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Activation of 4-α-Glucanotransferase Activity of Porcine Liver Glycogen Debranching Enzyme with Cyclodextrins

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Glycogen debranching enzyme (GDE) is a single polypeptide chain containing distinct active sites for 4-α-glucanotransferase and amylo-α-1,6-glucosidase activities. Debranching of phosphorylase limit dextrin from glycogen is carried out by cooperation of the two activities. We examined the effects of cyclodextrins (CDs) on debranching activity of porcine liver GDE using a fluorogenic branched dextrin, Glcα1-4Glcα1-4Glcα1-4(Glcα1-4Glcα1-4Glcα1-4Glcα1-6)Glcα1-4Glcα1-4Glcα1-4Glcα1-4GlcPA (B5/84), as a substrate. B5/84 was hydrolyzed by the hydrolytic action of 4-α-glucanotransferase to B5/81 and maltotriose. The fluorogenic product was further hydrolyzed by the amylo-α-1,6-glucosidase activity to the debranched product, Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4GlcPA (G8PA), and glucose. α-, β- and γ-CDs accelerated the liberation of B5/81 from B5/84, indicating that the 4-α-glucanotransferase activity was activated by CDs to remove the maltotriosyl residue from the maltotetraosyl branch. This led to acceleration of B5/84 debranching. The extent of 4-α-glucanotransferase activation increased with CD concentration before reaching a constant value. This suggests that there is an activator binding site and that the binding of CDs stimulates 4-α-glucanotransferase activity. In the porcine liver, glycogen degradation may be partially stimulated by the binding of a glycogen branch to this activator binding site.

Key words: activation of enzyme, branched dextrin, 4-α-glucanotransferase, glycogen debranching enzyme, HPLC.

Abbreviations: GDE, glycogen debranching enzyme; CD, cyclodextrin; Glc, D-glucose; GlcPA, 1-deoxy-1-[(2-pyridyl)amino]-D-glucitol; B5/81, Glcα1-4Glcα1-4Glcα1-4(Glcα1-6)Glcα1-4Glcα1-4Glcα1-4Glcα1-4GlcPA; B5/84, Glcα1-4Glcα1-4Glcα1-4(Glcα1-4Glcα1-4Glcα1-4Glcα1-6)Glcα1-4Glcα1-4Glcα1-4Glcα1-4GlcPA; G8PA, Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4GlcPA; EDTA, ethylenediaminetetraacetate.

Glycogen debranching enzymes (GDEs) are known to exist in mammalian muscle and liver, and they degrade glycogen together with glycogen phosphorylase. The amino acid sequences of GDEs from several species have been deduced based on their nucleotide sequences (*1–3*). GDEs have distinct active sites for 4-α-glucanotransferase activity [1,4-α-glucan:1,4-α-glucan 4-α-glycosyltransferase, EC 2.4.1.25] and amylo-α-1,6-glucosidase [dextrin 6-α-glucosidase, EC 3.2.1.33] activity on a single polypeptide chain (*4–10*).

Glycogen phosphorylase removes one glucose unit at a time from the non-reducing-end of glycogen, producing glucose-1-phosphate. This sequential phosphorolysis ceases near a branch point. GDE removes the maltooligosaccharide branch from the phosphorylase limit dextrin, restarting degradation by glycogen phosphorylase. The 4-α-glucanotransferase of GDE transfers the non-reducing-end maltooligosaccharide residue from the branch to the non-reducing-end of the other branch, exposing a 6-*O*-α-glucosyl residue, and then the amylo-α-1,6-glucosidase hydrolyzes the α-1,6-glycosidic linkage (*11–15*).

