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Note

Characterization, by the binding of D-mannonolactone, of the subsites adjacent to the catalytic site of glucoamylase from *Rhizopus niveus**

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In lysozyme, the 1,5-lactone form of sugar acids, having a half-chair conformation is thought to be an analog of the transition state in the enzyme-catalyzed reaction, and to be bound at Subsite D, which is situated at the catalytic site[1–3] of the enzyme. Moreover, Hehre *et al.*[4–5] pointed out that D-glucal, an unusual saccharide, is a good analog of the intermediate, rather than being simply a substrate analog, in the enzyme catalyzed reaction of glycosidases. Thus, studies on the interaction of lactones with glucoamylase, one of the glycosidases, could be useful for investigation of the enzyme mechanism and characterization of the subsites of the enzyme.

D-Glucono-1,5-lactone (D-gluconolactone), which has a half-chair conformation[6], is a potent inhibitor of glucoamylase, and is considered to be bound at Subsite 1, probably being mimic of the substrate conformation at the catalytic site during the transition state[7,8]. On the other hand, when D-glucose and D-mannose were investigated for the inhibition of glucoamylase, it was concluded[7,9] that these saccharides are bound at Subsite 2. Such binding is expected to result in purely competitive inhibition for substrates, including amylose[7].

We now report studies on the interaction of glucoamylase with D-mannono-1,5-lactone (D-mannonolactone), which has a conformation similar to that of D-gluconolactone, and might be expected to be a potent inhibitor by binding at Subsite 1. However, from our results with inhibition steady-state kinetics, difference spectrophotometry, and the fluorescence method, we conclude that D-mannonolactone is not bound at Subsite 1, but may be bound at Subsite 2.

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EXPERIMENTAL

Glucoamylase. — The enzyme from *Rhizopus niveus* was a product of Seikagaku Kogyo Co., and was used without further purification. The concentration of the enzyme was determined spectrophotometrically, taking the absorption unit $A^{1\%}_{280nm}$ to be 16.3 cm^{-1}, and the molecular weight, 56,000.

Saccharides. — Amylose having d.p. (average) of 17, and D-mannonolactone were respectively purchased from Hayashibara Biochemical Laboratories, Inc., and P-L Biochemicals, Inc.

Methods. — Hydrolytic reactions for substrate amylose (d.p. 17) catalyzed by glucoamylase were performed at 25° in 0.02M acetate buffer, pH 4.5. The time course of reaction was monitored by determination of the reducing value. Reducing power was measured by the $K_3Fe(CN)_6$ method with a Technicon Autoanalyzer[10].

U.v.-difference absorption spectra were measured at pH 4.5 and 25° by using a Cary-15 spectrophotometer, as described[11,12]. Fluorescence spectra, with excitation at 280 nm, were measured under the same conditions by using a Jarrell Ash and a Union Giken FS-401 fluorescence spectrophotometer[13]. The D-mannonolactone solution was used within 15 min after dissolving, to minimize the effects of its hydrolysis.

RESULTS AND DISCUSSION

Inhibition kinetics. — It was previously reported that D-gluconolactone shows mixed-type inhibition for an amylose (d.p. 17) substrate, suggesting binding at Subsite 1 of the enzyme[7,8]. D-Gluconolactone, which has as half-chair conformation[6], is also thought to be a transition-state analog for glucoamylase, by analogies with the lysozyme-catalyzed reaction[2,3]. D-Mannonolactone has a conformation similar to that of D-gluconolactone, except for the configuration of C-2, and might be expected to bind at Subsite 1. However, the experimental results with the

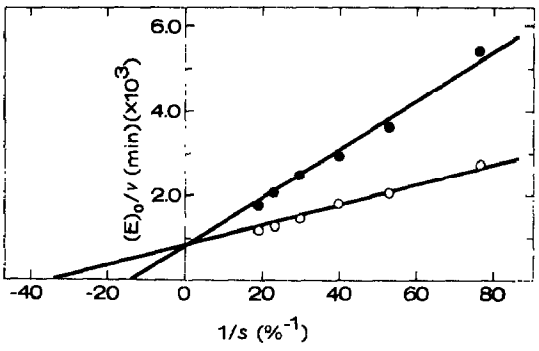

Fig. 1. Double reciprocal plot, $1/v$ *vs.* $1/s$ for amylose (d.p. 17)-glucoamylase system. Enzyme concentration, 46nM; open circles, absence of D-mannonolactone; closed circles, presence of D-mannonolactone (65.5mM); substrate, amylose (d.p. 17); pH 4.5; 25°C.

