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Donor Substrate Specificity of 4-α-Glucanotransferase of Porcine Liver Glycogen Debranching Enzyme and Complementary Action to Glycogen Phosphorylase on Debranching

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Glycogen debranching enzyme (GDE) has both 4-α-glucanotransferase and amylo-α-1,6-glucosidase activities. Here, we examined 4-α-glucanotransferase action of porcine liver GDE on four 6^4-*O*-α-maltooligosyl-pyridylamino(PA)-maltooctaoses, in the presence or absence of an acceptor, maltohexaose. HPLC analysis of digested fluorogenic branched dextrins revealed that in the presence or absence of acceptor, 6^4-*O*-α-glucosyl-PA-maltooctaose (B4/81) was liberated from 6^4-*O*-α-maltopentaosyl-PA-maltooctaose (B4/85), 6^4-*O*-α-maltotetraosyl-PA-maltooctaose (B4/84) and 6^4-*O*-α-maltotriosyl-PA-maltooctaose (B4/83), whereas 6^4-*O*-α-maltosyl-PA-maltooctaose (B4/82) was resistant to the enzyme. The fluorogenic product was further hydrolyzed by amylo-α-1,6-glucosidase to PA-maltooctaose (G8PA) and glucose. The ratio of the rates of 4-α-glucanotransferase actions on B4/85, B4/84 and B4/83 in the absence of the acceptor was 0.15, 0.42 and 1.00, respectively. The rates increased with increasing amounts of acceptor, changing the ratio of the rates to 0.09, 1.00 and 0.60 (with 0.5 mM maltohexaose) and 0.10, 1.00 and 0.58 (with 1.0 mM maltohexaose), respectively. Donor substrate specificity of GDE 4-α-glucanotransferase suggests complementary action of GDE and glycogen phosphorylase on glycogen degradation in the porcine liver. Glycogen phosphorylase degrades the maltooligosaccharide branches of glycogen by phosphorolysis to form maltotetraosyl branches, and phosphorolysis does not proceed further. GDE 4-α-glucanotransferase removes a maltotriosyl residue from the maltotetraosyl branch such that the α-1,6-linked glucosyl residue is retained.

Key words: 4-α-glucanotransferase, branched maltooligosaccharide, fluorogenic substrate, glycogen debranching enzyme, phosphorylase, substrate specificity.

Abbreviations: B4/81, 6^4-*O*-α-(-glucosyl-PA-maltooctaose; B4/82, 6^4-*O*-α-(-maltosyl-PA-maltooctaose; B4/83, 6^4-*O*-α-(-maltotriosyl-PA-maltooctaose; B4/84, 6^4-*O*-α-(-maltotetraosyl-PA-maltooctaose; B4/85, 6^4-*O*-α-(-maltopentaosyl-PA-maltooctaose; GDE, glycogen debranching enzyme; GlcPA, 1-deoxy-1-[(2-pyridyl)amino]-D-glucitol residue; G8PA, PA-maltooctaose; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; PA, pyridylamino.

Mammalian glycogen debranching enzyme (GDE) is found mainly in the liver and muscle and together with glycogen phosphorylase, degrades glycogen. Glycogen phosphorylase removes one glucose unit at a time from various non-reducing-ends of glycogen, producing glucose-1-phosphate. Sequential phosphorolysis by glycogen phosphorylase ceases near branch points until removal of maltooligosaccharide branches by GDE allows for further degradation (*1–5*). GDE has two distinct active sites, one with 4-α-glucanotransferase activity (1,4-α-glucan: 1,4-α-glucan 4-α-glycosyltransferase; EC 2.4.1.25) and the other with amylo-α-1,6-glucosidase activity (dextrin 6-α-glucosidase; EC 3.2.1.33) (*6–12*). The 4-α-glucanotransferase transfers the maltooligosaccharide moiety of the branch to the 4-position of a non-reducing-end glucosyl residue of another chains (the acceptor) to form an α-1,4-glucosidic linkage. The amylo-α-1,6-glycosidase then removes the remaining α-1,6-glucosyl residue to form α-1,4-glucan and glucose.

