Functional Roles of the Invariant Aspartic Acid 55, Tyrosine 306, and Aspartic Acid 309 in Glucoamylase from *Aspergillus awamori* Studied by Mutagenesis

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ABSTRACT: Three mutants, Asp55-Gly, Tyr306→Phe, and Asp309→Asn, of *Aspergillus awamori* glucoamylase (identical to *Aspergillus niger* glucoamylase) were constructed to elucidate the roles of two conserved regions within fungal glucoamylases. Kinetic studies indicate that both of these regions are closely associated with activity. The Asp55→Gly mutation decreases the k_{cat} approximately 200 times toward maltose and isomaltose, while K_M values remain similar to the wild-type. This localizes Asp55 to subsite 1 of glucoamylase where it affects catalytic activity, but not ground-state binding. The pK_a value of the catalytic general acid, Glu179, is 1 pH unit lower in that mutant compared to wild-type enzyme, confirming the proximity of Asp55 to the site of catalysis. Tyr306→Phe is highly active, but affects binding in subsite 2. It moreover shows enhanced binding in the fourth subsite, suggesting that the conserved region around residue 306 interacts with Trp120, a critical residue that directs conformational changes stabilizing the transition-state structure. Finally, the Asp309→Asn mutation decreases the k_{cat} for isomaltose hydrolysis around 200-fold, but only 30-fold for maltose. This specific effect on the hydrolysis of the α-1,6-linked substrate locates Asp309 to subsite 2. Substitution of Asp309 influences affinities of distant subsites, especially subsite 4, similar to mutations of other carboxylic acid residues situated near subsites 1 and 2.

Glucoamylase (GA)[1] (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) is an exohydrolase which cleaves D-glucose from the nonreducing ends of starch and related oligo- and polysaccharides. The active sites of GAs from various fungal sources are kinetically similar and contain six or seven subsites with the catalytic site located between subsites 1 and 2 (Hiromi, 1970; Savel'ev et al., 1982; Hiromi et al., 1983; Koyama et al., 1984; Abe et al., 1985; Fagerström, 1991). The individual subsite affinities toward glucosyl residues are also similar, subsite 2 having the highest affinity and subsites 3–7 having decreasing affinities, the last three subsites having very small values. The affinity of subsite 1 in the GAs studied varies considerably (–7.1 to 5 kJ/mol) (Savel'ev et al., 1982; Hiromi et al., 1983; Koyama et al., 1984; Abe et al., 1985; Maegher et al., 1989; Fagerström, 1991), most likely reflecting the inherent inaccuracy involved in calculating the subsite 1 affinity in the model used (Hiromi, 1970) rather than actual differences in affinity. GA cleaves both α-1,6- and α-1,4-linked glucosyl substrates at the same catalytic site, though activity is considerably lower toward α-1,6 linkages than toward α-1,4 linkages (Hiromi et al., 1966). Isomaltose, an α-1,6-linked disaccharide, thus binds more weakly and is hydrolyzed more slowly than the α-1,4-linked maltose.

Comparison of the amino acid sequences of fungal GAs indicates four short regions of high homology corresponding to *Aspergillus awamori* GA (identical to *Aspergillus niger* GA): residues 35–59 (I), 104–134 (II), 162–196 (III), and 300–311 (IV) (Itoh et al., 1987). Critical functions for regions II and III from *A. awamori* have been indicated by chemical modification and site-directed mutagenesis studies. Region II contains Trp120, a residue located near subsite 4 (Clarke & Svensson, 1984a,b) but crucial for directing protein conformational changes that stabilize the substrate transition state in subsites 1 and 2 (Sierks et al., 1989; Svensson & Sierks, 1992). Region III encompasses an essential carboxylic acid cluster (Svensson et al., 1990) containing the general acid catalyst, Glu179 (Sierks et al., 1990), Glu180, which forms a hydrogen bond with the 2-OH group of maltose in subsite 2 (Sierks & Svensson, 1992), and Asp176, suggested to have a role in the base-catalyzed water hydroxyl attack at the C-1 position of the glucose to be produced (Sierks et al., 1990). Functional roles associated with the remaining two conserved regions, I and IV, however, are not yet known. Site-directed mutations of five different residues from region I have been constructed in GA from *Saccharomycopsis fibuligera*; residues Ala81, Asp89, Trp94, Arg96, and Asp97 (Itoh et al., 1989) corresponding to *A. niger* residues Ala39, Asp47, Trp52, Arg54, and Asp55, respectively. Ala81 and Asp89 were shown to be important for thermal stability but not catalytic activity, and mutations at Trp94, Arg96, and Asp97 decreased or had no detectable enzyme activity.

