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Active Site Mapping of Amylo-α-1,6-glucosidase in Porcine Liver Glycogen Debranching Enzyme Using Fluorogenic 6-*O*-α-Glucosyl-maltooligosaccharides

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Glycogen debranching enzyme (GDE) has two enzymatic activities, 4-α-glucanotransferase and amylo-α-1,6-glucosidase. Products with 6-*O*-α-glucosyl structures formed from phosphorylase limit dextrin by the 4-α-glucanotransferase activity are hydrolyzed to glucose by the amylo-α-1,6-glucosidase activity. Here, we probed the active site of amylo-α-1,6-glucosidase in porcine liver GDE using various 6-*O*-α-glucosyl-pyridylamino (PA)-maltooligosaccharides, with structures $(Glc\alpha1\text{-}4)_m(Glc\alpha1\text{-}6)Glc\alpha1\text{-}4(Glc\alpha1\text{-}4)_nGlcPA$ (GlcPA, 1-deoxy-1-[(2-pyridyl)amino]-D-glucitol residue). Fluorogenic dextrins were prepared from 6-*O*-α-glucosyl-α-, β-, or γ-cyclodextrin through partial acid hydrolysis, followed by fluorescent tagging of the reducing-end residues of the hydrolysates and separation by gel filtration and reversed-phase HPLC. Porcine liver GDE hydrolyzed dextrins with the structure Glcα1-4(Glcα1-6)Glcα1-4Glc to glucose and the corresponding PA-maltooligosaccharides, whereas other dextrins were not hydrolyzed. Thus, substrates must have two glucosyl residues sandwiching the isomaltosyl moiety to be hydrolyzed. The rate of hydrolysis increased as m increased and reached maximum at m = 4. The rates were the highest when n = 1 but did not vary much with changes in n. Of the dextrins examined, Glcα1-4Glcα1-4Glcα1-4Glcα1-4(Glcα1-6)Glcα1-4Glcα1-4GlcPA (6^3-*O*-α-glucosyl-PA-maltoheptaose) was hydrolyzed most rapidly, suggesting that it fits the best in the amylo-α-1,6-glucosidase active site. It is likely that the active site accommodates 6^2-*O*-α-glucosyl-maltohexaose and that the interactions of seven glucosyl residues with the active site allow the most rapid hydrolysis of the α-1,6-glucosidic linkage of the isomaltosyl moiety.

Key words: active site, amylo-α-1,6-glucosidase, branched maltooligosaccharide, fluorogenic substrate, glycogen debranching enzyme.

Abbreviations: FD, fluorogenic dextrin; GDE, glycogen debranching enzyme; GlcPA, 1-deoxy-1-[(2-pyridyl)amino]-D-glucitol residue; PA, pyridylamino; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry.

Mammalian glycogen debranching enzyme (GDE) is found mainly in the liver and muscle and degrades glycogen in concert with glycogen phosphorylase. The enzyme is unique in that it 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) (*1–7*). This enzyme removes maltooligosaccharide branches of phosphorylase limit dextrin of glycogen to restart phosphorylase degradation (*8–12*). The 4-α-glucanotransferase activity transfers the maltooligosaccharide branch to the 4-position of the non-reducing-end glucosyl residue of the main chain to form an α-1,4-glucosidic linkage. The amylo-α-1,6-glucosidase activity then removes the remaining branching point α-1,6-glucosyl residue to give α-1,4-glucan and glucose.

Although the role of the GDE amylo-α-1,6-glucosidase in glycogen degradation is well established and the amino acid sequences of GDEs from several species have been deduced from their nucleotide sequences (*13–15*), very little is known about the active site and its substrate specificity. The active site is thought to be a space to accommodate a certain 6-*O*-α-glucosyl-maltooligosaccharide molecule tightly, and each glucosyl residue of the substrate must have specific interactions with the amino acid residues in the active site, allowing cleavage of the α-1,6-glycosidic linkage. Thus, 6-*O*-α-glucosyl-maltooligosaccharides and their derivatives will be useful as probes of the active site.

It should be possible to use a set of 6-*O*-α-glucosyl-maltooligosaccharides of various sizes to measure the size of the active site because the substrate that fits best should be hydrolyzed at the highest rate. We previously reported that fluorogenic 6^4-*O*-α-glucosyl-PA-maltopentaose and 6^4-*O*-α-glucosyl-PA-maltohexaose

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were useful substrates for GDE (*16*). These two dextrins were predominantly obtained from mono-6-*O*-α-glucosyl-α-cyclodextrin and glucose using *Bacillus macerans* cyclodextrin glucanotransferase. Partial acid hydrolysis of mono-6-*O*-α-glucosyl-cyclodextrins and fluorescent tagging of the hydrolysates are expected to allow production of a homologous series of fluorogenic dextrins (FD) with 6-*O*-α-glucosyl structures. Here, we prepared various FDs with 6-*O*-α-glucosyl structures and used them for active site mapping of the amylo-α-1,6-glucosidase of porcine liver GDE.