An early study of GDE reported that a rabbit muscle protein fraction had the ability to transfer maltotriosyl and maltosyl residues from a glycogen molecule to the acceptor maltotriose (*3*). Recently, we found that maltotriosyl and maltosyl residues were transferred to acceptors by porcine brain GDE from phosphorylase limit dextrins of glycogen but not from glycogen (*13*). Thus, the enzymes seem to have different donor substrate specificities. Glycogen and its phosphorylase limit dextrin are very large molecules of indefinite structure, and therefore, it is not clear how many and which size of maltooligosaccharide branches exist in these molecules. Thus, substrate specificity of enzymes can be determined more easily using donor substrates of definite structures, rather than glycogen or its limit dextrin.

We previously reported that a fluorogenic branched dextrin, 6^4-*O*-α-maltotetraosyl-PA-maltooctaose (B4/84), was debranched to form PA-maltooctaose (G8PA) by

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porcine skeletal muscle GDE (*14*). A set of fluorogenic branched dextrins with various size branches is likely to be useful for examining the donor substrate specificity of the 4-α-glucanotransferase activity of GDE. Here, we report the mode of action of porcine liver GDE on fluorogenic branched dextrins, Glcα1-4Glcα1-4Glcα1-4Glcα1-4[(Glcα1-4)$_n$Glcα1-6]Glcα1-4Glcα1-4Glcα1-4GlcPA (n = 1–4, GlcPA; 1-deoxy-1-[(2-pyridyl)amino]-**D**-glucitol residue), and a complementary action of glycogen phosphorylase on debranching.

MATERIALS AND METHODS

Materials—Maltohexaose, maltooctaose, Wakosil-II 3C18 HG (1 × 30 cm) and 5C18 HG (6 × 150 mm, 1 × 25 cm) columns, and *Klebsiella pneumoniae* pullulanase (EC. 3.2.1.41) were purchased from Wako Pure Chemicals (Osaka, Japan). G8PA was prepared by pyridylamination of maltooctaose as reported previously (*15*). B4/84 and B4/81, which were reported previously (*14*, *16*), were used. Porcine liver GDE was purified as described previously (*17*).

Preparation of 6^4-O-α-Maltooligosyl-PA-maltooctaose—6^4-*O*-α-Maltopentaosyl-PA-maltooctaose (B4/85), 6^4-*O*-α-maltotriosyl-PA-maltooctaose (B4/83) and 6^4-*O*-α-maltosyl-PA-maltooctaose (B4/82) were prepared from G8PA and maltopentaose, maltotriose and maltose, respectively, in a similar manner as for B4/84 (*14*). To a mixture of 80 μmol G8PA and 80 μmol maltooligosaccharide in 425 μl of 0.2 M 3,3-dimethylglutaric acid-NaOH buffer (pH 6.0), 75 μl of pullulanase (17 units) was added, and the mixture was incubated at 37°C for 2 days. After inactivation of the enzyme by heating at 100°C for 10 min, the reaction mixture was diluted with water, and then, subjected to gel-filtration on a Toyopearl HW40F column (2.8 × 280 cm) equilibrated with 10 mM ammonium acetate buffer (pH 6.0). Elution was monitored by measuring the absorbance at 320 nm and by analytical HPLC. Fractions eluting prior to G8PA were collected as the 6-*O*-α-maltooligosyl-PA-maltooctaose fraction and lyophilized. The lyophilized residue was dissolved in a small amount of water and subjected to preparative HPLC on a Wakosil-II 3C18 HG column (1 × 30 cm). The elution buffer for isolation of B4/85 was 50 mM ammonium acetate buffer (pH 5.5) containing 0.045% 1-butanol at 25°C and at a flow rate of 1.4 ml/min. For isolation of B4/83 and B4/82, the elution buffer was 50 mM ammonium acetate buffer (pH 4.3) containing 0.045% 1-butanol. Elution was monitored using a fluorescence detector (excitation at 320 nm; emission at 400 nm). Fluorescent peaks were collected as 6-*O*-α-maltooligosyl-PA-maltooctaose fractions and further purified by re-chromatography. Structures were determined by HPLC analysis of partial acid hydrolysates and pullulanase digests, and by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). The yields for B4/85, B4/83 and B4/82 were 150 nmol, 210 nmol and 180 nmol, respectively.