Chemical modification of carboxyl groups of *A. niger* GA with carbodiimide showed that Asp55 is partially reactive, suggesting that region I is at least partly exposed on the surface of GA (Svensson et al., 1990). Region IV, in contrast, seems to be buried since it did not react with carbodiimide or *N*-bromosuccinimide (Svensson et al., 1986, 1990). Site-directed mutations of three invariant amino acid residues from regions I and IV were constructed in *A. awamori* GA to elucidate roles of these regions. In the present work, the invariant residue in *A. awamori* GA Asp55 from region I (Itoh et al., 1987) was studied by constructing the nonconservative substitution to Gly. This substitution removes any hydrogen bond interactions that the Asp55 side chain may participate in. In region IV, two conservative changes, Tyr306→Phe and Asp309→Asn, were made since this region may be buried in the protein and more radical changes could alter the overall structure.

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[1] Abbreviations: GA, glucoamylase; TAA, Taka-amylase A.

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Table I: Kinetic Parameters for GA Mutants with Isomaltose and Maltooligodextrins of DP 2–7

	enzyme								
	wild-type			Asp55→Gly			Glu180→Gln		
substrate	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($s^{-1}\cdot mM^{-1}$)	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($s^{-1}\cdot mM^{-1}$)	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($s^{-1}\cdot mM^{-1}$)
isomaltose[a]	0.34 ± 0.01	30.3 ± 1.2	1.13×10^2	0.0017 ± 0.0001	38.5 ± 4.5	4.41×10^{-5}	0.184 ± 0.012	95 ±11.6	0.00193
maltose[a]	9.2 ± 0.4	1.4 ± 0.2	6.4	0.040 ± 0.001	1.5 ± 0.1	0.03	1.27 ± 0.09	35.3± 6.8	0.036
maltose[b]	14.4 ± 0.5	1.73 ± 0.14	8.3				1.53 ± 0.05	41.4 ± 4.6	0.0369
maltotriose[b]	62 ± 4	0.73 ± 0.09	86				2.94 ● 0.07	26 ± 2.0	0.113
maltotetraose[b]	87 ± 4	0.53 ± 0.07	165				10.25 ± 0.53	12.4 ± 2.3	0.825
maltopentaose[b]	94 ± 3	0.31 ± 0.03	300				11.24 ± 0.32	12.1 ± 1.0	0.932
maltohexaose[b]	86 ± 4	0.20 ± 0.03	430				12.65 ± 0.55	12.2 ± 1.5	1.041
maltoheptaose	84 ± 2.4	0.22 ± 0.04	390	0.23 ± 0.05	0.19 ± 0.01	1.23	11.66 ± 0.45	10.5 ± 1.2	1.11

	Tyr306→Phe			Asp309→Asn		
	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($s^{-1}\cdot mM^{-1}$)	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($s^{-1}\cdot mM^{-1}$)
isomaltose[a]	0.235 ± 0.006	47.2 ± 3.5	4.98×10^{-3}	0.0016 ± 0.0001	71.3 ± 9.0	2.23×10^{-5}
maltose[a]	8.2 ± 0.5	3.68 ± 0.67	2.23	0.32 ± 0.2	5.0 ± 1.1	6.44×10^{-2}
maltose[b]						
maltotriose[b]	34.7 ± 1.1	2.85 ± 0.26	12.2	1.39 ± 0.06	5.1 ± 0.5	0.27
maltotetraose[b]	108.2 ± 3.8	1.09 ± 0.14	99.3	3.6 ± 0.16	2.5 ± 0.32	1.44
maltopentaose[b]	100.2 ± 2.6	0.74 ± 0.08	135.4	5.1 ± 0.4	2.6 ± 0.5	1.96
maltohexaose[b]	103.3 ± 1.2	0.71 ± 0.03	145.5	4.61 ± 0.18	1.7 ± 0.2	2.71
maltoheptaose[b]	99 ± 3.7	0.67 ± 0.12	147.8	4.94 ± 0.24	0.99 ± 0.19	4.99

[a] At 45 °C, pH 4.5, using a 0.05 M sodium acetate buffer. [b] Determined for the GA mutants at 45 °C and for wild-type GA at 50 °C (Sierks et al., 1989), respectively.

