

Functional roles and subsite locations of Leu177, Trp178 and Asn182 of *Aspergillus awamori* glucoamylase determined by site-directed mutagenesis

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Fungal glucoamylases contain four conserved regions. One region from the *Aspergillus niger* enzyme contains three key carboxylic acid residues, the general acid catalytic group, Glu179, along with Asp176 and Glu180. Three site-directed mutations, Leu177 → His, Trp178 → Arg and Asn182 → Ala, were constructed near these acidic groups to reveal the function of other conserved residues in this region. Leu177 and Trp178 are strictly conserved among fungal glucoamylases, while an amide, predominantly Asn, always occurs at position 182. Substitutions of Leu177 or Trp178 cause significant decreases in k_{cat} with the substrates tested. Similar increases in activation energies obtained with Leu177 → His with both α -(1,4)- and α -(1,6)-linked substrates indicate Leu177 is located in subsite 1. K_M values obtained with the Trp178 → Arg mutation increase for an α -(1,6)-linked substrate, but not for α -(1,4)-linked substrates. Calculated differences in activation energy between substrates indicate Trp178 interacts specifically with subsite 2. The Asn182 → Ala mutation did not change k_{cat} or K_M values, indicating that Asn182 is not crucial for activity. These results support a mechanism for glucoamylase catalytic activity consisting of a fast substrate binding step followed by a conformational change at subsite 1 to stabilize the transition state complex.

Key words: catalytic mechanism/glucoamylase/kinetics/site-directed mutagenesis/transition state energy

Introduction

Glucoamylase (GA) (1,4- α -D-glucan glucohydrolase, EC 3.2.1.3) catalyzes the release of D-glucose from the non-reducing ends of starch or related oligo- and polysaccharide molecules. GAs from a variety of fungal sources have been sequenced and shown to have significant homology (Tanaka *et al.*, 1986; Itoh *et al.*, 1987), suggesting that they are all structurally and functionally related. Kinetic measurements on GAs from different sources have demonstrated that their active sites are also similar, containing a substrate binding area composed of seven consecutive glucosyl residue binding subsites (Hiromi, 1970; Savel'ev and Firsov, 1982; Sierks *et al.*, 1989). The catalytic site is located between subsites 1 and 2, with subsite 2 possessing the strongest subsite binding affinity.

Functional roles of several residues in the GA active site have been identified. Chemical modification studies have located Trp120 (Clarke and Svensson, 1984b), Asp176, Glu179 and Glu180 (Svensson *et al.*, 1988, 1990) in the active site of

Aspergillus niger GA. Site-directed mutagenesis (Sierks *et al.*, 1989, 1990) of GA has determined the function of these four residues. Glu179 is the general acid catalyst of pK_a 5.9, and Asp176, located near subsites 1 and 2, was suggested as the catalytic base of pK_a 2.7, which in its deprotonized state stabilizes the substrate transition state (Sierks *et al.*, 1990). The existence of two catalytic carboxyl groups with these pK_a 's has previously been demonstrated (Hiromi *et al.*, 1966a, b; Savel'ev and Firsov, 1982). Glu180 interacts with α -(1,4)-linked substrates forming a hydrogen bond with the 2-OH group of the reducing-end glucosyl group of maltose, which occupies subsite 2 of GA (Sierks and Svensson, 1992). Trp120, located near subsite 4 (Clarke and Svensson, 1984b), interacts with residues in subsites 1 and 2 affecting Asp176 (Sierks *et al.*, 1990) and induces a conformational change that stabilizes the transition state (Sierks *et al.*, 1990; Svensson and Sierks, 1992). Additional Trp residues have been identified in the active site, although their specific roles have not been identified (Clarke and Svensson, 1984a). Here the active site region, comprising the functionally important carboxylic acid residues, has been further investigated by site-directed mutagenesis studies of residues Leu177, Trp178 and Asn182. The kinetic properties of these mutant enzymes have provided additional information about the subsite locations of these residues as well as their involvement in the mechanism and the binding specificity of GA.

A drawback in the industrial use of GA is that D-glucose yields are limited to ~95% from concentrated starch solutions. This occurs because of the slow hydrolysis of α -(1,6)-D-glucosidic bonds in starch and the formation of various condensation products, mainly α -(1,6)-linked isomaltooligosaccharides, in a stepwise manner from D-glucose (Nikolov *et al.*, 1989). Therefore, if the residues involved in differential binding between α -(1,4)- and α -(1,6)-linked substrates can be identified, the rate of production of condensation products can probably be altered and the yield of glucose from starch improved. The hydrogen bond identified between Glu180 and α -(1,4)-linked, but not α -(1,6)-linked, substrates in subsite 2 of GA (Sierks *et al.*, 1990; Sierks and Svensson, 1992) provides an important clue to understanding the bond type specificity of GA.

