration of 1.5−4.2 μM. Aliquots (50 μL) were removed and added to 0.1 M sodium borate, pH 9.4 (200 μL), in microtiter wells to quench the reaction. The absorbance was read at 405 nm and quantitated using 4-nitrophenol as a standard.

Changes in activation energy for substrate hydrolysis were calculated from $\Delta(\Delta G^{\ddagger}) = -RT \ln[(k_{cat}/K_m)_{mut}/(k_{cat}/K_m)_{wt}]$ (Wilkinson et al., 1983). The affinities at individual substrate glucosyl binding subsites were calculated as earlier described from values of k_{cat} and K_m determined for maltose through maltoheptaose (Hiromi et al., 1983; Fagerström, 1991; Frandsen et al., 1994a). The pH activity dependence was determined using 50 mM citrate phosphate at 16 different pH values from pH 2.4 to 7.2. Where possible, maltose was used at ca. $^1/_{12}K_m$ and $12K_m$ to estimate pK_a of the catalytic groups in free and substrate-complexed enzyme, respectively. However, since Asp309 → Glu and Trp317 → Phe GAs have unusually high K_m values, pK_a of the catalytic groups in the enzyme−substrate complexes was estimated at $2K_m$ and $6K_m$, respectively. The enzyme concentration range was 0.062−0.346 μM.

Thermostability. GA (0.9 μM) was incubated at varying temperatures (25−80 °C) in 0.1 M sodium acetate, pH 4.3, for 5 min, and the residual activity toward maltose was determined using the glucose dehydrogenase assay (Svensson et al., 1986a; Stoffer et al., 1993). T_m is defined as the temperature corresponding to recovery of 50% of the initial activity.

GdnHCl Denaturation. Unfolding of GA (approximate concentration 0.24 μM) by GdnHCl from 0 to 8 M in 0.05 M sodium acetate, pH 4.5, was followed by fluorescence changes using a Perkin-Elmer LS50 or LS50B luminiscence spectrometer. Excitation and emission wavelengths were 280 and 320 nm, respectively, and the slit width was 5 mm. From the measured fluorescence intensity, the fraction of unfolded protein, f_u, was calculated from $f_u = F_N - F_{obs}/F_N - F_U$, where F_N and F_U are fluorescence intensities with the folded and unfolded protein, respectively, and F_{obs} is the fluorescence observed after 1 h incubation at room temperature. Standard free energy parameters were not calculated, since the unfolding of GA is not considered a reversible process (Williamson et al., 1992).

RESULTS

Kinetic Parameters. k_{cat} and K_m are determined for GA mutants using the α-1,4-linked maltose and maltoheptaose and the α-1,6-linked isomaltose as substrates. While k_{cat} for hydrolysis of maltose and maltoheptaose is reduced for Arg54 → Leu and Arg54 → Lys GAs to less than 0.1% of the wild-type value, K_m increased about 2-fold for Arg54 → Leu GA and decreased 2−4-fold for Arg54 → Lys GA (Table 1). Both mutants have extremely low activity toward isomaltose, and the corresponding kinetic parameters could not be determined. In contrast, replacement of Arg305 by Lys resulted in an exceptionally great loss of affinity for α-1,4-linked substrates, the rates of hydrolysis increasing linearly up to at least 500 mM maltose and 40 mM maltoheptaose. The second-order rate constant, k_{cat}/K_m, is $(2.5 \times 10^3-10^4)$-fold reduced compared to that of wild-type GA, but proper analysis of k_{cat} and K_m is impossible since substrate saturation was not achieved (Table 1). Most surprisingly, however, Arg305 → Lys and wild-type GAs have identical K_m for isomaltose, although the mutant displays only 0.1% of the wild-type k_{cat} value (Table 1).

