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Binding of isomaltose and maltose to the glucoamylase from *Aspergillus niger*, as studied by fluorescence spectrophotometry and steady-state kinetics*

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ABSTRACT

The binding of maltose, isomaltose, and D-glucono-1,5-lactone to the glucoamylase [E.C.3.2.1.3] from *Aspergillus niger* was monitored by the fluorescence-intensity change ($\triangle F$) based on the tryptophan residues of the enzyme, and the binding parameters (K_d and $\triangle F_{max}$) were evaluated from the dependence of $\triangle$F on the concentration of substrate and analogue. Maltose caused the fluorescence-intensity change, but isomaltose did not, although it is hydrolyzed by the enzyme. Both substrates bind to the glucoamylase of *Rhizopus niveus* and cause $\triangle F$, suggesting that some difference exists in the conformation of the isomaltose-binding subsites between the two glucoamylases.

INTRODUCTION

With the glucoamylase from *Rhizopus niveus*, we have studied statically and kinetically the interaction of malto-oligosaccharide substrates and analogues, to investigate the enzyme mechanism and the subsite structure at which the ligands are bound. The results indicate that linear malto-oligosaccharides and D-glucono-1,5-lactone are bound to subsite(s), including subsite 1, which is occupied by the non-reducing-end glucose residue of the substrate. There is a two-step mechanism in which a fast bimolecular association-process is followed by a slow, unimolecular isomerization[1–9]. One tryptophan residue out of the two at the active site appears to be located at subsite 1 and is involved in the observed changes in the u.v.-difference absorption and fluorescence intensities, along with "productive" binding of substrate and analogues[1–12].

Interaction of isomaltose with *Rhizopus* glucoamylase has been studied by steady-state kinetics[13–15] and by fluorescence spectrophotometry[16]. It is concluded that isomaltose is mainly bound "nonproductively", where subsites 2 and 3 are occupied, and the disaccharide is hydrolyzed very slowly. The second tryptophan residue may be located at subsite 3. The glucoamylase from *Aspergillus niger*, one of the most useful enzymes for the production of D-glucose, has been well studied as regards its primary structure,

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enzymic character, and practical usage[17-21]. However, the binding of saccharides to *Aspergillus* glucoamylase, has not been studied kinetically and statically, and hence its mechanism of action is not well understood, nor has the subsite structure of the enzyme yet been evaluated*.

Here, based on observations by steady-state kinetics and fluorescence spectrophotometry for *Aspergillus* glucoamylase, we have investigated the subsites at which the substrates maltose and isomaltose, and an analogue, D-glucono-1,5-lactone, are bound. Changes in fluorescence intensity, attributable to the tryptophan residue, were not observed in the binding of isomaltose, even though the steady-state kinetic parameters are nearly equal to those for the *Rhizopus* glucoamylase. These results suggest that the structure of one subsite of the *Aspergillus* glucoamylase, involved in the binding of isomaltose (maybe subsite 3) is different from that of the *Rhizopus* enzyme.

EXPERIMENTAL

Materials. — Glucoamylase from *A. niger*, a crystalline preparation, purchased from Fluka AG, Switzerland, was used without further purification. Enzyme concentrations were estimated spectrophotometrically, assuming an absorption unit $A^{1\%}_{280nm}$ of 14.2 cm^{-1} and a molecular weight of 77 000. Glucoamylase from *R. niveus*, which has $A^{1\%}_{280nm}$ of 16.3 and molecular weight of 58 000, was obtained from Toyobo Fine Chemicals Co., Tokyo, and was used without further purification. The purity of these enzyme preparations were confirmed by using a Pharmacia Phast System electrophoresis apparatus. Isomaltose, and maltose, and D-glucono-1,5-lactone of guaranteed grade were products of Makor Chemicals Ltd., Israel, and Kanto Chemicals Co. Tokyo, respectively. An enzymic reagent for determination of D-glucose was purchased from Toyobo Biochemicals Co., Tokyo.

