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Deciphering the Molecular Basis of the Broad Substrate Specificity of α-Glucosidase from *Bacillus* sp. SAM1606

Akio Noguchi[1], Masayasu Yano[1], Yasuhiro Ohshima[1], Hisashi Hemmi[1], Misa Inohara-Ochiai[2], Maki Okada[2], Kyong-Son Min[3], Toru Nakayama[*,1] and Tokuzo Nishino[1]

[1]Department of Biomolecular Engineering, Graduate School of Engineering, Tohoku University, Aobayama 07, Aoba-ku, Sendai, Miyagi 980-8579, [2]Institute for Advanced Technology, Suntory Ltd., Yamazaki, Mishima-gun, Shimamoto-cho, Osaka 618-0001, and [3]Department of Nutrition, Kobe Gakuin University, Nishi-ku, Kobe, Hyogo 651-2180

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The α-glucosidase of *Bacillus* sp. strain SAM1606 is a member of glycosyl hydrolase family 13, and shows an extraordinarily broad substrate specificity and is one of very few α-glucosidases that can efficiently hydrolyze the α-1,1-glucosidic linkage of α,α′-trehalose (trehalose). Phylogenetic analysis of family-13 enzymes suggests that SAM1606 α-glucosidase may be evolutionally derived from an α-1,6-specific ancestor, oligo-1,6-glucosidase (O16G). Indeed, replacement of Pro[273*] and Thr[342*] of *B. cereus* O16G by glycine and asparagine (the corresponding residues in the SAM1606 enzyme), respectively, was found to cause 192-fold enhancement of the relative catalytic efficiency for trehalose, suggesting that O16G may easily "evolved" into an enzyme with an extended substrate specificity by substitution of a limited number of amino acids, including that at position 273* (an asterisk indicates the amino-acid numbering of the SAM1606 sequence). To probe the role of the amino acid at position 273* of α-glucosidase in determination of the substrate specificity, the amino acid at position 273 of SAM1606 α-glucosidase was replaced by all other naturally occurring amino acids, and the resultant mutants were kinetically characterized. The results showed that substitution of bulky residues (*e.g.*, isoleucine and methionine) for glycine at this position resulted in large increases in the K_m values for trehalose and maltose, whereas the affinity to isomaltose was only minimally affected by such an amino-acid substitution at this position. Three-dimensional structural models of the enzyme-substrate complexes of the wild-type and mutant SAM1606 α-glucosidases were built to explore the mechanism responsible for these observations. It is proposed that substitution by glycine at position 273* could eliminate steric hindrance around subsite +1 that originally occurred in parental O16G and is, at least in part, responsible for the acquired broad substrate specificity of SAM1606 α-glucosidase.

Key words: α-amylase family, α-glucosidase, glycosyl hydrolase, molecular evolution, oligo-1,6-glucosidase, substrate specificity.

Abbreviation: O16G, oligo-1,6-glucosidase.
Amino-acid residues of enzymes (other than SAM1606 α-glucosidase) are numbered so as to correspond to the numbering of SAM1606 α-glucosidase on the basis of multiple alignment (see Fig. 1 for an example), and positional numbering according to this notation is indicated by an asterisk.
Subsites are numbered according to the nomenclature of Davies *et al*. (*31*).

α-Glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20) catalyzes the hydrolysis of 1-*O*-α-D-glucopyranosides with net retention of the anomeric configuration (*1*, *2*). Two phylogenetically distinct classes of α-glucosidases are known, one of which belongs to glycosyl hydrolase family 13 [also called "the α-amylase family" (*3–5*)] and the other to family 31 (*1*, *5*). The substrate specificities of α-glucosidases may be described in terms of the α-glucosidic linkage types of substrate diglucoses (*e.g*., α-1,4-specific or α-1,6-specific), aglycon specificity (*e.g*., aryl-specific *versus* alkyl-specific), and substrate chain-length specificity (*i.e.*, the ability to act on tri- and higher oligosaccharides), and these specificities greatly differ with the source of the enzyme (*1*, *2*, *6*). This may be, at least in part, due to a difference in the three-dimensional structures of subsites +1 and +2 of enzymes (*5*).