Table 1: Kinetic Parameters for Hydrolysis of Maltose, Isomaltose, Maltoheptaose, and 4-Nitrophenyl α-D-Glucopyranoside by Wild-Type and Mutant Glucoamylases from *A. niger*[a]

substrate	wild-type	Arg54 → Leu	Arg54 → Lys	Arg305 → Lys	Asp309 → Glu	Trp317 → Phe
maltose						
k_{cat} (s^{-1})	10.7 ± 0.6[b]	0.0094 ± 0.0002	0.0079 ± 0.0004		9.1 ± 0.2	6.0 ± 1.2
K_m (mM)	1.21 ± 0.14	3.03 ± 0.20	0.59 ± 0.13		52.4 ± 4.6	14.0 ± 0.64
k_{cat}/K_m (s^{-1} mM^{-1})	8.84 ± 1.14	0.0031 ± 0.0002	0.013 ± 0.003	0.0024[c]	0.174 ± 0.016	0.43 ± 0.08
isomaltose						
k_{cat} (s^{-1})	0.41 ± 0.04	*d*	*d*	$4.36 \times 10^{-4} \pm 4.0 \times 10^{-5}$	0.090 ± 0.003	0.17 ± 0.03
K_m (mM)	19.8 ± 2.8			21.0 ± 4.2	61.9 ± 5.3	153.0 ± 50.0
k_{cat}/K_m (s^{-1} mM^{-1})	0.021 ± 0.004			$2.1 \times 10^{-5} \pm 4.0 \times 10^{-6}$	0.0015 ± 0.0001	0.0011 ± 0.0004
maltoheptaose						
k_{cat} (s^{-1})	59.7 ± 1.6	0.043 ± 0.003	0.032 ± 0.006		67.1 ± 2.06	51.8 ± 2.3
K_m (mM)	0.12 ± 0.01	0.21 ± 0.05	0.031 ± 0.002		9.44 ± 0.70	3.30 ± 0.28
k_{cat}/K_m (s^{-1} mM^{-1})	498 ± 44	0.200 ± 0.05	1.03 ± 0.20	0.0658[c]	7.11 ± 0.57	15.70 ± 1.50
4-nitrophenyl α-D-glucopyranoside						
k_{cat} (s^{-1})	0.50 ± 0.01	*d*	*d*	*d*	0.36 ± 0.02	*d*
K_m (mM)	3.70 ± 0.19				27.5 ± 2.2	
k_{cat}/K_m (s^{-1} mM^{-1})	0.135 ± 0.007				0.013 ± 0.001	

[a] Determined at 45 °C in 0.05 M sodium acetate, pH 4.5. [b] Standard deviation. [c] Determination of the second-order rate constant, k_{cat}/K_m. Saturation of this mutant was not possible, i.e., $K_m \gg [S]$ (see text). [d] Not determined.

Table 2: Increase in Transition-State Binding Energies, $\Delta(\Delta G^{\ddagger})$[a] (kJ mol^{-1}), between Wild-Type and Mutant Glucoamylase from *A. niger*[b]

	enzyme				
substrate	Arg54 → Leu	Arg54 → Lys	Arg305 → Lys	Asp309 → Glu	Trp317 → Phe
maltose	21.0 ± 0.4[c]	17.3 ± 0.7	21.8	10.4 ± 0.4	8.0 ± 0.6
isomaltose	*d*	*d*	18.3 ± 0.7	7.1 ± 0.5	7.7 ± 1.1
maltoheptaose	20.7 ± 0.7	16.5 ± 0.6	23.6	11.2 ± 0.3	9.1 ± 0.3

[a] $\Delta(\Delta G^{\ddagger}) = -RT \ln[(k_{cat}/K_m)_{mut}/(k_{cat}/K_m)_{wt}]$. [b] Calculated from the data in Table 1. [c] Standard deviation. [d] Not determined.

Asp309 → Glu and Trp317 → Phe GAs possess substantial activity especially toward the α-1,4-linked substrates. The k_{cat} for Asp309 → Glu GA is within 10% of that for wild-type, while the values for Trp317 → Phe GA decreased moderately (Table 1). Both mutants, however, have poor affinity for maltose and maltoheptaose: K_m increased 28–80- and 12–45-fold, respectively (Table 1). For isomaltose hydrolysis, k_{cat} was about 20% and 40% and K_m increased 3–8-fold compared to the wild-type values (Table 1). Moreover, for Asp309 → Glu GA the effect was very similar toward 4-nitrophenyl α-D-glucopyranoside hydrolysis, k_{cat} being 70% and K_m 7-fold higher than that for wild-type GA (Table 1).