Methods. — The *Aspergillus* glucoamylase-catalyzed hydrolysis of isomaltose and maltose as substrates was measured in 0.02M acetate buffer at pH 4.5 and 25.0 with enzymic analysis employing the "Gluco-stat" reagent (D-glucose oxidase and peroxidase are used for the determination of D-glucose). The time course of the enzyme-catalyzed reaction for each concentration of substrate was observed, and confirmed the linearity sufficiently for evaluation of the initial velocity. Fluorescence spectrophotometry of the binding of ligands to the enzyme was performed in 0.02M acetate buffer at pH 4.5 and 25.0 using a Hitachi 850 fluorescence spectrophotometer and the procedures described in previous papers[9,12,16]. Data treatment and analysis for evaluation of the steady-state kinetic and static (binding) parameters, and graphics for those data were performed with an Epson PC 286V personal computer and "Bio-graph" software (Ookubo Mycom Co., Tokyo), as described elsewhere[12,16].

* Recently studied by B. Svensson *et al.* 1990 (unpublished data).

RESULTS AND DISCUSSION

Steady-state kinetic observation of the enzyme-catalyzed hydrolysis of isomaltose and maltose. — The time course of hydrolysis was observed at various concentrations of isomaltose to evaluate kinetic parameters, the Michaelis constant (K_m), and the molar activity (k_0) of some typical examples are illustrated in Fig. 1, where the ordinate records absorbance at 550 nm (release of D-glucose measured by the "Gluco-stat" reaction). The experimental points were fitted to a linear plot, as shown in Fig. 1; the slope gives the initial velocity v. A typical example of the v *vs.* [S] plots is shown in Fig. 2, where [Isomaltose] denotes the initial concentration of isomaltose. The v *vs.* [S] plots give the kinetic parameters for hydrolysis of isomaltose, as summarized in Table I. The kinetic parameters, K_m and k_0 for maltose were also evaluated and are presented in Table I, along with reported values[13–15,22] for hydrolysis of isomaltose and maltose by *Rhizopus* glucoamylase: the values indicate that isomaltose is a substrate not only for the *Rhizopus* enzyme but also for the *Aspergillus* enzyme. The k_0/K_m ratio (1.6) of the *Aspergillus* enzyme for isomaltose is only 1/60 000 of that (2600) for maltose; however, is not much different from that (4.8 for isomaltose) of the *Rhizopus* enzyme. Based on the same discussion as presented in the previous paper[16], we may also conclude here that one possible cause for the difference in the hydrolytic rates between isomaltose and maltose may be attributed to the difference in their binding modes: the main mode of isomaltose binding is "nonproductive", in which subsite 1 is not occupied by the glucose residue of isomaltose, whereas maltose binds "productively", with subsite 1 occupied; the catalytic site is located between subsites 1 and 2. The binding over Subsites 1 and 2 is thus "productive" (see Refs. 10 and 11 for "productive" and "nonproductive"). Fluorescence spectrophotometry is also useful for providing evidence on the binding mode of, and the subsites bound by, these ligands, as described next.

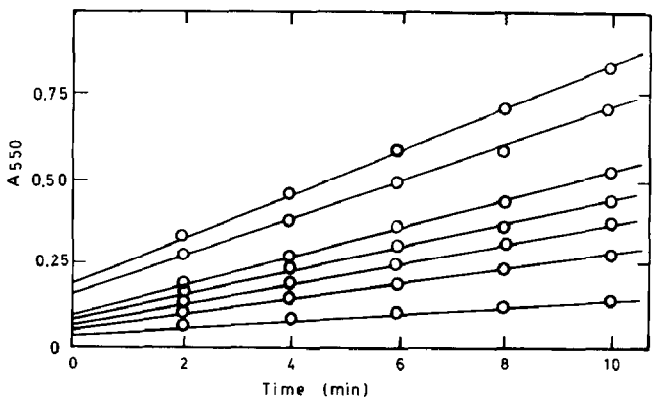

Fig. 1. Time courses of the *Aspergillus* glucoamylase-catalyzed hydrolysis of isomaltose. $[E]_0$; 3.3μM, $[S]_0$ (isomaltose); 3.2, 8.0, 12.0, 16.0, 24.0, 48.0, and 72.0mM (upper), 0.02M acetate buffer, pH 4.5, 25.0°. Straight lines are obtained using best-fit values, and initial velocities were evaluated from the slope of the line.