In the cell, GDE is located near glycogen, as it is associated with glycogen metabolism. It is no surprise that GDE interacts with these substances at a site distinct from the active site(s), and that this interaction affects the active site(s). The binding of various α-glucans to GDE was investigated by analyzing their inhibitory activities with regard to the interaction between GDE and glycogen or phosphorylase limit dextrin (*8*, *16*, *17*). Glycogen and phosphorylase limit dextrin are very large molecules of indefinite structure. They function as donor and acceptor substrates for 4-α-glucanotransferase, and substrate for amylo-α-1,6-glucosidase. Part of the molecule is bound to the active site, while a much larger part remains outside of it, and some other part may interact with a remote region of the enzyme, a binding site for α-glucans. Therefore, it is insufficient to evaluate this interaction based on inhibition of GDE activity. To ensure reliable results, smaller substrates having a defined structure must be used for inhibition experiments.

Recently, we proposed a fluorogenic branched dextrin, Glcα1-4Glcα1-4Glcα1-4(Glcα1-4Glcα1-4Glcα1-4Glcα1-6)Glcα1-4Glcα1-4Glcα1-4Glcα1-4GlcPA (B5/84), as substrate for assaying debranching activity (*18*). With the substrate, 4-α-glucanotransferase activity can be measured precisely by quantifying the fluorogenic products by HPLC.

This paper describes the activation of the 4-α-glucanotransferase action on B5/84 by α-, β- or γ-cyclodextrin (CD) and discusses aspects of an activator binding site distinct from the active site(s) of the enzyme.

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MATERIALS AND METHODS

Materials—Porcine liver was purchased from Matsubara Meat Plant (Matsubara, Japan). Toyopearl HW 40F, DEAE-Toyopearl 650M and Super Q column were from Tosoh (Tokyo, Japan), Sephacryl S-300 was from Amersham Pharmacia Biotech (Uppsala, Sweden). α-, β-, and γ-CDs and Wakosil-II 5C18 HG (6 × 150 mm) column were from Wako Pure Chemicals (Osaka, Japan). Fluorogenic dextrins, B5/84 and Glcα1-4Glcα1-4Glcα1-4(Glcα1-6)-Glcα1-4Glcα1-4Glcα1-4Glcα1-4GlcPA (B5/81), were prepared as described previously (*18*). Fluorogenic compounds were determined by measuring the absorbance at 310 nm in 0.1 M acetic acid, with the pyridylamino group at 0.17 mM giving an absorbance of 1.0 in a 1-cm light path (*19*).

Amylo-α-1,6-Glucosidase Assay—GDE amylo-α-1,6-glucosidase activity was measured using B5/81 as a substrate as reported previously (*18*). A mixture (120 μl) containing 10 μM B5/81, 50 mM maleic acid–NaOH buffer (pH 6.0), 0.05% gelatin, 5 mM EDTA, 10 mM β-mercaptoethanol and the enzyme preparation was incubated at 37°C for an appropriate period. The enzymatic reaction was stopped by adding 75 μl of 1 M acetic acid and heating at 100°C for 3 min. The fluorogenic product in the digest, Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4GlcPA (G8PA), was measured by HPLC as follows. A Wakosil-II 5C18 HG column (6.0 × 150 mm) was eluted with 50 mM ammonium acetate buffer, pH 4.3, containing 0.15% 1-butanol at a flow rate of 1.5 ml/min. The elution was monitored by observing the fluorescence at 400 nm (excitation at 320 nm). One unit of amylo-α-1,6-glucosidase was defined as the amount of enzyme releasing 1 μmol of G8PA per min. under the conditions employed.

Protein Assay—Protein was assayed by measuring the absorbance at 280 nm using bovine serum albumin as a standard.

Purification of GDE from Porcine Liver—Porcine liver GDE was purified from porcine liver by measuring amylo-α-1,6-glucosidase activity in five steps, similarly to porcine brain enzyme (*20*).

Step 1. Preparation of crude enzyme solution: Porcine liver (100 g) was homogenized in 1,130 ml of 7 mM sodium phosphate buffer, pH 6.5, containing 10 mM β-mercaptoethanol using a Potter-Elvehjem homogenizer. After the homogenate was centrifuged at 12,500 × *g* for 25 min, the resulting supernatant was filtered using Hyflo Super-Cel. The filtrate was used as crude enzyme solution.

