

Site-directed mutagenesis at the active site Trp120 of *Aspergillus awamori* glucoamylase

Michael R. Sierks^{1,2}, Clark Ford³, Peter J. Reilly^{1,4} and Birte Svensson⁵

Departments of ¹Chemical Engineering and ³Genetics, Iowa State University, Ames, IA 50011, USA and ⁵Department of Chemistry, Carlsberg Laboratory, DK-2500 Copenhagen Valby, Denmark

²Present address: Carlsberg Laboratory, Copenhagen, Denmark

⁴To whom correspondence should be addressed

Trp120 of *Aspergillus awamori* glucoamylase has previously been shown by chemical modification to be essential for activity and tentatively to be located near subsite 4 of the active site. To further test its role, restriction sites were inserted in the cloned *A. awamori* gene around the Trp120 coding region, and cassette mutagenesis was used to replace it with His, Leu, Phe and Tyr. All four mutants displayed 2% or less of the maximal activity (k_{cat}) of wild-type glucoamylase towards maltose and maltoheptaose. Michaelis constants (K_M) of mutants decreased 2- to 3-fold for maltose and were essentially unchanged for maltoheptaose compared with the wild type, except for a >3-fold decrease for maltoheptaose with the Trp120 → Tyr mutant. This mutant also bound isomaltose more strongly and had more selectivity for its hydrolysis than wild-type glucoamylase. A subsite map generated from malto-oligosaccharide substrates having 2–7 D-glucosyl residues indicated that subsites 1 and 2 had greater affinity for D-glucosyl residues in the Trp120 → Tyr mutant than in wild-type glucoamylase. These results suggest that Trp120 from a distant subsite is crucial for the stabilization of the transition-state complex in subsites 1 and 2.

Key words: glucoamylase/kinetics/site-directed mutagenesis/subsite mapping

Introduction

Glucoamylase (1,4- α -D-glucan glucohydrolase, EC 3.2.1.3) is an exohydrolase that cleaves D-glucose from the non-reducing ends of starch or related oligo- and polysaccharide molecules. Glucoamylases are produced by several filamentous fungi and yeasts, with those from *Aspergilli* being the most commercially important.

Fungal glucoamylases generally occur as multiple forms. *Aspergillus niger* produces two forms, of which the larger, GA I, is thought to consist of three main functional areas: (i) the catalytic region from residues 1 to 440; (ii) a serine- and threonine-rich section from residues 441 to 512, which is heavily O-glycosylated and may be involved in stabilizing the enzyme (Pazur *et al.*, 1970; Svensson *et al.*, 1983); and (iii) a carboxy-terminal tail from residues 513 to 616, which is lacking in the smaller form, GA II, and which is required for starch granule binding (Ueda, 1981; Svensson *et al.*, 1986b).

Kinetic studies on different glucoamylases, in which values of catalytic coefficient (k_{cat}) and Michaelis constant (K_M) were obtained for malto-oligosaccharide substrates of different chain lengths, have all shown the active site to consist of 6–7 subsites

accommodating substrate D-glucosyl residues, with the catalytic site located between subsites 1 and 2. These kinetic studies yielded binding energies of subsite 1 that were slightly negative or positive, of subsite 2 strongly negative, of subsite 3 less negative, and of the remaining subsites slightly negative (Hiromi, 1970; Savel'ev *et al.*, 1982; Tanaka *et al.*, 1983; Koyama *et al.*, 1984; Meagher *et al.*, 1989).

Chemical modifications of various fungal glucoamylases have also shown similarities in the amino acid residues in the active site. One or more tryptophanyl residues have been detected in subsites 1 and 2 in the *Rhizopus* and *A. niger* enzymes (Ohnishi *et al.*, 1975, 1983; Clarke and Svensson, 1984a; Svensson *et al.*, 1986a). Another tryptophanyl residue at or near subsite 4 is essential for activity (Savel'ev and Firsov, 1983; Clarke and Svensson, 1984a) and has been specifically identified as Trp120 in *A. niger* glucoamylase (Clarke and Svensson, 1984b).