MATERIALS AND METHODS

Mutant Preparation. Mutant GA enzymes were constructed using methods described previously (Sierks et al., 1989). The following mutagenic primers were used: Asp55→Gly, 5′-CGAGACCAGAGCCTCGAGTCCAGGT-3′; Tyr306→Phe, 5′-GTCCTCAGGGAACCGACCCAC-3′; and Asp309→Asn, 5′-GTAGTACGTGTTCTCAGGGTA-3′. Mutations were verified by DNA sequencing of the subcloned region. Enzymes, oligonucleotides, and reagents used for the mutagenesis were obtained as described elsewhere (Søgaard & Svensson, 1990). Plasmid purification, subcloning, sequencing, yeast transformation, fermentation, protein purification, and enzyme kinetic measurements at 45 °C were performed essentially as described previously (Sierks et al., 1989).

Enzyme Assays. The substrate maltose was obtained from Merck, FRG, isomaltose was from Sigma, and maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose were obtained from Boehringer Mannheim, FRG. Determination of the kinetic parameters k_{cat} and k_{cat}/K_M for maltose hydrolysis at different pH values was performed as described elsewhere (Sierks et al., 1990), and the pK_a values of the catalytic groups were calculated using the software Enzfitter (Elsevier-BIOSOFT, Amsterdam) and according to the methods of Dixon (1953). For the free enzyme and the enzyme–substrate complex, pK_a values were determined at maltose concentrations sufficiently low that the rate (v) was proportional to k_{cat}/K_M, and sufficiently high that v was proportional to k_{cat}, respectively. At the extreme pH values, 2.4 and 6.6, the activities were in addition determined at 2–4 maltose concentrations from 15 to 100 mM.

Subsite affinities were generated from the k_{cat} and K_M values obtained with the series of maltooligosaccharides of DP from 2 to 7 at 45 °C and pH 4.4 utilizing the subsite affinity model described by Hiromi (1970). Due to irregularities in the kinetic parameters obtained with the present mutant GAs, a reliable value for the subsite 1 affinity could not be obtained. In these cases, therefore, to estimate the subsite 2 affinity, a subsite 1 affinity identical to wild-type GA was assumed (2.6 kJ/mol) (Sierks et al., 1989). This approach should be valid if the mutated residue is not located in subsite 1. The sensitivity of the calculated subsite 2 affinity toward the assumed affinity of subsite 1 was tested using a subsite 1 affinity of 0.0 kJ/mol, resulting in only minor changes (less than 0.5 kJ/mol) in the affinity of subsite 2.

RESULTS

Kinetic Parameters. The kinetic parameters k_{cat} and K_M were determined for the three GA mutants Asp55→Gly, Tyr306→Phe, and Asp309→Asn, using two different disaccharide substrates, maltose (α-1,4-linked) and isomaltose (α-1,6-linked), and the series of α-1,4-linked maltooligodextrins from maltotriose to maltoheptaose (Table I). As compared to wild-type GA, the mutant retaining the highest activity, Tyr306→Phe, decreases k_{cat} marginally toward maltose and isomaltose and 50% toward maltotriose but increases k_{cat} slightly for the longer maltooligodextrins. This mutation also affects ground-state binding; K_M thus increases 3–5-fold for maltose through maltoheptaose, but only slightly for isomaltose. Asp309→Asn decreases k_{cat} 20–40-fold and increases K_M 4–7-fold for the α-1,4-linked substrates as compared to wild-type. For isomaltose hydrolysis, however, the k_{cat} value decreases 200-fold, while K_M doubles. Finally, the mutation Asp55→Gly decreases k_{cat} around 200-fold for both the α-1,4- and α-1,6-linked disaccharide substrates, and 300-fold for maltoheptaose, while all K_M values are similar to wild-type values (Table I).

