J. Biochem. 2009;145(5)585–590
doi:10.1093/jb/mvp012

Inspection of the Activator Binding Site for 4-α-Glucanotransferase in Porcine Liver Glycogen Debranching Enzyme with Fluorogenic Dextrins

Eriko Yamamoto, Yumiko Watanabe, Yasushi Makino and Kaoru Omichi*

Department of Chemistry, Graduate School of Science, Osaka Prefecture University, 1-1, Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan

Received November 17, 2008; accepted January 13, 2009; published online January 20, 2009

Recently, we found that α-, β- and γ-cyclodextrins accelerated the 4-α-glucanotransferase action of porcine liver glycogen debranching enzyme (GDE) on Glcα1-4Glcα1-4Glcα1-4(Glcα1-4Glcα1-4Glcα1-4Glcα1-6)Glcα1-4Glcα1-4Glcα1-4Glcα1-4GlcPA (B5/84), and proposed the presence of an activator binding site in the GDE molecule. In liver cells, the structures of α-glucans proximal to the site GDE acts are not cyclodextrins, but glycogen and its degradation products. To estimate the structural characteristics of intrinsic activators and to inspect the features of the activator binding site, we examined the effects of four fluorogenic dextrins, $(Glc\alpha1\text{-}6)_m Glc\alpha1\text{-}4(Glc\alpha1\text{-}4)_n GlcPA$ (B5/51, $m = 1$, $n = 3$; B6/61, $m = 1$, $n = 4$; B7/71, $m = 1$, $n = 5$; G6PA, $m = 0$, $n = 4$), on the debranching of B5/84 by porcine liver GDE. The GDE 4-α-glucanotransferase removed the maltotriosyl residue from the maltotetraosyl branch of B5/84, producing Glcα1-4Glcα1-4Glcα1-4(Glcα1-6)Glcα1-4Glcα1-4Glcα1-4Glcα1-4GlcPA (B5/81). In the presence of G6PA, the removed maltotriosyl residue was transferred to G6PA to give Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4GlcPA (G9PA). In the absence of G6PA, the removed maltotriosyl residue was transferred to water. B7/71, B6/61 and B5/51 did not undergo any changes by the GDE, but they accelerated the action of the 4-α-glucanotransferase in removing the maltotriosyl residue. Of the four fluorogenic dextrins examined, B6/61 most strongly accelerated the 4-α-glucanotransferase action. The activator binding site is likely to be a space that accommodates the structure of Glcα1-6Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glc.

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

Abbreviations: GDE, glycogen debranching enzyme; 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, Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4GlcPA; B7/71, Glcα1-6Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4GlcPA; B6/61, Glcα1-6Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4GlcPA; B5/51, Glcα1-6Glcα1-4Glcα1-4Glcα1-4Glcα1-4GlcPA; B5/61, Glcα1-4(Glcα1-6)Glcα1-4Glcα1-4Glcα1-4Glcα1-4GlcPA; G9PA, Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4GlcPA; EDTA, ethylenediaminetetraacetate.

Glycogen debranching enzyme (GDE) is a member of the α-amylase family. Mammalian GDE, together with glycogen phosphorylase, degrades glycogen in muscle and liver. 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 malto-oligosaccharide branch so that degradation by glycogen phosphorylase can resume (*1–5*). 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 activity (dextrin 6-α-glucosidase, EC 3.2.1.33) on the single polypeptide chain (*6–12*). The 4-α-glucanotransferase transfers the non-reducing-end malto-oligosaccharide residues 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 hydrolyses the α-1,6-glycosidic linkage. Deficiency of the activities causes a type III glycogen storage disease (Cori's disease), and many structural studies of the human gene have been reported (*13–19*).