inhibition kinetics indicated that the noncompetitive terms, K_i' and K_q in Eqs. *12* and *13*, respectively, of ref. 7, are essentially zero (see Fig. 1), thus excluding an enzyme–D-mannonolactone–amylose complex (enzyme–inhibitor–substrate) formation in which the analog is bound at Subsite 1 according to derivation (see ref. 7). Thus, the experimental results clearly show that D-mannonolactone is a purely competitive inhibitor for amylose (d.p. 17) substrate, as shown in Fig. 1.

The inhibitor constant K_i for D-mannonolactone was evaluated to be 47mM, which is much different from that for D-gluconolactone (K_i = 1.5mM). D-Glucose and D-mannose cause purely competitive inhibition for *p*-nitrophenyl α-D-glucoside[9] as substrate, and are thought[7] to be bound at Subsite 2. The inhibitor constants of D-glucose and D-mannose are evaluated to be 0.1M and 0.16M, respectively, indicating that the K_i value for D-mannonolactone is rather similar to those for D-glucose and D-mannose. Therefore, based on the results with inhibition kinetics, we reasonably conclude that D-mannonolactone is not bound at Subsite 1 of the enzyme.

Difference spectrophotometry. — U.v.-difference absorption spectrum, which has peaks at 285 and 302 nm, was observed when D-mannonolactone was added to glucoamylase solution (data not shown here). The difference spectrum is similar to that produced by the D-glucose binding, and is quite different from that produced by the D-gluconolactone binding, the spectrum of which has a trough at 302 nm, instead of a peak (see ref. 12).

Intensity of the difference absorption at 302 nm per mole of the enzyme ($\Delta\epsilon_{302}$) was evaluated on the basis of the difference absorption spectrum, and was plotted against the concentration of D-mannolactone [M], as shown in Fig. 2. The plot gives a hyperbolic curve, thus suggesting the following reaction scheme:

$$E + M \underset{K_d}{\rightleftharpoons} EM, \qquad (1)$$

where EM is the D-mannonolactone–glucoamylase complex, and K_d is dissociation

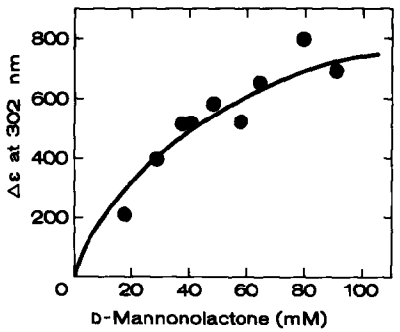

Fig. 2. Dependency of the molar difference absorption (Δε) upon the concentration of mannonolactone [M]. Enzyme concentration, 21μM; Δε evaluated at 302 nm of the difference absorption spectra produced by the D-mannonolactone binding; pH 4.5; 25°C.

constant of the EM complex. Under the conditions employed, the total concentration of the enzyme, $[E]_0$, is negligible in comparison with the concentration [M] of D-mannonolactone. Since $\Delta\epsilon$ is proportional to [EM], a linear relationship between $\Delta\epsilon$ and [M] can be obtained, as described[12]. Thus, the "binding parameters", $\Delta\epsilon_{max}$ (where all of the enzyme is saturated by the binding of D-mannonolactone) and K_d, can be evaluated from the linear plot of $1/\Delta\epsilon$ against 1/[M]. The K_d value for D-mannonolactone was found to be 45mM. This value is in good agreement with the K_i value already evaluated, 47mM, but is much different from K_d for D-gluconolactone (1.5mM).

