J. Biochem. **139**, 535–541 (2006)
doi:10.1093/jb/mvj056

Acceptor Specificity of 4-α-Glucanotransferases of Mammalian Glycogen Debranching Enzymes

Yasushi Makino and Kaoru Omichi*
Department of Chemistry, Graduate School of Science, Osaka Prefecture University, Sakai, Osaka 590-0035

Received September 28, 2005; accepted January 13, 2006

Glycogen debranching enzyme (GDE) has two distinct active sites for its 4-α-glucanotransferase and amylo-α-1,6-glucosidase activities. The GDE 4-α-glucanotransferases of mammals show stringent donor specificity; only α-glucans with an α-1,6–linked maltotetraosyl or maltotriosyl branch function as donors of a maltotriosyl or maltosyl residue. In this study, we investigated the acceptor specificity of the 4-α-glucanotransferases using methyl α-maltooligosides, *p*-nitrophenyl α-maltooligosides, and pyridylaminated maltooligosaccharides of various sizes as the acceptor substrates, and phosphorylase limit dextrin as the donor substrate. High-performance liquid chromatography analysis of the transfer products indicated that maltotriosyl and maltosyl residues were specifically transferred from phosphorylase limit dextrin to acceptors with a maltopentaosyl residue comprising a nonreducing-end. These results suggest that the acceptor binding sites in the active sites of mammalian GDE 4-α-glucanotransferases are composed of tandem subsites that are geometrically complementary to five glucose residues.

Key words: acceptor binding site, acceptor specificity, 4-α-glucanotransferase, glycogen debranching enzyme, transglycosylation.

Abbreviations: EDTA, ethylenediaminetetraacetic acid; GDE, glycogen debranching enzyme; Glc, D-glucose; GlcPA, 1-deoxy-1-[(2-pyridylamino)-D-glucitol]; HPLC, high-performance liquid chromatography; PA, pyridylamino; pNP, *p*-nitrophenyl.

Glycogen debranching enzyme (GDE) is a multicatalytic enzyme, containing distinct active sites for 4-α-glucanotransferase (1,4-α-glucan: 1,4-α-glucan 4-α-glycosyltransferase, EC 2.4.1.25) and amylo-α-1,6-glucosidase (glycogen phosphorylase limit dextrin α-1,6-glucohydrolase, EC 3.2.1.33) on a single polypeptide chain (*1*–*7*). This enzyme degrades glycogen in concert with glycogen phosphorylase (*8*). Glycogen phosphorylase catalyzes the removal of α-1,4-glucosyl residues from the outermost chains of the glycogen molecule until approximately four glucose residues remain on either side of the α-1,6-branching (*9*–*11*). GDE then removes the α-1,6–linked lateral branch to allow phosphorylase degradation to continue. The 4-α-glucanotransferase removes the maltooligosyl residue from the lateral branch by transglycosylation to expose a 6-*O*-α-glucosyl residue, and the amylo-α-1,6-glucosidase hydrolyzes the α-1,6-glycosidic linkage of the product.

A deficiency of GDE activity causes type III glycogen storage disease (*12*–*14*). Although measurement of GDE activity is very important for a clinical diagnosis, a universal assay method has not been established because the properties of GDE are not understood well enough. One of the most important characteristics of GDE is the substrate specificity of the 4-α-glucanotransferase. Knowledge of the substrate specificity is the basis for elucidating the catalytic mechanism, which will allow establishment of an enzyme assay.

It is well known that the GDE 4-α-glucanotransferases of mammals show stringent donor specificity (*15*, *16*). Only α-glucans with an α-1,6–linked maltotetraosyl or maltotriosyl branch function as donors of a maltotriosyl or maltosyl residue, and phosphorylase limit dextrin is considered to be the best donor substrate. Based on this donor specificity, the activity of a GDE 4-α-glucanotransferase has been assayed nonstoichiometrically by following changes in the spectrum of the complex between limit dextrin and iodine (*17*–*19*).

Although the donor specificity of the mammalian GDE 4-α-glucanotransferases is well-known, little is known about their acceptor specificity. In this study, we investigated their acceptor specificity by using phosphorylase limit dextrin as the donor substrate and maltooligosaccharide derivatives of various sizes as the acceptor substrates. By analyzing the transfer products directly, we showed that the chain length of the acceptor substrate significantly influences the rate of GDE transglycosylation.

MATERIALS AND METHODS

Materials—Glycogen, *p*-nitrophenyl α-D-glucopyranoside, α-cyclodextrin, maltose, and 2-aminopyridine were purchased from Wako Pure Chemicals (Osaka, Japan); methyl α-D-glucopyranoside was from Tokyo Kasei Kogyo (Tokyo, Japan); rabbit muscle phosphorylase a was from Sigma (St. Louis, Mo, USA); the Vivapore 10 concentrator (30,000 MW cut-off) was from Vivascience (Gloucestershire, UK); the Diaflo YM-30 membrane (30,000 MW cut-off) was from Millipore Japan (Tokyo, Japan); and the silver staining kit was from Bio-Rad (Richmond, CA, USA). Wakosil-II 5C18 HG columns were obtained from Wako Pure

*To whom correspondence should be addressed. Fax: +81-72-222-4791, E-mail: komichi@c.s.osakafu-u.ac.jp

Chemicals, the Shodex Asahipak NH2P-50 column from Showa Denko (Tokyo, Japan), the Super Q column and Toyopearl HW-40F from Tosoh (Tokyo, Japan), and DEAE-Sephacel and Sephacryl S-300 from Amersham Pharmacia Biotech (Uppsala, Sweden). Cyclodextrin glucanotransferase from *Bacillus macerans* was kindly donated by Amano Enzyme (Nagoya, Aichi, Japan).