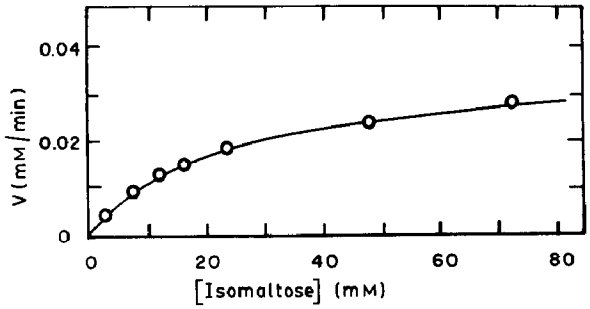

Fig. 2. Dependence of the initial rate v on the initial concentration $[S]_0$ of isomaltose. Experimental conditions are as described in Fig. 1. The solid line is the theoretical curve obtained using the kinetic parameters. [Isomaltose] denotes the initial concentration of isomaltose $[S]_0$.

Static observation of the binding of isomaltose and maltose to Aspergillus *glucoamylase by fluorescence spectrophotometry.* — A decrease in intensity of the fluorescence spectrum attributable to a tryptophan residue of the *Aspergillus* glucoamylase occurred upon binding of maltose. Figure 3 shows the change in fluorescence intensity ($\triangle F$) at 340 nm plotted against the initial concentration of maltose [Maltose]. The plots show a simple saturation curve in conformity with the Michealis equation, indicating the formation of a maltose–enzyme complex. On the basis of the $\triangle F$ *vs.* $[L]_0$ plot, the binding (static) parameters, the dissociation constant (K_d), and the maximum change in fluorescence intensity ($\triangle F_{max}$, the $\triangle F$ when the enzyme is saturated with ligand), may be evaluated, as described later. With *Rhizopus* glucoamylase, both maltose and isomaltose manifest the fluorescence change and give the binding parameters[16]. Such a change in fluorescence intensity was not observed, however, upon binding of isomaltose to *Aspergillus* glucoamylase. This is a major point of difference between the *Aspergillus* glucoamylase and that from *Rhizopus*.

Subsites occupied by maltose, isomaltose, and D-glucono-1,5-lactone were predicted on the basis of static observations by fluorescence spectrophotometry[7,8,12]. D-

TABLE I

Steady-state kinetic parameters for hydrolysis of isomaltose and maltose by the *Aspergillus* glucoamylase-catalyzed reaction[a]

Substrate	K_m(M)	k_0 (s^{-1})	k_0/K_m ($m^{-1}s^{-1}$)
Aspergillus glucoamylase			
Isomaltose	0.022 ±0.0017	0.035 ±0.0011	1.6
Maltose	0.00063 ±0.00003	1.65 ±0.53	2600
Rhizopus glucoamylase			
Isomaltose[b]	0.025	0.12	4.8
Maltose	0.0014 ±0.00005	2.83 ±0.57	2000
Maltose[c]	0.0012	3.01	2500

[a] In 0.02M acetate buffer, pH 4.5, 25.0°. The kinetic parameters are verages of 3–6 determinations. ± denotes standard deviation. [b] Ref. 15. [c] Ref. 22.

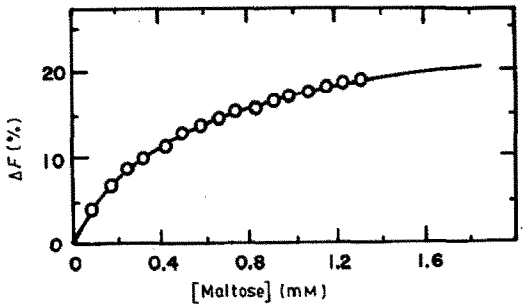

Fig. 3. Dependence of change in the fluorescence intensity of *Aspergillus* glucoamylase on the concentration of maltose. $[E]_0$; 0.58μM, λ_{EX}; 280 nm, 0.02M acetate buffer, pH 4.5, 25.0°. The solid line is the theoretical curve for the $\triangle F$ *vs.* $[L]_0$ plot obtained according to Eq. *2*. Data were analyzed by the procedures described in Fig. 1.