MATERIALS AND METHODS

Materials—Mono-6-*O*-α-glucosyl-α-cyclodextrin, mono-6-*O*-α-glucosyl-β-cyclodextrin, γ-cyclodextrin, maltose, 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). A Cosmosil 5C18-AR-II column (4.6 × 250 mm) was purchased from Nacalai Tesque (Kyoto, Japan). *Rhizopus delemar* glucoamylase (41 units/mg) was from Oriental Yeast (Tokyo, Japan). Porcine liver GDE was purified as described previously (*17*).

Preparation of Mono-6-O-α-glucosyl-γ-cyclodextrin—To a mixture of 2 g of γ-cyclodextrin and 540 mg of maltose in 2.5 ml of 0.1 M 3,3-dimethylglutaric acid-NaOH buffer (pH 6.0), 0.5 ml of *K. pneumoniae* pullulanase (110 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 applied to a Toyopearl HW 40F column (2.8 × 290 cm) equilibrated with 10 mM ammonium acetate buffer, pH 6.0. Elution was monitored by TLC using Kieselgel 60 plates (Merck). The solvent was 2-butanone: acetic acid: water, 3 : 1 : 1 (V/V/V), and the developing reagent was 5% sulphuric acid in methanol. The mono-6-*O*-α-maltosyl-γ-cyclodextrin fractions eluting prior to γ-cyclodextrin were collected and lyophilized. The mono-6-*O*-α-maltosyl-γ-cyclodextrin (96 mg) was incubated with *R. delemar* glucoamylase (4 units) in 6 ml of 0.1 M ammonium acetate buffer (pH 4.8) for 60 min at 37°C. TLC showed that all of the mono-6-*O*-α-maltosyl-γ-cyclodextrin was consumed but that γ-cyclodextrin was not formed. After heat-denaturation of the enzyme, the reaction mixture was separated by gel-filtration on the Toyopearl HW 40F column. The mono-6-*O*-α-glucosyl-γ-cyclodextrin fractions were collected and lyophilized.

Preparation of 6-O-α-Glucosyl-PA-maltooctaoses—A solution of 100 mg of mono-6-*O*-α-glucosyl-γ-cyclodextrin in 20 ml of 0.2 M hydrochloric acid was heated at 90°C for 45 min. Acid hydrolysis was stopped at an early stage, when a considerable amount of mono-6-*O*-α-glucosyl-γ-cyclodextrin remained, to suppress formation of lower molecular weight dextrins. The mixture was neutralized with 1 M NaOH and then concentrated to dryness under reduced pressure. Pyridylamination (*18*) of the hydrolysate was carried out as follows. The hydrolysate was dissolved in a mixture of 150 μl of acetic acid and 420 mg of 2-aminopyridine, and the resulting solution was heated at 90°C for 60 min. Next, 1.83 ml of a reducing reagent (prepared by dissolving 10 g of borane-dimethylamine complex in 4 ml acetic acid and 2.5 ml water) was added, and the solution was heated at 80°C for 50 min. Five milliliter of 3 M ammonium hydroxide was added to the reaction mixture, and excess reagents were removed by extraction three times with 3 ml of chloroform. The water layer was concentrated to dryness under reduced pressure. The residue was dissolved in a small volume of water and then separated by gel-filtration on a Toyopearl HW 40F column (2.3 × 115 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 containing 6-*O*-α-glucosyl-PA-maltooctaoses eluting prior to PA-maltooctaose were collected 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 compounds were eluted with 50 mM ammonium acetate buffer (pH 5.0) containing 0.02% 1-butanol at 25°C and at a flow rate of 1.0 ml/min. The elution was monitored by fluorescence detection (excitation at 320 nm; emission at 400 nm). Seven peaks (γ1–γ7) were detected. Peak γ6 was further separated into γ6a and γ6b by HPLC on a Cosmosil 5C18-AR-II column (4.6 × 250 mm) with the same buffer at a flow rate of 0.25 ml/min. The eight dextrins were further purified by rechromatography. The yields were 0.11, 0.15 0.17, 0.10, 0.14, 0.10, 0.10, and 0.16 μmol for dextrins γ1, γ2, γ3, γ4, γ5, γ6a, γ6b and γ7, respectively.