The sequences of six different microbial glucoamylases have been determined. Glucoamylases from *A. niger* (Svensson *et al.*, 1983) and *A. awamori* (Nunberg *et al.*, 1984) are identical, those from *Saccharomyces diastaticus* (Yamashita *et al.*, 1985) and *Saccharomyces cerevisiae* (Yamashita *et al.*, 1987) are highly homologous, and those from *Rhizopus oryzae* (Ashikari *et al.*, 1986) and *Saccharomycopsis fibuligera* (Itoh *et al.*, 1987) share four highly conserved regions with the others (Tanaka *et al.*, 1986; Itoh *et al.*, 1987). The essential Trp120 of *A. niger* is invariant in all six glucoamylases (Figure 1). Moreover, Trp120 is homologous with Trp83 of Taka-amylase A, which in the crystal structure is indicated to be in a loop that is involved in substrate binding (Clarke and Svensson, 1984b; Matsuura *et al.*, 1984). Despite the lack of overall Trp120 homology between Taka-amylase A and glucoamylase, the two enzymes are similar in this area (Figure 1).

In the present study the crucial role of Trp120 in the mechanism of action of glucoamylase was examined by site-directed mutagenesis, by using a previously cloned gene from *A. awamori* as expressed in yeast (Nunberg *et al.*, 1984; Innis *et al.*, 1985). Given the regional sequence homology, insights gained into the function of Trp120 should be applicable to other glucoamylases and possibly to Taka-amylase A as well. Therefore, the effects of substituting His, Leu, Phe and Tyr at Trp120 on values of k_{cat} and K_M were studied by using maltose (4-O- α -D-glucopyranosyl-D-glucose), maltoheptaose and, in some cases, isomaltose (6-O- α -D-glucopyranosyl-D-glucose) as substrates. In addition, values of k_{cat} and K_M were determined for Trp120 → Tyr and wild-type glucoamylases with malto-oligosaccharides having 2–7 D-glucosyl residues to compare their subsite maps. Changes in subsite binding energies caused by mutations allow an evaluation of the effects of individual amino acid residues on the active site region.

Materials and methods

Enzymes and reagents

Reagents were obtained from Sigma or Fisher. [α -³²P]ATP (800 Ci/mmol) and [γ -³²P]ATP (3000 Ci/mmol) were obtained from

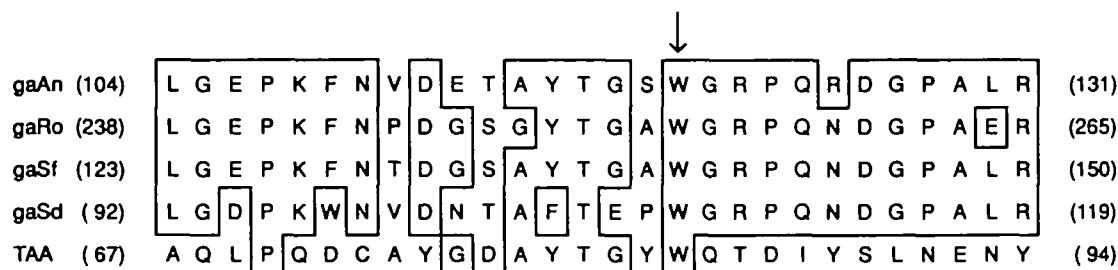


Fig. 1. Sequence homologies among fungal glucoamylases and Taka-amylase A centred around Trp120 of *A.niger* and *A.awamori* glucoamylases. Enzymes: gaAn, *A.niger* (Svensson *et al.*, 1983) and *A.awamori* (Nunberg *et al.*, 1984) glucoamylases; gaRo, *R.oryzae* glucoamylase (Ashikari *et al.*, 1986); gaSf, *S.fibuligera* glucoamylase (Itoh *et al.*, 1987); gaSd, *S.diastaticus* glucoamylase (Yamashita *et al.*, 1985); TAA, Taka-amylase A (Toda *et al.*, 1982). Intracellular *S.cerevisiae* glucoamylase (Yamashita *et al.*, 1987) is identical to gaSd in the region shown except for a substitution of D for P at residue 107 of gaSd. ↓, Trp120 in *A.niger* and *A.awamori* glucoamylases. Numbers in parentheses are those of amino acid residues at the beginning and end of each segment, counting from the N-terminus of the mature protein.