Preparation of Phosphorylase Limit Dextrin from Glycogen—Phosphorylase limit dextrin was prepared from glycogen essentially according to the method of Hers *et al.* (*20*). A mixture of 1.0 g glycogen and 60 units of phosphorylase a in 50 ml of 0.1 M sodium phosphate buffer, pH 7.4, was incubated in a cellophane bag and dialyzed against 0.1 M sodium phosphate buffer, pH 7.4. After 24 h, 20 units of phosphorylase a were added to the internal solution, and then the incubation and dialysis were continued for 24 h. The enzymatic reaction was stopped by heating at 100°C for 5 min, and the insoluble material produced was removed by centrifugation. The resulting solution was dialyzed against water and then lyophilized.

Preparation of Methyl α-Maltooligosides—Methyl α-maltooligosides (Glc_n-O-CH_3, where Glc = D-glucose residue and n = degree of polymerization) with a degree of polymerization ranging from 2 to 7 were prepared from α-cyclodextrin and methyl α-D-glucopyranoside using cyclodextrin glucanotransferase, and then isolated by gel filtration on Toyopearl HW-40F followed by size-fractionation high-performance liquid chromatography (HPLC) (*16*).

Preparation of p-*Nitrophenyl α-Maltooligosides*—*p*-Nitrophenyl α-maltooligosides (Glc_n-O-pNP) with a degree of polymerization ranging from 2 to 7 were prepared from α-cyclodextrin and *p*-nitrophenyl α-D-glucopyranoside using cyclodextrin glucanotransferase, and then isolated by gel filtration on Toyopearl HW-40F followed by size-fractionation HPLC (*16*).

Preparation of Pyridylaminated Oligosaccharides—Pyridylaminated (PA-) maltose (Glc-GlcPA, where GlcPA = 1-deoxy-1-[(2-pyridylamino)-D-glucitol] residue) was prepared by pyridylamination of maltose as reported previously (*21*). PA-maltooligosaccharides (Glc_{n-1}-GlcPA) with a degree of polymerization ranging from 3 to 7 were prepared from α-cyclodextrin and PA-maltose using cyclodextrin glucanotransferase, and then isolated by reversed-phase HPLC (*16*).

Glcα1-4Glcα1-4(Glcα1-6)Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4GlcPA (FD8-6) was prepared from a mixture of 6-*O*-α-glucosyl-α-cyclodextrin and PA-maltose by transglycosylation with cyclodextrin glucanotransferase, and then purified by gel filtration on Toyopearl HW-40F followed by reversed-phase HPLC (*16*).

Measurement of Amylo-α-1,6-Glucosidase Activity—Amylo-α-1,6-glucosidase activity was measured using FD8-6 as the substrate as described previously (*16*, *22*). A mixture (50 μl) containing 100 μM FD8-6, 100 mM maleic acid–NaOH buffer (pH 6.0), 2 mM ethylenediaminetetraacetic acid (EDTA), 5 mM β-mercaptoethanol, and the enzyme preparation was incubated at 37°C for an appropriate period. The enzymatic reaction was stopped by adding 30 μl of 1 M acetic acid and heating at 100°C for 5 min. The fluorogenic product in the digest, Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4GlcPA (PA-maltooctaose, Glc_7-GlcPA), was measured by HPLC. One unit of debranching enzyme was defined as the amount of enzyme that releases 1 nmol of PA-maltooctaose per h under the conditions employed.

Transglycosylation Reaction with a Maltooligosaccharide Derivative as the Acceptor Substrate—4-α-Glucanotransferase activity was measured by using phosphorylase limit dextrin and a maltooligosaccharide derivative (Glc_n-O-CH_3, Glc_n-O-pNP, or Glc_{n-1}-GlcPA) as the donor and acceptor substrates of the maltooligosyl residue, respectively. A mixture (200 μl) containing 3% phosphorylase limit dextrin, 10 mM maltooligosaccharide derivative, 100 mM maleic acid–NaOH buffer (pH 6.0), 2 mM EDTA, and 5 mM β-mercaptoethanol was incubated with 30 units of GDE at 37°C for an appropriate period. The enzymatic reaction was stopped by heating at 100°C for 5 min. The transfer products formed were analyzed by HPLC.

HPLC of Maltooligosaccharide Derivatives—Size-fractionation HPLC of methyl and *p*-nitrophenyl α-maltooligosides was performed on a Shodex Asahipak NH2P-50 column (4.6 × 150 mm) at a flow rate of 0.9 ml/min. The eluent was a 4:1 (v/v) mixture of acetonitrile and water. The elution was monitored with an Erma RI detector Model ERC-7510.

Reversed-phase HPLC of methyl α-maltooligosides was performed on a Wakosil-II 5C18 HG column (1.0 × 25 cm). The column was eluted with water at a flow rate of 3.0 ml/min, and the elution was also monitored with an Erma RI detector (*23*).