The α-glucosidase of *Bacillus* sp. strain SAM1606 is a member of family 13 that shows an extraordinarily broad substrate specificity—the enzyme can efficiently hydrolyze α-1,1-, α–1,3-, α-1,4-, and α-1,6-linked diglucoses as well as sucrose and 1-*O*-aryl α-D-glucosides (*7*, *8*). Indeed, it is one of very few α-glucosidases that can efficiently hydrolyze the α-1,1-glucosidic linkage of α,α′-trehalose (trehalose). The primary structure of this enzyme is similar to that of *Bacillus cereus* ATCC7064 oligo-1,6-glucosidase (dextrin 6-α-glucanohydrolase, EC 3.2.1.10; termed O16G; identity, 67%; see Fig. 1), which is highly specific for the α-1,6-linkage (*8*, *9*). Indeed, phylogenetic

[*]To whom correspondence should be addressed. Fax: +81-22-217-7293, E-mail: nakayama@seika.che.tohoku.ac.jp

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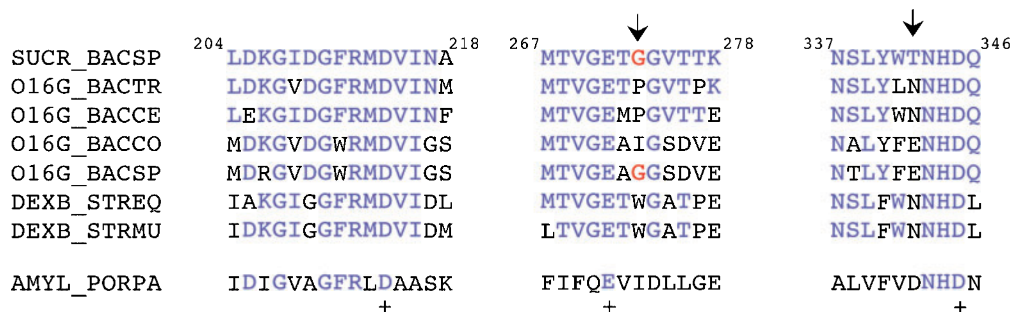

Fig. 1. **Comparison of the amino acid sequences containing putative catalytic residues of members of a subgroup of family-13 α-glucosidases.** The amino acid sequences of the suggested subgroup members of glycosyl hydrolase family 13 (upper 7 sequences, see also Fig. 2) are shown in a one-letter notation, along with that of porcine pancreatic α-amylase (bottom sequence). The enzymes are: SUCR_BACSP, *Bacillus* sp. SAM1606 α-glucosidase (*8*); O16G_BACTR; O16G of *B. thermoglucosidasius* (*19*); O16G_BACCE, O16G of *B. cereus* (*9*); O16G_BACCO, O16G of *B. coagulans* (*20*); O16G_BACSP, O16G of *Bacillus* sp. (*18*); DEXB_STREQ, dextran glucosidase of *S. equisimilis* (*21*); DEXB_STRMU, dextran glucosidase of *Streptococcus mutans* (*22*); and AMYL_PORPA, porcine pancreatic α-amylase (*23*). Amino acid residues identical to those of SUCR_BACSP are shown in red (for position 273*) and blue (for other positions). Putative catalytic residues demonstrated by X-ray crystallographic studies of several α-amylase family enzymes are indicated below the sequences by plus signs. Arrows above the sequences indicate the amino acid residues that were the targeted sites for mutagenesis in this study. The amino acid numbering is based on that of the SUCR_BACSP sequence.

analysis of SAM1606 α-glucosidase suggests that SAM1606 α-glucosidase is closely related to O16G in its molecular evolution (Fig. 2; Ref. *5*).

We previously showed, by means of comparative site-directed mutagenesis studies, that replacement of Gly^{273} of SAM1606 α-glucosidase with proline, the amino-acid residue located at the corresponding position in O16G, caused a specific and significant decrease in the enzyme's affinity to trehalose, although the catalytic efficiencies for its hydrolysis of isomaltose, maltose, and sucrose changed only minimally upon this mutation (*10*). This effect of the Gly^{273}→Pro substitution was specifically enhanced by the Thr^{342}→Asn substitution. Position 273 of SAM1606 α-glucosidase is involved in a sequence that is highly conserved among family-13 enzymes (Fig. 1) and is located in close proximity to the putative catalytic residues (Asp^{214}, Glu^{271}, and Asp^{345}) of this enzyme, as predicted on the basis of the published crystal structure of *B. cereus* O16G (*8*, *11*). Currently, the mechanistic details of specificity determination by the amino-acid residue at position 273 of SAM1606 α-glucosidase remain to be clarified and, hence, it is still unclear whether the amino acid at this position must exclusively be glycine for the enzyme's ability to act on trehalose.

