Subsite mapping of *Hormoconis resinae* glucoamylases and their inhibition by gluconolactone

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The subsite maps of two purified glucoamylases (P and S) from the fungus *Hormoconis resinae* were determined from kinetic data using sets of linear malto- and isomaltooligosaccharides as substrates. Glucoamylase P, which has an unusually high debranching (1,6-glucosidic) activity, showed a subsite map different from all known subsite maps of glucoamylases. The free energy of binding of maltooligosaccharides was negative at subsite 1, whereas all the others show a positive or zero energy at subsite 1. Inhibition of both glucoamylases P and S acting on either malto- or isomaltohexaose by gluconolactone [D-glucono-(1,5)-lactone] was investigated. Gluconolactone decreased the values of the maximum velocity, V , suggesting it can bind to subsite 1. The size of inhibition constants, identified as dissociation constants of gluconolactone from free enzyme, depended on whether the substrate was maltohexaose or isomaltohexaose. This suggests that gluconolactone has at least two binding sites, and that there are different subsites 1 (or 2) for 1,4- and 1,6-linked substrates. From previously reported results with other glucoamylases, an 'induced fit' model was constructed for glucoamylases hydrolysing oligosaccharides.

Introduction

Glucoamylases (1,4- α -D-glucan glucohydrolases, EC 3.2.1.3) are enzymes that degrade oligosaccharides such as amylose and amylopectin, releasing D-glucose from the nonreducing-end of the oligosaccharide. Usually the activity towards the branching points (1,6- and 1,3-glucosidic bonds) of natural oligosaccharides is low. However, some glucoamylases do contain high debranching activity, and these enzymes are of commercial interest for the food and fermentation industry in that they are capable of converting their oligosaccharide substrates almost completely to glucose.

Hiromi (Hiromi, 1970; Hiromi *et al.*, 1973a) has developed a subsite theory for analysing the substrate affinities of glucoamylases. The active site of glucoamylase is proposed to consist of several subsites, which are numbered starting from the binding site of the glucose at the nonreducing-end. The basic assumptions made are as follows. 1. The substrates bind in rapid equilibria. 2. The active site consists of n subsites, each having an affinity A_n for a residue of the substrate. 3. A substrate can be bound to the enzyme in either productive or nonproductive modes. 4. The intrinsic rate (k_{int}) of hydrolysis is independent of substrate length. 5. The binding affinity of an oligomeric substrate is equal to the sum of the individual subsite affinities.

The subsite structure of different glucoamylases seems to have a common arrangement, where subsite 2 has the highest affinity for the oligomeric substrates and glucose (Ohnishi *et al.*, 1975; Hiromi *et al.*, 1973a), followed by a decreasing affinity upon going towards subsites 3 to 7. Subsite 1 seems to show a negative or zero affinity (Tanaka *et al.*, 1983a).

The cleavage of different α -D-glycosidic bonds is considered to occur at the same active centre of glucoamylases (Hiromi *et al.*, 1966a,b,c), between subsites 1 and 2 (Hiromi *et al.*, 1973a).

Gluconolactone [D-glucono-(1,5)-lactone], having a distorted half-chair conformation, has been considered to be a transition state analogue in the hydrolysis reaction (Ohnishi *et al.*, 1975; Hiromi *et al.*, 1983). Studies of glucoamylases from *Rhizopus niveus* and *Rhizopus delemar* have suggested that this inhibitor binds to subsite 1 of these enzymes (Ohnishi *et al.*, 1975; Hiromi *et al.*, 1982, 1983; Tanaka *et al.*, 1982).

