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Article

Effect of polyethylene glycol on binding of a transition-state analogue glucono-1:5-lacton to glucoamylase from *Rhizopus niveus* *

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In the presence of polyethylene glycol (PEG), binding of a transition-state analogue, glucono-1:5-lactone (GLN) to glucoamylase from *Rhizopus niveus* was investigated by means of the transient-phase kinetics using a stopped-flow spectrophotometer in a 0.025 acetate buffer pH 4.5 at 5.0 °C. GLN was supposed to be bound by the two-step mechanism, a fast association process (first step, K_{-1}) followed with a slow isomerization process (second step, k_{+2} and k_{-2}). A transient-phase kinetic parameter, K_{-1} in the presence of high concentration PEG was found to be identical with that in the absence of PEG, suggesting that PEG does not effect on the association process of the ligand. Moreover the parameter of k_{-2} was increased in the high concentration of PEG, suggesting that EL complex was formed loosely.

Key words; *Rhizopus niveus*, glucoamylase, polyethylene glycol, glucono-1:5-lactone, transient-phase kinetics, viscosity.

Glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3, abbreviated to GA) catalyzes the release of β-D-glucose from the non-reducing ends of starch, amylose, amylopectin, and related malto-oligosaccharides [1]. Thus, GA is one of the most important enzymes for the production of glucose in the fields of processing industries for starch and food materials. Based on the steady-state kinetic observations with a series of malto-oligosaccharides Gn (degree of polymerization n=2-7) as substrates, the subsite

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theory has been proposed by Hiromi, who has adequately applied its idea to GA from *Rhizopus niveus* and analyzed the subsite structure in the enzyme active site at the first time in the world [2,3].

Binding reactions of the ligands (substrates, analogues, and inhibitors) to the enzyme active site are observed to investigate its mechanism using the kinetic and static methods [4-14]. Especially, the transient-phase kinetics using a stopped-flow apparatus is inevitable for evaluation of the elementary process in the binding reaction [4,7-13]. For study on the binding mechanism, glucono-1:5-lactone (gluconolactone, GLN) is one of the best ligands for enzyme mechanism, because GLN is thought to be an analogue of the transition-state, which is a decisive process in the enzyme-catalyzed reaction. Thus the transient-phase kinetics on the GLN binding is an interesting and important project of the studies on the GA-catalyzed reaction.

Enzyme-catalyzed reactions are usually carried out at the dilute solution, of which reaction vessel is under not viscous environment, at the most laboratories of industry and research institutes. However, in cytosol of biological cells and in a reaction vessel of the manufacture process for making food and industrial materials, almost all the reactions cannot proceed under the dilute environment [15]. Thus, it could be very interesting and important that enzyme-catalyzed reactions are carried out in the unusual conditions, for instance, in the presence of high concentration of reactants and reagents, and are investigated about the environmental factor such as viscosity. Here we were interested in polyethylene glycol (PEG) for providing of a viscous environment around the enzyme-catalyzed reaction. Thus, the transient-phase kinetics on the binding reaction of GLN to GA was tried to analyze its mechanism in the presence and absence (0～10%) of PEG using the stopped-flow apparatus. The steady-state kinetics on the GA-catalyzed reactions for maltose as a substrate in the presence and absence (0～20%) of PEG could be useful for investigation of its effect on the kinetic parameters, the Michaelis constant K_m and molar activity k_0.

In this paper we aimed to elucidate the effect of PEG on what process of the ligand binding to GA using the transient-phase kinetics.

EXPERIMENTAL

Materials

GA form *Rhizopus niveus*, pure grade, was purchased from Toyobo Co., Osaka, and used without further purification. Concentration of the enzyme preparation was determined spectrophotometrically with an absorption unit $AU^{1\%}_{280nm}$ of 16.3 cm^{-1} and the molecular weight of 58000 [11,13]. GLN of a product of Nacalai Tesque Inc., Kyoto, was used within 10 min after dissolution to minimize its hydrolysis into gluconic acid and conversion into gluconic

acid-1:4-lactone. The hydrolysis rate of GLN in the solution at 10 min was confirmed to be less than 2% of the initial concentration under the experimental conditions employed. PEG-6000, maltose, and other chemicals, guranteed grade products, were purchased from Wakenyaku Inc., Kyoto, and used without further purification.