Reversed-phase HPLC of *p*-nitrophenyl α-maltooligosides was also performed on a Wakosil-II 5C18 HG column (1.0 × 25 cm). The column was eluted with 50 mM ammonium acetate buffer, pH 4.5, containing 0.5% 1-butanol at a flow rate of 3.0 ml/min. The elution was monitored by measuring the absorbance at 307 nm due to the *p*-nitrophenyl residue with a Hitachi Variable Wavelength UV monitor Model 638-41.

PA-maltooligosaccharides were loaded on a Wakosil-II 5C18 HG column (6.0 × 150 mm) and eluted with 50 mM ammonium acetate buffer, pH 4.5, containing 0.05% 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).

Purification of GDE from Skeletal Muscles—The skeletal muscle enzymes were purified from porcine, bovine, and rabbit skeletal muscles, respectively, through the following five steps:

Step 1. Preparation of a crude enzyme solution: Skeletal muscle (50 g) was homogenized in 450 ml of 20 mM phosphate buffer, pH 6.5, containing 10 mM β-mercaptoethanol using a Potter-Elvehjem homogenizer. After the homogenate had been centrifuged at 10,000 × *g* for 25 min, the resulting supernatant was filtered through a Hyflo Super-Cel, and the filtrate was used as the crude enzyme solution.

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

Step 3. DEAE-Sephacel anion-exchange chromatography: The dialyzed solution (80 ml) was applied to a DEAE-Sephacel column (2.2 × 95 cm) equilibrated with 7 mM phosphate buffer, pH 6.5, containing 10 mM β-mercaptoethanol, and the column was washed with 200 ml of the same buffer. Amylo-α-1,6-glucosidase was eluted with a linear gradient of 7 mM to 0.65 M phosphate buffer (1.0 liter). The pooled solution containing amylo-α-1,6-glucosidase was concentrated by ultrafiltration using a Diaflo YM-30 membrane.

Step 4. Sephacryl S-300 gel filtration: The solution obtained in Step 3 was applied to a Sephacryl S-300 column (3.0 × 140 cm) equilibrated with 20 mM phosphate buffer, pH 6.5, containing 5 mM EDTA and 10 mM β-mercaptoethanol, and 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 YM-30 membrane and a Vivapore 10 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 5 mM β-mercaptoethanol at a flow rate of 1.0 ml/min. The solution obtained in Step 4 was injected into the column, and the column was then washed with 5 ml of the same buffer. Amylo-α-1,6-glucosidase was eluted with a linear gradient of 20 to 180 mM phosphate buffer delivered at 1.0 ml/min for 45 min. The pooled solution containing amylo-α-1,6-glucosidase was concentrated to a small volume using a Vivapore 10 membrane.

The enzymes purified from porcine, bovine, and rabbit skeletal muscles gave a single band on native polyacrylamide gel electrophoresis (PAGE), respectively (*24*).

Partial Purification of GDE from Livers—The porcine, bovine, and rabbit liver enzymes were partially purified, respectively, by ammonium sulfate precipitation, DEAE-Sephacel anion-exchange chromatography, and Sephacryl S-300 gel filtration, as for the skeletal muscle enzymes. Each enzyme preparation hydrolyzed FD8-6 to yield only PA-maltooctaose, and was free of α-glucosidase, α-amylase, and phosphorylase activities. Although the enzyme preparations were not completely purified, they were considered sufficiently pure for examining the enzymatic properties.

RESULTS

Transfer of Maltooligosyl Residues from Phosphorylase Limit Dextrin to Methyl α-Maltooligosides by Porcine Skeletal Muscle GDE—First of all, the transglycosylation reaction of porcine skeletal muscle GDE was examined using phosphorylase limit dextrin as the donor substrate and methyl α-maltoheptaoside (Glc_7-O-CH_3) as the acceptor substrate. The reaction mixture was analyzed by reversed-phase HPLC, as shown in Fig. 1A. Transfer products were detected at the elution positions of authentic Glc_{10}-O-CH_3 and Glc_9-O-CH_3; however, chain-shortened products (Glc-O-CH_3–Glc_6-O-CH_3) were not detected. These results indicated that maltotriosyl (Glc_3-) and maltosyl (Glc_2-) residues of phosphorylase limit dextrin were transferred to the nonreducing-end glucosyl residues of Glc_7-O-CH_3 to give Glc_{10}-O-CH_3 and Glc_9-O-CH_3, respectively. The time course of the action of porcine skeletal muscle GDE on phosphorylase limit dextrin and Glc_7-O-CH_3 is shown in Fig. 2A, where it can be seen that Glc_{10}-O-CH_3 and Glc_9-O-CH_3 increased linearly with time at a constant ratio of 97:3. The relationships between the enzyme concentration and the amounts of Glc_{10}-O-CH_3 and Glc_9-O-CH_3 were also linear under the conditions used (Fig. 2B).