The amino acid sequences of six different microbial glucoamylases have been determined. The complete sequences of *Aspergillus niger* (Svensson *et al.*, 1983) and *Aspergillus awamori* (Nunberg *et al.*, 1984) glucoamylases are identical. The glucoamylases from *Rhizopus oryzae* (Ashikari *et al.*, 1986), *Saccharomyces diastaticus* (Yamashita *et al.*, 1985), *Saccharomyces cerevisiae* (Yamashita *et al.*, 1987) and *Saccharomycopsis fibuligera* (Itoh *et al.*,

1987) share four highly conserved regions with the *Aspergillus* enzyme (Tanaka *et al.*, 1986; Itoh *et al.*, 1987). These regions, numbered according to the sequence of *A. awamori* glucoamylase, include Asp-176, Glu-179, Glu-180 and Trp-120. Sierks *et al.* (1990) have, on the basis of site-directed mutation experiments, suggested Glu-179 as the general acid catalyst and Asp-176 as the general base catalyst in the hydrolysis reaction of oligosaccharides. Glu-180 plays, according to their interpretation, a role in raising the pK_a of Glu-179. Fluorescence quenching of one or more tryptophanyl residues upon binding of substrate to glucoamylases has been demonstrated (Hiromi *et al.*, 1982; Clarke & Svensson, 1984). Trp-120 is proposed to be needed for the stabilization of the transition-state complex in subsites 1 and 2 (Sierks *et al.*, 1989).

The fungus *Hormoconis resinae* (ATCC 20495) produces two extracellular glucoamylases (P and S), one of them (P) having an exceptionally high activity towards 1,6-glycosidic bonds in addition to the 1,4-glycosidic activity (McCleary & Anderson, 1980). These glucoamylases seem to be highly homologous (Fagerström *et al.*, 1990), and also to show high homology to other glucoamylases (to be published elsewhere), especially in the four highly conserved regions.

Methods

Materials. Linear oligosaccharides ranging from maltose (4-*O*- α -D-glucopyranosyl-maltose) to maltoheptaose (4-*O*- α -D-glucopyranosyl-maltoheptaose) (Sigma) and isomaltose (6-*O*- α -D-glucopyranosyl-isomaltose) to isomaltoheptaose (6-*O*- α -D-glucopyranosyl-isomaltoheptaose) (Biocarb, Uppsala, Sweden) were used as substrates. To determine released glucose, a kit based on glucose dehydrogenase (Merck) was used. In the case of gluconolactone inhibition, released glucose was measured with a kit based on hexokinase (Boehringer).

Enzyme preparations. Glucoamylase P and S were purified from *Hormoconis resinae* (ATCC 20495) culture media as described in Fagerström *et al.* (1990). The preparations were homogeneous on SDS-PAGE. The apparent molecular masses estimated from SDS-PAGE were 71 kDa (glucoamylase P) and 78 kDa (glucoamylase S).

The concentration of purified protein was determined spectrophotometrically at 205 nm using the method described by Scopes (1974); $A_{1\text{cm}}^{1\%}$ values of 307 for glucoamylase P and 314 for glucoamylase S were used.

Determination of subsite energies. The kinetic parameters, K_m and V were determined by incubating enzyme with the respective linear oligosaccharides. Reactions were performed at 25 °C. The incubation buffer was 50 mM-sodium acetate, pH 4.30, containing 150 μ g ovalbumin (Sigma grade V) ml^{-1} . In order to minimize the formation of oligosaccharides from D-glucose by the condensation reaction of glucoamylase (Nikolov *et al.*, 1989), short incubation times (0.5 to 15 min) were used. In the case of glucoamylase S with isomaltooligosaccharides, however, incubation times of up to 40 min had to be used. Samples were withdrawn at 0.5 to 10 min intervals, heated at 100 °C for 3 min and then transferred to an ice-water bath.

The amount of glucose released was measured with the glucose dehydrogenase kit. All incubations with the different substrate concentrations were run as duplicates, and in the case of glucoamylase P with malto- and isomaltooligosaccharides, two independent experiments were performed.

To minimize the effect of substrate hydrolysis producing a new shorter substrate for the next hydrolysis round, only points at less than 14% complete theoretical hydrolysis were accepted. Substrate concentrations ranged from about half K_m to at least $10 \times K_m$, except for glucoamylase S with isomaltooligosaccharides, where substrate concentrations of up to $5 \times K_m$ were used. The kinetic parameters were calculated from the initial velocity data according to Wilkinson (1961) (steady-state kinetics) using nonlinear regression analysis (Gauss-Newton, no weighting, SAS software). Values for molecular activity, k_0 [from the molar concentration of enzyme (e_0) and maximum velocity (V), $k_0 = V/e_0$ and k_0/K_m were calculated and used to determine the free energies of binding for each subsite 3 to 7 by using equation (1). For estimating the intrinsic rate, k_{int} , and the free energy of substrate binding at subsite 1 (A_1), equation (2) was used [by plotting $e^{(A_n+1)/RT}$ against $(1/k_0)_n$].