In this study, Gly^{273} of SAM1606 α-glucosidase was replaced by all other naturally occurring amino acids, and some of the resultant mutant enzymes were kinetically characterized to probe the molecular basis of the broad substrate specificity of SAM1606 α-glucosidase. It was found that the substrate specificity of this enzyme was significantly affected by the nature of the amino acid at position 273. The results are explained on the basis of the modeled three-dimensional structures of the enzyme-substrate complexes, and the role of the amino-acid residue at position 273 in determination of the specificity of this enzyme is discussed.

MATERIALS AND METHODS

Chemicals—*p*-Nitrophenyl 1-*O*-α-D-glucopyranoside, maltose, isomaltose, and sucrose, all of analytical grade, were obtained from Nacalai Tesque, Kyoto. Trehalose was the product of Merck, Darmstadt, Germany. Maltose was freed from contaminating glucose by high-performance liquid chromatography on an Asahipak NH2P50 column (1 cm × 25 cm; Shoko, Tokyo) using a Shimadzu LC9A system in which 70% (v/v) CH_3CN in H_2O was isocratically developed at a flow rate of 2.0 ml/min by monitoring using thin layer chromatography. To determine the exact concentration of isomaltose, which was supplied as syrup containing water, isomaltose was hydrolyzed in 1 M HCl at 100°C for 24 h. After neutralization with NaOH, the glucose formed was determined by the method of Pütter and Becker (*12*) using a kit (Roche Diagnostics, Mannheim, Germany). For all other chemicals, the purest reagents available were used.

Bacterial Strains and Plasmids—Construction of plasmid pGBSU5, which encodes the gene coding for the wild-type SAM1606 α-glucosidase, was described previously (*10*). *B. cereus* ATCC7064 (*9*) was obtained from the American Type Culture Collection (Manassas, Virginia, USA). The gene encoding O16G (*9*) was amplified by PCR using chromosomal DNA of *B. cereus* ATCC7064 as a template, and primers O16G-Fw (5′-CTAATGATGAATGAAATGGATCCCATAATGG-3′) and O16G-Rev (5′-CCAAAACGGTATAAGGATCCTTCTTATTTC-3′), where the underlining indicates the *Bam*HI site. The thermal cycling conditions were 94°C for 5 min for denaturation, followed by 30 cycles of 94°C for 30 s for denaturation, 45°C for 30 s for annealing, and 72°C for 1 min for extension, and then 72°C for 10 min for extension. The entire nucleotide sequence of the amplified DNA was confirmed by sequencing in both orientations using a CEQ2000XL DNA Analysis system (Beckman Coulter, California, USA). The amplified DNA was digested with *Bam*HI and then inserted into the *Bam*HI site of the pET15b vector (Novagen, Madison, Wisconsin, USA) to obtain plasmid pO16G, which encodes the full-length O16G gene.

Site-Directed Mutagenesis and Expression—*In vitro* mutagenesis of the SAM1606 α-glucosidase gene was carried out with the *Hinc*II/*Eco*RI fragment of the α-glucosidase gene [*i.e.*, fragment III of plasmid pGBSU5 (*10*)] by