Step 2. Ammonium sulfate precipitation: To the crude enzyme solution, ammonium sulfate was added to give 15% saturation. The precipitate was removed by centrifugation, and ammonium sulfate was then added to the supernatant to give 45% saturation. The resultant precipitate was collected by centrifugation and dialyzed against 7 mM phosphate buffer, pH 6.5, containing 10 mM β-mercaptoethanol.

Step 3. DEAE-Toyopearl chromatography: The dialyzed solution (140 ml) was applied onto a DEAE-Toyopearl 650M column (2.2 × 80 cm) equilibrated with 7 mM phosphate buffer, pH 6.5, containing 10 mM β-mercaptoethanol, and the column was washed with 100 ml of the same buffer. GDE was eluted with a linear gradient of phosphate buffer from 7 mM to 0.3 M (600 ml). The pooled solution containing the amylo-α-1,6-glucosidase was concentrated by ultrafiltration using a Diaflo-membrane YM-30.

Step 4. Sephacryl S-300 gel filtration: The solution obtained from Step 3 was applied to a Sephacryl S-300 column (3.0 × 130 cm) equilibrated with 20 mM phosphate buffer, pH 6.5, containing 5 mM EDTA and 10 mM β-mercaptoethanol, and was then eluted with the same buffer. The amylo-α-1,6-glucosidase fractions were pooled and concentrated to a small volume by ultrafiltration using a Diaflo-membrane YM-30 and a Vivapore 5 membrane.

Step 5. Super Q HPLC: Anion-exchange HPLC was performed on a Super Q column (7.5 × 75 mm). The column was equilibrated with 20 mM phosphate buffer, pH 7.5, containing 10 mM β-mercaptoethanol, at a flow rate of 1.0 ml/min. The solution obtained in Step 4 was injected into the column, which was then washed with 5 ml of the same buffer, and the concentration of phosphate buffer was linearly increased from 20 to 100 mM over 20 min, and was then maintained at 100 mM for 10 min. The amylo-α-1,6-glucosidase fractions were pooled and concentrated to a small volume using a Vivapore 5 membrane.

Polyacrylamide Gel Electrophoresis (PAGE)—PAGE was carried out according to the method of Williams and Reisfeld using a 5–20% gradient gel (*21*). Proteins were stained with a Bio-Rad silver staining kit.

Digestion of CDs with GDE and Product Analysis—α-, β- or γ-CD (5 μmol) was incubated with 1.4 units of porcine liver GDE at 37°C in 100 μl of 50 mM sodium maleate buffer (pH 6.0) containing 0.05% gelatin, 5 mM EDTA and 10 mM β-mercaptoethanol for 20 h. The reaction mixture was heated at 100°C for 5 min, and 0.02 mg of mannose was added to the mixture as an internal standard before lyophilization. Pyridylamination of the reducing-end glucosyl residues in the reaction mixture was carried out as reported previously (*22*). Pyridylaminated samples were analyzed by HPLC using PA-maltooligosaccharides as standard compounds as described above.

Digestion of B5/84 with GDE in the Presence of CDs and HPLC Analysis—A mixture of B5/84 (1.0 nmol) and α-, β- or γ-CD (5 μmol) was incubated with 1.4 units of porcine liver GDE at 37°C in 100 μl 50 mM sodium maleate buffer (pH 6.0) containing 0.05% gelatin, 5 mM EDTA and 10 mM β-mercaptoethanol for 20 min. Then, 200 μl of 0.2 M acetic acid was added to stop the enzymatic reaction before the solution was heated at 100°C for 5 min. Reaction mixtures were analyzed by HPLC as described above.

For examining the influence of the CD concentration on the rate of GDE action, enzymatic reactions were carried out at various CD concentrations.

RESULTS AND DISCUSSION

Purification of GDE from Porcine Liver—Porcine liver GDE was purified to homogeneity by ammonium sulfate precipitation, DEAE-Toyopearl chromatography, Sephacryl S-300 gel-filtration, and Super Q anion-exchange HPLC as described in "MATERIALS AND METHODS," by monitoring amylo-α-1,6-glucosidase activity (Figs. 1 and 2). The process of purification is summarized in Table 1.