Preparation of 6-O-α-Glucosyl-PA-maltoheptaoses—Mono-6-*O*-α-glucosyl-β-cyclodextrin was partially hydrolyzed with hydrochloric acid, and the hydrolysate was fluorescently tagged in a similar manner as for mono-*6-O*-α-glucosyl-γ-cyclodextrin. The reaction mixture was subjected to gel-filtration, and 6-*O*-α-glucosyl-PA-maltoheptaose fractions eluting prior to PA-maltoheptaose were collected as 6-*O*-α-glucosyl-PA-maltooctaoses. Six peaks (β1, β2, β3, β4, β5 and β6) of 6-*O*-α-glucosyl-PA-maltoheptaoses were isolated by HPLC on a Wakosil-II 3C18 HG column (1 × 30 cm) under the same elution conditions as used for isolation of 6-*O*-α-glucosyl-PA-maltooctaoses. Peak β4 was further separated into β4a and β4b using the same column but with the buffer at pH 4.5 with 0.05% 1-butanol. The seven dextrins were further purified by rechromatography. The yields from 100 mg mono-6-*O*-α-glucosyl-β-cyclodextrin were 0.13, 0.29, 0.24, 0.18, 0.08, 0.31 and 0.22 μmol for β1, β2, β3, β4a, β4b, β5 and β6, respectively.

Preparation of 6-O-α-Glucosyl-PA-maltohexaoses—Mono-6-*O*-α-glucosyl-α-cyclodextrin was partially hydrolyzed with hydrochloric acid, and the hydrolysate was fluorescently tagged in a similar manner as described for mono-*6-O*-α-glucosyl-γ-cyclodextrin and mono-*6-O*-α-glucosyl-β-cyclodextrin. The reaction mixture was subjected to gel-filtration, and 6-*O*-α-glucosyl-PA-maltohexaose fractions eluting prior to PA-maltohexaose were collected. Five peaks (α1, α2, α3, α4 and α5) of 6-*O*-α-glucosyl-PA-maltohexaoses were isolated by HPLC on a Wakosil-II 3C18 HG column (1 × 30 cm) under the same elution conditions as 6-*O*-α-glucosyl-PA-maltooctaoses or 6-*O*-α-glucosyl-PA-maltoheptaoses. The fractions were

further purified by rechromatography. The yields from 100 mg mono-6-*O*-α-glucosyl-α-cyclodextrin were 0.15, 0.76, 0.56, 0.35 and 0.70 μmol for α1, α2, α3, α4 and α5, respectively.

Preparation of 6^3-O-α-Glucosyl-PA-maltotetraose and 6^3-O-α-Glucosyl-PA-maltopentaose—6^3-*O*-α-Glucosyl-PA-maltohexaose (40 nmol) was incubated with 0.16 units glucoamylase for 2 h at 37°C in 1.4 ml of 50 mM ammonium acetate buffer (pH 4.5). After heating at 100°C for 5 min, the digest was injected onto a Wakosil-II 5C18 HG HPLC column (1 × 25 cm). 6^3-*O*-α-Glucosyl-PA-maltotetraose (peak a) and 6^3-*O*-α-glucosyl-PA-maltopentaose (peak b) were eluted with 50 mM ammonium acetate buffer (pH 4.5) containing 0.05% 1-butanol at 25°C and at a flow rate of 2.0 ml/min.

Digestion of Fluorogenic Dextrins by GDE—FDs (0.5 nmol) were incubated with 4.2 units of porcine liver GDE for 20 h at 37°C in 30 μl of 50 mM sodium maleate buffer (pH 6.0) containing 0.05% gelatin, 5 mM EDTA, and 10 mM β-mercaptoethanol. One unit was defined as the amount of enzyme that produces 1 nmol of PA-maltoheptaose per minute using Glcα1-4Glcα1-4Glcα1-4Glcα1-4(Glcα1-6)Glcα1-4Glcα1-4GlcPA (6^3-*O*-α-glucosyl-PA-maltoheptaose) as the substrate. To stop the reaction, the mixtures were heated at 100°C for 5 min. The mixtures were then analysed by reversed-phase HPLC.

Analytical HPLC—HPLC was used to analyse the digests and the acid hydrolysates of the FDs. 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 0.05% 1-butanol at 25°C and at a flow rate of 1.5 ml/min.

Partial Acid Hydrolysis of Fluorogenic Dextrins—FDs (1.0 nmol) were hydrolyzed at 90°C for 30 min in 30 μl of 0.1 M hydrochloric acid. The mixtures were neutralized with 30 μl of 0.1 M NaOH, followed by the addition of 30 μl of 0.2 M ammonium acetate buffer (pH 4.5).

Matrix-assisted Laser Desorption/ionization Mass Spectrometry (MALDI–MS)—The molecular weights of the FDs that were resistant to GDE 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 analysed in linear positive ion mode using a Voyager-DE STR BioSpectrometry Workstation (PE Biosystems, Foster City, CA, USA). The instrument was calibrated using the external standard pyridylaminated xyloglucans (Xyl_3Glc_3GlcPA, M_r 1140.41 and $FucGal_2Xyl_3GlcGlcPA$, M_r 1610.57).

Measurement of the Rate of Hydrolysis by GDE—FDs (0.50 or 0.17 nmol) were incubated for 10 min at 37°C with porcine liver GDE in 30 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 10% of each substrate. To stop the enzymatic reaction, 30 μl of 0.2 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 were separated and quantified by reversed-phase HPLC. The rate of hydrolysis was calculated from the amount of the fluorogenic product.