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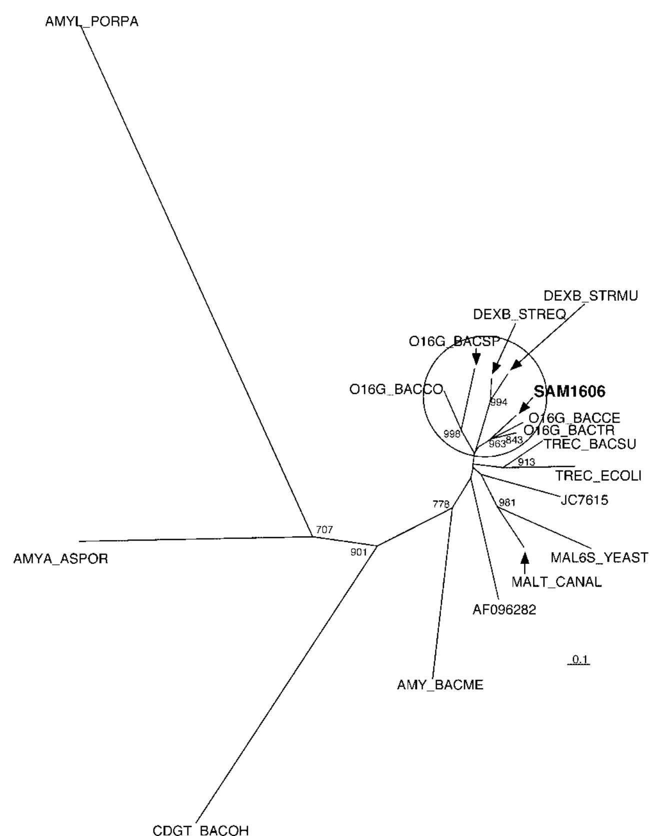

Fig. 2. **A non-rooted phylogenetic tree derived from the amino acid sequences of family-13 α-glucosidases and related enzymes.** The sequence names are as follows: O16G_BACCO, O16G of *Bacillus coagulans* (*20*); O16G_BACSP, "O16G" of *Bacillus* sp. strain F5 (*18*); DEXB_STRMU, dextran glucosidase of *Streptococcus mutans* (*22*); DEXB_STREQ, dextran glucosidase of *S. equisimilis* (*21*); SUCR_BACSP, *Bacillus* sp. SAM1606 α-glucosidase (*8*); O16G_BACCE, *B. cereus* ATCC7064 (*9*); O16G_BACTR, O16G of *B. thermoglucosidasius* KP1006 (*20*); TREC_BACSU, trehalose-6-phosphate hydrolase of *B. subtilis* (P39795); TREC_ECOLI, trehalose-6-phosphate hydrolase of *Escherichia coli* (*24*); JC7615, α-glucosidase of *Penicillium minioluteum* (*25*); MALS6_YEAST, α-glucosidase of *Saccharomyces cerevisiae* (*26*); MALT_CANAL, α-glucosidase of *Candida albicans* (*27*); AF096282, α-glucosidase of *Thermus aquaticus caldophilius* (Genbank accession number, AF096282); AMY_BACME, α-amylase of *B. megaterium* (*28*); CGT_BACOH, cyclomaltodextrin glucanotransferase of *B. ohbensis* (*29*); AMYL_ASPOR, α-amylase of *Aspergillus oryzae* (*30*); and AMYL_PORPA, porcine pancreatic α-amylase (*23*). The numbers indicate bootstrap values greater than 700. Bar, 0.1 nucleotide substitutions per site.

the method of Kunkel (*13*) using a kit (Mutan K; Takara Shuzo, Kyoto). Eighteen mutants having an amino-acid substitution at position 273 were prepared using specific mutagenesis oligonucleotides (not shown). To facilitate the identification of mutated fragments, base substitutions that result in a Gly^{273}→Ser substitution were first introduced, producing an *Spe*I site in the fragment. All other mutations were made with this fragment; therefore, individual mutations could be easily identified from the loss of the *Spe*I site. Individual mutations were verified by DNA sequencing in both orientations. Replacement of fragment III in pGBSU5 with the mutated fragment III allowed us to obtain mutated α-glucosidase genes. *E. coli* strain W3110 was used as a host for expression of the wild-type and all of the mutant α-glucosidases under the control of the *icp* promoter of the insecticidal protein gene from *B. thuringiensis* subsp. *sotto* (*8*). *E. coli* W3110 transformant cells were grown to the stationary phase at 37°C in 3 liters of Luria-Bertani broth containing 50 μg/ml ampicillin, and then the cells were collected by centrifugation. For preliminary evaluation of K_m values of mutants, enzymes were partially purified from crude extracts of the transformant cells by polyethyleneimine treatment and heat treatment, as described previously (*7*, *8*), which could effectively eliminate the endogenous trehalase, maltase, sucrase, and isomaltase activities of *E. coli*. The wild-type and some mutant enzymes (the Gly^{273}→Ile, Gly^{273}→Met, Gly^{273}→Val, and Gly^{273}→Ser mutants) were further purified to homogeneity by ion-exchange and gel-filtration chromatographies, as described previously (*10*).