Purification of GDE from porcine liver has not been reported to date. Some characterization was necessary to identify the purified enzyme as GDE. Its molecular weight was estimated to be 170,000 by gel-filtration,

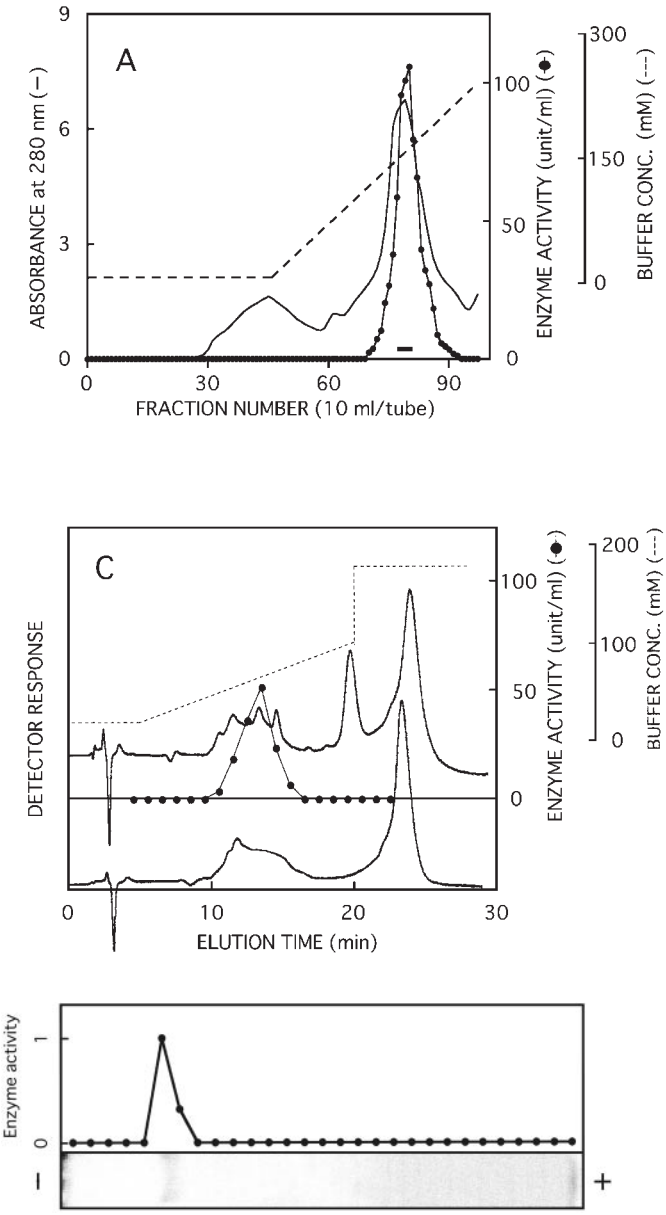

Fig. 1. **Purification of GDE from porcine liver.** Chromatography was carried out as described in "MATERIALS AND METHODS"; (—) protein; (. . .) amylo-α-1,6-glucosidase activity. Fractions indicated by bars were collected for further purification. A: DEAE-Toyopearl chromatography of the precipitate obtained following 15–45% saturation with ammonium sulfate. B: Sephacryl S-300 gel filtration of the amylo-α-1,6-glucosidase fraction obtained by DEAE-Toyopearl chromatography. C: Super Q HPLC of the amylo-α-1,6-glucosidase fraction obtained by Sephacryl S-300 gel filtration. Protein profile in the bottom was the control with the starting buffer.

Fig. 2. **Polyacrylamide gel electrophoresis of purified enzyme.** Purified enzyme was applied to two lanes, which were separated after electrophoresis. One lane was stained with a silver stain kit, and the other was sliced into 2.0-mm sections, from which the amylo-α-1,6-glucosidase activity was extracted with 200 μl of 50 mM sodium maleate buffer (pH 6.0) containing 0.05% gelatin, 5 mM EDTA and 10 mM β-mercaptoethanol at 37°C for 1 h. Activity was measured as described in "MATERIALS AND METHODS." A: Stained with silver stain kit. B: amylo-α-1,6-glucosidase activity. The value of the section with the highest activity was taken as unity.

which is similar to those of human muscle enzyme (172,614) and rabbit muscle enzyme (177,542), calculated from cDNA sequences (*1*, *2*). Large molecular weight is one of the characteristics of GDE.