Changes in transition-state stabilization energy, $\Delta(\Delta G^{\ddagger})$, are calculated (Table 2) from the data in Table 1. Arg54 → Leu/Lys and Arg305 → Lys GA increased the activation energy, $\Delta(\Delta G^{\ddagger})$, by 16.5–23.6 kJ mol^{-1}, suggesting that the mutation eliminated a charged hydrogen bond to the substrate intermediate (Fersht et al., 1985). $\Delta(\Delta G^{\ddagger})$ of 7.1–11.2 kJ mol^{-1} for Asp309 → Glu and Trp317 → Phe GAs is compatible with loss of one or more uncharged hydrogen bonds for stabilization in the mutant compared to the wild-type substrate transition state.

Subsite Mapping. Arg54 → Lys and Trp317 → Phe GAs are selected for characterization of the binding affinity throughout the extended active site area as representatives of subsite 1 and subsite 2 mutants having reasonable substrate affinity. The individual kinetic parameters determined for the different maltodextrins show that the effect of the mutation Arg54 → Leu GA is confined to k_{cat} and is large for all substrates—maltose through maltoheptaose (Table 3). Trp317 → Phe GA in contrast has a very modest effect on k_{cat}, while the K_m values increase about 10-fold. The three outermost of the seven identified subsites contribute very little binding energy (Hiromi et al., 1983), and for both mutants the effect is clearly associated with the innermost subsites. Binding energy calculations (Table 4) thus show that the highly favorable interaction between GA and substrate at subsite 2 is decreased by about 6 and 2 kJ mol^{-1} in Trp317 → Phe and Arg54 → Leu GAs, respectively. Otherwise the subsite affinities are very similar to those of wild-type GA.

pH–Activity Dependence. pH–activity curves for Asp309 → Glu and Trp317 → Phe GAs in their substrate-complexed forms closely resemble the pH profiles for wild-type GA, indicating that the catalytic residues, having pK_a values of 2.7 and 5.9 in wild-type GA (Hiromi et al., 1966; Sierks et al., 1990), are essentially unperturbed by the mutations (Figure 2a). The uncomplexed Asp309 → Glu GA in contrast shows a narrow, more bell-shaped pH–activity curve; the pK_a of its catalytic acid is thus suppressed by 0.5–1 unit, whereas that of the catalytic base appears to be elevated compared to the wild-type enzyme value (Figure 2b). The Arg54 → Leu GA in both free and complexed states behaves as if the ionization of the catalytic acid is not influenced (data not shown).

Thermostability. The recombinant *A. niger* wild-type GA has T_m of 71 °C (Figure 3) and is slightly more thermostable than GA purified from a commercial enzyme preparation, for which T_m is 67 °C (Svensson et al., 1986a; Stoffer et al., 1993). Trp317 → Phe and Asp309 → Glu GAs have considerably lower stability, T_m being reduced by 7 and 13 °C, respectively (Figure 3). Because of the low activity of Arg54 → Leu GA at the protein concentration used in heat stability measurements, only a rough estimate in T_m was determined, being 6 °C less than the T_m of the wild-type enzyme.

Stability in GdnHCl. Unfolding of the Asp309 → Glu GA mutant is compared at increasing concentrations of GdnHCl to that of recombinant wild-type GA (Figure 4).