Subsite Maps. A subsite map, reflecting the changes in the free energy of binding at each of the subsites of the GA mutants, can be calculated from the k_{cat} and K_M data obtained with the maltooligodextrin substrates (Hiromi, 1970). The subsite energies for the Tyr306→Phe and Asp309→Asn GA mutants as well as wild-type GA (Sierks et al., 1989) are given in Table II. Subsite affinities were not determined for the Asp55→Gly mutant enzyme because of its very low activity. Wild-type GA consistently displays an increase in

Table II: Subsite Binding Energies (kJ/mol) for GA Mutants[a]

	subsite						
enzyme	1	2	3	4	5	6	7
WT[b]	2.6	−21.4	−6.3	−1.8	−1.6	−1.0	0.3
Y306F	2.6	−18.2	−6.1	−5.6	−0.8	−0.2	−0.03
D309N	2.6	−17.2	−3.8	−4.4	−0.8	−0.9	−1.6
E180Q	2.6	−13.4	−3.0	−5.3	−0.3	−0.3	−0.2
D176N[c]	3.8	−17.4	−7.4	−3.7	1.2	−1.4	−1.2

[a] At 45 °C, pH 4.5, using a 0.05 M sodium acetate buffer. [b] From Sierks et al. (1989) at 50 °C. [c] From Sierks et al. (1990) at 50 °C.

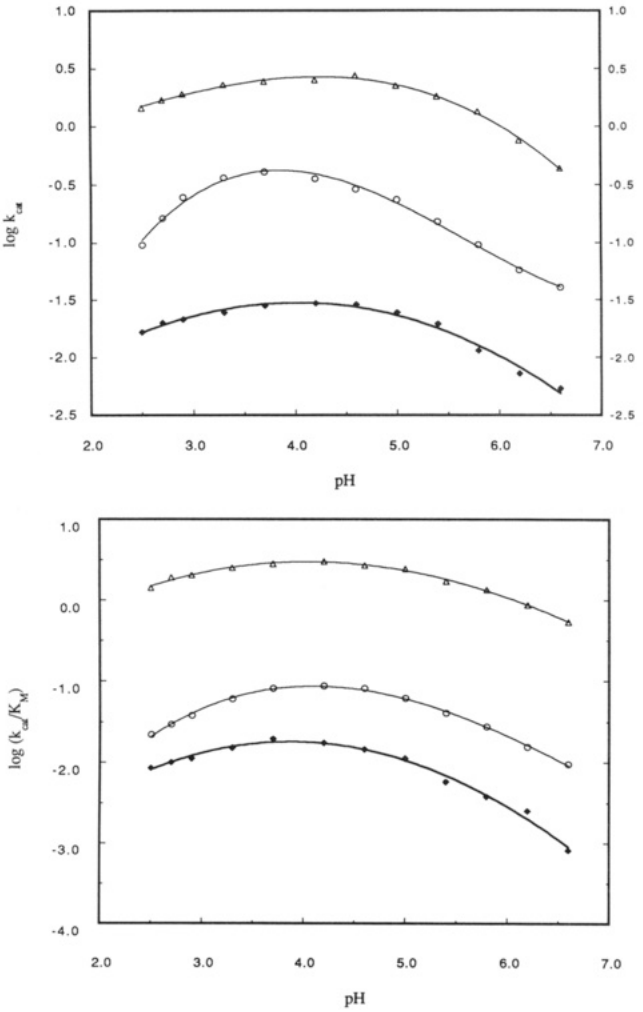

FIGURE 1: Determination of pK_a values for (top) maltose-complexed and (bottom) uncomplexed GA of wild-type (△), Asp55-Gly (♦), and Asp309→Asn (○) *A. awamori* glucoamylases at 45 °C. Substrate concentrations were 5.0, 5.0, and 60 mM for the top panel and 0.2, 0.2, and 0.6 mM for the bottom panel for wild-type, Asp55→Gly, and Asp309→Asn GA, respectively.

k_{cat} and a decrease in K_M with longer substrate chain length due to better stabilization of the transition-state structure, resulting in subsites with decreasing affinities as they get further from the catalytic site, although changes beyond subsite 4 are small. The affinities at subsites 2–7 of the Tyr306→Phe mutant are very similar to those obtained for wild-type GA except for a noticeably large increase in the affinity of subsite 4 and a slight decrease at subsite 2. The subsite 1 affinity was assumed identical to wild-type with all the mutant GAs studied here to enable calculation of their subsite 2 affinities. The k_{cat} values for the Asp309→Asn mutant display a trend similar to wild-type GA, increasing with substrate length up to five glucosyl residues and then leveling off. The K_M values, however, do not decrease consistently with increasing substrate