GDE should exert debranching activity for branched dextrins through the cooperation of 4-α-glucanotransferase and amylo-α-1,6-glucosidase activities. A fluorogenic branched dextrin, B5/84, was found to be a suitable substrate for assaying the debranching activity of GDE by using crude enzyme preparations of porcine muscle GDE (*18*).

B5/84 was incubated with purified enzyme, and the reaction mixture at early stages of the enzyme action was analyzed by HPLC, as described in "MATERIALS AND METHODS." As shown in Fig. 3A, two fluorogenic products were formed by the enzyme, and their structures were confirmed to be B5/81 and G8PA, as reported previously (*18*). The maltotriosyl residue of the maltotetraosyl branch was removed by the hydrolytic action of the 4-α-glucanotransferase, producing B5/81. Some of the fluorogenic product was immediately hydrolyzed by the amylo-α-1,6-glucosidase to G8PA and glucose. Debranching of B5/84 indicates that the purified enzyme was a GDE.

The removed maltotriosyl residue is thought to be transferred to the non-reducing-end glucosyl residue of the main chain, to the non-reducing-end residues of another B5/84 molecule, or to water. However, the expected transfer products could not be detected in the enzymatic reaction mixture under the expected elution conditions (with buffer solution containing 1% 1-butanol, data not shown). Thus, the maltotriosyl residue was transferred to water; hydrolysis occurred.

Acceleration of 4-α-Glucanotransferase Action by CDs—α-Glucans, which do not function as GDE substrates, are considered to be effective probes to identify binding

Table 1. **Summary of GDE purification from porcine liver.**

Purification step	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (-fold)
Crude enzyme	10,800	8,870	0.821	100	1.0
$(NH_4)_2SO_4$ precipitation	4,480	5,830	1.30	66	1.6
DEAE-Toyopearl chromatography	320	4,080	12.8	46	15.6
Sephacryl S-300 gel filtration	0.63	245	389	2.8	476
Super Q HPLC	0.050	68.0	1,360	0.80	1,690

One unit of GDE was defined based on amylo-α-1,6-glucosidase activity, as described in "MATERIALS AND METHODS."

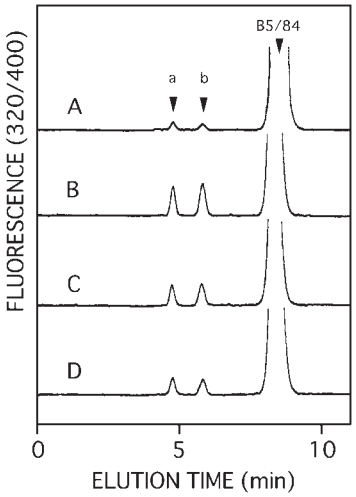

Fig. 3. **HPLC of B5/84 digest by porcine liver GDE without or with CDs.** Enzymatic reaction and HPLC of reaction mixture were carried out as described in "MATERIALS AND METHODS." Arrowheads indicate the elution positions of a, G8PA and b, B5/81. A: B5/84 digest without CDs; B: B5/84 digest with 5 mM α-CD; C: B5/84 digest with 5 mM β-CD; D: B5/84 digest with 5 mM γ-CD.