Recently, we found that α-, β- and γ-cyclodextrins accelerated the action of porcine liver GDE 4-α-glucanotransferase on a fluorogenic branched dextrin, B5/84. This suggested the existence of a binding site for cyclodextrins in the enzyme, and that binding of cyclodextrins to the site, called the activator binding site, activated GDE (*20*). The activation of GDE 4-α-glucanotransferase suggests that glycogen degradation is controlled by modification of GDE activity. However, cyclodextrins are not essential for the activation, because cyclodextrins are not present in liver cells. Certain oligosaccharides derived from glycogen or its oligosaccharide moieties must be intrinsic activators that can be bound to the activator binding site with higher affinity to strongly affect glycogen degradation.

*To whom correspondence should be addressed. Tel: +81-72-254-9191, E-mail: komichi@c.s.osakafu-u.ac.jp

There are two distinct active sites in the porcine liver GDE molecule. It is difficult to determine whether a dextrin accelerates the enzymatic action when it is the substrate for one of the two activities itself. Fluorogenic dextrins with non-reducing-end isomaltosyl residues, Glcα1-6Glcα1-4(Glcα1-4)$_n$GlcPA, are not hydrolysed by the GDE amylo-α-1,6-glucosidase found in porcine liver, although they have the Glcα1-6Glc structure (*21*). Furthermore, they have no malto-oligosaccharide branches susceptible for 4-α-glucanotransferase. Thus, a molecule that accelerates the enzymatic action can be identified as an activator.

This study describes the acceleration of GDE 4-α-glucanotransferase action by homologous fluorogenic dextrins and discusses the features of the activator binding site for GDE 4-α-glucanotransferase.

MATERIALS AND METHODS

Materials—A Wakosil-II 3C18 HG column (1 × 30 cm), a Wakosil-II 5C18 HG column (1 × 25 mm), maltohexaose and malto-octaose were purchased from Wako Pure Chemicals (Osaka, Japan). A Shodex Asahipak NH2P-50 column (4.0 × 250 mm) was purchased from Showa Denko (Tokyo Japan). Porcine liver GDE was purified as described previously (*20*). *Bacillus stearothermophilus* α-glucosidase was purchased from Sigma. B5/84 was prepared as described previously (*22*). G6PA and Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4GlcPA (G8PA) were prepared by pyridylamination of maltohexaose and malto-octaose, respectively, as reported previously (*23*). Glcα1-4(Glcα1-6)Glcα1-4Glcα1-4Glcα1-4Glcα1-4GlcPA (B5/61), B6/61, B7/71 and B5/81 were prepared as described previously (*21*). The standard mixture of PA-malto-oligosaccharides was prepared by partial acid hydrolysis of G8PA.

Preparation of B5/51—B5/51 was prepared from B5/61 by taking advantage of the preferential action of *Bacillus stearothermophilus* α-glucosidase for an α-1,4-glucosidic linkage. B5/61 (1.2 μmol) was incubated with 13 U of α-glucosidase for 10 h at 37°C in 25 ml of 50 mM phosphate buffer (pH 6.8). To stop the enzymatic reaction, 1 ml of 0.2 M acetic acid was added to the reaction mixture, and the mixture was heated at 100°C for 15 min. B5/51 was isolated as the predominant product from the digest by preparative HPLC. The column used was a Wakosil-II 3C18 HG column (1 × 30 cm), and the elution buffer was 0.05 M ammonium acetate buffer, pH 4.3, containing 0.07% 1-butanol at a flow rate of 2 ml/min. The elution was monitored by measuring the fluorescence (excitation wavelength, 320 nm; emission wavelength, 400 nm). Isolated B5/51 was further purified with a Shodex Asahipak NH2P column. The eluent was acetonitrile:water:acetic acid (666:333:3, v/v/v) titrated to pH 7.0 with aqueous ammonia at a flow rate of 0.8 ml/min. The elution was monitored by measuring the fluorescence (excitation wavelength, 310 nm; emission wavelength, 380 nm).