Table 3: Kinetic Parameters for Wild-Type and Mutant Glucoamylases from *A. niger* with Maltooligodextrins of DP 2–7[a]

substrate	wild-type			Arg54 → Leu			Trp317 → Phe		
	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})
maltose	10.7 ± 0.6[b]	1.21 ± 0.14	8.84 ± 1.14	0.0094 ± 0.0002	3.03 ± 0.20	0.0031 ± 0.0002	6.03 ± 1.15	14.01 ± 0.64	0.43 ± 0.08
maltotriose	33.5 ± 0.7	0.28 ± 0.04	120 ± 17	0.027 ± 0.001	0.725 ± 0.086	0.0372 ± 0.0046	32.0 ± 7.4	5.4 ± 0.9	5.93 ± 1.69
maltotetraose	41.3 ± 1.4	0.12 ± 0.01	344 ± 31	0.037 ± 0.001	0.279 ± 0.020	0.133 ± 0.010	22.3 ± 0.6	1.5 ± 0.1	14.87 ± 1.07
maltopentaose	48.0 ± 3.1	0.11 ± 0.01	436 ± 49	0.041 ± 0.001	0.319 ± 0.095	0.129 ± 0.038	42.3 ± 3.0	1.7 ± 0.2	24.88 ± 3.42
maltohexaose	56.2 ± 5.0	0.11 ± 0.01	511 ± 65	0.042 ± 0.001	0.235 ± 0.071	0.179 ± 0.054	41.5 ± 2.2	1.7 ± 0.1	24.41 ± 1.93
maltoheptaose	59.7 ± 1.6	0.12 ± 0.01	498 ± 44	0.043 ± 0.001	0.214 ± 0.049	0.201 ± 0.046	51.8 ± 2.3	3.3 ± 0.3	15.70 ± 1.59

[a] Determined at 45 °C in 0.05 M sodium acetate, pH 4.5. [b] Standard deviation.

Table 4: Subsite Binding Energies (kJ mol^{-1}) for Hydrolysis of Maltooligosaccharides by Wild-Type and Mutant Glucoamylases from *A. niger*[a]

subsite	wild-type	Arg54 → Leu	Trp317 → Phe
1	−2.33	−1.43	−0.75
min[b]	−1.53	−1.34	0.25
max	−3.13	−1.77	−2.41
2	−20.45	−18.38	−14.01
min	−19.38	−17.83	−12.37
max	−21.53	−19.00	−14.17
3	−6.90	−6.57	−6.94
min	−6.17	−6.06	−6.60
max	−7.61	−7.06	−8.15
4	−2.79	−3.51	−3.12
min	−2.19	−3.37	−2.43
max	−3.42	−3.91	−3.50
5	−0.63	0.08	−1.36
min	−0.08	1.20	−0.79
max	−1.16	−0.81	−1.90
6	−0.42	−0.87	0.05
min	0.22	0.77	0.61
max	−1.06	−2.49	−0.54
7	0.07	−0.31	1.17
min	0.63	1.08	1.65
max	−0.52	−1.80	0.69

[a] Calculated from the data in Table 3. [b] The minimum and maximum estimates given show the effect of worst case combinations of errors on k_{cat}/K_m.

Whereas the G1 and G2 forms of wild-type GA unfold in an apparent one-state transition with a midpoint around 5.6 M GdnHCl, the denaturation of Asp309 → Glu GA G1 has two distinct states with midpoints at 3 and 6.2 M GdnHCl, respectively. Since the G1 form contains both the catalytic and the starch-binding domains connected by a highly *O*-glycosylated linker region (Svensson et al., 1983, 1986b), we assume that this two-state unfolding represents denaturation of individual domains. The G2 form of Asp309 → Glu GA lacks the starch-binding domain and accordingly displays a one-state unfolding process with midpoint at 3 M GdnHCl. The unfolding of Arg305 → Lys GA (not shown) closely resembled that of Asp309 → Glu GA.