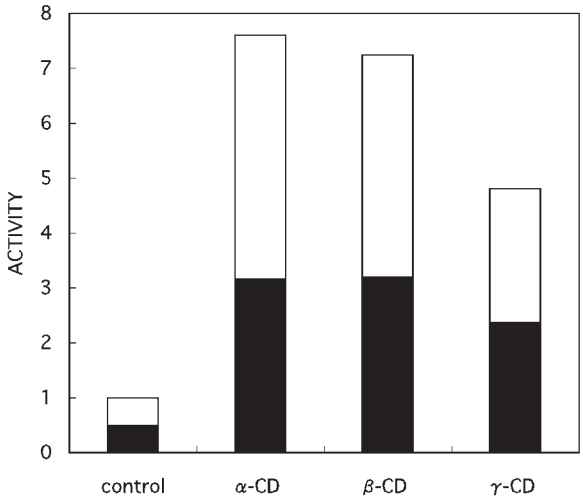

Fig. 4. **Comparison of debranching and 4-α-glucanotransferase activities for B5/84 with or without CDs.** Debranching activity was calculated based on the amount of G8PA liberated, and is represented by the black regions. The 4-α-glucanotransferase activity was calculated based on the total amounts of G8PA and B5/81, and represented by combined total of the black and white regions. The value of the 4-α-glucanotransferase activity without CD was taken as unity.

sites for α-glucans. α-, β- and γ-CDs are suitable, as their structures are defined, and they have no maltooligosaccharide branches, no α-1,6-linked glucosyl residues, and no non-reducing-end glucosyl residues that can accept maltooligosaccharide residues on transglycosylation by 4-α-glucanotransferase. In order to confirm that GDE does not act on CDs, α-, β-, or γ-CD was incubated with GDE, and product analysis of each reaction mixture by HPLC was carried out after reductive amination of reducing-end residues with 2-aminopyridine, as described in "MATERIALS AND METHODS." No pyridylaminated maltooligosaccharide was detected (data not shown), indicating that GDE does not act on CDs.

GDE action on B5/84 was examined in the presence of α-, β-, or γ-CD. Enzymatic reactions and HPLC of the reaction mixtures were carried out, as described in "MATERIALS AND METHODS." Figure 3, B–D, shows chromatograms of the reaction mixtures with 5 mM α-, β-, and γ-CD, respectively. B5/81 and G8PA were produced as in the absence of CDs; however, the rates of G8PA formation with α-, β- and γ-CD were 6.5-fold, 6.6-fold and 4.9-fold higher, respectively, than those without, indicating that debranching of B5/84 was accelerated by CDs. Debranching was carried out by cooperation of the two activities of GDE. The rate of 4-α-glucanotransferase action can be calculated from the total amounts of G8PA and B5/81 produced, as B5/81 was hydrolyzed by the amylo-α-1,6-glucosidase to G8PA (Fig. 4). The total amounts of G8PA and B5/81 with α-, β- and γ-CD were 7.6-fold, 7.3-fold and 4.8-fold larger, respectively, than those without. This indicates that CDs activate the 4-α-glucanotransferase. Activation of 4-α-glucanotransferase by CDs resulted in acceleration of debranching.

Liu *et al*. reported that the 4-α-glucanotransferase of rabbit muscle GDE was inhibited by CDs, when the activity was measured based on changes in the iodine-limit dextrin spectrum using phosphorylase limit dextrin from glycogen as the substrate (*17*). It is notable that CDs are activators of 4-α-glucanotransferase activity in the porcine liver enzyme, while they were inhibitors of the rabbit muscle enzyme.

Activator Binding Site—The relationship between the rate of 4-α-glucanotransferase action on B5/84 and CD concentration was examined. The rates increased with CD concentration, and reached constant values (Fig. 5). The results indicate that there is a binding site for CDs, an "activator binding site," within the enzyme, and that saturation of the activator binding site fully activates the enzyme. At high CD concentrations, the rates lower slightly. This phenomenon is similar to the substrate inhibition seen at high substrate concentrations.