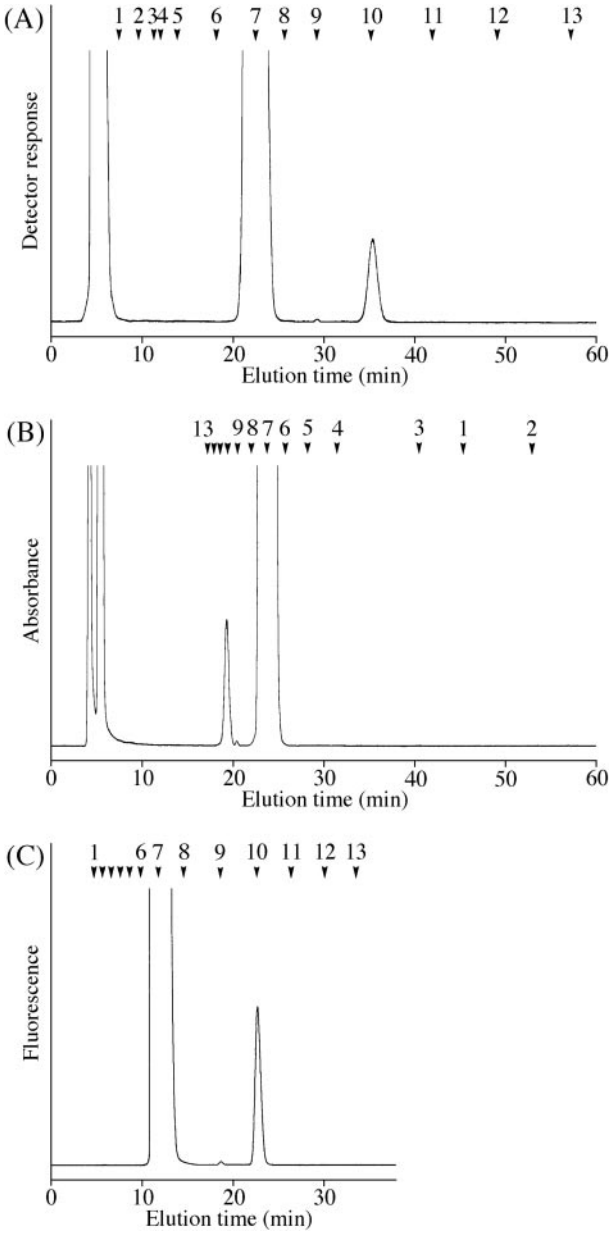

Fig. 1. **HPLC analysis of the products formed by GDE 4-α-glucanotransferase.** A mixture of phosphorylase limit dextrin and a maltoheptaose derivative (Glc_7-O-CH_3, Glc_7-O-pNP, or Glc_6-GlcPA) was incubated with porcine skeletal muscle GDE, and then the enzymatic reaction mixture was analyzed by reversed-phase HPLC. HPLC of the produced (A) methyl α-maltooligosides, (B) *p*-nitrophenyl α-maltooligosides, and (C) PA-maltooligosaccharides. Arrowheads 1–13 indicate the elution positions of (A) Glc-O-CH_3–Glc_{13}-O-CH_3, (B) Glc-O-pNP–Glc_{13}-O-pNP, and (C) GlcPA–Glc_{12}-GlcPA.

Next, methyl α-maltooligosides with a degree of polymerization ranging from 1 to 6 (Glc-O-CH_3–Glc_6-O-CH_3) were

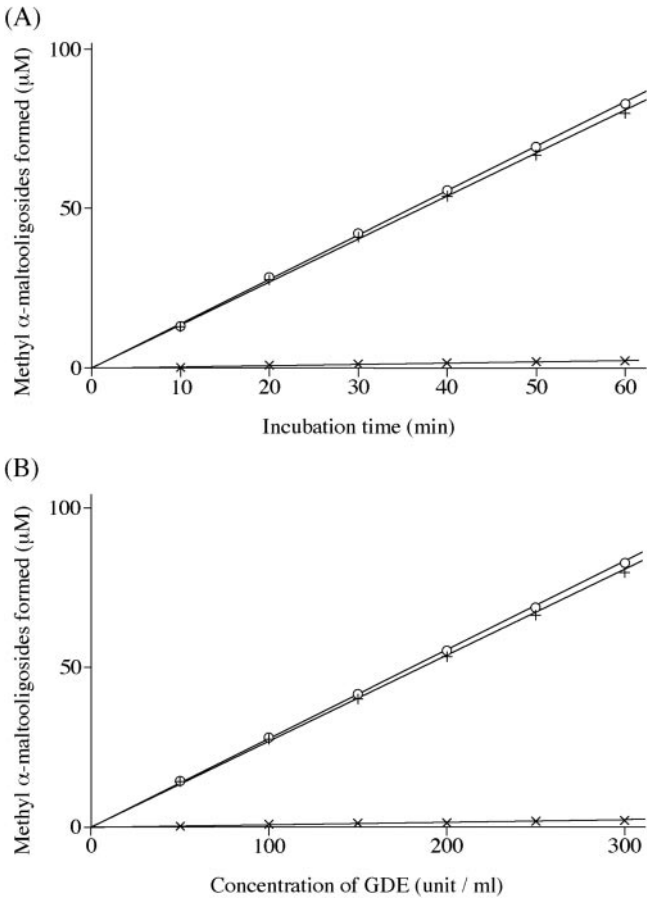

Fig. 2. **Transglycosylation from phosphorylase limit dextrin to Glc_7-O-CH_3 by porcine skeletal muscle GDE.** Glc_3- and Glc_2-residues of phosphorylase limit dextrin were transferred to the nonreducing-end glucosyl residues of Glc_7-O-CH_3 to give Glc_{10}-O-CH_3 and Glc_9-O-CH_3, respectively. These products were separated and quantified by HPLC. (A) Time courses of the formation of transfer products. (B) Relationships between the enzyme concentration and the amounts of transfer products. +, Glc_{10}-O-CH_3; ×, Glc_9-O-CH_3; O, total.