The structure of B5/51 was confirmed by HPLC analysis of its partial acid hydrolysate as reported previously (*21*). HPLC was carried out with a Wakosil-II 5C18 HG column (1 × 25 cm). The elution buffer was 50 mM ammonium acetate buffer, pH 4.5, containing 0.05% 1-butanol, and the flow rate was 2.5 ml/min. The hydrolysate of B5/51 contained GlcPA, PA-maltose, PA-maltotriose, PA-maltotetraose, PA-maltopentaose and B5/51, but not G6PA. These data indicated that B5/51 was Glcα1-6Glcα1-4Glcα1-4Glcα1-4Glcα1-4GlcPA, taking into account that it was obtained from B5/61.

Measurement of the Initial Velocity of 4-α-Glucanotransferase Action on B5/84—B5/84 (0.6 nmol) was incubated at 37°C with 0.26 U of porcine liver GDE in 40 μl of 50 mM sodium maleate buffer (pH 6.0) containing 0.05% gelatin, 5 mM EDTA and 10 mM β-mercaptoethanol, with or without the fluorogenic dextrins. One unit of GDE was defined as the amount producing 1 pmol of B5/81 per minute at 37°C and pH 6.0 from 40 μl of 15 μM B5/84. The incubation period was adjusted so that the total amount of B5/81 and G8PA liberated was below 15% of the initial amount of B5/84. To stop the enzymatic reaction, 100 μl of 0.1 M acetic acid was added to the reaction mixture, and the mixture was heated at 100°C for 5 min. The fluorogenic products in the enzymatic reaction mixtures, except for the reaction mixture with B5/51, were separated and quantified with a Wakosil-II 5C18 HG column (1.0 × 25 cm) as described above. Product analysis of the mixture with B5/51 was carried out with a Shodex Asahipak NH2P column (4.0 × 250 mm). The eluent was acetonitrile:water:acetic acid (710:290:3, v/v/v) titrated to pH 7.0 with aqueous ammonia at a flow rate of 0.8 ml/min. The rate of 4-α-glucanotransferase action was calculated from the total amount of the fluorogenic products G8PA and B5/81.

RESULTS AND DISCUSSION

Mode of Action of GDE 4-α-Glucanotransferase on B5/84—B5/84 was incubated with porcine liver GDE for an appropriate period (3–25 min) in the presence or absence of 80 μM G6PA, B7/71, B6/61 or B5/51, and the mixture was analysed by HPLC at an early stage in the enzymatic reaction as described in MATERIALS AND METHODS section.

G8PA and B5/81 were found in the reaction mixture in the absence of the fluorogenic dextrins (Fig. 1A). The product analysis of the enzymatic reaction mixture indicates that GDE 4-α-glucanotransferase removed the maltotriosyl residue of the maltotetraosyl branch of B5/84, producing B5/81. B5/81 was then immediately hydrolysed by GDE amylo-α-1,6-glucosidase to the debranched product, G8PA and glucose (Fig. 2). The removed maltotriosyl residue was transferred to water because of the absence of a suitable acceptor. The total amount of G8PA and B5/81 liberated is equal to the amount of B5/84 that the GDE 4-α-glucanotransferase acted on.

G8PA, G9PA and B5/81 were found in the reaction mixture with 80 μM G6PA (Fig. 1B). The product analysis indicates that GDE 4-α-glucanotransferase removed the maltotriosyl residue, producing B5/81. The removed maltotriosyl residue was transferred to the non-reducing-end glucosyl residue of the acceptor, G6PA, producing G9PA. With the donor substrate B5/84, G6PA