New England Nuclear. Restriction endonucleases and DNA modifying enzymes were purchased from either Bethesda Research Laboratories (BRL), Promega Biotech, Boehringer Mannheim Biochemicals or Stratagene. Culture media were obtained from Difco, Sigma or Fisher. Bacteriophage M13mp19 and *Escherichia coli* strain JM101 were purchased from BRL. A plasmid containing the glucoamylase gene in a yeast expression vector, pGAC9 (Figure 2), and a laboratory strain of *S.cerevisiae*, C468 (α , *leu2-3*, *leu2-112*, *his3-11*, *his3-15*, *mal*⁻) (Innis *et al.*, 1985), were generous gifts from Cetus. Oligonucleotides were purchased from the Iowa State University Nucleic Acid Facility. Spectra/Por dialysis membranes (Fisher) with a molecular weight cutoff of 12 000–14 000 were used for protein purification.

Plasmid purification, subcloning and sequencing

Plasmid DNA was isolated according to established procedures (Maniatis *et al.*, 1982). Fragments isolated for subcloning were purified on an International Biotechnologies #UEA electroeluter. Plasmid DNA for sequencing was prepared following Maniatis *et al.* (1982) with an additional polyethylene glycol precipitation. Plasmid sequencing and single-stranded DNA preparation and sequencing followed procedures of Chen and Seeburg (1985) and Sanger *et al.* (1977) respectively.

Construction of vector for mutagenesis

Construction of a restriction site cassette around Trp120 was accomplished by introducing silent mutations at residue 112 to create a *SalI* site with the primer 5'-GCAGTCTCGTCGAC-ATT-3' and at residue 121 to create a *NotI* site with the primer 5'-TGCGGCCGCCCCAAGA-3' (bold denotes mutations; Figure 3). Both mutations were constructed by using the two-primer method (Norris *et al.*, 1983), the second primer in each case being the 17-mer universal primer obtained from BRL. Mutations were identified by colony hybridization screening (Grunstein and Hogness, 1975), using the respective radiolabeled mutagenic oligonucleotides as probes. Positive clones were verified by DNA sequencing (Sanger *et al.*, 1977). A 0.78 kb *HindIII*–*PstI* fragment containing the two mutations was sequenced, verifying that no additional, spurious mutations had arisen during mutagenesis. This 0.78 kb fragment was then subcloned into the glucoamylase gene, replacing the wild-type 0.78 kb fragment.

Cassette mutagenesis

For each mutation, 3 μ g of the cassette primer containing either a 9- or 17 bp overlap with the mutant primers were annealed with 3 μ g of the mutant oligonucleotide and polymerized

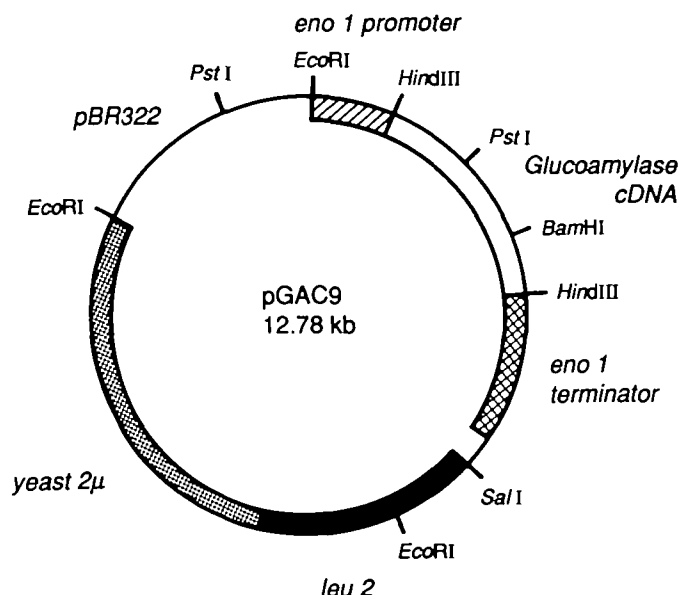


Fig. 2. Plasmid pGAC9 (Innis *et al.*, 1985). The pBR322 sequence allows autonomous replication of the plasmid in *E.coli* and contains the ampicillin gene. The *eno 1* promoter and terminator are two regions from the yeast *enolase* gene that allow expression of the glucoamylase gene in yeast. The *leu 2* sequence allows selection of yeast transformants on leucine-deficient media. The yeast 2 μ sequence allows autonomous replication of the plasmid in yeast. *PstI*, *EcoRI*, *HindIII*, *BamHI* and *SalI* are restriction endonuclease sites.