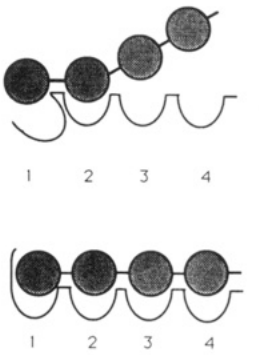

FIGURE 2: Schematic two-step glucoamylase hydrolytic mechanism with a fast initial recognition step controlled primarily by the strong affinity at subsite 2 followed by a conformational change at subsite 1 to stabilize the transition-state complex (Fagerström, 1991).

length. The values for maltose and maltotriose hydrolysis are similar as are the values for maltotetraose and maltopentaose hydrolysis. Slight decreases in the binding constant were obtained for maltohexaose and maltoheptaose. The subsite map for this mutant GA thus shows a discontinuity indicating a stronger affinity at subsite 4 than at subsite 3. The subsite 4 affinity is also 2.6 kJ/mol larger than the value for the wild-type GA (Table II).

Mutation of another carboxylic acid group in the active site, Asp176→Asn (Sierks et al., 1990), previously had caused changes in the kinetic parameters obtained with the maltooligodextrin series similar to those found here for the Asp309→Asn GA. The Asp176→Asn GA also displayed a discontinuity in the k_{cat} and K_M values obtained with substrates of increasing length. A significantly increased affinity at subsite 4 and decreased affinity at subsite 5 were evident as compared to wild-type GA (Sierks et al., 1990). The location of Asp176 near the catalytic site (Svensson et al., 1990) along with effects on distant subsite affinities caused by mutation suggested an interaction between subsites 1 and 2 with distant subsites. A third conserved GA carboxylic acid group in the active site, Glu180, was localized to the second subsite of GA (Sierks et al., 1990) where it binds to the 2-OH group of maltose (Sierks & Svensson, 1992). The Glu180→Gln mutation (Sierks et al., 1990) is shown here to also have effects similar to the Asp176→Asn and Asp309→Asn mutants on the subsite map of GA (Table II). The kinetic parameters obtained with Glu180→Gln GA display the same trend as seen with wild-type GA, the k_{cat} values increasing with chain length and the K_M values decreasing, though k_{cat} is much lower and K_M much higher in general than for wild-type (Table I). The subsite map of the Glu180→Gln mutant GA, however, reveals perturbations in the binding affinities of distant subsites when compared to wild-type (Table II). As expected for Glu180→Gln GA, the subsite map indicates the affinity of subsite 2 is substantially lower (8 kJ/mol) than for wild-type GA, reflecting the lost hydrogen bond between Glu180 and maltose at subsite 2 (Sierks & Svensson, 1992). The affinity of subsite 3 is also lower than in the wild-type GA, while the subsite 4 affinity (−5.3 kJ/mol) is much higher (Sierks et al., 1989). Subsites 5–7 have low affinities resembling the wild-type pattern.

pK_a of Catalytic Groups. The large changes in the kinetic parameters caused by mutation of Asp55 and Asp309 suggest that these two residues are located near the catalytic site. If this is true, the resulting loss of an acidic side chain in the two mutants, Asp55→Gly and Asp309→Asn, should alter the charge environment of the two catalytic groups. Therefore, the effects of these mutations on the pK_a values of the general

Table III: Changes in Binding Energies [$\Delta(\Delta G)$][a] (kJ/mol) between Wild-Type and Mutant GA Enzymes[b]

	enzyme			
substrate	Asp55→Asn	Glu180→Gln	Tyr306→Phe	Asp309→Asn
isomaltose	14.7	4.7	2.2	16.5
maltose	14.5	13.7	2.8	12.2
maltotriose		17.6	5.2	15.3
maltotetraose		14.0	1.3	12.5
maltopentaose		15.3	2.1	13.3
maltohexaose		15.9	2.9	13.4
maltoheptaose	15.2	15.5	2.6	11.5

[a] $\Delta(\Delta G) = -RT \ln [(k_{cat}/K_M)_{mut}/(k_{cat}/K_M)_{wt}]$. [b] At 45 °C, pH 4.5, using a 0.05 M sodium acetate buffer.