The free energy of substrate binding at subsite 2 was calculated from K_m by using equation (3) and also from k_{int} and k_0/K_m using equation (4). For the derivation of these equations, the reader is referred to Hiromi *et al.* (1973a).

$$A_{n+1} = RT[\ln(k_0/K_m)_{n+1} - \ln(k_0/K_m)_n] \quad (1)$$

$$e^{(A_{n+1}/RT)} = \{[k_{\text{int}}/(k_0)_n] - 1\} e^{(A_1/RT)} \quad (2)$$

$$A_2 = -RT \ln \{0.018(K_m)_n [e^{(A_1+A_3+\dots+A_7)/RT} + e^{(A_1+A_4+\dots+A_{n+1})/RT}]\} \quad (3)$$

$$A_2 = RT \ln [(k_0/K_m)_n / (0.018 \times k_{\text{int}})] - (A_1 + A_3 + \dots + A_n) \quad (4)$$

Inhibition by D-glucono-(1,5)-lactone. Maltohexaose and isomaltohexaose were incubated with enzyme at 25 °C with or without the inhibitor D-glucono-(1,5)-lactone (gluconolactone) in 50 mM-sodium acetate, pH 4.3, containing ovalbumin (150 μ g ml^{-1}). Samples of 60 μ l were withdrawn at 3 to 20 min intervals, heated at 100 °C for 3 min and cooled in an ice-water bath. The samples were assayed for released glucose by the hexokinase method. Gluconolactone solutions were prepared just before use (less than 5 min) to minimize the possible conversion to D-glucono-(1,4)-lactone or hydrolysis to gluconic acid (Shimihara & Takahashi, 1970).

Results

The K_m , k_0 , k_0/K_m and k_{int} values of the glucoamylases with linear maltooligosaccharides (G2 to G7) and linear isomaltooligosaccharides (IG2 to IG7) as substrates are listed in Table 1. The calculated free energies of substrate binding (A_n), together with the estimates of the 'worst case' combinations of errors in the six estimates of K_m ($\pm 20\%$) and six estimates of V ($\pm 7\%$), are listed in Table 2 (assuming A_8 to be zero). Values for the second subsite calculated by the two different methods were essentially the same, and their average was used in each case for A_2 .

The recalculated kinetic parameters from the free energies of substrate binding were in good agreement with the observed ones (not shown; the deviations were between 0% and 20%). Thus the theory yields an internally consistent set of subsite affinities for *H. resinae* glucoamylases.

Table 1. Kinetic data for glucoamylases P and S

The kinetic values K_m and k_0 ($=V/e_0$) for both glucoamylases acting on either malto- or isomaltooligosaccharides of different length (n), were obtained from initial velocity data using nonlinear regression analysis as described in the text. In addition, the calculated intrinsic rates of hydrolysis (k_{int}) are shown.

	Maltooligosaccharides (G)			Isomaltooligosaccharides (IG)		
Value of <i>n</i>	<i>K_m</i> (mM)	<i>k₀</i> (s ⁻¹)	<i>k₀/K_m</i> (s ⁻¹ mM ⁻¹)	<i>K_m</i> (mM)	<i>k₀</i> (s ⁻¹)	<i>k₀/K_m</i> (s ⁻¹ mM ⁻¹)
<i>Glucoamylase P</i>						
2	1.108	3.92	3.53	14.55	1.92	0.13
3	0.174	11.60	66.58	4.523	2.76	0.61
4	0.145	19.43	133.67	3.158	5.35	1.70
5	0.133	13.26	99.45	2.840	6.53	2.29
6	0.111	17.07	153.27	2.585	7.37	2.85
7	0.086	19.08	222.67	2.127	5.52	3.07
	<i>k_{int}</i> = 19.4			<i>k_{int}</i> = 30.8		
<i>Glucoamylase S</i>						
2	1.420	5.03	3.54	36.50	0.160	0.0044
3	0.609	29.41	48.29	22.24	0.523	0.0234
4	0.395	40.27	101.94	24.08	0.825	0.0342
5	0.235	42.66	181.51	22.65	0.754	0.0333
6	0.177	48.33	273.06	24.34	0.814	0.0334
7	0.206	49.83	241.87	24.15	0.753	0.0312
	<i>k_{int}</i> = 261.6			<i>k_{int}</i> = 5.4		