Fluorescence spectrophotometry. — The fluorescence spectrum of the enzyme, which has a peak at ~340 nm, was also observed, and the difference intensity-change in fluorescence, ΔF, was measured by the procedures described for D-gluconolactone[13]. The plot of ΔF against [M] is a hyperbola, suggesting the same reaction scheme as Eq. *1*. Since ΔF is proportional to the D-mannonolactone–glucoamylase complex EM, the binding parameters, ΔF_{max} (where all of the enzyme is saturated by D-mannonolactone) and K_d, can also be evaluated from a linear plot of $1/\Delta F$ – 1/[M]. The K_d value was found to be 0.095M, which is much different from that for D-gluconolactone (K_d = 1.1mM). These K_d and K_i values are summarized in Table I.

The standard free-energy change, ΔG^0, for the formation of the D-mannonolactone–enzyme complex can be evaluated from K_i and K_d values, according to the following equations.

$$\Delta G^0 = -RT\ln(1/K_i) \quad (2)$$

$$= -RT\ln(1/K_d) \quad (3)$$

The results are summarized in Table I. ΔG^0 for the D-mannonolactone–glucoamylase complex-formation is much less than that for that of the maltose- or D-gluconolactone–glucoamylase complex ($\Delta G^0 \simeq 4$ kcal/mol), and is rather similar

TABLE I

DISSOCIATION OR INHIBITOR CONSTANT AND STANDARD FREE-ENERGY CHANGE FOR THE D-MANNONOLACTONE–GLUCOAMYLASE COMPLEX FORMATION[a]

K_d or K_i (mM)[b]	$-\Delta G^0$ (kcal/mol)[c]	Method[d]
47 ±6.3	1.8	kinetics
45 ±16	1.9	d. s.
95 ±16	1.4	fl. s.

[a]0.02M Acetate buffer, pH 4.5, 25°C. The Michaelis constant K_m for substrate amylose (d.p. 17) was evaluated to be 0.023 ±0.0086% (s.d.). [b]K_d, dissociation constant; K_i, inhibitor constant. [c]$-\Delta G^0$, standard free-energy change. [d]Kinetics, the inhibition steady-state kinetic method; d. s., difference absorption spectrophotometry; fl. s., fluorescence spectrophotometry.

to ΔG^0 for the D-glucose- or D-mannose-glucoamylase complex formation[9] ($\Delta G^0 \simeq$ 1–2 kcal/mol).

Binding subsite for D-*mannonolactone.* — Taken together, all of our findings lead us to conclude that D-mannonolactone does not bind at Subsite 1. Based on steady-state kinetic studies, Hiromi *et al.*[14,15] concluded that the catalytic site of glucoamylase lies between Subsite 1 and Subsite 2. In previous studies[7–9], it was concluded that D-gluconolactone is bound at Subsite 1, whereas D-glucose and D-mannose are bound at Subsite 2, a conclusion that may prove to be an important point for characterization of the subsites adjoining the catalytic site (Subsite 1 and Subsite 2); that is, these conclusions mean that the configuration of C-2 of the lactone is crucial for binding Subsite 1, because the difference between D-gluconolactone and D-mannonolactone, and also between D-glucose and D-mannose, is only the position of the OH-2 group. Inasmuch as the lactone mimics the conformation of the putative transition-state, the failure of D-mannonolactone to bind at Subsite 1 carries a strong implication as regards the role of OH-2 in inducing a transition-state conformation. Previous studies using *O*-2-(hydroxyethyl)malto-oligosaccharides also support this suggestion; that is, (hydroxyethyl)ation at O-2 of the (nonreducing) D-glucosyl group blocks hydrolysis by glucoamylase, presumably because such substitution sterically interferes[16] with binding at Subsite 1.

All of the analogs, including D-glucose, D-mannose, D-galactose, and D-xylose result in purely competitive inhibition for substrate *p*-nitrophenyl α-D-glucopyranoside, and are consistent with their being bound[9] at Subsite 2. These findings suggest that the specificity for binding at Subsite 2 is not particularly exact; therefore, the conformation of this subsite is presumed to be certainly flexible. Subsite 1 may, however, not recognize any kind of exception to the structure for the D-glucose residue, and should be called a "recognizer"; thus specificity for the binding at this subsite may be extremely exact, and limited to just D-*glucose* residues.

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