The highly conserved GA region, encompassing Asp176, Glu179 and Glu180, has a distant resemblance to active site sequences in other starch hydrolases and related enzymes, including α -amylases, α -glucosidases and glucosyltransferases (Figure 1) (Svensson, 1988). These sequence similarities were utilized to design mutations at Leu177, Trp178 and Asn182. Enzymes active on α -(1,6)-D-glucosidic bonds characteristically contain amino acid residues with small aliphatic side chains at the position corresponding to Leu177 in GA, while enzymes active only on α -(1,4)-D-glucosidic bonds contain primarily the large aromatic side chains Phe or Trp. His was selected instead of the latter residues based on the rationale that its ring structure may favor activity on α -(1,4)-D-glucosidic bonds, while the hydrophilic nature of the ring may disfavor α -(1,6)-D-glucosidic bond cleavage or formation. Trp178 was mutated to Arg because Arg is invariably present in all the α -amylases, having activity

Table 1. Kinetic parameters for the hydrolysis of maltose, maltoheptaose and isomaltose by mutant and wild-type *A. awamori* glucoamylases at pH 4.4 and 50°C

Enzyme	Leu177 → His	Trp178 → Arg	Asn182 → Ala	Wild-type
Maltose (G2)				
k_{cat} (s ⁻¹)	2.6 ± 0.1	1.86 ± 0.11	11.6 ± 0.3	14.4 ± 0.5
K_M (mM)	5.3 ± 0.5	1.52 ± 0.28	1.38 ± 0.10	1.73 ± 0.14
k_{cat}/K_M (mM ⁻¹ s ⁻¹)	0.49	1.22	8.4	8.3
$\Delta(\Delta G)$ (kJ/mol)	8.0	5.4	0.0	—
Maltoheptaose (G7)				
k_{cat} (s ⁻¹)	15.9 ± 0.8	16.6 ± 0.4	65 ± 4	84 ± 2
K_M (mM)	0.30 ± 0.04	0.26 ± 0.02	0.26 ± 0.04	0.22 ± 0.02
k_{cat}/K_M (mM ⁻¹ s ⁻¹)	53	63	250	390 ± 0.02
$\Delta(\Delta G)$ (kJ/mol)	5.6	5.2	1.2	—
Isomaltose (iG2)				
k_{cat} (s ⁻¹)	0.0438 ± 0.018	0.0728 ± 0.0027	0.422 ± 0.021	0.487 ± 0.031
K_M (mM)	49.7 ± 5.7	73.1 ± 6.6	34.7 ± 4.8	36.0 ± 4.7
k_{cat}/K_M (mM ⁻¹ s ⁻¹)	0.000881	0.000997	0.0122	0.0135
$\Delta(\Delta G)$ (kJ/mol)	7.7	7.4	0.3	—
k_{cat}/K_M (G7)	110	52	30	47
k_{cat}/K_M (G2)				
k_{cat}/K_M (G2)	550	1230	690	620
k_{cat}/K_M (iG2)				

(Fersht *et al.*, 1985; Street *et al.*, 1986). The increase in activation energy resulting from the Leu177 mutation is only slightly higher than the value expected for elimination of a hydrogen bond between an uncharged protein group and a maltose or isomaltose hydroxyl group. The Leu177 side chain, however, cannot form a hydrogen bond with the substrate. The increased activation energy caused by the mutation can thus be attributable to either a change in conformation that disrupts a hydrogen bond between the substrate and the peptide backbone or to effects on crucial catalytic groups situated nearby.