Glucono-1,5-lactone, considered to be a transition-state analogue, also causes a decrease in fluorescence intensity; a typical $\triangle F$ *vs.* $[L]_0$ plot is presented in Fig. 4, where the initial concentration of gluconolactone $[L]_0$ is denoted by [D-Glucono-1,5-lactone]. From these $\triangle F$ *vs.* $[L]_0$ plots and the following scheme, the static (binding) parameters K_d and $\triangle F_{max}$ were evaluated as follows:

$$E + L \underset{K_d}{\rightleftharpoons} (EL) \qquad (1),$$

where L is ligand and K_d is the dissociation constant ([E][L]/[(EL)]) of the ligand–enzyme complex (EL). The value is proportional to [(EL)]. These measurements of fluorescence intensity were performed under experimental conditions where $[L]_0 >> [E]_0$, and thus K_d is determined as follows:

$$\triangle F = \triangle F_{max}[L]_0/(K_d + [L]_0) \qquad (2)$$

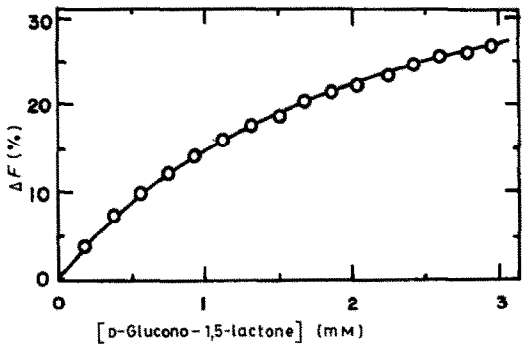

Fig. 4. Dependence of change in the fluorescence intensity of *Aspergillus* glucoamylase on the concentration of D-glucono-1,5-lactone. $[E]_0$; 0.58μM, λ_{EX}; 280 nm, 0.02M acetate buffer, pH 4.5, 25.0°. The solid line is the theoretical curve for the $\triangle F$ *vs.* $[L]_0$ plot obtained according to Eq. *2*. Data were analyzed as described in Fig. 1.

TABLE II

Static parameters for the interaction of isomaltose, maltose, and D-glucono-1,5-lactone with *Aspergillus* glucoamylase[a]

Ligand	K_d (mM)	$\triangle F_{max}$ (%)
Aspergillus glucoamylase		
Isomaltose	–	0
Maltose	0.53	25.5
	0.56	22.6
D-Glucono-1,5-lactone	2.1	47.7
Rhizopus glucoamylase		
Isomaltose	18	23
Maltose	1.3	29
D-Glucono-1,5-lactone	0.77	28

[a] In 0.02M acetate buffer, pH 4.5, 25.0°, $[E]_0$; 0.58μM, Ex λ; 280 nm. The measurements were repeated 6–9 times and average binding parameters are presented; deviation ~10%. [b] Reported in ref. 6.

where $[L]_0$ denotes the initial concentration of ligands such as isomaltose, maltose, and D-glucono-1,5-lactone. Based on the linear plot according to Eq. *2*, the K_d and $\triangle F_{max}$ values for those ligands were evaluated and are summarized in Table II. The binding parameters of the *Aspergillus* enzyme for maltose are almost equal those of *Rhizopus* enzyme, and these values for the *Aspergillus* enzyme with D-glucono-1,5-lactone are somewhat larger than those with the *Rhizopus* but the difference is not large. The results suggest that the subsites of the *Aspergillus* glucoamylase for binding of these ligands are identical with those of the *Rhizopus* glucoamylase; maltose over subsites 1 and 2, and D-glucono-1,5-lactone at subsite 1. Analysis of the binding type in the presence of another ligand, using fluorescence spectrophotometry, should be effective for confirming the subsites for these ligands; the binding of isomaltose does cause fluorescence changes, and hence the static parameters were not evaluated for the *Aspergillus* enzyme.

Binding of maltose and D-*glucono-1,5-lactone to* Aspergillus *glucoamylase in the presence of isomaltose.* — In the presence of isomaltose, the binding of maltose or D-glucono-1,5-lactone to the *Aspergillus* enzyme was observed by fluorescence spectrophotometry and the binding subsite of these ligands was predicted by the same procedure as already described[12–16]. The dependence of $\triangle F$ upon the concentration of maltose or D-glucono-1,5-lactone was examined at a fixed concentration of isomaltose, because pure isomaltose is scarce. D-Glucono-1,5-lactone has been regarded as a transition-state analogue for the glucoamylase reaction because of its half-chair conformation; it may be bound at subsite 1, by analogy with *Rhizopus* glucoamylase[7,12].