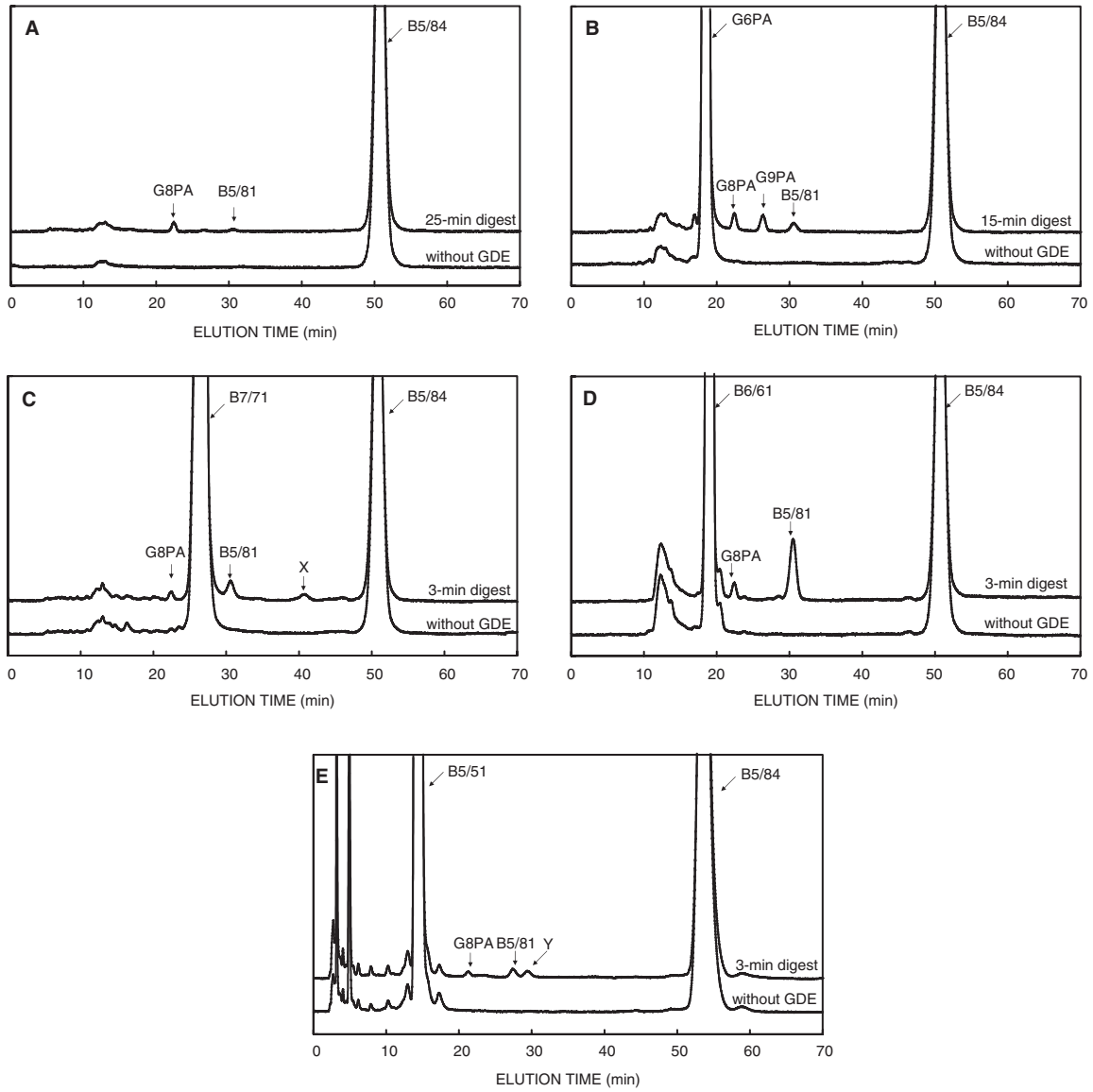

Fig. 1. **HPLC of B5/84 digest by porcine liver GDE.** Enzymatic reaction and HPLC of the reaction mixture were carried out as described in MATERIALS AND METHODS section. The chromatograms of the reaction mixture without GDE are at the bottom. (A) A 25-min digest of B5/84; (B) 15-min digest of B5/84 with 80 μM G6PA; (C) 3-min digest of B5/84 with 80 μM B7/71; (D) 3-min digest of B5/84 with 80 μM B6/61; (E) 6-min digest of B5/84 with 80 μM B5/51.

functioned as an acceptor substrate, similarly as with the phosphorylase limit dextrin of glycogen (*24*). Some of the B5/81 produced was hydrolysed to G8PA and glucose (Fig. 2). The ratio of the amount of G9PA to the total amount of G8PA and B5/81 was 0.68, indicating that 68% of the removed maltotriosyl residue was transferred to G6PA. The other 32% was transferred to water—i.e. hydrolysis occurred—although the GDE 4-α-glucanotransferase is classified as a transferase.