RESULTS AND DISCUSSION

Preparation of 6-O-α-Glucosyl-PA-maltooligosaccharides—The isomaltosyl structure is indispensable for the substrates of GDE amylo-α-1,6-glucosidase. Mono-6-*O*-α-glucosyl-cyclodextrins containing the isomaltosyl structure were used as starting materials to prepare substrates of various sizes. 6-*O*-α-Glucosyl-PA-maltooctaoses were prepared from mono-6-*O*-α-glucosyl-γ-cyclodextrin through partial acid hydrolysis, followed by fluorescence tagging. Pyridylaminated oligosaccharides were separated by gel-filtration from the reaction mixture. Eight fluorogenic compounds (γ1, γ2, γ3, γ4, γ5, γ6a, γ6b and γ7) were isolated from the pyridylaminated oligosaccharide mixture by preparative reversed-phase HPLC (Fig. 1,A and γ6). These are expected to be 6-*O*-α-glucosyl-PA-maltooligosaccharides because they were derived from mono-6-*O*-α-glucosyl-γ-cyclodextrin.

Structural analysis of the fluorogenic compounds was carried out by HPLC of the digests with porcine liver GDE and of the partial acid hydrolysates. Figure 2 shows the chromatograms of the digest and the hydrolysate of peak γ3. Peak γ3 was completely hydrolyzed by GDE to produce PA-maltooctaose (Fig. 2A and B), indicating that it was one of 6-*O*-α-glucosyl-PA-maltooctaoses.

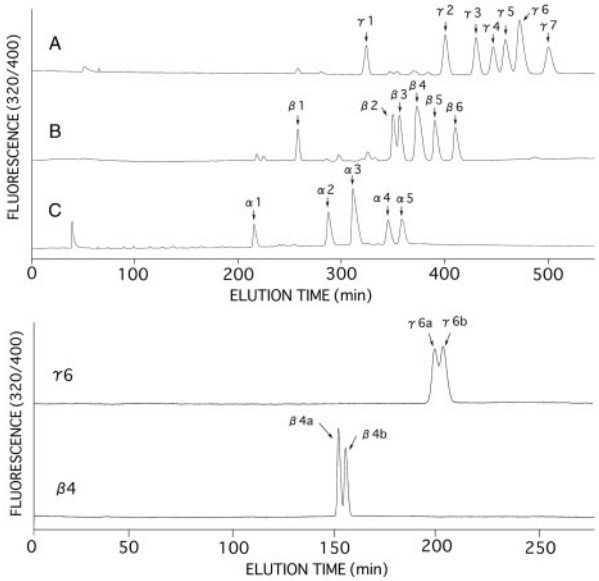

Fig. 1. **Isolation of 6-*O*-α-glucosyl-PA-maltooligosaccharides by preparative HPLC.** Mono-6-*O*-α-glucosyl-cyclodextrins were partially hydrolyzed with hydrochloric acid, and the hydrolysates were pyridylaminated. The reaction mixtures were subjected to gel-filtration. PA-oligosaccharide mixtures obtained by gel-filtration were subjected to HPLC as described under 'MATERIALS AND METHODS'. (A) PA-oligosaccharide mixture from 6-*O*-α-glucosyl-γ-cyclodextrin; (B) PA-oligosaccharide mixture from 6-*O*-α-glucosyl-β-cyclodextrin; (C) PA-oligosaccharide mixture from 6-*O*-α-glucosyl-α-cyclodextrin. Peaks γ6 in Fig. 1A and β4 in Fig. 1B were subjected to preparative HPLC as described under 'MATERIALS AND METHODS'. (γ6) peak γ6; (β4) peak β4.