as above. The following oligonucleotides were used: 5'-ATAGATGTTCGACGAGACTGCC-3' (cassette primer), 5'-CAGATGCGGCCGCCCCGAAAGAACCAAGTGTAGGCAGTCTC-3' (Trp120 → Phe), 5'-CAGATGCGGCCGCCCCGTAAGAACCAAGTGTAGGCAGTCTC-3' (Trp120 → Tyr), 5'-CAGATGCGGCCGCCCCAGAGAACCAGTGTAGGCAGTCTCGTCGACAT-3' (Trp120 → Leu) and 5'-CAGATGCGGCCGCCCCGTGAGAACCAGTGTAGGCAGTCT-3' (Trp120 → His). The oligonucleotides were designed to extend beyond the cassette restriction sites to allow binding by the respective restriction enzymes (Figure 3). The polymerized fragments were digested with *NotI* and *SalI* and then ligated as a cassette into the vector. Mutants were confirmed by sequencing the cassette and then were subcloned into pGAMS10 for yeast transformation. The plasmid pGAMS10 was constructed from pGAC9 by removal of the carboxy-terminal *HindIII* site in the glucoamylase gene, performed by digestion with *HindIII* and

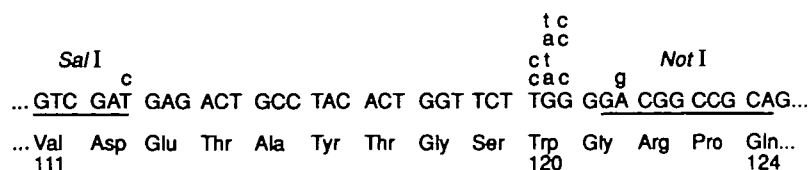


Fig. 3. Mutations of the *A. awamori* glucoamylase Trp120 cassette. Two silent mutations were made by site-directed mutagenesis to create *SalI* and *NotI* restriction sites flanking the Trp120 coding region. Cassette mutagenesis was used to change the TGG Trp codon to TCC (Phe), TAC (Tyr), CTG (Leu) and CAC (His). Mutations are shown in small letters above the wild-type nucleotide sequence.

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

	Trp120 → His	Trp120 → Leu	Trp120 → Phe	Trp120 → Tyr	Wild type
Maltose (G ₂)					
k_{cat} (s ⁻¹)	0.0659 ± 0.0036 ^a	0.255 ± 0.013	0.178 ± 0.012	0.118 ± 0.006	14.4 ± 0.05
K_M (mM)	0.919 ± 0.124	0.530 ± 0.086	0.909 ± 0.147	0.634 ± 0.095	1.73 ± 0.14
k_{cat}/K_M (s ⁻¹ mM ⁻¹)	0.0717	0.481	0.195	0.186	8.3
Maltoheptaose (G ₇)					
k_{cat} (s ⁻¹)	1.40 ± 0.12	1.81 ± 0.11	1.57 ± 0.10	0.440 ± 0.016	84 ± 2
K_M (mM)	0.319 ± 0.069	0.242 ± 0.041	0.216 ± 0.033	0.0594 ± 0.0077	0.22 ± 0.02
k_{cat}/K_M (s ⁻¹ mM ⁻¹)	4.38	7.49	7.28	7.40	390
Isomaltose (iG ₂)					
k_{cat} (s ⁻¹)	—	—	—	0.00588 ± 0.00049	0.487 ± 0.031
K_M (mM)	—	—	—	3.17 ± 0.68	36.0 ± 4.7
k_{cat}/K_M (s ⁻¹ mM ⁻¹)	—	—	—	0.00186	0.0135
k_{cat}/K_M (G ₇)	61.1	15.6	37.3	39.9	47
k_{cat}/K_M (G ₂)	—	—	—	99.9	620
k_{cat}/K_M (iG ₂)	—	—	—	—	—

^aStandard deviation.

subsequent filling in of the overhanging bases (Maniatis *et al.*, 1982).