For both enzymes, the effect of increasing chain length in either oligosaccharide series was to increase k_0 (by 3- to 10-fold) and decrease K_m (by 7- to 11-fold, except that the K_m of glucoamylase S for isomaltooligosaccharides did not decrease further after isomaltotriose). These trends were usually monotonic, but, for example, glucoamylase P showed a peak k_0 at both maltotetraose and maltoheptaose. However, in general the larger substrates were both more tightly bound and more rapidly hydrolysed.

The differences between the k_0 values for the oligosaccharides of different length, compared to the k_{int} value of the respective enzyme, can be explained by the distribution of productive and nonproductive binding (Suetsugu *et al.*, 1973). In the cases of glucoamylase P and S with isomaltooligosaccharides and glucoamylase S with maltooligosaccharides, the k_0 values are much smaller than the corresponding k_{int} . The substrates seem to be bound preferentially in nonproductive modes. Only glucoamylase P with maltooligosaccharides as substrate showed a k_0 (at G4 and G7) of the same size as k_{int} , indicating that in these cases productive complexes overwhelm nonproductive complexes.

Because for all substrates A_2 (the free energy of binding at subsite 2) is much more negative than A_3 , even than the sum of $A_3 + A_4 + \dots + A_7$, the most important nonproductive complex will occupy subsite 2. The

Table 2. Free energy of binding of substrate to glucoamylases P and S

The minimum and maximum estimates given show the effect of 'worst case' combinations of errors in the six estimates of K_m ($\pm 20\%$) and the six estimates of V ($\pm 7\%$) and generate each series of subsite binding energies.

Value of A_n	Substrate binding energy (kJ mol^{-1})					
	Glucoamylase P			Glucoamylase S		
	Mean	Max.	Min.	Mean	Max.	Min.
<i>Maltooligosaccharides</i>						
1	-3.83	-5.63	-0.98	+3.27	-1.48	+25.08
2	-19.04	-22.43	-16.88	-19.70	-22.08	-17.22
3	-7.27	-8.62	-5.92	-6.47	-7.82	-5.12
4	-1.73	-3.08	-0.37	-1.85	-3.20	-0.50
5	+0.73	-0.62	+2.08	-1.43	-2.78	-0.08
6	-1.07	-2.42	+0.28	-1.01	-2.36	+0.34
7	-0.92	-2.28	+0.44	+0.30	-1.05	+1.65
<i>Isomaltooligosaccharides</i>						
1	+3.00	-1.72	+22.57	+4.49	-0.81	+23.6
2	-16.57	-18.89	-14.02	-13.95	-15.89	-11.07
3	-3.80	-5.15	-2.45	-4.16	-5.52	-2.81
4	-2.53	-3.88	-1.18	-0.93	-2.28	-0.42
5	-0.75	-2.10	+0.60	+0.07	-1.28	+1.42
6	-0.54	-1.89	+0.81	-0.01	-1.36	+1.35
7	-0.18	-1.53	+1.17	+0.17	-1.18	+1.52

simplest reaction scheme for glucoamylase (E) binding and degrading oligosaccharide substrate (S) in the presence of an inhibitor (I), that can bind to subsites 1 and 2, can therefore be written

where ES and ES' are productive and nonproductive enzyme-substrate complexes respectively, $K_p + K'_p$ describe inhibitor binding at subsite 1, and K_q describes inhibitor binding at subsite 2. From this reaction scheme (5), the rate equation is

$$\begin{aligned}
 e/v = & (1/k)[1 + (K_s/K'_s)(1 + i/K'_p)] \\
 & + (K_s/k)[1 + (i/K_q) + (i/K_p)] 1/s
 \end{aligned} \quad (6)$$

Thus, if gluconolactone would bind only to subsite 2 (to form EI' in the scheme), it would inhibit both productive and nonproductive binding, with no effect on V . Binding of gluconolactone to subsite 1 in free enzyme would inhibit only the productive binding, but the formation of $ES'I$ would stabilize nonproductive binding and so decrease V .