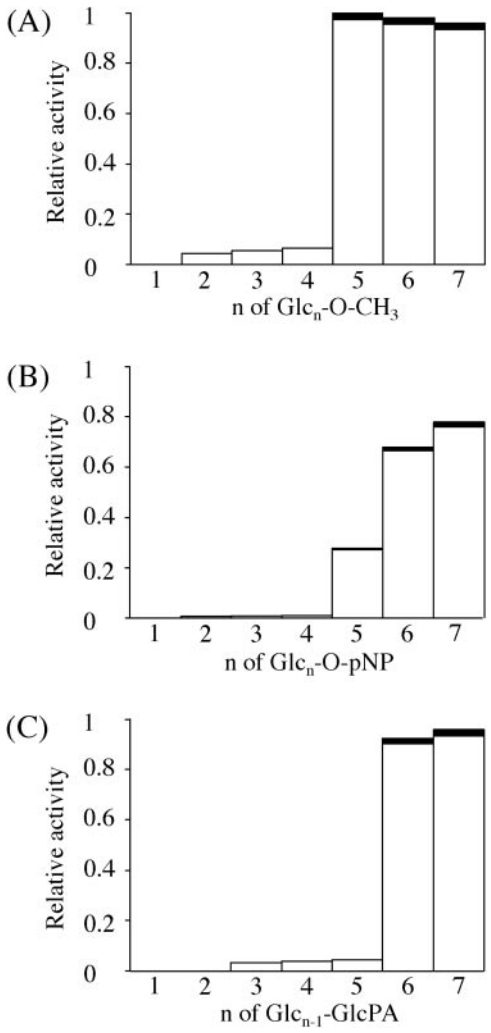

Fig. 3. **Eligibility of various sized maltooligosaccharide derivatives as acceptors for transglycosylation by porcine skeletal muscle GDE.** Acceptor specificity was examined using phosphorylase limit dextrin as the donor substrate and maltooligosaccharide derivatives of various sizes as the acceptor substrates. Transglycosylation activity toward (A) methyl α-maltooligosides, (B) *p*-nitrophenyl α-maltooligosides, and (C) PA-maltooligosaccharides. Activities were normalized by that toward Glc_5-O-CH_3. White and black columns indicate the relative levels of transfer of Glc_3- and Glc_2-residues, respectively.

used as the acceptor substrates (Fig. 3A). Glc_2-O-CH_3 and larger methyl α-maltooligosides were effective as acceptors, whereas Glc-O-CH_3 was not. Glc_3- and Glc_2-residues of the donor substrates were transferred to the nonreducing-end glucosyl residues of Glc_n-O-CH_3 to give Glc_{n+3}-O-CH_3 and Glc_{n+2}-O-CH_3, respectively. The ratio of the Glc_3- and Glc_2-transfer was 97:3, irrespective of the chain lengths of the acceptor substrates. The rates of transglycosylation to Glc_5-O-CH_3 and larger methyl α-maltooligosides were approximately 15-fold higher than those to Glc_2-O-CH_3, Glc_3-O-CH_3, and Glc_4-O-CH_3. Although the transglycosylation reaction was examined in the concentration ranges of 0.5–5% for the donor and 5–50 mM for the acceptor, the ratios of the reaction rates toward Glc-O-CH_3–Glc_7-O-CH_3 remained constant (data not shown).

Transfer of Maltooligosyl Residues from Phosphorylase Limit Dextrin to p*-Nitrophenyl α-Maltooligosides and PA-Maltooligosaccharides by Porcine Skeletal Muscle GDE*— To examine the transglycosylation reaction of porcine skeletal muscle GDE, *p*-nitrophenyl α-maltooligosides (Glc_n-O-pNP) of various sizes were used as the acceptor substrates (Figs. 1B and 3B). A series of *p*-nitrophenyl α-maltooligosides can be sensitively detected and conveniently quantified using a UV monitor, although the *p*-nitrophenyl group is bulky compared to a methyl group. Glc_2- and larger derivatives were effective as acceptors, and the ratio of the Glc_3- and Glc_2-transfer was 97:3, as in the case of methyl α-maltooligosides. The results shown in Fig. 3B are basically similar to those in Fig. 3A; however, the transglycosylation activities toward *p*-nitrophenyl α-maltooligosides, especially those with a degree of polymerization ranging from 2 to 5, were relatively low. These results were also obtained irrespective of the concentrations of the donor (0.05–5%) and acceptor (0.5–50 mM) (data not shown).

We further examined the transglycosylation reaction using PA-maltooligosaccharides (Glc_{n-1}-GlcPA) of various sizes as the acceptor substrates (Figs. 1C and 3C). Excellent separation, highly sensitive detection, and convenient quantification can be achieved using a series of PA-maltooligosaccharides, although the reducing-end GlcPA-residue cannot form a pyranose-ring (*25*). The results shown in Fig. 3C are almost the same as those in Fig. 3A if reducing-end GlcPA-residues of PA-maltooligosaccharides are not included in the count of the degree of polymerization. These results were also obtained irrespective of the concentrations of the donor (0.05–5%) and acceptor (0.05–50 mM) (data not shown).