Expression and purification of mutant enzymes

Competent cells of *S. cerevisiae*, prepared by using lithium acetate (Ito *et al.*, 1983), were transformed with the mutated plasmids. Wild-type and mutant glucoamylases were produced by using 5 l fermentations at 30°C and pH 4.5 for 48 h in selective medium (Innis *et al.*, 1985). Broth supernatants were concentrated 20-fold by using an Amicon hollow-fiber filter with a molecular weight cutoff of 10 000 and then were precipitated with 3 vol of ethanol. Precipitates were resuspended in 75 ml of 0.5 M NaCl–0.1 M NaOAc buffer at pH 4.4, recentrifuged to remove cell debris and then loaded onto a 10 mm i.d. × 20 mm long acarbose–Sephacrose affinity column (Clarke and Svensson, 1984b). After loading, the column was rinsed with the same buffer until the eluant reached a low but constant absorbance at 280 nm. The bound enzymes were eluted with a 1.7 M Tris buffer, pH 7.6, at a flow rate of 3 ml/min. The eluted enzymes were dialyzed against water to remove salts and then lyophilized.

Protein concentrations were determined by using the Bio-Rad dye-binding kit (Bradford, 1976) and purified *A. niger* glucoamylase as a standard.

Determination of kinetic parameters

Reactions to determine the kinetic parameters were performed with 4 ml of liquid volume in 5 ml stirred Pierce Reacti-Vials with substrates of the malto-oligosaccharide series from maltose to maltoheptaose and with isomaltose. Conditions for all kinetic

studies were 50°C and pH 4.4 in 0.05 M sodium acetate buffer. Five to six samples of 0.2 ml were taken at specified time intervals at each of 6–9 substrate concentrations between roughly 0.2 and 4 K_M , the reaction being quenched by addition of Tris buffer, pH 7.0, to a final concentration of 1 M. Glucose concentrations were determined by a glucose oxidase method (Fleming and Pegler, 1963). The Wilkinson (1961) method was used to determine K_M and V_{max} values. Values of k_{cat} were determined by dividing V_{max} by the molar enzyme concentration, using a GA I mol. wt of 95 000 instead of the 82 700 found for *A. niger* glucoamylase (Svensson *et al.*, 1983). This value was used since *S. cerevisiae* glycosylates glucoamylase to a greater extent than *A. niger* (Innis *et al.*, 1985), as indicated by SDS gel electrophoresis.

Results and discussion

A series of four mutations, replacing Trp120 with His, Leu, Phe and Tyr, were constructed in the cloned glucoamylase gene from *A. awamori* and expressed in *S. cerevisiae* to investigate the role of the tryptophanyl side chain. Two restriction sites flanking the Trp120 coding region were first created by site-directed mutagenesis so that the mutations could easily be made by cassette mutagenesis (Figure 3).

Kinetic properties of the mutants and wild-type glucoamylase were measured by using maltose and maltoheptaose as substrates, as they productively bind only to the first two and to all seven subsites respectively. All four mutants had significantly smaller K_M values with maltose than the wild-type enzyme (Table I).

Table II. Kinetic parameters for hydrolysis of malto-oligosaccharides by Trp120 → Tyr and wild-type *A. awamori* glucoamylases at pH 4.4 and 50°C

Substrate	Trp120 → Tyr			Wild type		
	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	0.118 ± 0.006*	0.634 ± 0.095	0.186	14.4 ± 0.5	1.73 ± 0.14	8.3
Maltotriose	0.256 ± 0.009	0.121 ± 0.013	2.11	62 ± 4	0.73 ± 0.09	86
Maltotetraose	0.568 ± 0.017	0.132 ± 0.009	4.30	87 ± 4	0.53 ± 0.07	165
Maltopentaose	0.534 ± 0.030	0.0748 ± 0.0111	7.14	94 ± 3	0.31 ± 0.03	300
Maltohexaose	0.466 ± 0.020	0.0742 ± 0.0071	6.29	86 ± 4	0.20 ± 0.03	430
Maltoheptaose	0.440 ± 0.016	0.0594 ± 0.0077	7.40	84 ± 2	0.22 ± 0.02	390

*Standard deviation.