Fig. 1. Lineweaver-Burk double reciprocal plots of the inhibition of glucoamylases by gluconolactone. Inhibition of: (a) glucoamylase P (65.2 nM) degrading maltohexaose by 0 mM (●), 0.4 mM (△) and 4 mM (□) gluconolactone; (b) glucoamylase p (81.6 nM) degrading isomaltohexaose by 0 mM (●), 4 mM (△) and 10 mM (□) gluconolactone; (c) glucoamylase S (56.4 nM) degrading maltohexaose by 0 mM (●), 0.4 mM (△), 2 mM (□) and 4 mM (▼) gluconolactone; (d) glucoamylase S (1.8 μM) degrading isomaltohexaose by 0 mM (●), 1 mM (△) and 4 mM (□) gluconolactone.

Inhibition by gluconolactone of the degradation of malto- and isomaltohexaose by glucoamylase P and S decreases V (Fig. 1), suggesting that both enzymes bind gluconolactone at subsite 1, like *R. niveus* and *R. delemar* glucoamylases (Hiromi *et al.*, 1983; Ohnishi *et al.*, 1976). For both enzymes the $1/s$ intercepts are nearly constant for maltohexaose, but change greatly for isomaltohexaose.

By plotting the ordinate intercepts and slopes of the Lineweaver-Burk plots against i (example in Fig. 2), the values of the following set of constants can be obtained for each data set.

From the intercepts

$$k_0 = k K'_s / (K_s + K'_s) \quad (7)$$

$$K'_i = K'_p (K_s + K'_s) / K_s \quad (8)$$

From the slopes

$$K_i = K_p K_q / (K_p + K_q) \quad (9)$$

From combination of slopes and intercepts

$$K_m = K_s K'_s / (K_s + K'_s) \quad (10)$$

$$K''_i = K'_s K'_p \quad (11)$$

The values are listed in Table 3. Higher concentrations of inhibitor could not be used, because of the sensitivity

Fig. 2. Example of secondary plots of intercepts (a) and slopes (b) against gluconolactone concentration. The plots are for glucoamylase P with isomaltohexaose.

Table 3. Kinetic constants extracted from the rate equation (6) and estimated from the secondary plots (Fig. 2).

M6, maltohexaose; IM6, isomaltohexaose.

	Glucoamylase P		Glucoamylase S	
	M6	IM6	M6	IM6
k_0 (s ⁻¹)	11.5	6.7	50.5	0.84
K_m (mM)	0.11	2.04	0.23	18.0
K_i (mM)	1.03	0.28	2.41	1.20
K'_i (mM)	0.78	6.25	1.94	3.60
K''_i (mM)	0.086	12.8	0.45	63.6

limits of the assay and the need to avoid long incubations, which could effect the conversion of gluconolactone (see Methods). Because of the high price of the oligosaccharides, assays using intermediate concentrations of inhibitor were not performed. The slightly different values of K_m and k_0 compared to those of malto- and isomaltohexaose in Table 1 are due to the use of linear regression analysis in the former and nonlinear regression analysis in the later case. For both enzymes K'_i and K''_i are much bigger for isomaltohexaose than for maltohexaose. The K_i values are also different for the

different substrates. According to scheme (5) they should be the same, since they involve only the dissociation constants of *I* from free enzyme (namely K_p and K_q). Apparently this simple reaction scheme does not fully fit the empirical data.

Discussion

Glucoamylases probably have basically similar reaction mechanisms, because they have very similar amino acid sequences at the active site proposed by Tanaka *et al.* (1986). Site-directed mutagenesis studies (Sierks *et al.*, 1990) confirm the importance of these sequences.