Acceptor Specificity of 4-α-Glucanotransferase of Porcine Skeletal Muscle GDE—The results in Fig. 3 indicate that the chain length of the acceptor substrate significantly influences the rate of GDE transglycosylation. In particular, it has been shown that a Glc_2-residue comprising the nonreducing-end of an acceptor substrate is indispensable for GDE transglycosylation. Furthermore, it should be pointed out that the rates of transglycosylation to Glc_5- and larger derivatives were approximately 15-fold higher than those to Glc_2-, Glc_3-, and Glc_4-derivatives. These results indicate that the acceptor binding sites in the active sites of GDE 4-α-glucanotransferases accommodate five glucose residues and interact with a Glc_5-residue comprising the nonreducing-end of the acceptor.

Because maltooligosaccharides (Glc_n∼OH) in aqueous solution are in equilibrium between α- and β-anomers, a series of methyl α-maltooligosides (Glc_n-O-CH_3) is generally regarded as the best acceptor substrates for mapping the acceptor binding site of GDE 4-α-glucanotransferase. The bulky *p*-nitrophenyl group is expected to influence the binding of *p*-nitrophenyl maltooligoside to the acceptor binding site, and it should be preferable that the bulky group is outside of the active site. Although the PA-group is also bulky, a long and flexible glucitol group may keep it away from the acceptor binding site.

Comparison of Acceptor Specificities of Mammalian GDE 4-α-Glucanotransferases—In mammals, GDE is reported to exist in two isoforms; muscle and liver ones (*26*). The acceptor specificities of GDE 4-α-glucanotransferases from porcine muscle and liver were compared by using methyl α-maltooligosides (Glc_n-O-CH_3) of various sizes as the acceptor substrates and phosphorylase limit dextrin as the donor substrate. No difference was detected between the acceptor specificities of these isozymes (data not shown). Furthermore, the acceptor specificities of the bovine and rabbit GDE 4-α-glucanotransferases are almost the same as that of the porcine enzymes (data not shown). These results suggest that the acceptor specificity is basically common to mammalian GDE 4-α-glucanotransferases.

DISCUSSION

GDE has the active site of amylo-α-1,6-glucosidase as well as that of 4-α-glucanotransferase. The active site of amylo-α-1,6-glucosidase also interacts with both donor and acceptor molecules. Thus, it is difficult to extract the interaction of the maltooligosaccharide derivatives with the acceptor binding site only, and to evaluate it exactly as the kinetic parameters, K_m and V_{max}. In this study, we estimated the interaction from the experimental values of the rates of transglycosylation reactions with maltooligosaccharide derivatives of various sizes at constant concentrations. The ratios of the reaction rates obtained were independent of both the donor and acceptor concentrations, indicating that the estimation is appropriate.

It is conceivable that the acceptor binding sites of mammalian GDE 4-α-glucanotransferases are composed of tandem subsites geometrically complementary to several glucose residues, similar to that of Taka-amylase A (*27*) or hen egg white lysozyme (*28*) (Fig. 4). Subsite AS1 is a binding site for the nonreducing-end glucosyl residue of the acceptor substrate, and the maltooligosyl residue of the donor substrate is transferred to the AS1-binding glucosyl residue of the acceptor substrate. Following subsite AS1, there should be four subsites (AS2–AS5) in the acceptor binding sites of mammalian GDE 4-α-glucanotransferases because the enzymes showed a strong preference for acceptors with a Glc_5-residue comprising a nonreducing-end. Filling of subsite AS2 with the corresponding glucosyl residue of the acceptor substrate should be indispensable for GDE transglycosylation based on the fact that Glc-O-CH_3, Glc-O-pNP, and Glc-GlcPA were ineffective as acceptor substrates. The relatively low rates of transglycosylation to *p*-nitrophenyl maltooligosides with a degree of polymerization ranging from 2 to 5 can be explained by the idea that filling of subsites AS1–AS5 with the corresponding glucosyl residues of these substrates may be somewhat difficult because of steric hindrance by the *p*-nitrophenyl group (Fig. 4B). Although the PA-group is also bulky, a long and flexible glucitol group may keep it away from the acceptor binding site (Fig. 4, C-I). The glucitol group cannot fit into subsite AS5 because each of the subsites accommodates a rigid pyranose-ring residue (Fig. 4, C-II).

Previously, Takrama and Madsen studied the stabilization of sulfhydryl groups of rabbit muscle GDE by binding a series of maltooligosaccharides (Glc_n∼OH) (*29*). Their studies indicated that the enzyme contains at least two binding sites for maltooligosyl residues. They further speculated that one of these may be the acceptor binding site for the 4-α-glucanotransferase and that it may have four subsites, but this hypothesis has not been substantiated by further experiments. In the present study, we examined the transglycosylation reactions of mammalian GDE 4-α-glucanotransferases by using phosphorylase limit dextrin as the donor substrate and maltooligosaccharide derivatives (Glc_n-O-CH_3, Glc_n-O-pNP, and Glc_{n-1}-GlcPA) of various sizes as the acceptor substrates. By analyzing the transfer products directly, we elucidated that the acceptor binding sites of the enzymes are composed of five subsites.