A more complete picture of the binding interactions in subsite 1 of GA can be obtained by using information obtained through molecular recognition studies with GA. The 4- and 6-OH groups of the non-reducing end glucosyl residue of maltose are crucial for GA activity (Bock and Pedersen, 1987). The changes in activation energy, $\Delta(\Delta G)$, resulting from the replacement of each hydroxyl group with a hydrogen atom in this glucosyl ring, are ~0, 9, 19 and 17 kJ/mol for the 2-, 3-, 4- and 6-OH groups respectively (Sierks *et al.*, 1992). The 4 and 6-OH groups play a critical role in catalysis and the magnitudes of the changes in activation energy caused by the removal of each of the hydroxyl groups imply that they probably form hydrogen bonds with charged groups in the protein. The comparatively smaller increase in the transition state energy resulting from the Leu177 → His mutation suggests that this residue does not interact directly with the 4- or 6-OH groups. The 2-OH group, furthermore, does not contribute significantly to the binding energies. Therefore, if a hydrogen bond is formed between a Leu177 main chain peptide group and the substrate, the most likely candidates are the 3-OH group or the glucosyl-ring oxygen. Alternatively, the Leu177 → His mutation may perturb a closely situated GA residue that hydrogen bonds with the substrate in subsite 1. The increase in the activation energy for maltose hydrolysis, obtained by substitution of Leu177 with His (8.0 kJ/mol), is in the same range as that obtained by substitution of the substrate 3-OH group by a hydrogen atom (9 kJ/mol), supporting an interaction between a Leu177 main chain group and the 3-OH which is lost in the

Leu177 → His GA. This would indicate that a conformational change involving the protein backbone at position 177 has taken place.

The k_{cat} values for the Trp178 → Arg mutation decrease 5- to 8-fold for all three substrates compared with wild-type GA (Table 1). K_M values for maltose and maltoheptaose are not significantly different from that obtained with the wild-type enzyme, while the K_M value for isomaltose doubles, leading to a 2-fold increase in the selectivity for maltose over isomaltose hydrolysis. Selectivity for maltoheptaose over maltose cleavage is unchanged. The changes in transition state energy, $\Delta(\Delta G)$, resulting from the Trp178 → Arg mutation are higher for isomaltose hydrolysis (7.4 kJ/mol) than for the hydrolysis of the two α -(1,4)-linked substrates, maltose and maltoheptaose (5.4 and 5.2 kJ/mol respectively). These results suggest that Trp178 is located in subsite 2 where the transition state structures of isomaltose and maltose should be quite distinct. The change of Trp178 to Arg increases the preference for the hydrolysis of α -(1,4)-linked over the α -(1,6)-linked substrate, although the catalytic rates in general are substantially lower than with the wild-type enzyme.

Molecular recognition studies using maltose analogs have also clarified the binding pattern of the substrate-reducing end glucosyl ring which occupies subsite 2 of GA. The 3-OH group is crucial for activity and replacement of the 1- and 2-OH groups retains residual activity, while replacement of the 6-OH group with a hydrogen atom slightly increases GA activity (Bock and Pedersen, 1987). The changes in activation energy resulting from the replacement of each OH group by a hydrogen atom are 11 kJ/mol for 3-OH, 5 kJ/mol for 2-OH and -2 kJ/mol for 1-OH and 6-OH (Sierks and Svensson, 1992). The Glu180 residue hydrogen bonds to the 2-OH group of this glucosyl ring, accounting for the change in binding energy for that group (Sierks and Svensson, 1992), leaving Trp178 to interact with either the 1-, 3- or 6-OH groups, the ring oxygen or to provide van der Waals interactions with the substrate. Because the $\Delta(\Delta G)$ value for isomaltose hydrolysis is higher than those obtained for maltose or maltoheptaose

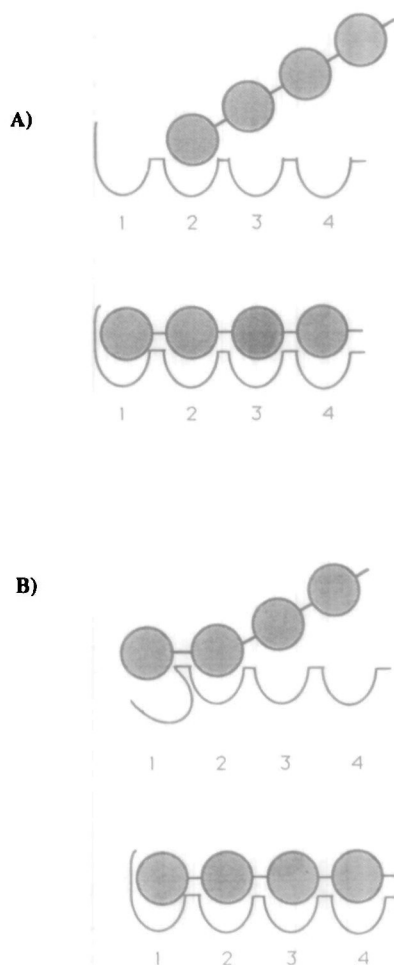


Fig. 2. (A) Depiction of the proposed GA hydrolytic mechanism (Hiromi *et al.*, 1983) showing an initial fast recognition step of substrate to subsite 2 followed by a unimolecular rearrangement to the active configuration. (B) Depiction of a second proposed hydrolytic mechanism (Fagerstrom, 1991) showing an initial fast recognition step of substrate primarily controlled by the large affinity of subsite 2 of GA followed by a conformational change at subsite 1 to obtain the active configuration.