Partial Acid Hydrolysis of Fluorogenic Dextrins—In order to examine branching points, fluorogenic branched dextrins (0.2 nmol) were hydrolyzed at 90°C for 20 min in 50 μl of 0.2 M hydrochloric acid. The mixtures were neutralized with 50 μl of 0.2 M NaOH, followed by addition of 50 μl of 0.2M ammonium acetate buffer (pH 4.5).

Analytical HPLC—HPLC was used to analyze the digests and acid hydrolysates of the fluorogenic branched dextrins. HPLC was carried out using a Wakosil-II 5C18 HG column (6 × 150 mm) and elution with 50 mM ammonium acetate buffer (pH 4.5) containing 0.07% 1-butanol at 25°C and at a flow rate of 1.5 ml/min.

MALDI-MS—The molecular weights of the fluorogenic branched dextrins were determined by MALDI-MS. Samples (2 μl) were mixed with 2 μl of the matrix solution containing 10 mg 2,5-dihydroxybenzoic acid, 300 μl acetonitrile and 700 μl water and 1 μl of the mixture was loaded onto the target plate and allowed to dry before analysis. Molecular ions were analyzed in linear positive ion mode using a Voyager-DE STR BioSpectrometry Workstation (PE Applied Biosystems, Foster City, CA, USA). The instrument was calibrated using the external standard, pyridyl aminated nonasaccharide, (Gal)$_2$(GlcNAc)$_2$(Man)$_3$GlcNAcGlcNAcPA (MW 1,718.65).

Measurement of the Rate of 4-α-Glucanotransferase Action on Fluorogenic Branched Dextrins—Fluorogenic branched dextrins (20 μM) with or without maltohexaose were incubated for 10 min at 37°C with 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. The concentration of the enzyme was adjusted to hydrolyze less than 7% of each substrate. To stop the enzymatic reaction, 200 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 B4/81 and G8PA in the enzymatic reaction mixtures were separated and quantified by reversed-phase HPLC. The rate of 4-α-glucanotransferase action was calculated from the total amount of the fluorogenic products.

RESULTS AND DISCUSSION

Preparation of 6^4-O-α-Maltooligosyl-PA-maltooctaoses—A set of 6^4-*O*-α-maltooligosyl-PA-maltooctaoses with maltooligosaccharide branches of various sizes is effective to examine the donor substrate specificity of GDE 4-α-glucanotransferase. B4/85, B4/83 and B4/82 were prepared in a similar manner as for B4/84, taking advantage of the reverse reaction of *Klebsiella pneumoniae* pullulanase. G8PA and maltooligosaccharides at high concentrations were incubated with pullulanase, and the reaction mixtures were subjected to gel-filtration as described in the MATERIALS AND METHODS. The 6-*O*-α-maltooligosyl-PA-maltooctaose mixtures were obtained by collection of the peaks that eluted prior to elution of G8PA (data are not shown).

Preparative reversed-phase HPLC of the mixtures was carried out as described in the MATERIALS AND METHODS. A chromatogram of the 6-*O*-α-maltopentaosyl-PA-maltooctaose mixture obtained from the enzymatic reaction mixture of maltopentaose and G8PA by gel-filtration is shown in Fig. 1A. Though separation of each peak was not sufficient, it was possible to identified six 6-*O*-α-maltopentaosyl-PA-maltooctaoses. Taking it into account that *Klebsiella pneumoniae*