The relatively high 1,6/1,4-activity ratio of glucoamylase P results from both its higher k_{int} towards isomalto-oligosaccharides and its lower k_{int} towards maltooligosaccharides compared with glucoamylase S (Table 1). The high negative free energy of binding of maltooligosaccharides at subsite 1 in glucoamylase P is unique among known glucoamylases (Hiromi, 1970; Tanaka *et al.*, 1983a; Koyama *et al.*, 1984; Monma *et al.*, 1989; Ono *et al.*, 1988; Meagher *et al.*, 1989). For the five glucoamylases purified from *Aspergillus niger*, this free energy decreases from about +2.3 kJ mol⁻¹ to about 0 kJ mol⁻¹ (calculated from the data of Ono *et al.*, 1988) in the same order as increasing relative activity towards dextran 80 (a 1,6-glycosidically linked oligosaccharide). Possibly a large negative value for this free energy favours relatively high activity towards 1,6-linked oligosaccharides.

The gluconolactone inhibition pattern shows that no simple (rigid) enzyme mechanism adequately describes glucoamylases P and S. This is because the change in the slopes (i.e. the K_i values in Table 3) of the Lineweaver-Burk plots with inhibitor concentration experimentally depends on the nature of the substrate. For any simple scheme, such as scheme (5), the expression for these slopes should include only inhibitor terms involving dissociation constants of the inhibitor from free enzyme. Additional nonproductive enzyme-substrate complexes, involving subsites 3 to *n* but not subsite 2, probably occur, but at a much smaller concentration than that utilizing subsite 2, because A_2 is about three times bigger than the sum of A_3 to A_7 (Table 2). But consideration of such complexes does not alter the expected inhibition pattern: additional terms appear in the intercept of the Lineweaver-Burk plot, but the slope is not changed. If there are two subsites 2, one for 1,4- and another (possibly overlapping) for 1,6-glucosidically linked substrates, they may bind inhibitor with different dissociation constants, to form two species of *EI*, which, respectively, prevent binding of 1,4- or 1,6-linked substrate. Then there would be two K_q 's in the reaction scheme, and this

would explain the observed variation of K_i with substrate. Clearly, the same effect could be obtained with a common subsite 2 for 1,4- and 1,6-linked substrates but distinct subsites 1, which bind *I* to form two different *EI* species.

For both glucoamylases P and S, K'_i [$=K'_s K'_p$ in scheme (5)] was over 100-fold bigger for isomaltohexaose than maltohexaose, so that either nonproductive substrate binding (K'_s) or binding of gluconolactone to the nonproductive complex (K'_p) is much less favourable for isomaltohexaose than for maltohexaose. Possibly, there is steric interference between gluconolactone bound at subsite 1 and the terminal glucose of isomaltohexaose bound at subsite 2 in the nonproductive mode.

Meagher *et al.* (1989) and Sierks *et al.* (1990) have suggested that subsite 2 of glucoamylase from *A. awamori* binds isomaltose in a different way than maltose. This may also be the case with glucoamylases P and S from *H. resinae*. The free energy of binding of maltooligosaccharides at subsite 2 of glucoamylases is between -19 and -22 kJ mol $^{-1}$. The results from *A. niger* (Meagher *et al.*, 1989) and the results shown in this paper indicate that the free energy of binding of isomaltooligosaccharides to subsite 2 of these enzymes is between -14 and -15 kJ mol $^{-1}$.

This difference may be due to a difference of one or more hydrogen bonds (corresponding to 2 to 6 kJ mol $^{-1}$ each) and/or a difference in the type of hydrogen bonds (e.g. between two charged groups, 14 to 18 kJ mol $^{-1}$) as outlined by Fersht *et al.* (1985). Thus, it seems justified to assume that the two differently linked oligosaccharides bind differently to the active site of glucoamylase.