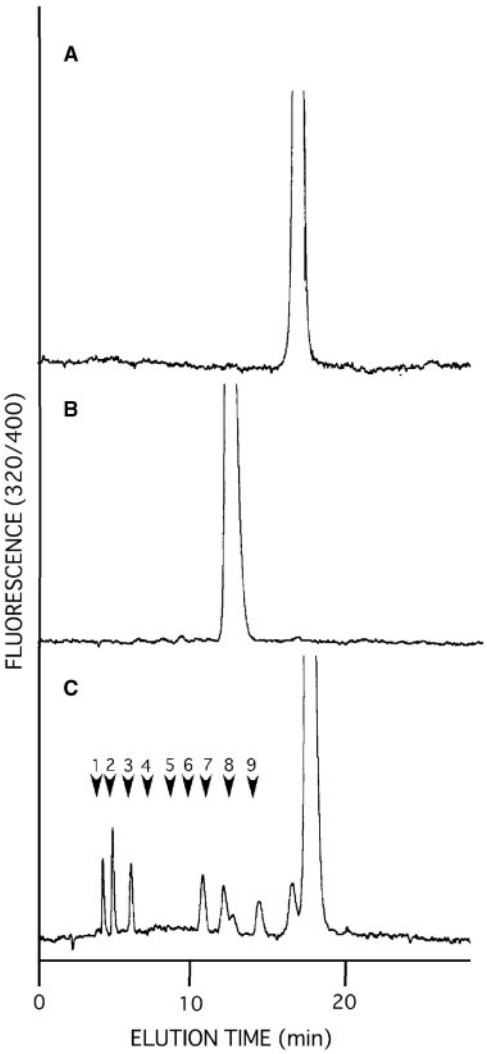

Fig. 2. **HPLC of the GDE digest and the partial acid hydrolysate of peak γ3.** Digestion with GDE and partial acid hydrolysis of peak γ3 were carried out as described under 'MATERIALS AND METHODS'. The digest and the hydrolysates were analyzed by HPLC as described under 'MATERIALS AND METHODS'. (A) peak γ3; (B) GDE digest of peak γ3; (C) partial acid hydrolysate of peak γ3. Arrowheads 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. The standard mixture of PA-maltooligosaccharides was obtained by partial acid hydrolysis of PA-maltononaose.

PA-glucose, PA-maltose and PA-maltotriose were found in the hydrolysate, but significant amounts of PA-maltotetraose and the higher PA-maltooligosaccharides were not (Fig. 2C). The distribution of the products indicates that PA-glucose, PA-maltose and PA-maltotriose were formed through only a single cleavage of the corresponding α-1,4-glycosidic linkage, while PA-maltotetraose and the higher PA-maltooligosaccharides were formed through successive cleavage of the α-1,4- and α-1,6-glycosidic linkages. Thus, the branch point glucosyl residue was at the fourth residue from the reducing-end. The structure of peak γ3 was therefore Glcα1-4Glcα1-4Glcα1-4Glcα1-4(Glcα1-6)Glcα1-4Glcα1-4Glcα1-4GlcPA (6^4-*O*-α-glucosyl-PA-maltooctaose). Similarly, the structures of peaks, γ2, γ5, γ6a and γ6b were determined to be 6^3-*O*-α-glucosyl-PA-maltooctaose, 6^5-*O*-α-glucosyl-PA-maltooctaose, 6^7-*O*-α-glucosyl-PA-maltooctaose and 6^6-*O*-α-glucosyl-PA-maltooctaose, respectively.

Peaks γ1, γ4 and γ7 were resistant to GDE. MALDI–MS showed that the molecular weights for these three compounds were all 1554, indicating that they must be pyridylaminated glucose nonamers. As for peak γ3, HPLC analysis of the partial acid hydrolysates were used to determine the branch point glucosyl residues of peaks γ1, γ4 and γ7. Peaks γ4 and γ7 were Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4(Glcα1-6)Glcα1-4GlcPA (6^2-*O*-α-glucosyl-PA-maltooctaose) and Glcα1-6Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4GlcPA (6^8-*O*-α-glucosyl-PA-maltooctaose), respectively. PA-isomaltose, Glcα1-6GlcPA, was detected in the partial acid hydrolysate of peak γ1, indicating that it was Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4(Glcα1-6)GlcPA (6^1-*O*-α-glucosyl-PA-maltooctaose). All 6-*O*-α-glucosyl-PA-maltooctaoses expected were identified and named FD 8-x (FD8-x, 6^x-*O*-α-glucosyl-PA-maltooctaose) as shown in Table 1.

Similarly, seven 6-*O*-α-glucosyl-PA-maltoheptaoses and five 6-*O*-α-glucosyl-PA-maltohexaoses were prepared from mono-6-*O*-α-glucosyl-β-cyclodextrin and mono-6-*O*-α-glucosyl-α-cyclodextrin, respectively. They were separated by preparative HPLC (Figs. 1B, 1β4, and 1C), and their structures were determined (Table 1). 6^1-*O*-α-Glucosyl-PA-maltohexaose was not found in the reaction mixture from 6-*O*-α-glucosyl-α-cyclodextrin.

6^3-*O*-α-Glucosyl-PA-maltotetraose and 6^3-*O*-α-glucosyl-PA-maltopentaose were prepared by partial glucoamylase digestion of 6^3-*O*-α-glucosyl-PA-maltohexaose. They were separated from the digest by preparative reversed-phase HPLC, and their structures were determined by HPLC analysis of the GDE digests and the partial acid hydrolysates (Table 1).

Susceptibility of 6-O-α-Glucosyl-PA-maltooligosaccharides to Hydrolysis by GDE—6-*O*-α-Glucosyl-PA-maltooligosaccharides were incubated with porcine liver GDE, and digests obtained at an early stage were analysed by reversed-phase HPLC. Most of the FDs were hydrolyzed by the enzyme to produce glucose and the corresponding PA-maltooligosaccharides. The rates of hydrolysis were calculated from the amounts of PA-maltooligosaccharides produced (Table 2).