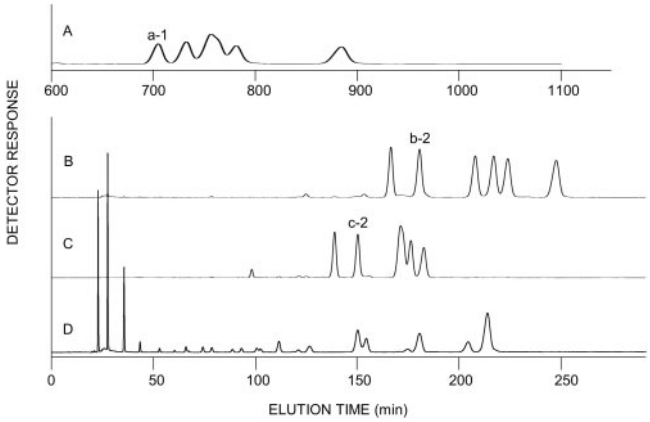

Fig. 1. **Preparative HPLC for isolation of B4/85, B4/83 and B4/82.** HPLC of fractions eluting prior to G8PA on gel-filtration of the enzymatic reaction mixtures were carried out as described in the MATERIALS AND METHODS. A, fraction obtained from the enzymatic reaction mixture containing G8PA and maltopentaose; B, fraction from the G8PA and maltotriose mixture; C, fraction from the G8PA and maltose mixture; D, partial acid hydrolysate of B4/84.

pullulanase can produce six different 6-*O*-α-maltotetraosyl-PA-maltooctaoses other than 6^1-*O*-α-maltotetraosyl-PA-maltooctaose and 6^2-*O*-α-maltotetraosyl-PA-maltooctaose from G8PA and maltotetraose (*14*), it seems reasonable that the number of 6-*O*-α-maltopentaosyl-PA-maltooctaoses observed was six. The first peak (peak a-1) was identified to be B4/85 as follows. The result of MALDI-MS showed that the molecular weight of peak a-1 was 2,203, consistent with a pyridylaminated glucose tridecamer. Peak a-1 could be hydrolyzed by pullulanase to produce G8PA, indicating that it was 6-*O*-α-maltopentaosyl-PA-maltooctaose (Fig. 2A). In the partial acid hydrolysate of peak a-1, PA-glucose, PA-maltose and PA-maltotriose were found, whereas PA-maltotetraose and higher PA-maltooligosaccharides were not detected, suggesting that the maltopentaosyl branch was linked to the fourth glucosyl residue of G8PA from the reducing-end (Fig. 2B). Based on these data, the structure of peak a-1 was determined to be Glcα1-4Glcα1-4Glcα1-4Glcα1-4(Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-6)Glcα1-4Glcα1-4Glcα1-4GlcPA.

Preparative HPLC of the mixtures of 6-*O*-α-maltotriosyl-PA-maltooctaoses and 6-*O*-α-maltosyl-PA-maltooctaoses obtained by gel-filtration are shown in Fig. 1B and C. B4/83 and B4/82 should be found in the partial acid hydrolysate of B4/84. Peak b-2 (Fig. 1B) and peak c-2 (Fig. 1C) eluted at the same elution positions as the hydrolysates of B4/84 (Fig. 1D). The molecular weights of peaks b-2 and c-2 were 1,879 and 1,717, respectively. They were hydrolyzed by pullulanase to produce the fluorogenic product G8PA, and the major PA-maltooligosaccharide in partial acid hydrolysates of peaks b-2 and c-2 were PA-glucose, PA-maltose and PA-maltotriose. Thus, peaks b-2 and c-2 were Glcα1-4Glcα1-4Glcα1-4Glcα1-4(Glcα1-4Glcα1-4Glcα1-6)Glcα1-4Glcα1-4Glcα1-4GlcPA and Glcα1-4Glcα1-4Glcα1-4Glcα1-4(Glcα1-4Glcα1-6)Glcα1-4Glcα1-4Glcα1-4GlcPA, respectively.