The product analysis of the enzymatic reaction mixture with 80 μM B7/71 showed that the products were G8PA, B5/81 and an unknown peak X (Fig. 1C). Peak X was collected and incubated with porcine liver GDE and the enzymatic reaction mixture was analysed by HPLC. Peak X was slowly converted to a fluorogenic peak very close to the B7/71 peak (data not shown). It suggested that peak X is not a transfer product to B7/71, but probably to a contaminating substance of B7/71.

The products of the enzymatic reaction with 80 μM B6/61 were B5/81 and G8PA. No other products were found (Fig. 1D). The removed maltotriosyl residue was not transferred to B6/61, but to water. It is important to note that the ratio of G8PA to B5/81 in the enzymatic reaction mixture when B6/61 was included was smaller than in the absence of B6/61. This indicates that the liberation of B5/81 by the GDE 4-α-glucanotransferase was accelerated with B6/61, compared to its hydrolysis by the GDE amylo-α-1,6-glucosidase. We cannot exclude the possibility that B6/61 may inhibit the hydrolysis of B5/81 to G8PA by the GDE amylo-α-1,6-glucosidase.

The enzymatic reaction mixtures with 80 μM B5/51 were analysed by size-fractionation HPLC as described in

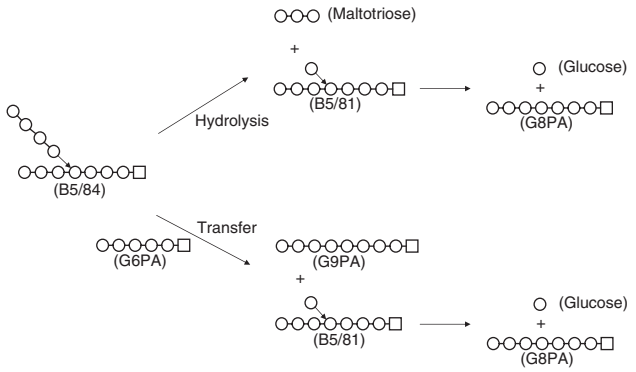

Fig. 2. **Mode of action of porcine liver GDE on B5/84.** O, D-glucose residue; -, α-1,4-glycosidic linkage; □, 1-deoxy-1-[(2-pyridyl)amino]-D-glucitol residue; ↓, α-1,6-glycosidic linkage.

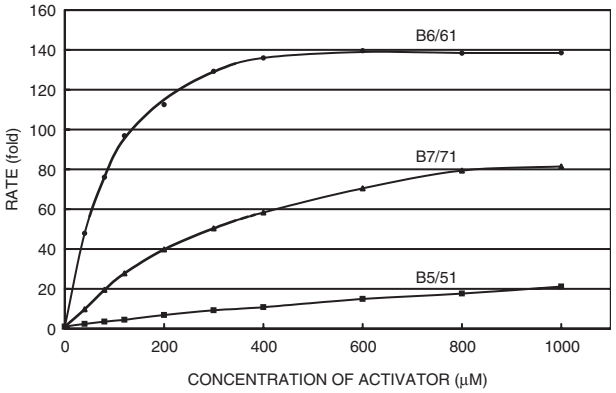

Fig. 3. **Relationship between the rate of 4-α-glucanotransferase action on B5/84 and the concentration of Glcα1-6Glcα1-4(Glcα1-4)$_n$GlcPA.** B5/84 was digested with GDE in the presence of various concentrations of Glcα1-6Glcα1-4(Glcα1-4)$_n$GlcPA, and HPLC of the reaction mixtures was carried out as described in MATERIALS AND METHODS section. The 4-α-glucanotransferase activity was calculated based on the total amount of G8PA and B5/81. The value of the 4-α-glucanotransferase activity without Glcα1-6Glcα1-4(Glcα1-4)$_n$GlcPA was taken as unity.