One of the major benefits of five subsites is that α-1,6–linked Glc_4- and Glc_3-residues of phosphorylase limit dextrin function as donors of Glc_3- and Glc_2-residues rather than acceptors of them. This improves the efficiency of removing the phosphorylase limit branch, thereby contributing to a rapid restart of phosphorylase degradation. The ability to rapidly mobilize glucose residues from glycogen is essential for mammals, for example, in muscle during exercise. Although phosphorylase degradation of glycogen results in the release of many glucose 1-phosphate (Glc-O-PO_3^{2-}) molecules, GDE does not transfer

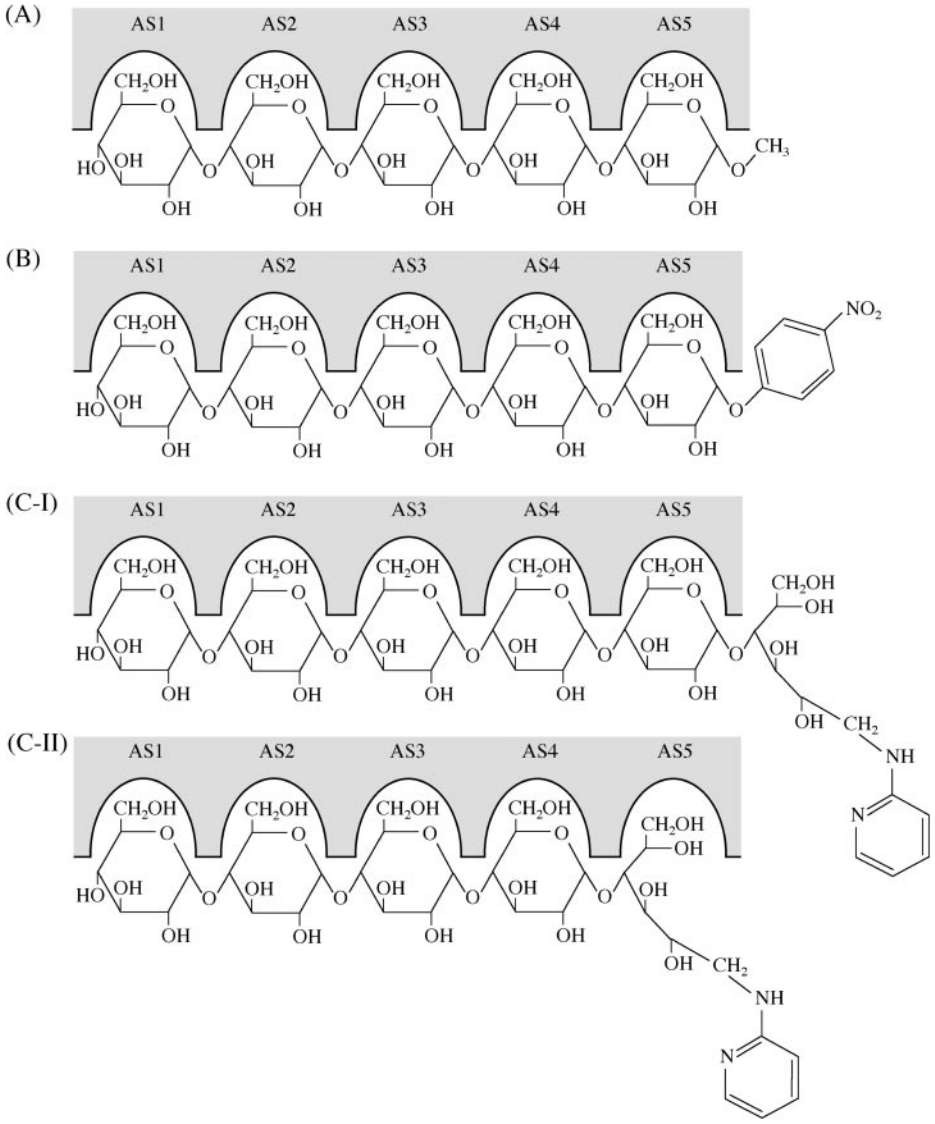

Fig. 4. **Schematic representation of acceptor binding to the active site of mammalian GDE 4-α-glucanotransferase.** Our results suggest that the acceptor binding site in the active site of mammalian GDE 4-α-glucanotransferase consists of five subsites (AS1–AS5). Subsite AS1 is a binding site for the nonreducing-end glucosyl residue of the acceptor substrate, and the maltooligosyl residue of the donor substrate is transferred to the AS1-binding glucosyl residue of the acceptor substrate. Binding of (A) Glc_5-O-CH_3, (B) Glc_5-O-pNP, (C-I) Glc_5-GlcPA, and (C-II) Glc_4-GlcPA.

a maltooligosyl residue to them because the binding between subsite AS2 and the corresponding glucosyl residue of the acceptor substrate is indispensable for transglycosylation.