A gene encoding an O16G mutant having the Pro^{273*}→Gly substitution was obtained using a kit (Quick Change Mutagenesis kit, Stratagene, La Jolla, California, USA) with pO16G (see above) as a template, and PCR primers 5′-CGGTTGGTGAAATGGGTGGTGTAACGACAG-3′ and 5′-CAGTCGTTACACCACCCATTTCACCAACCG-3′. The resultant plasmid, pO16G-Pro^{273*}→Gly, was then used as a template for PCR-based mutagenesis with primers m2-F (5′-CTGTATTGGACTAACCATGATCAGCCTCGC-3′) and m2-R (5′-GCGAGGCTGATCATGGTTAGTCCAATACAG-3′) to produce a plasmid, pO16G-Pro^{273*}→Gly/Asn^{342*}→Thr, encoding an O16G mutant with both the Pro^{273*}→Gly and Asn^{342*}→Thr substitutions. A gene encoding the Asn^{342*}→Thr mutant of O16G was created by PCR using pO16G as a template, and PCR primers m2-F and m2-R. The O16G and its mutants were each expressed as an N-terminal in-frame fusion with a His_6-tag under the control of the T7 RNA polymerase transcription (pET) system (Novagen, Madison, WI, USA) in cells of *E. coli* strain BL21(DE3). The transformant cells were grown at 37°C in Luria-Bertani broth containing 50 μg/ml ampicillin until OD_{600} reached 0.6. Isopropyl β-thiogalactoside was then added to a final concentration of 1 mM, followed by further cultivation for 4 h. Cells were collected by centrifugation, suspended in a 0.05 M potassium phosphate buffer, pH 6.8 (termed buffer A), and then disrupted at 4°C by 10 cycles of ultrasonication (where one cycle corresponds to 20 kHz for 30 s followed by an interval of 1 min). Polyethyleneimine was added to a final concentration of 0.12%, and then the mixture was allowed to stand at 4°C for 30 min. After centrifugation, the supernatant was applied to a His-Trap™ column (1 ml; Amersham, Piscataway, New Jersey, USA) equilibrated with buffer A. The column was washed with buffer A containing 60 mM imidazole, followed by elution of the enzyme with buffer A containing 300 mM imidazole.

Non-denaturing polyacrylamide gel electrophoresis (PAGE; acrylamide concentration, 7.5%) was performed according to the procedure of Davis (*14*). Sodium dodecyl sulfate (SDS) PAGE (acrylamide concentration, 7.5%) was performed as described by Laemmli (*15*). Protein was stained by silver staining or with Coomassie Brilliant Blue R250, with destaining in a destaining solution (a 2:1:7 mixture of methanol, acetic acid, and water).

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Table 1. **Kinetic properties of the wild-type *B. cereus* ATCC7064 O16G and mutants.**

A: K_m (mM)[a]				
Substrate	Wild-type O16G	$Pro^{273*}\rightarrow Gly$	$Pro^{273*}\rightarrow Gly/Asn^{342*}\rightarrow Thr$	$Asn^{342*}\rightarrow Thr$
Trehalose	320	14	2.0	–[c]
Isomaltose	5.3	14	20	13
B: V_{max} (U mg^{-1})[a]				
Substrate	Wild-type O16G	$Pro^{273*}\rightarrow Gly$	$Pro^{273*}\rightarrow Gly/Asn^{342*}\rightarrow Thr$	$Asn^{342*}\rightarrow Thr$
Trehalose	1.8	0.10	0.11	–[c]
Isomaltose	26	6.2	4.5	5.4
C: V_{max}/K_m (U mg^{-1} mM^{-1})				
Substrate	Wild-type O16G	$Pro^{273*}\rightarrow Gly$	$Pro^{273*}\rightarrow Gly/Asn^{342*}\rightarrow Thr$	$Asn^{342*}\rightarrow Thr$
Trehalose	0.0056 (0.12)[b]	0.0071 (1.6)[b]	0.055 (23)[b]	0.0051 (1.2)[b,c]
Isomaltose	4.9 (100)[b]	0.44 (100)[b]	0.23 (100)[b]	0.42 (100)[b]