In this experiment, two binding modes, *competitive* and *noncompetitive*, are possible, as follows:

(*1*) *Competitive type.* When a ligand L (maltose or D-glucono-1,5-lactone) is assumed to compete for subsite 1 with another ligand S (isomaltose) without formation of the ligand–isomaltose–glucoamylase complex ESL, the change of fluorescence intensity ($\triangle F$) is represented as follows:

$$\triangle F = \frac{\triangle F^{L}_{max}[L]_0/K_L + \triangle F^{S}_{max}[S]_0/K_S}{1 + [L]_0/K_L + [S]_0/K_S} \quad (3),$$

where $[S]_0$ and $[L]_0$ are the initial concentrations of isomaltose and maltose or D-glucono-1,5-lactone, respectively, $\triangle F^{S}_{max}$ and $\triangle F^{L}_{max}$ are $\triangle F_{max}$ values for isomaltose and maltose or D-glucono-1,5-lactone, respectively, and K_S and K_L denote K_d for the isomaltose–enzyme and the maltose– or D-glucono-1,5-lactone–enzyme complexes, respectively. As the values of each term in the right-hand side of Eq. *3* are known (Table II), $\triangle F$ may be calculated theoretically, using K_m for isomaltose instead of K_S, because no change in fluorescence intensity of *Aspergillus* glucoamylase was observed upon binding of isomaltose, and therefore, K_S cannot be evaluated by fluorescence spectrophotometry. In this experiment $[S]_0$ was selected to be 18mM, which is almost equal to its K_m value (Table I).

(*2*) *Noncompetitive type*. When S (isomaltose) is bound to form the isomaltose–ligand–enzyme ternary complex ESL, $\triangle F$ is expressed as follows:

$$\triangle F = \frac{\triangle F^{L}_{max}[L]_0/K_L + \triangle F^{S}_{max}[S]_0/K_S + \triangle F^{SL}_{max}[S]_0[L]_0/K_{SK'L}}{1 + [L]_0/K_L + [S]_0/K_S + [L]_0[S]_0/K_S K'_L} \quad (4)$$

where K'_L is the dissociation constant (K_d) of L (maltose or D-glucono-1,5-lactone) from the ESL complex, and $\triangle F^{SL}_{max}$ is the $\triangle F_{max}$ value at which the enzyme is saturated with both L and S. The K'_L and $\triangle F^{SL}_{max}$ values are obtained by data-fitting with the least-squares method, and K'_S, which is the K_d value of isomaltose from the ESL complex, is given by $K'_S = K_S K'_L/K_L$.

A typical example of the $\triangle F$ *vs.* $[L]_0$ plots, where $[L]_0$ is the initial concentration of D-glucono-1,5-lactone, obtained in the presence of isomaltose (18mM), is illustrated in Fig. 5a, open circles; the initial concentration of D-glucono-1,5-lactone is denoted by [D-glucono-1,5-lactone]. The solid line (C) is a theoretical curve for competitive binding between isomaltose and D-glucono-1,5-lactone, obtained according to Eq. *3*. Curve C deviates distinctly from the experimental points, indicating that the competitive binding type is not valid for this case. Another curve (NC) is a theoretical one for noncompetitive binding obtained according to Eq. *4*, using the values presented in Table III; for example, for D-glucono-1,5-lactone $K'_L = 2.25$mM and $\triangle F^{SL}_{max} = 35.8\%$. The curve NC is in agreement with the experimental points for isomaltose and D-glucono-1,5-lactone, suggesting that isomaltose does not compete with D-glucono-1,5-lactone for a subsite (subsite 1).