REFERENCES

1. Abdullah, M. and Whelan, W.J. (1963) A new pathway in rabbit muscle for the enzymatic debranching of glycogen. *Nature* **197**, 979–980
2. Brown, D.H., Illingworth, B., and Cori, C.F. (1963) Combined action of oligo-1,4→1,4-glucantransferase and amylo-1,6-glucosidase in debranching glycogen. *Nature* **197**, 980–982
3. Gordon, R.B., Brown, D.H., and Brown, B.L. (1972) Preparation and properties of the glycogen-debranching enzyme from rabbit liver. *Biochem. Biophys. Acta* **289**, 97–107
4. Bates, E.J., Heaton, G.M., Taylor, C., Kernohan, J.C., and Cohen, P. (1975) Debranching enzyme from rabbit skeletal muscle; evidence for the location of two active centres on a single polypeptide chain. *FEBS Lett.* **58**, 181–185
5. Taylor, C., Cox, A.J., Kernohan, J.C., and Cohen, P. (1975) Debranching enzyme from rabbit skeletal muscle; purification, properties and physiological role. *Eur. J. Biochem.* **51**, 105–115
6. Liu, W., Madsen, N.B., Braun, C., and Withers, S.G. (1991) Reassessment of the catalytic mechanism of glycogen debranching enzyme. *Biochemistry* **30**, 1419–1424
7. Nakayama, A., Yamamoto, K., and Tabata, S. (2001) Identification of the catalytic residues of bifunctional glycogen debranching enzyme. *J. Biol. Chem.* **276**, 28824–28828
8. Roach, P.J. (2002) Glycogen and its metabolism. *Curr. Mol. Med.* **2**, 101–120
9. Walker, G.T. and Whelan, W.J. (1960) The mechanism of carbohydrate action. 8. Structures of the muscle-phosphorylase limit dextrins of glycogen and amylopectin. *Biochem. J.* **76**, 264–268
10. Newgard, C.B., Hwang, P.K., and Fletterick, R.J. (1989) The family of glycogen phosphorylase: structure and function. *Crit. Rev. Biochem. Mol. Biol.* **24**, 69–99
11. Johnson, L.N. (1992) Glycogen phosphorylase: control by phosphorylation and allosteric effectors. *FASEB J.* **6**, 2274–2282
12. Howell, R.R. and Williams, J.C. (1983) The glycogen storage disease in *The Metabolic Basis of Inherited Disease* (Stanbury, J.B., Wyngaarden, J.B., Fredrickson, D.S., Goldstein, J.L., and Brown, M.S., eds.) pp. 141–166, McGraw-Hill, New York

13. Chen, Y.T. and Burchell, A. (1995) Glycogen storage disease in *The Metabolic and Molecular Bases of Inherited Disease* (Scriver, C.R., Beaudet, A.L., Sly, W.S., and Valle, D., eds.) pp. 935–965, McGraw-Hill, New York
14. Shen, J.J. and Chen, Y.T. (2002) Molecular characterization of glycogen storage disease type III. *Curr. Mol. Med*. **2**, 167–175
15. Lee, E.Y.C. and Whelan, W.J. (1972) Glycogen and starch debranching enzymes in *Enzymes* (Boyer, P.D., ed.) pp. 191–234, Academic Press, New York
16. Watanabe, Y., Makino, Y., and Omichi, K. (2005) Fluorogenic substrates of glycogen debranching enzyme for the assaying debranching activity. *Anal. Biochem.* **340**, 279–286
17. Nelson, T.E., Palmer, D.H., and Larner, J. (1970) An investigation of properties of rabbit muscle oligo-1,4→1,4-glucantransferase. *Biochim. Biophys. Acta*. **212**, 269–280
18. Gillard, B.K. and Nelson, T.E. (1977) Amylo-1,6-glucosidase/4-α-glucanotransferase: use of reversible substrate model inhibitors to study the binding and active sites of rabbit muscle debranching enzyme. *Biochemistry* **16**, 3978–3987
19. Liu, W., Madsen, N.B., Fan, B., Zucker, K.A., Glew, R.H., and Fry, D.E. (1995) Effects of oligosaccharide binding on glycogen debranching enzyme activity and conformation. *Biochemistry* **34**, 7056–7061
20. Hers, H.G., Verhue, W., and Hoof, F.V. (1967) The determination of amylo-1,6-glucosidase. *Eur. J. Biochem*. **2**, 257–264
21. Kuraya, N. and Hase, S. (1992) Release of *O*-linked sugar chains from glycoproteins with anhydrous hydrazine and pyridylamination of the sugar chains with improved reaction conditions. *J. Biochem*. **112**, 122–126
22. Omichi, K. and Hase, S. (1998) An assay method for glycogen debranching enzyme using new fluorogenic substrates and its application to detection of the enzyme in mouse brain. *J. Biochem*. **123**, 932–936
23. Omichi, K. and Ikenaka, T. (1986) Inspection of active sites of human salivary α-amylase isozymes by means of non-reducing-end substituted maltooligosaccharides with 2-pyridylamino residue. *J. Biochem*. **99**, 1245–1252
24. Davis, B.J. (1964) Disk electropholesis. II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci*. **121**, 404–427
25. Hase, S., Ikenaka, T., and Matsushima, Y. (1978) Structure analyses of oligosaccharides by tagging of the reducing end sugars with a fluorescent compound. *Biochem. Biophys. Res. Commun*. **85**, 257–263
26. Bao, Y., Dawson, T.L., and Chen, Y.T. (1996) Human glycogen debranching enzyme gene (AGL): complete structural organization and characterization of the 5′ flanking region. *Genomics* **38**, 155–165
27. Matsuura, Y., Kusunoki, M., Harada, W., and Kakudo, M. (1984) Structure and possible catalytic residues of Taka-amylase A. *J. Biochem*. **95**, 697–702
28. Blake, C.C.F., John, L.N., Mair, G.A., North, A.C.T., Phillips, D.C., and Sarma, V.R. (1967) Crystallographic studies of the activity of hen egg-white lysozyme. *Proc. R. Soc*. **167**, 378–388
29. Takrama, J. and Madsen, N.B. (1988) Binding of glycogen, oligosaccharides and glucose to glycogen debranching enzyme. *Biochemistry* **27**, 3308–3314