This demonstrated the influence of Trp120 on subsites 1 and 2, further clarifying why it was previously found to be critical for catalytic activity when located in a distant subsite (Clarke and Svensson, 1984b). When maltoheptaose was used as substrate, K_M values for three of the mutants, Trp120 → His, Trp120 → Leu and Trp120 → Phe, were roughly the same as or greater than that of wild-type glucoamylase, but the value for the Trp120 → Tyr mutant was significantly lower (Table I).

Values of k_{cat} with both maltose and maltoheptaose for all four mutants were between 0.005 and 0.02 times that of wild-type glucoamylase. Mutant enzymes with the more hydrophobic side chains at residue 120 (Leu and Phe) had larger k_{cat} values on maltose than those with His or Tyr. Because the mutants have some residual activity and because the *S. cerevisiae* strain expressing them is *mal*⁻ and therefore does not produce glucoamylase itself, Trp120 is not essential. However, it clearly has a key role in enzymatic activity, even from a location near subsite 4.

As the Trp120 → Tyr mutant showed stronger binding and lower catalytic activity with maltoheptaose than did the other three mutants, it and wild-type glucoamylase were further characterized by constructing their subsite maps by using linear malto-oligosaccharides with 2–7 D-glucosyl residues as substrates. With both forms, values of k_{cat} increased from maltose to maltotetraose and were essentially constant at longer chain lengths, whereas values of K_M decreased from maltose to maltoheptaose (Table II). Binding energies for each subsite, generated from the kinetic data (Hiromi *et al.*, 1973), are shown in Table III. The recombinant wild-type enzyme shows a subsite structure similar to those reported earlier for glucoamylases secreted by natural hosts (Hiromi, 1970; Savel'ev *et al.*, 1982; Tanaka *et al.*, 1983; Koyama *et al.*, 1984; Meagher *et al.*, 1989). The Trp120 → Tyr mutant has binding energies very similar to those of wild-type glucoamylase in all the subsites except 1 and 2, which are significantly more negative, subsite 1 by 4.6 kJ/mol and subsite 2 by 2.3 kJ/mol. This indicates that the Trp120 → Tyr mutant binds D-glucosyl residues in subsites 3–7 as efficiently as does wild-type glucoamylase.

The increase in strength of substrate binding at subsites 1 and 2 by the other three mutants is negated when the entire active site is filled by maltoheptaose (Table I). This is most probably due to a decreased affinity for D-glucosyl residues at remote subsites of the enzyme caused by substitution of His, Leu and Phe for Trp120.

Wild-type and Trp120 → Tyr glucoamylases were further compared by using isomaltose as substrate. Isomaltose contains two D-glucosyl units linked in an α-(1→6) configuration, as opposed to the α-(1→4) linkage of maltose. This implies a different orientation of isomaltose in the active site, as evidenced

Table III. Subsite energies for hydrolysis of malto-oligosaccharides by Trp120 → Tyr and wild-type *A. awamori* glucoamylases at pH 4.4 and 50°C

Subsite	Binding energy (kJ/mol)	
	Trp120 → Tyr	Wild type
1	-2.0	2.6
2	-23.7	-21.4
3	-6.5	-6.3
4	-1.9	-1.8
5	-1.4	-1.6
6	0.3	-1.0
7	-0.4	0.3

Table IV. Increases in activation energy of substrate hydrolysis by *A. awamori* glucoamylase at pH 4.4 and 50°C caused by mutations of residue 120

Substrate	$\Delta(\Delta G)$ (kJ/mol)			
	Trp120 → His	Trp120 → Leu	Trp120 → Phe	Trp120 → Tyr
Maltose	12.8	7.7	10.1	10.2
Maltoheptaose	12.0	10.6	10.7	10.6
Isomaltose	—	—	—	5.3

by its K_M value with wild-type glucoamylase being 20 times that of maltose (Table I). However, isomaltose seemed to adapt better to the active site of the Trp120 → Tyr mutant, as its K_M value was <10% that of wild-type glucoamylase. This caused the selectivity for maltose over isomaltose of the Trp120 → Tyr mutant to decrease 6-fold compared with wild-type glucoamylase, as seen from the ratios of k_{cat}/K_M values for maltose over isomaltose for the two enzyme forms (Table I).