6-*O*-α-Glucosyl-PA-maltooligosaccharide, $(Glc\alpha1\text{-}4)_m(Glc\alpha1\text{-}6)Glc\alpha1\text{-}4(Glc\alpha1\text{-}4)_nGlcPA$, is thought to be composed of the isomaltosyl moiety, the non-reducing-end maltooligosaccharide moiety $(Glc\alpha1\text{-}4)_m$ and the reducing-end PA-maltooligosaccharide moiety $(Glc\alpha1\text{-}4)_nGlcPA$. The α-1,6-glycosidic linkages of dextrins with $m=0$ or $n=0$ could not be hydrolyzed by the enzyme, indicating that at least the two glucosyl residues sandwiching the isomaltosyl moiety are indispensable for hydrolysis by the GDE amylo-α-1,6-glucosidase activity.

Table. 1. **Structural analysis of 6-*O*-α-glucosyl-PA-maltooligosaccharides.**

Peak	PA-maltooligosaccharide in the GDE digest	Molecular weight	The largest PA-maltooligosaccharide of the major products in the acid hydrolysate	Structure	Name
γ1	—[a]	1554	G[b] GPA	G GGGGGGGGPA	FD8-1[c]
γ2	GGGGGGGGPA	n.d.	GGPA	G GGGGGGGGPA	FD8-3
γ3	GGGGGGGGPA	n.d.	GGGPA	G GGGGGGGGPA	FD8-4
γ4	—	1554	GPA	G GGGGGGGGPA	FD8-2
γ5	GGGGGGGGPA	n.d.	GGGGPA	G GGGGGGGGPA	FD8-5
γ6a	GGGGGGGGPA	n.d.	GGGGGGPA	G GGGGGGGGPA	FD8-7
γ6b	GGGGGGGGPA	n.d.	GGGGGPA	G GGGGGGGGPA	FD8-6
γ7	—	1554	GGGGGGGPA	G GGGGGGGGPA	FD8-8
β1	—	1392	G[b] GPA	G GGGGGGGPA	FD7-1
β2	GGGGGGGPA	n.d.	GGPA	G GGGGGGGPA	FD7-3
β3	GGGGGGGPA	n.d.	GGGGGPA	G GGGGGGGPA	FD7-6
β4a	GGGGGGGPA	n.d.	GGGGPA	G GGGGGGGPA	FD7-5
β4b	—	1392	GPA	G GGGGGGGPA	FD7-2
β5	GGGGGGGPA	n.d.	GGGPA	G GGGGGGGPA	FD7-4
β6	—	1392	GGGGGGPA	G GGGGGGGPA	FD7-7
α1	—	1230	GGGGGPA	G GGGGGGPA	FD6-6
α2	GGGGGGPA	n.d.	GGGGPA	G GGGGGGPA	FD6-5
α3	GGGGGGPA	n.d.	GGGPA	G GGGGGGPA	FD6-4
α4	GGGGGGPA	n.d.	GGPA	G GGGGGGPA	FD6-3
α5	—	1230	GPA	G GGGGGGPA	FD6-2
a	GGGGPA	n.d.	GGPA	G GGGGPA	FD4-3
b	GGGGGPA	n.d.	GGPA	G GGGGGPA	FD5-3

[a]Resistant to GDE. [b]PA-isomaltose. [c]FD8-1 means fluorogenic dextrin, 6^1-*O*-α-glucosyl-PA-maltooctaose.
n.d., not determined; GPA, PA-glucose; GGPA, PA-maltose; GGGPA, PA-maltotriose; GGGGPA, PA-maltotetraose; GGGGGPA, PA-maltopentaose; GGGGGGPA, PA-maltohexaose; GGGGGGGPA, PA-maltoheptaose; GGGGGGGGPA, PA-maltooctaose;
G
G; isomaltosyl residue.

The α-1,6-glycosidic linkage can only be hydrolyzed when the core structure, Glcα1-4(Glcα1-6)Glcα1-4Glc, is recognized by the active site.

GDE has not only an amylo-α-1,6-glucosidase active site but also a 4-α-glucanotransferase active site, and 6-*O*-α-glucosyl-maltooligosaccharide structures are formed from the phosphorylase limit dextrin of glycogen by the GDE 4-α-glucanotransferase activity. We reasoned that if the FDs interact with the 4-α-glucanotransferase active site, the extent of the interaction would depend on the sizes of the dextrins and their concentrations. If this was correct, the relative rates of hydrolysis for some dextrins would vary according to their concentration; however, changing the concentration had little effect on the relative rate of hydrolysis for all of the dextrins (Table 2), suggesting that the dextrins did not interact strongly with the 4-α-glucanotransferase active site.