Methods

Stopped-flow fluorescence spectrophotometry

Binding reactions were observed using a Photal RA-451 stopped-flow apparatus, which is a gas-pressure driven type with a 150W-D2 lamp, as the same procedures described elsewhere [7,9,11]. Change in the fluorescence intensity produced by the excitation at 280nm was followed through a Toshiba Kasei Kogyo cut-off filter (UV-31, 50% transmittance at 310 nm) from right angle to incident beam [7,9,11]. The dead-time of this apparatus was determined to be 1.0 msec under the 5 kg/cm^2 nitrogen gas pressure for driving using the method with ascorbic acid and 2,6-dichlorophenol-indophenol [16-18]. Reaction curves were usually accumulated 9 times by a Union Giken RA-450 data-averaging processor to improve the signal to noise ratio. A fixed concentration (6.1 μM) of glucoamylase (GA) was used for the binding reaction of GLN.

Concentration of GLN was examined finally 0 to 80 mM in an 0.025 M acetate buffer pH 4.5 at 5.0 ℃.

Steady-state kinetics for a maltose substrate

The GA-catalyzed hydrolysis of maltose as a substrate was observed in a 0.025 M acetate buffer pH 4.5 at 5.0 ℃ as the same procedures described elsewhere [14]. Amount of glucose released from maltose by the hydrolysis reaction was routinely measured using a commercially available glucose determination kit, Glucose C-II Test Wako. Based on the reaction time curves and the initial velocity v, the kinetic parameters, the Michaelis constant K_m and molar activity k_o were evaluated using an NEC PC-9801 personal computer and a Kyoto Soft Bio-graph software [10].

Measurement of viscosity.

Viscosity of the 0-10% PEG solutions made with an 0.025 M acetate buffer pH 4.5 was measured at 5 ℃ using a Toki Sangyo Co.

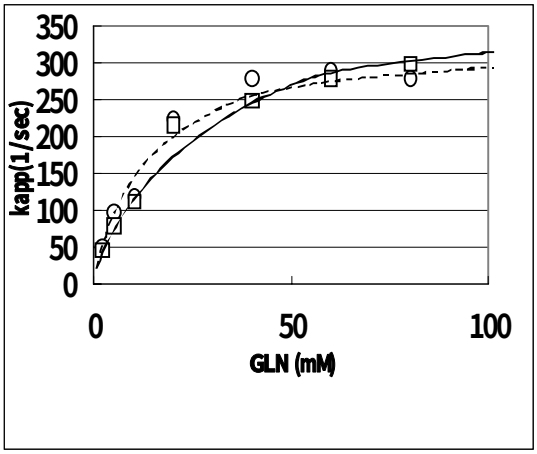

Fig.1. Dependence of the first-order rate constant k_{app} on the initial concentration of GLN, evaluated in 0% or 3% PEG-6000 solution. □; 0% PEG, ○; 3% PEG, 0.025M acetate buffer, pH 4.5, 5.0 ℃

viscometer model RE100L.

High performance liquid chromatography (HPLC)

A Shimadzu liquid chromatograph LC-4A and a Hitachi 833 data processor were used for the HPLC analysis with a Nacalai Tesque Cosmosil Packed Column 5C18-AR-II of 4.6 mm I.D. x 150 mm length. Detection was performed with a JASCO UVIDEC-100-IV UV spectrophotometer. A mixture of 2.5 mM ammonium dihydrogen phosphate and 20% acetonitrile was used for elution of the sample at flow rate of 1.0 ml/min, 40 °C. A precolumn labeling with 1-phenyl-3-methylpyrazolone (PMP) was prepared with the same procedures as described elsewhere [19].

RESULTS AND DISCUSSION

Transient-phase kinetics on the binding of gluconolactone

Binding reaction of glucono-1:5-lactone (gluconolactone, GLN) to gluco-amylase (GA) has been observed using a stopped-flow spectrophotometer, where only one relaxation is confirmed to obey first-order kinetics [8,11]. Here, using the same procedures, the binding reaction was observed in the presence of PEG to investigate its effect on the reaction of GLN with GA at pH 4.5 and 5.0 °C. The apparent first-order rate constant k_{app} was obtained for the binding reactions of GLN and dependence of k_{app} on the initial concentration of GLN was analyzed as shown in Fig 1, where the