hydrolysis, a specific transition state hydrogen bond interaction in subsite 2 would explain this difference. The most likely interaction between Trp178 and a substrate functional group situated in subsite 2 can be identified as follows. Because the mutation causes essentially equivalent increases in the transition state energies of maltose and maltoheptaose, 5.4 and 5.2 kJ/mol respectively, the 178 residue is not likely to interact with the 1-OH group, which is not present in maltoheptaose. Therefore, Trp178 should specifically interact with either 3-OH or 6-OH or the glucosyl-ring oxygen. Removal of the 6-OH group decreases the activation energy for maltose hydrolysis by nearly 2 kJ/mol and replacement of Trp178 significantly increases the activation energy for substrate hydrolysis, so Trp178 is not likely to interact with 6-OH. Replacement of the 3-OH group of maltose increases the $\Delta(\Delta G)$ by 11 kJ/mol, while replacement of Trp178 by Arg increases the activation energy by only 5 kJ/mol. If Trp178 is interacting with the 3-OH group, then either the Trp178 → Arg mutant enzyme retains significant contact with the 3-OH group or another protein group must also hydrogen bond with 3-OH. Alternatively, Trp178 may interact with the ring oxygen or the neighboring general acid group, Glu179. Because of the increased selectivity between maltose and isomaltose resulting from the

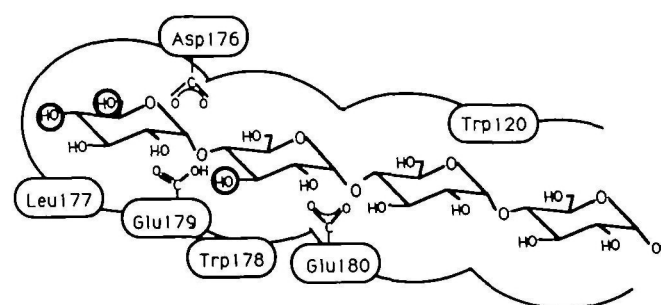


Fig. 3. Schematic drawing of the glucoamylase active site from *A. niger* complexed with maltotetraose. Circled substrate hydroxyl groups represent the critical groups for activity (Bock and Pedersen, 1987; Sierks and Svensson, 1992; Sierks *et al.*, 1992). Asp176 interacts with subsites 1 and 2, Leu177 is located in subsite 1, Arg178 is located in subsite 2, Glu179 is the general acid catalyst and Glu180 binds to the 2-OH in subsite 2 (Sierks and Svensson, 1992).

Trp178 → Arg mutation, Trp178 is expected to have a role in binding rather than an interaction with the catalytic Glu179. The magnitude of the $\Delta(\Delta G)$ values obtained for the three substrates was between 5 and 7 kJ/mol. Because the magnitude of the change in activation energy for all the substrates was similar, van der Waals interactions in subsites 1 or 2, between the glucosyl units and the large Trp178 side chain, may account for the non-specific increases in the $\Delta(\Delta G)$ values and seem to be the dominant influence of Trp178, while the specific subsite 2 interaction, possibly with the 3-OH group, is less important.

The kinetic parameters, k_{cat} and K_M , obtained with the Asn182 → Ala mutation are very similar to wild-type GA on the three substrates tested (Table I). Therefore, the 182 position in GA is not as intimately involved with the catalytic process as are residues 176–180, and it is reasonable that other substitutions at position 182 or at residues closer to the C-terminal can alter the properties of GA without significantly decreasing the catalytic rate. It is somewhat surprising that the rather highly conserved residue, Asn182, found in four GAs and present as the closely related Gln in the fifth (Itoh *et al.*, 1987), can be substituted by a relatively non-conservative replacement such as Ala without affecting enzyme function.