The hydrolysis rates at 16.7 μM substrate were approximately 3-fold higher than those at 5.67 μM (Table 2), indicating that the enzymatic reactions were carried out at substrate concentrations much lower than the Michaelis constants (K_m), where the rate of hydrolysis, v, was expressed by $v = (V_{max}/K_m) \times s$

Table 2. **Rates of hydrolysis of 6-*O*-α-glucosyl-PA-maltooligosaccharides by porcine liver GDE.**

Name	Structure	The rate of hydrolysis[a] at 16.7 μM substrate	at 5.67 μM substrate	
FD4-3	G GGGGPA	1.00[b]	0.340	(1.00)[c]
FD5-3	G GGGGGPA	10.1	3.51	(10.4)
FD6-2	G GGGGGGPA	0.00	0.00	(0.00)
FD6-3	G GGGGGGPA	17.1	5.43	(16.1)
FD6-4	G GGGGGGPA	5.67	1.89	(5.59)
FD6-5	G GGGGGGPA	0.278	0.966	(0.286)
FD6-6	G GGGGGGPA	0.00	0.00	(0.00)
FD7-2	G GGGGGGGPA	0.00	0.00	(0.00)
FD7-3	G GGGGGGGPA	25.0	7.80	(23.1)
FD7-4	G GGGGGGGPA	6.97	2.57	(7.59)
FD7-5	G GGGGGGGPA	3.91	1.38	(4.08)
FD7-6	G GGGGGGGPA	0.322	0.0949	(0.280)
FD7-7	G GGGGGGGPA	0.00	0.00	(0.00)
FD8-2	G GGGGGGGGPA	0.00	0.00	(0.00)
FD8-3	G GGGGGGGGPA	14.9	4.71	(13.9)
FD8-4	G GGGGGGGGPA	18.9	6.14	(18.2)
FD8-5	G GGGGGGGGPA	8.53	2.85	(8.43)
FD8-6	G GGGGGGGGPA	3.13	1.00	(2.97)
FD8-7	G GGGGGGGGPA	0.482	0.170	(0.503)
FD8-8	G GGGGGGGGPA	0.00	0.00	(0.00)

[a]Values are the rates relative to the rate for FD4-3 at 16.7 μM. The values are the means of triplicate measurements. The deviations of the three values from the means were <5%. [b]Taken as unity. [c]Values in parentheses are the rates relative to the rate for FD4-3 at 5.67 μM.

(*s*: the substrate concentration). $1/K_m$ is the equilibrium constant for formation of the enzyme-substrate complex, and V_{max}/e_0 is the rate constant (k_{+2}) for formation of the products from the enzyme-substrate complex (e_0: the enzyme concentration). It is, therefore, reasonable to evaluate the interactions between the substrates and the active site of amylo-α-1,6-glucosidase, based on the rates at a constant substrate concentration (16.7 μM or 5.67 μM).

Table 3 presents the results shown in Table 2 in a manner that highlights the dependence of the rate of hydrolysis on m or n. The rates increased with m and reached maximum at m = 4. Thus, some glucosyl residues other than the core structure likely interact with the active site. In contrast, the rates were the highest at n = 1 and did not vary much with changes in n. Among the homologous FDs, FD7-3 was the most readily hydrolyzed by GDE.

Characteristics of the Amylo-α-1,6-glucosidase Active Site—The active site of the GDE amylo-α-1,6-glucosidase must tightly bind certain 6-*O*-α-glucosyl-maltooligosaccharides. The active site can be considered to be composed of subsites (S_g, S_0, S_1, S_2, S_3, …, S_{-1}, S_{-2}, …) that are geometrically complementary to glucosyl residues in 6-*O*-α-glucosyl-maltooligosaccharides. S_g and S_0 are for the isomaltosyl residue and the α-1,6-glycosidic linkage is split between them (Fig. 3). The substrate binds to the active site, and each glucosyl residue interacts with the corresponding subsite. When G_g, G_0, G_1 and G_{-1} of the core structure interact with subsites S_g, S_0, S_1 and S_{-1}, respectively, the α-1,6-glycosidic linkage is hydrolyzed. Substrates that fit more tightly in the active site and interact with all of subsites are hydrolyzed the most rapidly. Among the homologous series, Glcα1-4Glcα1-4Glcα1-4Glcα1-4(Glcα1-6)Glcα1-4Glcα1-4GlcPA (FD7-3) was hydrolyzed most rapidly,

Table 3. **Dependence of the relative rate of hydrolysis at 16.7 μM substrate on m and n.**