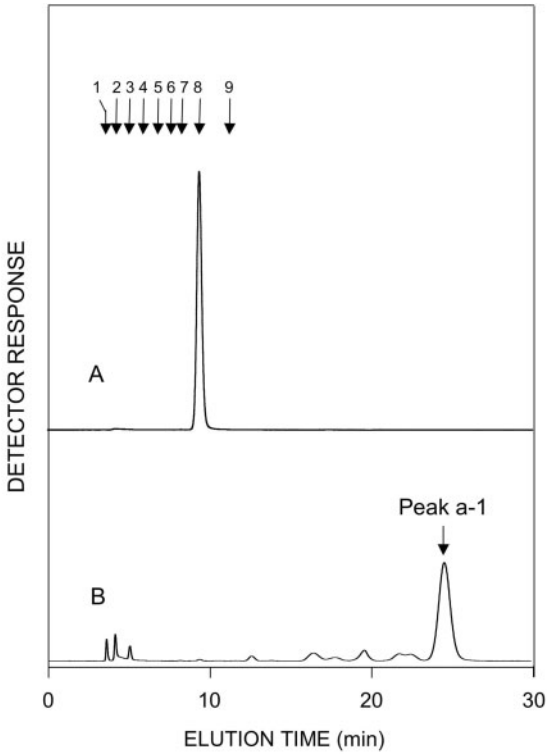

Fig. 2. **HPLC of the pullulanase digest and partial acid hydrolysate of peak a-1.** Pullulanase digested products and partial acid hydrolysates were obtained and analyzed by HPLC as described in the MATERIALS AND METHODS. A, pullulanase digest of peak a-1; B, partial acid hydrolysate of peak a-1. Arrows indicate the elution positions of the following compounds: 1, PA-glucose; 2, PA-maltose; 3, PA-maltotriose; 4, PA-maltotetraose; 5, PA-maltopentaose; 6, PA-maltohexaose; 7, PA-maltoheptaose; 8, PA-maltooctaose; 9, PA-maltononaose. A standard mixture of PA-maltooligosaccharides was obtained by partial acid hydrolysis of PA-maltononaose.

Action of Porcine Liver GDE on 6^4-O-α-Maltooligosyl-PA-maltooctaoses—The action of porcine liver GDE on B4/85, B4/84, B4/83 and B4/82 was examined. Maltohexaose was used as an acceptor substrate, because maltopentaose and higher maltooligosaccharides function as an acceptor for transglycosylation of porcine liver GDE (*18*). The four 6^4-*O*-α-maltooligosyl-PA-maltooctaoses were incubated with the enzyme in the presence or absence of maltohexaose, and the enzymatic reaction mixtures were analyzed at an early stage by reversed phase HPLC as described in the MATERIALS AND METHODS. The chromatograms of the enzymatic reaction mixtures in the presence of 1 mM maltohexaose are shown as examples (Fig. 3). HPLC analysis revealed that in the presence or absence of maltohexaose, B4/85, B4/84 and B4/83 were converted to B4/81 and G8PA, whereas there was little change in the case of B4/82. This indicates that GDE 4-α-glucanotransferase removed the maltotetraosyl residue from the maltopentaosyl branch of B4/85, the maltotriosyl residue from the maltotetraosyl branch of B4/84 and the maltosyl residue from the maltotriosyl branch of B4/83. The maltooligosaccharide residues that had been removed would then be transferred to the non-reducing-end residue of the