Tanaka *et al.* (1982, 1983b) have suggested that the observed biphasic binding of substrate to the glucoamylase of *R. niveus* represents a fast bimolecular binding of a glucose moiety to subsite 2 followed by a slow, unimolecular 'relocation' to subsite 1. However, an 'induced fit' mechanism seems equally likely, as shown in Fig. 3. Here, the fast step of productive binding is interaction of the penultimate glucose moiety (from the nonreducing end) with subsite 2 to form *ES*. The large negative value of A_2 makes it reasonable that subsite 2 will have the greatest on-velocity constant. The slow, unimolecular step would represent a conformational change in the enzyme (converting *ES* to *ES**) by which a tryptophan (presumably Trp-120 in *A. awamori*; Sierks *et al.*, 1989) and other residues fold around the nonreducing-end glucose to form a stable subsite 1 and change the fluorescence quenching of tryptophan. Hiromi *et al.* (1982) found that glucose and glucosides, binding to subsite 2, stabilize the gluconolactone-enzyme complex. This can be explained by a conformational change in the enzyme, with gluconolactone binding to a newly formed subsite 1 induced by the binding of glucose or glucosides

Fig. 3. Schematic representation of the proposed 'induced fit' model for binding of oligosaccharides to the subsites of glucoamylases. The numbers refer to the subsites starting from the nonreducing-end of the oligosaccharide chain: '1', unformed subsite 1; 1, newly formed subsite 1; *E*, free enzyme; *S*, free substrate; *ES*, loosely bound enzyme-substrate complex; *ES**, tightly bound productive enzyme-substrate complex; (Trp), unquenched fluorescence of tryptophan; [Trp], quenched fluorescence of tryptophan.

at subsite 2, to form a ternary gluconolactone-glucose (or glucoside)-enzyme complex. This would also explain the finding (Ono *et al.*, 1964), that the sum of the relative hydrolysis rates of different individual bonds in oligosaccharides is not equal to the overall relative activity. In other words, the environment of the bond affects the hydrolysis rate (Abdullah *et al.*, 1963). This environment could affect the formation of the 'induced fit' structure, as well as its flexibility. A thorough study of the inhibition of the hydrolysis of *p*-nitrophenyl α -D-glucoside, phenyl 6-*o*-acetyl- α -maltoside and maltose by different mono- and disaccharides was made by Hiromi *et al.* (1973b) with the glucoamylase from *R. delemar*. The type of inhibition was competitive for all analogues examined, indicating that they bind to subsite 2. None of them resembled gluconolactone. All disaccharides tested had D-glucose as one component, and showed a free energy of binding comparable to that of D-glucose, but much lower than that for maltose. The difference in free energy of binding of these analogues could be explained by the 'induced fit' model, with the formation of subsite 1 being affected by the nature of the non-D-glucose moiety.

It has been proposed that the fluorescence quenching of *Rhizopus* glucoamylase upon binding of glucono-

lactone and other substrate analogues may result from a single tryptophan residue that is located near subsite 1 of glucoamylases (Ohnishi *et al.*, 1975; Ohnishi & Hiromi, 1976; Hiromi *et al.*, 1982). Sierks *et al.* (1989) found that site-directed mutagenesis of Trp-120 to tyrosine in *A. awamori* glucoamylase decreased the K_m for isomaltose 11-fold and changed the free energy of binding of maltooligosaccharides at subsite 1 from +2.6 to -2.0 kJ mol⁻¹. They propose that Trp-120 is located near subsite 4, and that it influences binding of D-glucosyl residues at subsites 1 and 2. Asp-176 has been shown to be located at subsite 1 and to influence the binding energies of maltooligosaccharides at subsite 4 and 5 (Sierks *et al.*, 1990). These results suggest that Trp-120 and Asp-176 are responsible for a stabilization of the transition state.

In conclusion, although the physical interpretation of the subsite maps of glucoamylases P and S (and those of other enzymes) depends upon the assumptions made (see Introduction), it seems to be characteristic of enzymes with a high 1,6/1,4- activity ratio that they have a large, negative value for A_1 with maltooligosaccharides. Gluconolactone binds both enzymes as an inhibitor at subsite 1, but binding to other subsites cannot be excluded. Further, there seem to be distinct gluconolactone binding sites that interfere, respectively, with maltohexaose and isomaltohexaose; i.e. there seem to be distinct subsites 1 (and/or subsites 2) for 1,4- and 1,6-linked substrates. Finally, the simple subsite concept for glucoamylases is probably complicated by a stabilization of subsite 1 by occupancy of subsite 2.

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