DISCUSSION

The effects of the present active site mutations in GA from *A. niger* on enzymatic and stability properties are interpreted using the three-dimensional structure of uncomplexed GA (Aleshin et al., 1992) and two pseudotetrasaccharide complexes of GA from *Aspergillus awamori* var. *X100* solved to 2.4 and 2.2 Å resolution (Aleshin et al., 1994; Stoffer et al., 1995) in conjunction with molecular recognition studies using monodeoxy maltosides (Bock & Pedersen, 1987; Sierks & Svensson, 1992; Sierks et al., 1992; Svensson et al., 1994). The mutated Arg54 and Arg305 in wild-type GA bind substrate OH groups directly, whereas Trp317 and Asp309 influence these hydrogen bonds indirectly via side chain interactions with Arg305 (Figure 1). The relevant hydrogen bonds in subsite 1 are between NH2 of Arg54 and 4-OH and NH1 of Arg305 to 2-OH of the inhibitor (Figure 1) and at subsite 2 between the latter group and 3-OH (Figure 1). On the basis of a 1.7 Å resolution structure of D-*gluco*-dihydroacarbose with GA (A. E. Aleshin, B. Stoffer, L. M. Firsov, B. Svensson, and R. B. Honzatko, unpublished data), the hydrogen bond angle between Arg305 and the 2-OH of the nonreducing end of the substrate infers a weak interaction. The result is fully consistent with studies using substrate deoxy analogs (Bock & Pedersen, 1987; Sierks et al., 1992),

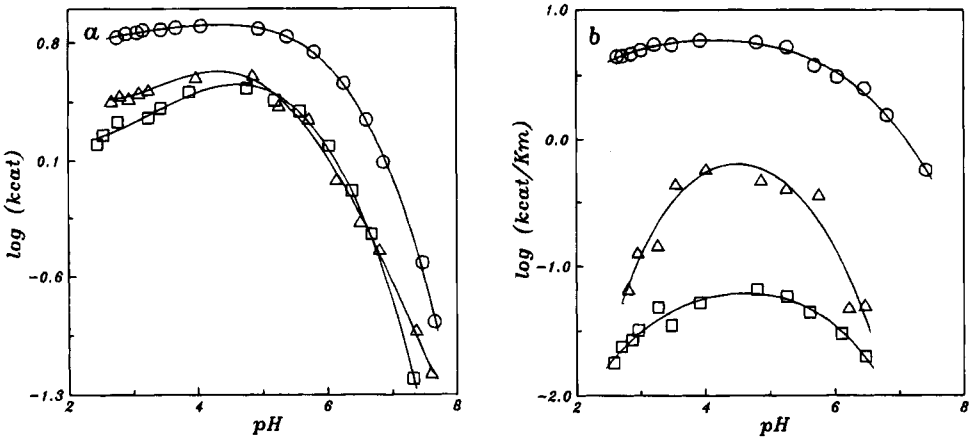

FIGURE 2: pH−activity profiles for maltose-complexed (a) and uncomplexed (b) wild-type (○), Asp309 → Glu (△), and Trp317 → Phe (□) glucoamylases from *A. niger* at 45 °C. Substrate concentrations were 12.7, 89.5, and 90.5 mM for panel a and 0.18, 4.47, and 2.68 mM for panel b for wild-type, Asp309 → Glu, and Trp317 → Phe GA, respectively. Lines drawn through points were fitted using a fourth degree polynomial and are intended to illustrate the shape of the plots.

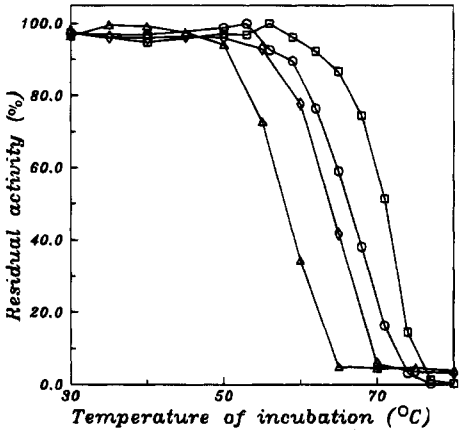

FIGURE 3: Temperature dependence for heat inactivation of G1 forms of recombinant (AnMT 833) wild-type (□), commercial (AMG 200L) wild-type (○), Trp317 → Phe (◇), and Asp309 → Glu (△) GAs.