The binding of maltose in the presence of isomaltose (18.0mM) is shown in Fig. 5b, where the initial concentration of maltose is denoted by [Maltose]. Obviously, the experimental points for the isomaltose–maltose example are much different from those for isomaltose-D-glucono-1,5-lactone: The experimental points are very close to curve C for the competitive type, but are much different from those for the noncompetitive type NC, indicating that there is considerable competition between maltose and isomaltose

TABLE III

Static parameters for the interaction of *Aspergillus* glucoamylase and ligands in the presence of isomaltose[a]

Ligand	K_d (mM)	$\triangle F_{max}$ (%)
Maltose	1.1	22.4
D-Glucono-1,5-lactone	2.3	35.8

[a] In the presence of isomaltose, [S] = 18.0 mM. Acetate buffer (0.02M), pH 4.5, 25.0°. The fluorescence spectrophotometric measurements were repeated 3–6 times and average values are presented, binding parameters thus include deviation of ~10%.

for a subsite (subsite 2). Thus it is reasonably concluded that maltose and isomaltose are mainly bound over subsites 1 and 2 and over subsites 2 and 3, respectively as illustrated in Fig. 6, and D-glucono-1,5-lactone is bound at subsite 1. The subsite structure of the *Aspergillus* glucoamylase has not yet been evaluated, but it may be analogous to that of the *Rhizopus* enzyme[10,11,22] as shown in Fig. 6. Based on these experimental results, we may suppose that a difference exists between the *Aspergillus* and *Rhizopus* enzymes in the conformation of one subsite, maybe subsite 3, which is occupied by the binding of

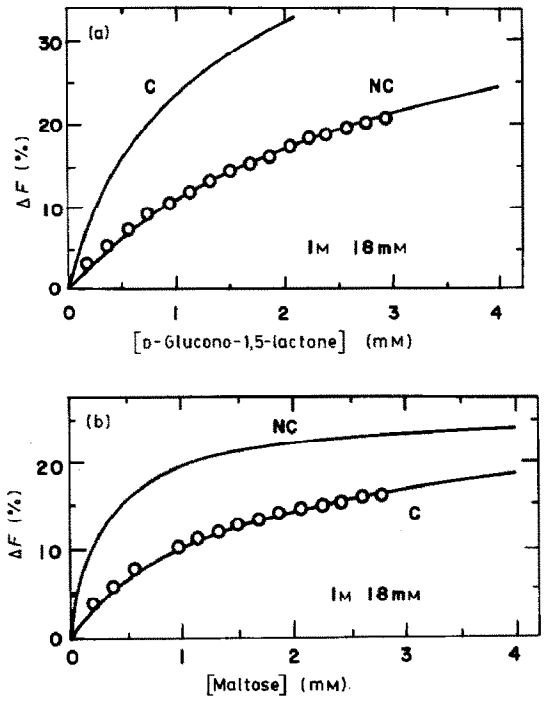

Fig. 5. Dependence of $\triangle F$ of *Aspergillus* glucoamylase on $[L]_0$ in the presence of isomaltose [S]. (*a*) Dependence of $\triangle F$ on D-glucono-1,5-lactone; (*b*) Dependencey of $\triangle F$ on maltose. In the presence of isomaltose at 18.0mM, [S] = 18.0mM. The solid lines C and NC are the theoretical curves obtained according to Eqs. *3* and *4*, respectively. C; competitive-type binding, NC; noncompetitive-type binding, 0.02M acetate buffer, pH 4.5 and 25.0°. Data were analyzed as described in Fig. 1.

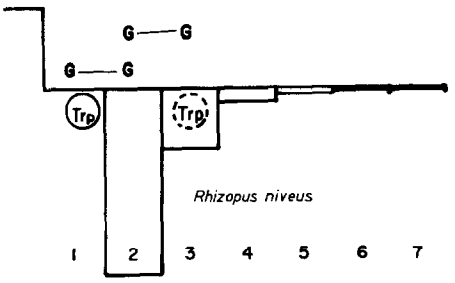

Fig. 6. Subsite structure of glucoamylase. The subsite structure of the *Aspergillus* glucoamylase was predicted by analogy with the *Rhizopus* glucoamylase subsite evaluated previously[22]. Subsites occupied by the binding of maltose and isomaltose are suggested as shown. One residue of tryptophan is proposed to be located at subsite 1. Another tryptophan residue may not be located at this subsite of the *Aspergillus* enzyme (dotted circle).

isomaltose; another tryptophan residue may not be located at the subsite of the *Aspergillus* enzyme (Fig. 6).

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