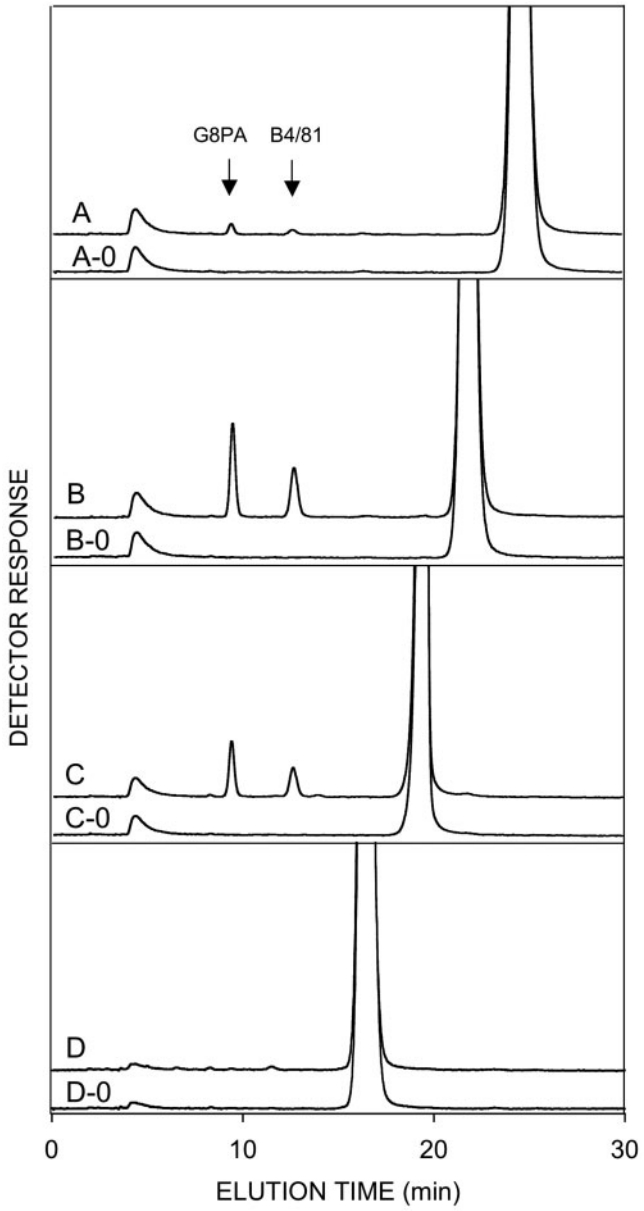

Fig. 3. **HPLC of the digests of the fluorogenic branched dextrins with porcine liver GDE in the presence of 1 mM maltohexaose.** B4/85, B4/84, B4/83 and B4/82 were incubated with porcine liver GDE in the presence of 1 mM maltohexaose and the digests were analyzed by HPLC as described in the MATERIALS AND METHODS. A, the digest of B4/85; A-0, control of A (without enzyme); B, the digest of B4/84; B-0, control of B; C, the digest of B4/83; C-0, control of C; D, the digest of B4/82; D-0, control of D.

acceptor, maltohexaose, though the transfer products are not fluorescent, such that the presence of transfer products cannot be confirmed. In the absence of the acceptor, it seems likely that the maltooligosaccharide residues that had been removed were transferred to water (that is, hydrolysis occurred), as the donor substrates, B4/85, B4/84 and B4/83, themselves did not function as an acceptor. Independent of transglycosylation or hydrolysis, GDE 4-α-glucanotransferase removed

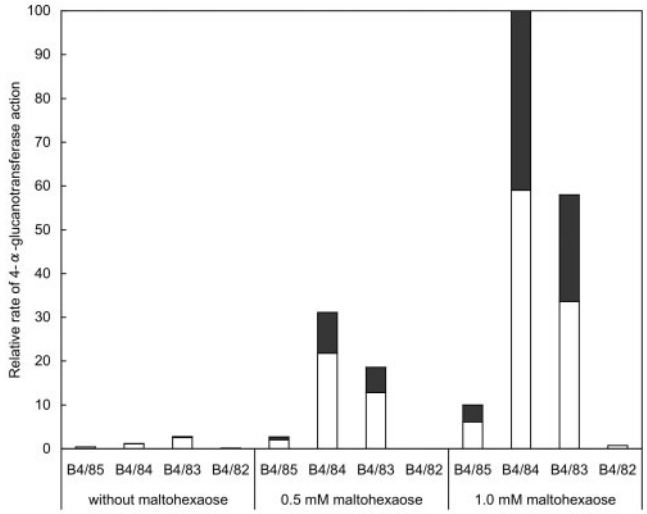

Fig. 4. **Comparison of the rate of 4-α-glucanotransferase action for the fluorogenic branched dextrins.** Fluorogenic branched dextrins were incubated with porcine liver GDE in the presence or absence of maltohexaose, and the enzymatic reaction mixtures were analyzed by HPLC as described in the MATERIALS AND METHODS. The 4-α-glucanotransferase activity was calculated based on the total amounts of B4/81 and G8PA, and represented by combined total of the black (B4/81) and white (G8PA) regions. The rate of 4-α-glucanotransferase action on B4/84 in the presence of 1.0 mM maltohexaose was set to 100.