It has been demonstrated that substitution of Trp120 in glucoamylase by His, Leu, Phe or Tyr improves substrate binding in subsites 1 and 2. However, the large decreases in k_{cat} values for these mutants on all substrates employed indicated that Trp120 has an integral role in forming the enzyme–substrate transition state. This agrees with earlier chemical modification results, which showed that Trp120, even though located in a distant subsite, was required for catalytic activity (Clarke and Svensson, 1984b). Therefore, it seems that the large size of the tryptophanyl side chain and not just its hydrophobic or aromatic character is required for stabilizing the transition state. Clarke and Svensson (1984b) previously suggested local structural similarity between Trp120 and Taka-amylase A Trp83, which Matsuura *et al.* (1984) had located in the three-dimensional structure of the enzyme at a binding subsite remote from the catalytic site. Although there is no experimental evidence at present for the function of Trp83,

this residue is strikingly free of steric hindrances in the active site cleft, which would allow it to undergo conformational changes. Consequently, given its large size, it may participate in long-range interactions, further supporting the location of the related Trp120 near subsite 4 in glucoamylase.

All Trp120 mutants bound maltose more tightly than did wild-type glucoamylase, but with comparatively little hydrolytic activity. The mutant enzymes therefore associated more strongly than wild-type glucoamylase with the maltose ground state, but not with the maltose transition state. The increase in activation energy for substrate hydrolysis by the Trp120 mutants can be estimated from the ratio of mutant and wild-type specificity constants for each substrate (Fersht, 1985):

$$\Delta(\Delta G) = -RT \ln [(k_{\text{cat}}/K_M)_{\text{mut}}/(k_{\text{cat}}/K_M)_{\text{wt}}]$$

where ΔG is the activation energy, R is the universal gas constant and T is the absolute temperature. Activation energies for maltose hydrolysis are 7.7–12.8 kJ/mol larger for the mutants than for wild-type glucoamylase (Table IV). This increase in activation energy for all the mutations can be attributed to either ground state stabilization or transition state destabilization, or both. Since the K_M values for maltose decreased for all the mutants, the maltose ground state is bound better than in wild-type glucoamylase. However, the mutation with the least affinity for maltose, that with the highest K_M value, Trp120 → His, also had the largest increase in activation energy. This suggests that it is not ground state stabilization, but rather transition state destabilization, that is primarily reflected by the $\Delta(\Delta G)$ values. Decreased stabilization of the maltoheptaose transition state occurred to about the same extent as with the maltose transition state, except for an increase in $\Delta(\Delta G)$ of ~3 kJ/mol with the Trp120 → Leu mutant. Therefore, Trp120 has a minor role in binding the transition state near subsite 4 but plays a critical role in binding at subsites 1 and 2. Finally, the effect of Trp120 in stabilizing the isomaltose transition state is not as large as with the maltose and maltoheptaose transition states, the $\Delta(\Delta G)$ value between Trp120 → Tyr and wild-type glucoamylases being 5.3 kJ/mol. This suggests that different binding interactions may be important for isomaltose hydrolysis.

The subsite maps for Trp120 → Tyr and wild-type glucoamylase indicate that subsites 1 and 2 have an increased affinity of 6.9 kJ/mol for D-glucosyl residues when the Trp120 side chain is not present. This may represent some of the energy used for adaption of the active site to fit the substrate transition state. We conclude that Trp120 directs the necessary conformational change.

Acknowledgements

The authors are grateful to Cetus Corporation for the gift of the glucoamylase gene, yeast expression vector and *S. cerevisiae* strain. This project was supported by a grant from the Biotechnology Council of Iowa State University. Journal Paper no. 13427 of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA. Project no. 2820.

References

- Ashikari, T., Nakamura, N., Tanaka, Y., Kiuchi, N., Shibano, Y., Tanaka, T., Amachi, T. and Yoshizumi, H. (1986) *Agric. Biol. Chem.*, **50**, 957–964.
 Bradford, M.M. (1976) *Anal. Biochem.*, **72**, 248–254.
 Chen, E.J. and Seeburg, P.H. (1985) *DNA*, **4**, 165–170.
 Clarke, A.J. and Svensson, B. (1984a) *Carlsberg Res. Commun.*, **49**, 111–122.
 Clarke, A.J. and Svensson, B. (1984b) *Carlsberg Res. Commun.*, **49**, 559–566.
 Fersht, A. (1985) *Enzyme Structure and Mechanism*, 2nd edn. W.H. Freeman, New York, p. 350.
 Fleming, I.D. and Pegler, H.F. (1963) *Analyst*, **88**, 967–968.