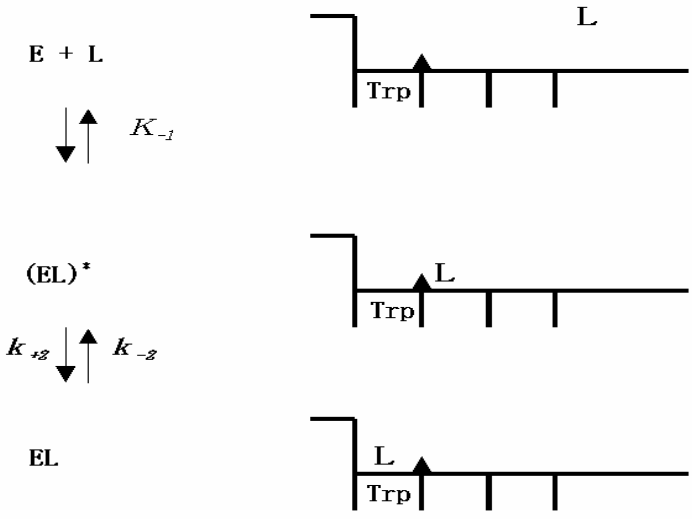

Fig.2. Two-step mechanism on binding a ligand to the subsite of GA.
L; ligand. The wedge indicates the catalytic site. A tryptophan residue is expected to locate at subsite 1.

concentration of gluconolactone is represented with GLN. Such a hyperbolic dependence of k_{app} on ligand ($[L]_o$) implies that the reaction obeys the two-step mechanism, which comprises of a fast bimolecular association process followed by a slow unimolecular isomerization process as follows,

$$E + L \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} (EL)^{*} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftarrows}} EL \qquad (1)$$

where EL is a tightly bound form of the GLN-GA complex and isomerized to $(EL)^{*}$, which is a less tightly bound form of the GLN-GA complex, and k_{+1}, k_{-1}, k_{+2} and k_{-2} are the rate constants of each elementary process as shown in Eq. (1). For this mechanism, two relaxation times (τ) can be most expected, then the reciprocal of slower relaxation time ($1/\tau$) is given under the condition $[L]_o >> [E]_o$ by the following equation,

$$1/\tau = k_{app} = k_{+2}[L]_o/(K_{-1} + [L]_o) + k_{-2} \qquad (2),$$

where K_{-1} is the dissociation constant of $(EL)^{*}$ defined as

$$K_{-1} = [E][L]/[(EL)^{*}] = k_{-1}/k_{+1} \qquad (3).$$

The solid and dotted lines in Fig. 1 are theoretical curves, which were drawn according

Table 1. Effects of PEG on the transient-phase kinetic and static parameters K_{-1},k_{+2},k_{-2}, and K_d for the GA-GLN binding reaction

PEG-6000	0%	1.5%	3.0%	5.0%	10.0%
K_{-1}(mM)	20	25	19	21	22
k_{+2}(sec^{-1})	380	430	340	370	300
k_{-2}(sec^{-1})	20	20	22	29	28
K_d (mM)	1.0	1.1	1.2	1.5	1.8

Table 2. Effects of PEG on the steady-state kinetic parameters K_m and k_o of the GA-catalyzed hydrolysis for maltose (pH4.5, 5.0℃)

PEG-6000 (%)	K_m (mM)	k_o (min^{-1})	k_o/K_m ($min^{-1}mM^{-1}$)
0	0.92	15	17
1.5	0.90	18	21
3	1.09	19	19
5	1.02	16	16
10	0.81	14	17
20	0.98	11	11

to Eq. (2) with the values thus obtained, indicating that these lines are fairly well covered the experimental results.

In the two-step mechanism, the overall dissociation constant of the EL complex K_d is represented as

$$K_d = [E][L]/([(EL)^*]+[EL])$$
$$= K_{-1}/[1+(k_{+2}/k_{-2})] \quad (4).$$

Based on the subsite structure of GA, the most possible mechanism of the GLN binding was illustrated in Fig. 2, where Trp residue(s) could be located at subsite 1 [5,9,10,13]. That is to say, GLN transiently attaches to subsite 2 and immediately relocates to subsite 1 accompany by change in the fluorescence intensity of the Trp residue.

The kinetic parameters, K_{-1}, k_{+2}, k_{-2}, and K_d for the binding reaction were evaluated in the presence and absence of PEG as the results are summarized in Table 1. The dissociation constant of the $(EL)^*$ complex, K_{-1} is almost identical and is not dependent on PEG in the presence of PEG, whereas k_{-2} and K_d are increased in high concentration of PEG. Thus, it will be concluded that PEG brings little effect on the first bimolecular association process but in the high concentration of PEG, EL complex is loosely formed.