MATERIALS AND METHODS section, because G8PA could not be separated from B5/51 by a reversed-phase column. The fluorogenic products were G8PA, B5/81 and unknown peak Y (Fig. 1E). The peak Y was resistant to GDE, suggesting that it was not a transfer product of B5/51.

The rate of the GDE 4-α-glucanotransferase action on B5/84 can be estimated from the total amounts of G8PA and B5/81 produced, regardless of transglycosylation or hydrolysis. The rates with 80 μM G6PA, 80 μM B7/71, 80 μM B6/61 and 80 μM B5/51 were ~4-fold, 20-fold, 76-fold and 4-fold, respectively, higher than in their absence. B7/71, B6/61 and B5/51 did not function as an acceptor and were not changed by the enzyme. However, these molecules, especially B6/61, accelerated the action of the GDE 4-α-glucanotransferase on B5/84.

Dependence of Acceleration on the Concentration of the Activator—The GDE action on B5/84 in the presence of various concentrations of B7/71, B6/61 and B5/51 was examined. The relationship between the rates of 4-α-glucanotransferase action and the concentrations of Glcα1-6Glcα1-4(Glcα1-4)$_n$GlcPA are shown in Fig. 3.

The rate increased with increasing concentrations of B6/61, and reached a maximum. This result is consistent with the existence of the activator binding site that was proposed previously (*20*). B6/61 fitted in the activator binding site, which caused an acceleration of the hydrolytic action of the 4-α-glucanotransferase. Increasing concentration of B6/61 resulted in an increase in the activated form of the enzyme. At the saturation point, all enzyme molecules are in the activated form and maximum activity is observed.

The rates increased with increasing concentrations of B7/71 and B5/51, similar to B6/61; however, the extent of the increase was lower. Measurement of the rate would have to be done in the presence of higher concentrations of B5/51 to obtain the maximum activity. However, this was not done because the amounts of fluorogenic impurities in the B5/51 preparation increase with increasing B5/51 concentration and the impurities of higher concentrations would affect considerably on the interaction between the enzyme and B5/51.

The maximum activity with B6/61 was ~140-fold that without the activator. Its concentration at half maximum activity was 70 μM. Comparing these values with the corresponding values from α-cyclodextrin (6-fold, 2 mM) (*20*), B6/61 with a terminal isomaltosyl residue has higher affinity for the activator binding site than α-cyclodextrin. This is probably due to its flexible structure and the isomaltosyl residue.

The measurement of the rate with various concentrations of G6PA was not carried out. Because G6PA is the acceptor substrate, it would not be correct to evaluate its interaction with the activator binding site by measuring the dependence of the rate on the concentration of G6PA.

Features of the Activator Binding Site—The activator binding site can be considered to be composed of several subsites (S_0, S_1, S_2, S_3, ..., S_{-1}, S_{-2}, ..., S_{b1}, S_{b2}, ...) that are geometrically complementary to glucosyl residues in the activator molecule. S_{b1} and S_0 bind the isomaltosyl residue (Fig. 4). Each glucosyl residue of the activator molecule interacts with the corresponding subsite. The dextrins, which fit more tightly in the site and interact with it strongly, accelerate the 4-α-glucanotransferase action more strongly. Acceleration by B6/61 reached the point of saturation at the lowest concentration and the rate at the point of saturation was the highest among the dextrins tested, suggesting that the activator binding site accommodates Glcα1-6Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glc best.