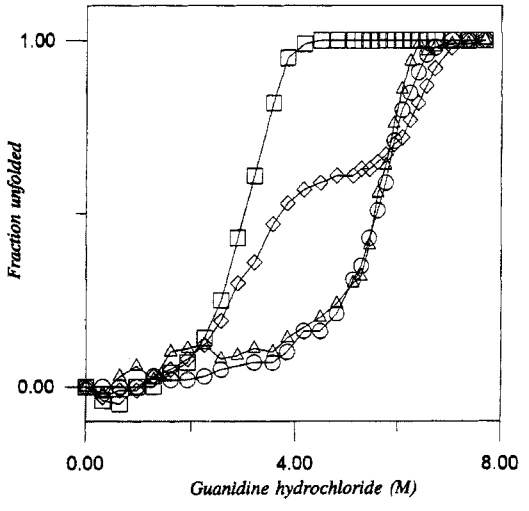

FIGURE 4: Denaturation curves for wild-type GA G1 (○), wild-type GA G2 (△), Asp309 → Glu GA G1 (◇), and Asp309 → Glu GA G2 (□). The fraction of unfolded protein is plotted as a function of [GdnHCl].

which indicate a poor interaction between the enzyme and the 2′-OH but a strong interaction with the 3-OH.

Arg54 → Leu/Lys and Arg305 → Lys GAs. Although the rate of catalysis is reduced substantially for Arg54 → Leu/Lys, only minor effects are found on substrate affinity. Interestingly the neutral Leu54 mutant has a slightly lower K_m than that of either the Lys54 mutant or the wild-type enzyme. The loss of the charged hydrogen bond contributed by Arg54 in the transition-state complex elicits an increase in transition-state stabilization energy, $\Delta(\Delta G^{\ddagger})$, of 17−21 kJ mol^{-1} for hydrolysis of maltose or maltoheptaose. The complementary replacement of the 4′-OH in maltose by hydrogen resulted in a similar $\Delta(\Delta G^{\ddagger})$ value of 18.8 kJ mol^{-1} in hydrolysis catalyzed by wild-type GA (Sierks et al., 1992). Apparently a hydrogen bond at subsite 1 between Arg54 (NH2) and the 4′-OH of the sugar ring (Figure 1) contributes significantly to the transition-state stabilization. Even in the case of the Lys54 mutant, a hydrogen bond between the lysine and the 4′-OH is unlikely. Arg54 of the wild-type enzyme interacts with the 4′-OH and the side-chain of Asp126. The interaction of Lys54 with the 4′-OH and Asp126 is mutually exclusive. We suggest that Lys54 would favor a salt link with Asp126 over a hydrogen bond to the 4′-OH. The mutation of Arg305 to Lys results in a $\Delta(\Delta G^{\ddagger})$ of 22−24 kJ mol^{-1}, the value for hydrolysis of isomaltose being slightly lower, 18 kJ mol^{-1}. Since hydrogen substitution in maltose of 2′-OH caused an insignificant and of 3-OH quite a large (11.3 kJ mol^{-1}) increase in $\Delta(\Delta G^{\ddagger})$ (Sierks & Svensson, 1992; Sierks et al., 1992), $\Delta(\Delta G^{\ddagger})$ for Arg305 → Lys GA is thus significantly higher (22.8 − 11.3 = 11.5 kJ mol^{-1}) than the contributions estimated using corresponding monodeoxy analogs of the substrate. This discrepancy may indicate that the mutation severely perturbs the hydrogen bond network or that a difference in solvation between either analog and parent substrate or between wild-type and mutant GA elicits a difference in entropy contribution from displacement of bound water to the bulk solvent as the ligand binds. Thermodynamic analysis of the binding of acarbose to Arg305 → Lys GA indicated a loss in binding energy of 40 kJ mol^{-1}, caused exclusively by a decrease in entropy, suggesting a decrease in complementarity resulting in that additional water being retained in the mutant compared to the wild-type GA complex (Berland et al., 1995).