Steady state kinetics on the GA-catalyzed reactions for maltose

Reaction time course of the GA-catalyzed hydrolysis for a substrate maltose was observed in the presence and absence of PEG at pH 4.5 and 5.0 ℃, then the initial velocity v was obtained on the basis of these reaction time-curves. Dependency of v on concentration of the substrate maltose s (the s-v plot) gives the steady-state kinetic parameters, the Michaelis constant K_m and molar activity k_o, by according to the Michaelis-Menten kinetics and the non-liner least-squares method [11]. The experimental results are summarized in Table 2, indicating that the kinetic parameters in the presence of PEG (1.5 - 10%) are almost identical with those in the absence of PEG, especially the k_o/K_m value is quite identical. Based on the subsite theory, k_o/K_m refers to the productive binding of substrates [2,14]. Thus, these kinetic results suggest that PEG (1.5 - 10%) does not effect on the productive binding of substrates but PEG (20%) effects on. It is agreement with the result showing in Table 1, of which k_{-2} and K_d are increased in high concentration of PEG.

Viscosity of the polyethylene glycol solutions

In the biological cells, especially in cytosol viscosity is one of the most important factors, which affect on the various biological functions, and the degree of viscosity (usually 3～5 cP in cytosol) is higher than that in water (1.5 cP at 5.0 ℃) [15]. Viscosity of the PEG solutions was determined at 5.0 ℃ as the results are illustrated in Fig. 3, clearly indicating that a 5% PEG solution gives around 8cP under the experimental conditions employed. In the relation with viscosity, we may conclude that the binding mechanism of ligands found in the presence of PEG (1.5-10%) is almost the same

with that in cytosol. Viscosity effects not so much on the association process of the substrate binding under the experimental conditions emplyed.

HPLC analysis of the GA-catalyzed reaction

Many carbohydrolases catalyze transglucosylation in addition to the hydrolysis. Here high concentration of PEG was employed for the experiments, therefore the GA-catalyzed reaction has to be confirmed for transglucosylation.

Because PEG has many OH groups in its molecule, thus it is very possible that OH becomes an acceptor instead of water (hydrolysis) for the enzyme-catalyzed reaction. The HPLC procedures were employed to observe the existence of transglucosylation reaction. A typical example of the results is illustrated in Fig. 4, where a substrate maltose was examined in the presence of 10% PEG at pH 4.5 and 5.0 ℃. The substrate maltose and

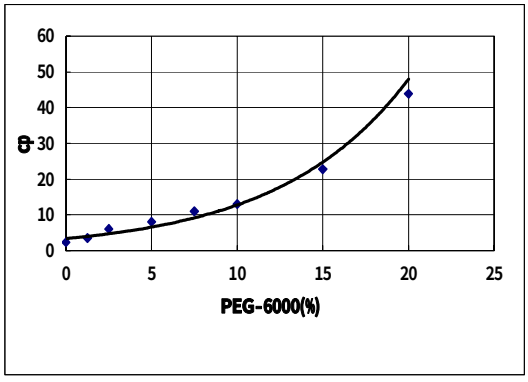

Fig.3. The viscosity of PEG-6000 solutions.

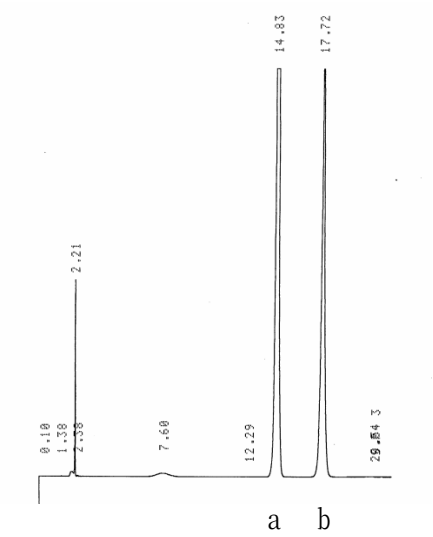

Fig.4. HPLC of the reaction mixture. [E]o; 3 μM, [S]o; 50 mM, pH 4.5, 5.0 ℃ and 60 min in the presence of 10% PEG. Elutant, 20v/v% CH_3CN in 2.5 mM ammonium dihydrogenphosphate,.Flow rate, 1 ml/min. Saccharides: a; maltose, b; glucose

a product glucose can be clearly seen, however, other products cannot be confirmed in the HPLC chromatogram, indicating that there is not a transfer product in the GA-catalyzed reaction mixture. Thus, it can be recognized that no transglucosylation was occurred at the GA-catalyzed reaction in the presence of PEG.

In this study, PEG was used for a viscous system of the enzyme-catalyzed reaction and it was shown that viscosity effected not so much on the association process of the substrate binding in the presence of PEG 1.5 - 10%, of which viscosity is almost the same with that in cytosol. However, in cytosol, many factors could be concerned to the enzyme-catalyzed reactions. Thus, It should be very important to

investigate the enzyme-catalyzed reactions in unusual conditions such as high viscosity.

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