maltooligosaccharide residues, $(Glc\alpha1\text{-}4)_n$, from the branches, $(Glc\alpha1\text{-}4)_nGlc\alpha1\text{-}6$, so as to retain the glucosyl residue, Glcα1-6, that is linked to the branching point glucosyl residue and liberate B4/81. GDE amylo-α-1,6-glucosidase hydrolyzed the liberated B4/81 to produce G8PA and glucose (*16*). The proportions of B4/81 to G8PA in the digests of B4/85, B4/84 and B4/83 without the acceptor were 0.00 to 1.00, 0.04 to 0.96 and 0.05 to 0.95, respectively (data not shown by the figure), and they increased with the acceptor concentration (Fig. 3). Probably, the rates of liberation of B4/81 by transglycosylation were so fast that amylo-α-1,6-glucosidase could not hydrolyze it to G8PA immediately. Another possibility is that maltohexaose may inhibit somewhat amylo-α-1,6-glucosidase activity.

Susceptibility of 6^4-O-α-Maltooligosyl-PA-maltooctaoses to GDE—As B4/81 was hydrolyzed by amylo-α-1,6-glucosidase to G8PA, the rate of 4-α-glucanotransferase action can be calculated from the total amount of B4/81 and G8PA produced. Figure 4 is a comparison of the rates of 4-α-glucanotransferase action on the fluorogenic branched dextrins with or without the acceptor. GDE 4-α-glucanotransferase is a transferase and an acceptor substrate is indispensable for transglycosylation. In the absence of the acceptor, hydrolysis occurred very slowly. The ratio of the rates of hydrolytic action on B4/85, B4/84 and B4/83 was 0.15, 0.42 and 1.00, respectively. As expected, the rates of 4-α-glucanotransferase action in the presence of the acceptor substrate were very fast, and the ratio of the rates was 0.09, 1.00 and 0.60 (with 0.5 mM maltohexaose) and 0.10, 1.00 and 0.58 (with 1.0 mM maltohexaose), respectively. Notably, the ratio