Even though very similar $\Delta(\Delta G^{\ddagger})$ values result from replacement of either Arg54 or Arg305 by lysine, these mutations influence k_{cat} and K_m differently. While Arg54 contributes little to ground-state binding of α-1,4-linked substrates, and supposedly also of isomaltose as suggested

from results of molecular recognition of deoxy analogs of isomaltose by wild-type GA (Frandsen et al., 1994b), Arg305 is essential for binding of the α-1,4-linked, but not the α-1,6-linked, substrates. Besides the hydrogen bond between NH1 of Arg305 and inhibitor OH groups at subsites 1 and 2 (Figure 1), NH2 hydrogen bonds OE2 of Glu180 which in turn hydrogen bonds to the 2-OH at subsite 2 (Figure 1). Because the hydrophilic side of ring b in acarbose faces Glu179, Glu180, and Arg305, and the hydrophobic side faces Tyr48 and Tyr311 (Aleshin et al., 1994), we conclude from the present mutational analysis that Arg305 specifically stabilizes α-1,4-linked substrates from the hydrophilic side of the substrate-binding pocket in subsites 1 and 2.

Alternatively the retained affinity for isomaltose in Arg305 → Lys GA may reflect that a hydrogen bond maintained in the transition-state complex of isomaltose is lost in that of maltose. A larger degree of conformational flexibility around the C5−C6 linkage in isomaltose presumably accommodates the substrate aglycon in the mutant GA−isomaltose transition-state complex, without significant hydrogen bond breaking. Pre-steady-state kinetic analysis of substrate binding to wild-type GA indicated distinct differences between isomaltose and maltose interactions (Olsen et al., 1992). The mechanism for isomaltose binding may need at least one additional kinetically significant step compared to maltose binding. As Arg305 is involved in the formation of these complexes, pre-steady-state analysis of binding to Arg305 → Lys GA may reveal kinetic steps that are substrate bond-type specific.

Asp309 → Glu and Trp317 → Phe GAs. Side chains of Asp309 and Trp317 play a role in stabilization of Arg305, Asp309 interacting electrostatically with the δ-guanido group and Trp317 stacking against the hydrophobic part of Arg305 (Aleshin et al., 1994; Stoffer et al., 1995; Figure 1). Both Asp309 → Glu and Trp317 → Phe GAs retain their catalytic capacity but have reduced affinity. Trp317 also faces the general base Glu400 (Figure 1), but Trp317 → Phe GA retains activity. The unusually large decrease in affinity of Asp309 → Glu GA for α-1,4-linked substrates is less and much less pronounced toward 4-nitrophenyl α-D-glucopyranoside and isomaltose, respectively. This behavior may be related to the substrate bond-type discrimination of Arg305 → Lys GA. The side chains of Asp309 and Trp317 do not bind directly to substrate, and polar interactions are not involved in the binding of the 4-nitrophenyl aglycon moiety. This further emphasizes the special significance of the direct contact of Arg305 to the second ring of α-1,4-linked substrates. Probably this interaction is perturbed because the extra CH_2 group in the Glu309 side chain disrupts enzyme−substrate interactions by the displacement of Arg305. 3-Deoxymaltoside as a substrate, then, should exhibit an increased K_m similar to the effect found with 4-nitrophenyl α-D-glucopyranoside.

When Asp309 is replaced by asparagine we previously found a 30-fold decrease in k_{cat} for hydrolysis of maltose (Sierks & Svensson, 1993), while substitution by glutamic acid in the present study results in k_{cat} of wild-type level. Although Asp309 has no direct interaction to the catalytic residues, the pH−activity profiles for Asp309 → Asn (Sierks & Svensson, 1993) and uncomplexed Asp309 → Glu GAs both reveal a decrease in pK_a of the catalytic acid. A disrupted interaction to Arg305 perhaps enhances the positive charge in the active site region, and although crystallographic results questioned the state of ionization of the general acid Glu179 (Aleshin et al., 1994), an uncompensated positive charge would depress the pK_a of Glu179. The positive charge of Arg305, offset by the interaction with Asp309 in wild-type GA, is probably disturbed in Asp309 → Asn/Glu GA, resulting in the perturbed pK_a of the catalytic residues.