acid catalyst and the base catalyst were determined for the complexed and free GA's from the pH dependence of the rate constant, k_{cat} (Figure 1, top), and of the second-order rate constant, k_{cat}/K_M, respectively (Figure 1, bottom). The Asp309→Asn mutation lowers the pK_a of the general acid, Glu179, from 5.9 in the wild-type (Sierks et al., 1990) to 5.3 in both the complexed and free forms of the mutant GA, while the pK_a of the general base slightly increases to 3.0 and 2.7 compared to the values of 2.7 and 2.3 obtained for wild-type complexed and uncomplexed GA forms, respectively. The Asp55→Gly mutation does not alter the pK_a of the base group, but decreases the pK_a value of the general acid to 5.5 in the complexed form and 5.0 in the free form. These effects are considered to be due to changes in k_{cat} and not K_M for Asp55→Gly, since the specific activity was essentially independent of the substrate concentration in the range 5–100 mM at pH 2.4 and 6.6. Asp309→Asn similarly had unchanged activity at pH 2.4, while the K_M at pH 6.6 was estimated to have increased 2-fold compared to pH 4.5 (data not shown).

DISCUSSION

Similar increases in the binding affinity of subsite 4 compared to wild-type GA (1.9–3.8 kJ/mol) caused by mutation of Asp176, Glu180, or Asp309 (Sierks et al., 1990), which are all located near the catalytic site, indicate that an interplay exists between subsites close to and more distant from the site of catalysis. Ligand-induced spectral perturbation of Trp120→Phe GA (Svensson & Sierks, 1992) reveals that Trp120, located near subsite 4 (Clarke & Svensson, 1984a,b), during catalysis approaches a negative environment, presumably the catalytic site region that contains Asp55, Asp176, Glu179, Glu180, and Asp309. This conformational change may affect subsite 1 and force the substrate into a lactone-type transition state (Lászlo et al., 1978). Information on distant subsites of GA may be gained from related enzymes (Svensson, 1988). GA residues 112–120 align with residues 75–83 in the α-amylase Taka-amylase A (TAA) and similar sequences in CGTases (Svensson, 1988). In the TAA crystal structure, this segment spans four subsites on one side of the catalytic site (Matsuura et al., 1984). If the GA counterpart shares this function, carboxyl groups near the catalytic site influence distant subsites controlled by the Trp120 region. The increased affinity at subsite 4 and varying effects on subsites 3, 5, 6, and 7, seen with Asp176→Asn, Glu180→Gln, Tyr306→Phe, and Asp309→Asn GAs, reflect such interactions. Changes at multiple subsites in mutant GAs do not invalidate the theory, assuming independent association of glucosyl residues with individual subsites (Hiromi, 1970), but rather illustrate that a mutation can affect a particular subsite directly as well as other more distant subsites indirectly through protein structural changes.

Asp309→Asn changes the kinetic parameters of maltose and isomaltose substantially, and shifts the pK_a values of the general acid and base catalysts. Asp309 is more critical for stabilization of the isomaltose than the maltose transition-state complex evidenced by the 10-fold greater decrease in k_{cat} for isomaltose. Since GA hydrolyzes α-1,4 and α-1,6 bonds at one catalytic site (Hiromi et al., 1966), Asp309 either preferably binds to the isomaltose transition state or disturbs the catalytic groups more in the isomaltose than in the maltose complex. This discrimination localizes Asp309 near subsite 2 where the protein interactions with α-1,4- and α-1,6-linked substrates differ. As K_M values do not decrease consistently with the longer maltooligodextrins for Asp309→Asn as seen for wild-type GA, Asp309 affects distant subsites, probably via the Trp120 region.

The k_{cat} values for Asp55→Gly GA dropped 200–300-fold compared to wild-type. The corresponding Asp97→Asn in the *Saccharomycopsis fibuligera* GA caused loss of detectable activity (Itoh et al., 1989), indicating the carboxyl group at this position is crucial for activity. The catalytic pathway of GA comprises (Hiromi et al., 1983) a fast initial substrate association at subsite 2, followed by a slow unimolecular rearrangement leading to the productive enzyme–substrate complex (Figure 2). Because Asp55→Gly decreased k_{cat} without affecting K_M, Asp55 is involved in transition-state rather than ground-state binding and might participate in the slow rearrangement to obtain productive binding in subsite 1. Alternatively, Asp55 could contribute to the electrostatic field in the transition-state complex. The shift of 1 pH unit in the pK_a value of the general acid catalyst in the free enzyme suggests Asp55 is close to Glu179. Glu180→Gln in subsite 2 caused a similar effect on the pK_a (Sierks et al., 1990).