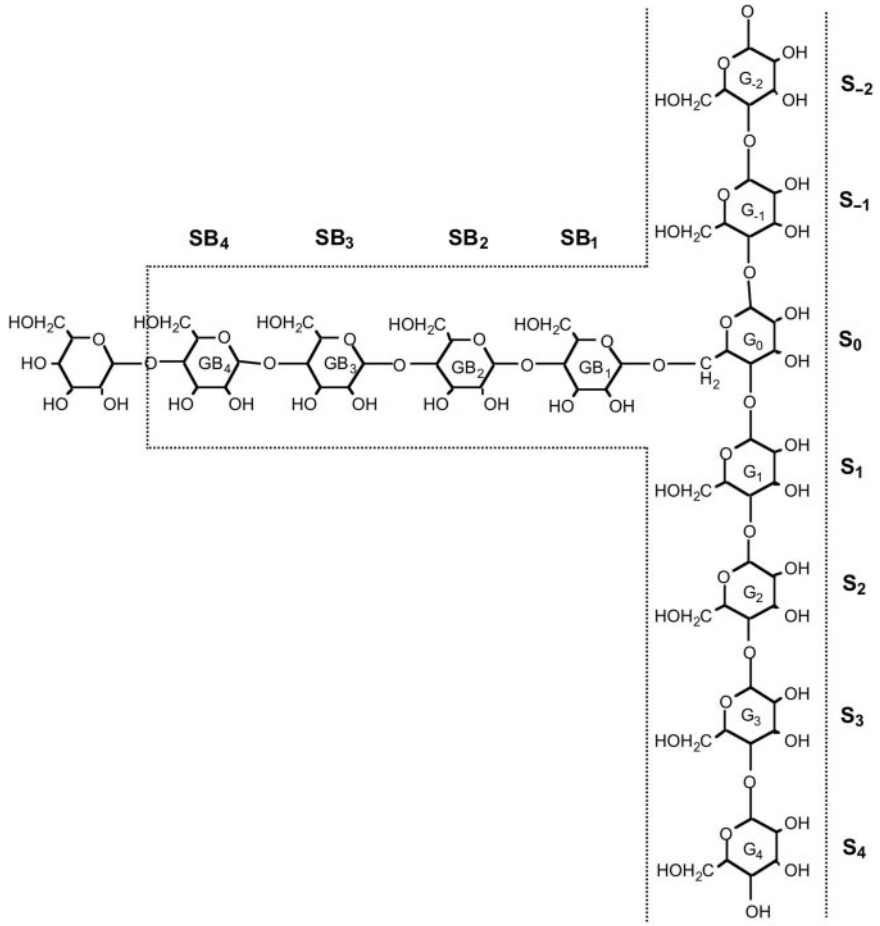

Fig. 5. **Schematic representation of the interaction of 6-*O*-α-maltooligosyl-maltooligosaccharide with the active site of GDE 4-α-glucanotransferase.** The active site is composed of several subsites (S_0, SB_1, SB_2, SB_3, SB_4,..., S_1, S_2,..., S_{-1}, S_{-2},...). SB_1 and S_0 are for the isomaltosyl residue. The α-1,4-glycosidic linkage between GB_1 and GB_2 is split.

did not vary with changes in the concentration of the acceptor. In the absence of the acceptor, hydrolysis by the transferase would proceed similarly as transglycosylation. However, every step is not the same as that of transglycosylation, because B4/83 was hydrolyzed most rapidly.

The active site of 4-α-glucanotransferase can be considered to be composed of several subsites (S_0, SB_1, SB_2, SB_3, SB_4,..., S_1, S_2,..., S_{-1}, S_{-2},...) that are geometrically complementary to glucosyl residues (G_0, GB_1, GB_2, GB_3, GB_4,..., G_1, G_2,..., G_{-1}, G_{-2},....) in 6-*O*-α-maltooligosyl-maltooligosaccharide, and the α-1,4-glycosidic linkage between GB_1 and GB_2 is split (Fig. 5). Comparing the susceptibility of B4/82, B4/83 and B4/84, an interaction between SB_3 and GB_3 seem mostly likely to cause cleavage of the α-1,4-glycosidic linkage between GB_1 and GB_2 to liberate B4/81. Moreover, the interaction between SB_4 and GB_4 is also important for the enzyme action. The non-reducing-end glucosyl residue of the maltopentaosyl branch of B4/85 would protrude from the active site. An extra glucosyl residue is likely to destabilize the enzyme-substrate complex, resulting in lower susceptibility than what is observed for B4/84. It is also probable that the active site would accommodate the B4/85 molecule only with some degree of steric hindrance, due to the presence of a glucosyl residue. The enzyme did not remove maltooligosaccharide residues from the main chain of the dextrins, suggesting that 4-α-glucanotransferase is likely to recognize G_0 and its neighbor residues G_1 and G_{-1}.

Complementary Actions of GDE 4-α-Glucanotransferase and Glycogen Phosphorylase—Glycogen phosphorylase removes one glucose unit at a time from the non-reducing-ends of glycogen, producing glucose-1-phosphate. This sequential phosphorolysis ceases near a branch point. The rate of 4-α-glucanotransferase action for B4/85 was approximately one-tenth of that for B4/84. Donor substrate specificity in terms of the size of the branch suggests that GDE 4-α-glucanotransferase acts on maltooligosaccharide branches in a manner complementary to the specificity of glycogen phosphorylase on debranching. GDE 4-α-glucanotransferase is unlikely to attack the maltopentaosyl branch to remove the maltotetraosyl residue, though it can perform this activity at a slow rate. Maltopentaosyl and larger branches are degraded by phosphorolysis of glycogen phosphorylase to the maltotetraosyl branch, after which phosphorolysis would not proceed further. GDE 4-α-glucanotransferase must alternate and remove the maltotriosyl residue from the maltotetraosyl branch, leaving behind the glucosyl

residue linked to the branching point glucosyl residue such that it can be acted on by GDE amylo-α-1,6-glucosidase. In this way, it appears that a maltosyl branch is not produced. Thus, maltooligosaccharide branches can be debranched and degraded via the complementary actions of GDE and glycogen phosphorylase.

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