The large binding energy usually associated with subsite 2 is decreased for Trp317 → Phe GA. Since Trp317 does not bind substrate directly, it most likely exerts its effect through Arg305. A small number of mutants so far has shown significant decreases in binding energy at subsite 2. They include Glu180 → Gln, which influences the hydrogen bond between Glu180 and the 2-OH (Sierks & Svensson, 1992; Harris et al., 1993) and Glu400 → Gln and Tyr48 → Trp, which influence the stacking of Tyr311 to the sugar residue at subsite 2 (Aleshin et al., 1994; Frandsen et al., 1994a; Stoffer et al., 1995). Mutations at subsite 2 greatly weaken binding affinity. Pre-steady-state kinetic analysis of Trp317 → Phe GA indicates that the first association complex between GA and the ligand is weakend, as seen by the equilibrium constant, K_1, of its formation being increased by approximately 10-fold (T. Christensen, K. Olsen, B. Svensson, and U. Christensen, unpublished results). This seems to be the most important feature of Trp317 → Phe GA, while the unimolecular rearrangement of the initial encounter complex to the productive complex is affected little by the mutation of Trp317.

Stability and Unfolding. The heat stabilities of Asp309 → Glu and Trp317 → Phe GAs were much decreased. The side chains of Arg305, Asp309, and Trp317 are near an element of unusual secondary structure (Aleshin et al., 1992; Stoffer et al., 1995), where strain is imposed upon Asn313 and Ser411 and the segment Ser310−Gly314 forms two overlapping reverse turns resembling a left handed 3_{10}-helix. OD2 of Asp309 is directly involved in a hydrogen bond to the backbone amide of Tyr311. The salt link between Asp309 and Arg305 and hydrophobic stacking of Trp317 against Arg305 may be critical to the stability of this element of secondary structure.

The appearance of two steps in the unfolding of Asp309 → Glu and wild-type GAs in GdnHCl clearly demonstrates that the catalytic and starch-binding domains have different conformational stabilities. Whereas unfolding of wild-type G1 and G2 occurs at similar [GdnHCl], the catalytic domain, being destabilized by the mutation Asp309 → Glu, unfolds at low [GdnHCl] followed by the unfolding of the starch-binding domain at a higher [GdnHCl]. The presence of the starch-binding domain does not stabilize the catalytic domain of the mutant, since both the G1 and G2 forms unfold at the same [GdnHCl]. It was previously demonstrated for GA G1 by differential scanning calorimetry that unfolding of the catalytic domain is irreversible, whereas unfolding of the starch-binding domain is a reversible process (Williamson et al., 1992). The mechanism of GA unfolding is poorly understood; it deviates from a two-state model (Williamson et al., 1992) and is probably initiated by unfolding of the catalytic domain, which through a number of metastable intermediates unfolds in an irreversible manner.

CONCLUSION

We have identified Arg305 as a key determinant in GA for the recognition of α-1,4-linked substrates. This side chain

hydrogen bonds substrate OH groups in the sugar rings adjacent to the glycosidic bond to be cleaved. However, a strong discrimination was observed for the substrate bond type. Thus the α-1,6-linked substrate isomaltose was bound with virtually wild-type affinity even though it was hydrolyzed so slowly that the transition-state stabilization energy for the two types of substrates came to very similar values. Replacement of either Arg54 or its interacting substrate OH group 4′-OH in maltoside led to similar $\Delta(\Delta G^{\ddagger})$ values, indicating a direct and presumably local effect in the replacement of the side chain in subsite 1. Also an effect was not detected in the pH dependence of the ionization of the catalytic acid when Arg54 was replaced by leucine, and only a minor loss in subsite affinity was obtained for the subsite 2 that interacts most strongly with substrate. Even the minor structural change of replacing Trp317, which stacks with Arg305, by phenylalanine had a clear effect both on the substrate recognition and in the protein stability. We anticipate that further mutational analysis of this particular region of the active site will lead to a basis for design of active enzymes with engineered substrate bond-type specificity. The importance of certain substrate OH groups (Lemieux, 1984; Hindsgaul et al., 1985; Nikrad et al., 1992; Lemieux & Spohr, 1994) has been strongly supported by the present GA study and by an earlier one dealing with Asp55 → Gly GA (Sierks et al., 1993; Svensson et al., 1994). In fact, the possibility for recovery almost as a rule of functional GA mutants with reasonable conformational stability, motivates continued studies on GA as a prototype for inverting exo-carbohydrases.

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