Changes in k_{cat}/K_M can be expressed in terms of the free energy of binding: $\Delta(\Delta G) = -RT \ln [(k_{cat}/K_M)_{mut}/(k_{cat}/K_M)_{wt}]$ (Wilkinson et al., 1983) where mut and wt refer to mutant and wild-type enzyme, respectively. Loss of a hydrogen bond with a charged or an uncharged residue results in an expected $\Delta(\Delta G)$ of around 15–19 or 2–6 kJ/mol, respectively (Fersht et al., 1985; Street et al., 1986). For Asp55→Gly, the $\Delta(\Delta G)$ values of approximately 15 kJ/mol (Table III) for hydrolysis of both α-1,4- and α-1,6-linked substrates suggest hydrogen bonding of substrate in subsite 1 to the carboxylate of Asp55. For wild-type GA, the $\Delta(\Delta G)$ contributions attributed to the 2-, 3-, 4-, and 6-OH groups of the nonreducing glucosyl ring of maltose are 0, 9, 19, and 17 kJ/mol, respectively (Sierks et al., 1992). Since subsite 1 accommodates this ring, Asp55 should bind with either the 4- or the 6-OH group. This also explains the large decrease in pK_a of the general acid only in the free and not in the substrate-complexed mutant GA, as the 4- and 6-OH groups are oriented away from the catalytic site.

Molecular recognition studies on wild-type GA using maltose analogues indicate $\Delta(\Delta G)$ contributions for the 1-, 2-, 3-, and 6-OH groups of the reducing-end glucosyl ring of -2, 5, 11, and −2 kJ/mol, respectively (Sierks & Svensson,

1992). Since $\Delta(\Delta G)$ values of 12–15 kJ/mol were calculated for Asp309→Asn GA acting on α-1,4-linked substrates, Asp309 and the 3-OH group may interact. However, loss of a hydrogen bond to the 3-OH group would be expected to substantially increase K_M, such as the 25-fold increase seen with the mutation of Glu180 which hydrogen bonds to the 2-OH group (Sierks et al., 1990; Sierks & Svensson, 1992). Furthermore, the 200-fold decrease in k_{cat} for isomaltose shows that Asp309 is important for catalysis, possibly by maintaining the high pK_a of the catalytic general acid, Glu179 (Sierks et al., 1990). Asp309, therefore, should not hydrogen bond to the transition state of α-1,4-linked substrates to a greater extent than the α-1,6-linked substrate.

Finally, Tyr306, despite being conserved, is not critical for GA activity, since Tyr306→Phe GA shows little change in kinetic parameters and small $\Delta(\Delta G)$ values. The Tyr306→Phe mutation, however, adversely affects binding of the α-1,6-linked substrates to a smaller extent than the α-1,4-linked substrates. This mutation thus influences subsite 2, though less so than replacements at Glu180 or Asp309. Since k_{cat} increased for maltotetraose and longer substrates, Tyr306 does not play a role in transition-state stabilization, but the increased affinity of subsite 4 suggests that Try306, like the nearby Asp309, interacts either directly or indirectly with the Trp120 segment. Noticeably, the $\Delta(\Delta G)$ for maltotriose is 2–4 kJ/mol higher than for the other substrates, as seen also with Glu180→Gln and Asp309→Asn GAs. This specific loss in transition-state stabilization for maltotriose may be due to a disrupted contact with the Trp120 region, which is reestablished by the longer substrates.

In summary, the kinetic properties of these GA mutants indicate that Asp55 is located in subsite 1, does not affect ground-state binding, and stabilizes the transition state by hydrogen-bonding with either the 4- or the 6-OH group of the nonreducing substrate glucosyl ring. Asp309, located in or near subsite 2, contributes to maintaining the proper charge and geometry of the catalytic site, and stabilizes the isomaltose transition-state complex. Tyr306 is not crucial for activity, but influences ground-state binding for α-1,4-linked substrates and interacts with the critical Trp120 region. Molecular recognition studies using the three mutants and relevant substrate analogues may help to identify the interacting atoms or functional groups in the GA substrate transition-state complex as demonstrated with the Glu180→Gln mutant of GA from *A. niger* (Sierks & Svensson, 1992). The recently constructed model of the closely related GA from *A. awamori* var. X100 (Aleshin et al., 1992) supports our conclusion.

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