The acceptor substrate G6PA must interact with the activator binding site in a similar manner as B6/61; however, it did not accelerate the 4-α-glucanotransferase action as much as B6/61. The interaction between subsites S_{b1} and G_{b1} of B6/61 is considered to be important for converting the enzyme into the activated form. The isomaltosyl structure of B6/61 must be essential for an effective activator. Replacement of the Glcα1-4GlcPA residue of B6/61 with a GlcPA residue resulted in decreased affinity for the activator binding site compared with B6/61 and B5/51. The GlcPA residue probably could

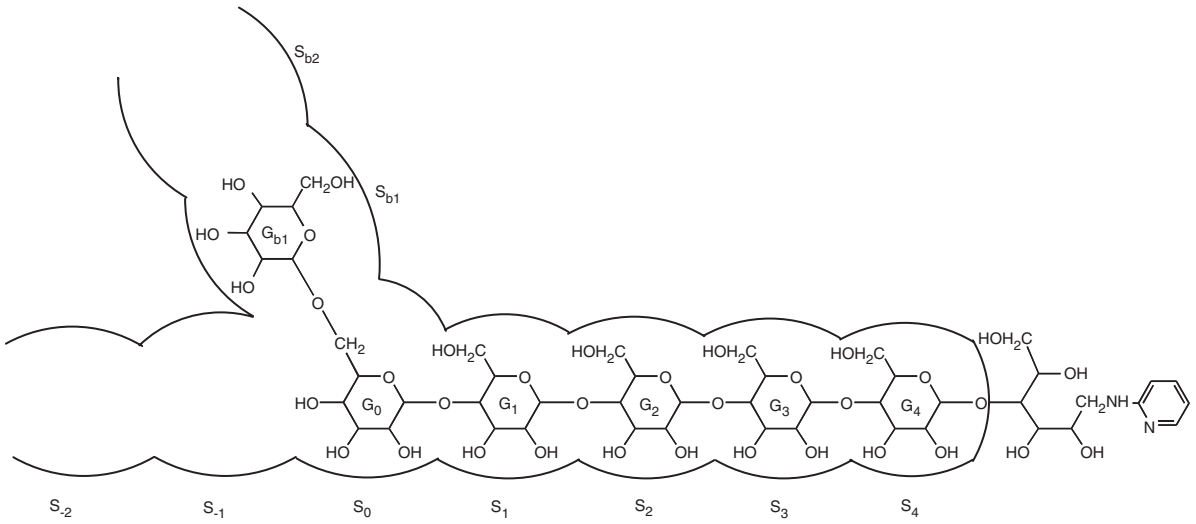

Fig. 4. **Schematic representation of the binding of B6/61 to the activator binding site of GDE.** The activator binding site is composed of several subsites (S_0, S_1, S_2, S_3, ..., S_{-1}, S_{-2}, ..., S_{b1}, S_{b2}, ...), with S_{b1} and S_0 binding the isomaltosyl residue. The existence of the subsites, S_{-1}, S_{-2}, ..., S_{b2}, ..., is hypothetical and has not been shown experimentally.

not interact with subsite S_4, since it is not a rigid glucopyranose ring but rather a flexible glucitol derivative. Thus, the interactions of the maltopentaosyl moiety (G_0, G_1, G_2, G_3, and G_4) with the subsites, S_0, S_1, S_2, S_3 and S_4 are important for activation, and the maltopentaosyl structure is also essential for an effective activator. The Glcα1-6Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glc moiety of B7/71 would be bound to the activator binding site, while the other Glcα1-4GlcPA moiety would protrude from the activator binding site. The protruding moiety is likely to destabilize the enzyme-activator complex, resulting in a lower value for the maximum activity (83-fold) and a higher concentration at half the maximum activity (200 μM).

B6/61 was the most powerful activator of the fluorogenic dextrins with a terminal isomaltosyl residue. The activator binding site does not always interact with dextrins of a terminal isomaltosyl residue. Some dextrins that are substrates would bind to the activator binding site and accelerate the 4-α-glucanotransferase action. The activation of GDE 4-α-glucanotransferase suggests that glycogen degradation is controlled by the modification of GDE activity as well as glycogen phosphorylase activity.

CONFLICT OF INTEREST

None declared.

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