The data obtained by kinetic and mutagenesis studies can help elucidate the catalytic mechanism of GA. Two models for catalysis by GA have been hypothesized, both representing a two-step process. In the first model the substrate undergoes a fast initial recognition step by binding loosely to subsite 2 of GA, followed by a slower unimolecular rearrangement of the enzyme and bound substrate to shift the substrate into subsite 1 in an active configuration (Figure 2A) (Hiromi *et al.*, 1983). The second model postulates an initial recognition step where the substrate occupies the entire binding site and the active conformation is obtained through a conformational change at subsite 1 of GA (Figure 2B) (Fagerstrom, 1991). The kinetic results obtained with the various mutations of residues located in GA subsites 1 and 2 provide evidence to select between these two models. Assuming the substrates bind initially to subsite 2 (Figure 2A), then a substitution of a binding residue at subsite 2 (Glu180 → Gln or Trp178 → Arg) should alter K_M values for all substrates (i.e. maltose and isomaltose) similarly, because it is only the non-reducing end that is recognized, while a change in a binding residue at subsite 1 (Leu177 → His) should not alter the K_M values. However, the Glu180 → Gln mutation has a much larger increase in the K_M value for α -(1,4)-linked substrates than for α -(1,6)-linked substrates, while the Trp178 → Arg mutation has

the opposite effect. The Leu177 → His mutation increases the K_M value 3-fold towards maltose, even though this residue is situated in subsite 1. These results suggest that the catalytic mechanism can be better represented by an initial fast binding step, primarily at subsite 2, followed by a unimolecular rearrangement based on a conformational change in subsite 1 of GA (Figure 2B), rather than dislocation of the substrate (Figure 2A). In this model the initial substrate recognition is still controlled by the very large affinity of subsite 2 for α -(1,4)-linked glucosyl residues (~ 22 kJ/mol) (Hiromi, 1970; Savel'ev and Firsov, 1982; Sierks *et al.*, 1989), but the slower rate-controlling step involves a protein conformational change at subsite 1. Here the replacement of Trp178 by Arg can affect ground state binding of isomaltose without affecting the binding of maltose or maltoheptaose, implying that Trp178 is not involved in initial substrate recognition of α -(1,4)-linked substrates, but does contribute to transition state stabilization. Replacement of Leu177 with His slightly weakens initial substrate recognition and further weakens transition state stabilization. The Trp120 residue, located near subsite 4, is essential for directing the conformational changes necessary to obtain a transition state structure in subsites 1 and 2 (Sierks *et al.*, 1990; Svensson and Sierks, 1992). The Trp120 residue may thus be involved in the conformational change of subsite 1, as depicted in Figure 2B.

All these results can begin to define a picture of the GA active site. Based on chemical modification studies (Clarke and Svensson, 1984b; Svensson *et al.*, 1990), kinetic data obtained on wild-type GA and the various mutations discussed here and previously (Sierks *et al.*, 1989, 1990), and on data obtained using maltose analogs (Sierks and Svensson, 1992; Sierks *et al.*, 1992), a scheme of the GA active site can be constructed locating certain key residues in the active site (Figure 3). The most important substrate hydroxyl groups (Bock and Pedersen, 1987), the 4- and 6-OH groups of the non-reducing end ring of maltose and the 3-OH group of the reducing ring, are circled. A four unit long substrate is pictured to indicate the relative position of the Trp120 residues. Because only maltose analogs were tested in the molecular recognition studies, no conclusions can be made about the hydroxyl groups of the third and fourth glucosyl rings from the non-reducing end. However, the Trp120 residue is located in a distant subsite (Clarke and Svensson, 1984b) and directs conformational changes in subsites 1 and 2 (Sierks *et al.*, 1989). Glu179 is the catalytic acid group and Asp176 interacts with subsites 1 and 2 (Sierks *et al.*, 1990). Glu180 binds to the 2-OH group of the glucosyl residue in subsite 2 (Sierks and Svensson, 1992). Trp178 is located in subsite 2 where it probably interacts with the 3-OH group or the glucosyl-ring oxygen in the transition state complex, but not the ground state, and Leu177 is located in subsite 1 where it is postulated to provide a transition state contact with the 3-OH group of the non-reducing end glucosyl residue through a main chain peptide hydrogen bond. Substitution of any of these residues results in a significant decrease in catalytic activity. The Asn182 residue, however, can be substituted without changing the catalytic parameters of GA, suggesting that it does not interact with the substrate in the catalytic site.

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