The mode of activation of the 4-α-glucanotransferase action varied with the CDs. Activation by β-CD reached

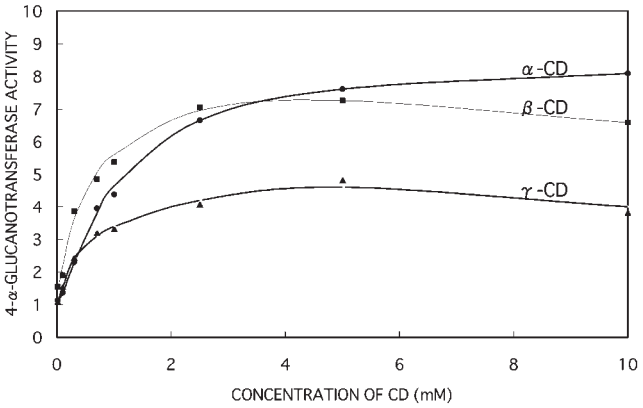

Fig. 5. **Relationship between the rate of 4-α-glucanotransferase action on B5/84 and CD concentration.** B5/84 was digested with GDE in the presence of various CD concentrations, and HPLC of the reaction mixtures was carried out as described for Fig. 3. The rate of the 4-α-glucanotransferase action was calculated as described for Fig. 4. The value of 4-α-glucanotransferase activity without CD was taken as unity.

the point of saturation at the lowest concentration, suggesting that its affinity for the activator binding site was strongest among the CDs tested. The extent of activation at the point of saturation is associated with activity of the activated form. The activity of the β-CD activated form was almost the same as that of the α-CD activated form, while the γ-CD activated form had lesser activity. Because the cyclic structure of CDs is rather rigid when compared with flexible linear α-glucans, which have both reducing-end and non-reducing-end residues, the three-dimensional features of the activator binding site may be estimated from the mode of activation. The activator binding site would be such a space that CDs or their partial structures can be accommodated and β-CD or its partial structure would fit more neatly than α- or γ-CD.

It is conceivable that the active site of 4-α-glucanotransferase is composed of the glycon binding site, the aglycon binding site, and the acceptor binding site. The aglycon binding site may be identical to the acceptor binding site. The maltotriosyl moiety of the B5/84 maltotetraosyl branch is bound to glycon binding site. The aglycon binding site is for B5/81 moiety of B5/84. When the α-1,4-glycosidic linkage between the maltotriosyl and B5/81 moieties is split, B5/81 falls away from the aglycon binding site, and water and the acceptor on the acceptor binding site competitively attack the activated maltotriosyl residue on the glycon binding site. The non-reducing-end glucosyl residue of the acceptor should be located in the vicinity of the reducing-end glucosyl residue of the activated glycon for transglycosylation. B5/81 and B5/84 could not be bound to the acceptor binding site in the binding mode to form the transfer products, particularly considering that the maltopentaosyl and larger maltooligosaccharide structures comprising the non-reducing-end glucose residue are suitable acceptors for transglycosylation of 4-α-glucanotransferases of mammalian GDE (*23*). CDs could not be bound to the glycon binding site and the acceptor binding site in a similar manner as the linear maltooligosaccharides having the non-reducing-end glucosyl residues. Actually, CDs did not inhibit the 4-α-glucanotransferase action on B5/84. It is likely that the activator binding site is separated from the active site of the 4-α-glucanotransferase.

The B5/84 molecule can also be bound to the activator binding site, and this would accelerate the 4-α-glucanotransferase action on another B5/84 molecule bound to the active site, in proportion to its concentration. In order to reduce the effects of B5/84 binding to the activator binding site, enzymatic reactions were carried out at low B5/84 concentration when compared to CD concentrations. The use of the fluorogenic substrate and HPLC allowed the quantification of small amounts of products in the enzymatic reaction mixture.

In porcine liver cells, the α-glucans proximal to GDE are not CDs, but glycogen. The activator binding site is thus likely to interact with a branch of glycogen or phosphorylase limit dextrin. Glycogen is degraded by glycogen phosphorylase and GDE. It is known that glycogen breakdown is controlled by modification of phosphorylase activity with allosteric effectors, such as AMP and glucose-6-phosphate, and with phosphorylase kinases and phosphoprotein phosphatases (*24–31*). On the other hand, little is known about the control associated with GDE. Binding of α-glucans to the activator binding site and their subsequent dissociation affect the active site of 4-α-glucanotransferase, and contribute to control of glycogen degradation.

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