Name	Structure	Rate at 16.7 μM	Name	Structure	Rate at 16.7 μM
	n = 1			m = 1	
FD8-3	G GGGGGGGGPA	14.9	FD8-7	G GGGGGGGGPA	0.482
FD7-3	G GGGGGGGPA	25.0	FD7-6	G GGGGGGGPA	0.322
FD6-3	G GGGGGGPA	17.1	FD6-5	G GGGGGGPA	0.278
FD5-3	G GGGGGPA	10.1			
FD4-3	G GGGGPA	1.00	FD4-3	G GGGGPA	1.00
	n = 2			m = 2	
FD8-4	G GGGGGGGGPA	18.9	FD8-6	G GGGGGGGGPA	3.13
FD7-4	G GGGGGGGPA	6.97	FD7-5	G GGGGGGGPA	3.91
FD6-4	G GGGGGGPA	5.67	FD6-4	G GGGGGGPA	5.67
			FD5-3	G GGGGGPA	10.1
	n = 3			m = 3	
FD8-5	G GGGGGGGGPA	8.53	FD8-5	G GGGGGGGGPA	8.53
FD7-5	G GGGGGGGPA	3.91	FD7-4	G GGGGGGGPA	6.97
FD6-5	G GGGGGGPA	0.278	FD6-3	G GGGGGGPA	17.1
				m = 4	
			FD8-4	G GGGGGGGGPA	18.9
			FD7-3	G GGGGGGGPA	25.0

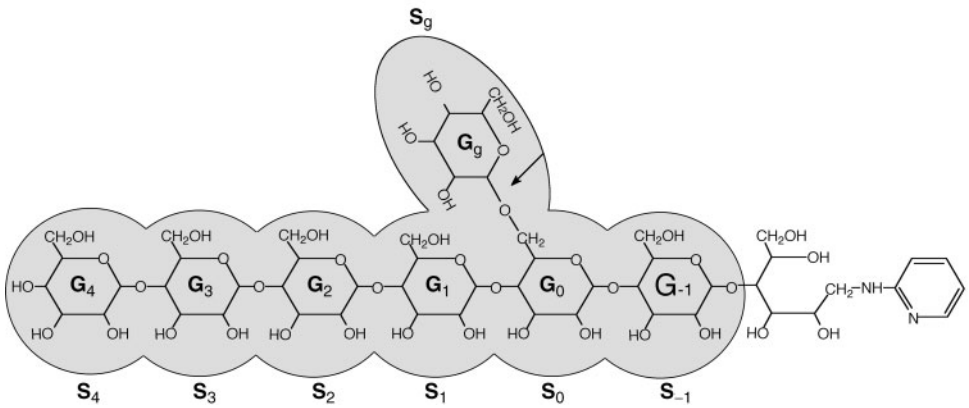

Fig. 3. **Schematic representation of the binding of 6^3-*O*-α-glucosyl-PA-maltoheptaose to the active site of GDE amylo-α-1,6-glucosidase.** The active site is composed of several subsites (S_g, S_0, S_1, S_2, S_3, . . ., S_{-1}, S_{-2}, . . .), and S_g and S_0 are for the isomaltosyl residue. When G_g, G_0, G_1 and G_{-1} of the substrate interact with subsites S_g, S_0, S_1 and S_{-1}, respectively, the α-1,6-glycosidic linkage is hydrolyzed. Our results suggested that the active site accommodates 6^2-*O*-α-glucosyl-maltohexaose.

suggesting that the active site accommodates Glcα1-4Glcα1-4Glcα1-4Glcα1-4(Glcα1-6)Glcα1-4Glc (6^2-*O*-α-glucosyl-maltohexaose) and is composed of seven subsites, S_g, S_0, S_1, S_2, S_3, S_4 and S_{-1}. The 1-deoxy-1-[(2-pyridyl)amino]-D-glucitol (GlcPA) residue is not a rigid glucopyranose ring but rather a flexible glucitol derivative. As shown in Fig. 3, it is expected to protrude from the active site. Notably, the interaction between the subsite S_2 and G_2 of the substrate strongly affects the rate of hydrolysis.

The rate of hydrolysis for FD8-3 was lower than that for FD7-3. Its 6^2-*O*-α-glucosyl-maltohexaose moiety is expected to bind tightly to the active site, while the other glucosyl moiety would protrude from the active site. The extra glucosyl moiety is likely to destabilize the enzyme-substrate complex, resulting in a lower rate of hydrolysis. Similarly, the higher rate of hydrolysis of FD4-3 ($m=1$, $n=1$), FD5-3 ($m=2$, $n=1$), FD6-3 ($m=3$, $n=1$) and FD7-3 ($m=4$, $n=1$) compared with the corresponding dextrins of $n=2$, 3 or 4 can be explained by the presence of moieties that protrude from the active site (Table 3).

X-ray crystallographic analysis of enzyme-inhibitor complexes is very useful for understanding the interaction between the active site and the substrate, but a study of a mammalian GDE-inhibitor complex has not yet been carried out. Instead, in the current study, the active site of porcine liver GDE was probed using a homologous series of fluorescently labelled 6-*O*-α-glucosyl-PA-maltooligosaccharides as substrates. Further mapping of the active site, including the role of ionic charges and hydrophobicity, can be accomplished using derivatives of 6^2-*O*-α-glucosyl-maltohexaoses containing ionic groups or hydrophobic atoms.

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