- Grunstein, M. and Hogness, D.S. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 3961–3968.
 Hiromi, K. (1970) *Biochem. Biophys. Res. Commun.*, **40**, 1–6.
 Hiromi, K., Nitta, Y., Numata, C. and Ono, S. (1973) *Biochim. Biophys. Acta*, **302**, 362–375.
 Innis, M.A., Holland, M.J., McCabe, P.C., Cole, G.E., Wittman, V.P., Tal, R., Watt, K.W.K., Gelfand, D.H., Holland, J.P. and Meade, J.H. (1985) *Science*, **228**, 21–26.
 Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983) *J. Bacteriol.*, **153**, 163–168.
 Itoh, T., Ohtsuki, I., Yamashita, I. and Fukui, S. (1987) *J. Bacteriol.*, **169**, 4171–4176.
 Koyama, T., Inokuchi, N., Kikuchi, Y., Shimada, H., Iwama, M., Takahashi, T. and Irie, M. (1984) *Chem. Pharm. Bull.*, **32**, 757–761.
 Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
 Matsuura, Y., Kusunoki, M., Harada, W. and Kakudo, M. (1984) *J. Biochem.*, **95**, 697–702.
 Meagher, M.M., Nikolov, Z.L. and Reilly, P.J. (1989) *Biotechnol. Bioeng.*, in press.
 Norris, K., Norris, F., Christiansen, L. and Fiil, N. (1983) *Nucleic Acids Res.*, **11**, 5103–5112.
 Nunberg, J.H., Meade, J.H., Cole, G., Lawyer, F.C., McCabe, P., Schweickart, V., Tal, R., Wittman, V.P., Flatgaard, J.E. and Innis, M.A. (1984) *Mol. Cell. Biol.*, **4**, 2306–2315.
 Ohnishi, M., Kogai, H. and Hiromi, K. (1975) *J. Biochem.*, **77**, 695–703.
 Ohnishi, M., Taniguchi, M. and Hiromi, K. (1983) *Biochim. Biophys. Acta*, **744**, 64–70.
 Pazur, J.H., Knull, H.R. and Simpson, D.L. (1970) *Biochem. Biophys. Res. Commun.*, **40**, 110–116.
 Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
 Savel'ev, A.N. and Firsov, L.M. (1983) *Biochemistry (USSR)*, **48**, 1125–1132.
 Savel'ev, A.N., Sergeev, V.R. and Firsov, L.M. (1982) *Biochemistry (USSR)*, **47**, 330–336.
 Svensson, B., Larsen, K., Svendsen, I. and Boel, E. (1983) *Carlsberg Res. Commun.*, **48**, 529–544.
 Svensson, B., Clarke, A.J., Møller, H., Svendsen, I. and Håkansson, K. (1986a) *13th International Carbohydrate Symposium, Ithaca, NY*. Abstract C76.
 Svensson, B., Larsen, K. and Gunnarsson, A. (1986b) *Eur. J. Biochem.*, **154**, 497–502.
 Tanaka, A., Fukuchi, Y., Ohnishi, M., Hiromi, K., Aibara, S. and Morita, Y. (1983) *Agric. Biol. Chem.*, **47**, 573–580.
 Tanaka, Y., Ashikari, T., Nakamura, N., Kiuchi, N., Shibano, Y., Amachi, T. and Yoshizumi, H. (1986) *Agric. Biol. Chem.*, **50**, 965–969.
 Toda, H., Kondo, K. and Narita, K. (1982) *Proc. Jpn. Acad.*, **58B**, 208–212.
 Ueda, S. (1981) *Trends Biochem. Sci.*, **5**, 89–90.
 Wilkinson, G.N. (1961) *Biochem. J.*, **80**, 324–332.
 Yamashita, I., Suzuki, K. and Fukui, S. (1985) *J. Bacteriol.*, **161**, 567–573.
 Yamashita, I., Nakamura, M. and Fukui, S. (1987) *J. Bacteriol.*, **169**, 2142–2149.

Received on March 16, 1989; accepted on April 24, 1989