[a]The standard errors of kinetic parameters were within ± 16%, except that those of the K_m values for trehalose were within ±35% (for wild type) and ±38% (for the $Pro^{273*}\rightarrow Gly/Asn^{342*}\rightarrow Thr$ mutant). [b]The values shown in parentheses indicate relative catalytic efficiencies for trehalose, the catalytic efficiencies for isomaltose being taken as 100%. [c]A linear relationship between initial velocity (v) and substrate concentration ([S]) was obtained in the range of [S] examined (up to 600 mM), suggesting that the K_m value of this mutant for trehalose should be significantly larger than 600 mM, because the enzyme-catalyzed reactions proceeds with first-order kinetics under the conditions of [S] $\ll K_m$. Therefore, only the V_{max}/K_m value was determined from slope of v *versus* [S] plots.

Enzyme Assay—Method I: The enzymatic hydrolysis of *p*-nitrophenyl 1-*O*-α-D-glucopyranoside was monitored as to the amount of *p*-nitrophenol released at 55°C (for SAM1606 α-glucosidase and its mutants) or 35°C (for O16G and its mutants). The standard assay mixture contained 3.0 μmol of *p*-nitrophenyl 1-*O*-α-D-glucopyranoside, 30 μmol of a sodium phosphate buffer (pH 7.2), and the enzyme, in a final volume of 3.0 ml. The mixture without the enzyme was brought to the specified temperature. The reaction was started by the addition of the enzyme, and then changes in absorbance at 405 nm were recorded with a spectrophotometer (Hitachi U-2000) equipped with a temperature-controlled cell holder. The extinction coefficient for *p*-nitrophenol under these conditions was 13,400 cm^{-1} M^{-1}.

Method II: For kinetic analysis of the hydrolysis of trehalose, maltose, sucrose, and isomaltose, the reaction mixture contained varying amounts of sugar, 16 μmol of a potassium phosphate buffer, pH 6.4, and the enzyme, in a final volume of 500 μl. The substrate concentrations used for kinetic studies generally ranged from $0.2K_m$ to $5K_m$, except that the highest substrate concentrations used were 400 mM (for maltose for $Gly^{273}\rightarrow Ile$ and $Gly^{273}\rightarrow Met$ mutants) and 600 mM (for trehalose for wild-type O16G and its $Asn^{342*}\rightarrow Thr$ mutant). The mixture without the enzyme was brought to 55°C (for SAM1606 α-glucosidase and its mutants) or 35°C (for O16G and its mutants). The reaction was started by the addition of the enzyme. After incubation for 10 min, the reaction was stopped by the addition of 60 μl of 1 M HCl, followed by incubation of the mixture at 30°C for 1 h to ensure enzyme inactivation (for trehalose, maltose, and isomaltose) or by heating of the mixture at 95°C for 5 min, followed by the addition of 60 μl H_2O (for sucrose). The blank did not contain the enzyme. To a 100-μl portion of the mixture, 690 μl of a 1 M potassium phosphate buffer, pH 7.2, 50 μl of 1 mg/ml glucose oxidase (*Aspergillus niger*, Nacalai Tesque), 60 μl of 10 mM 4-aminoantipyrine, 50 μl of 20 mM 2,4-dichlorophenol, and 150 μl of 0.01 mg/ml horseradish peroxidase were added, in that order, and then the reaction mixture was incubated at 30°C for 30 min. Glucose in the mixture was determined from the increase in absorbance at 505 nm. One unit of the enzyme is defined as the amount of enzyme that catalyzes the hydrolysis of one μmol of substrate per minute. K_m and V_{max} values, and their standard errors were estimated by fitting the initial velocity data to the Michaelis-Menten equation by nonlinear regression method (*16*). The absorption coefficient of the purified SAM1606 α-glucosidase and its mutants, $A_{280,\,1\%}$ of 25.5 (*10*), which was calculated from the amino-acid sequence, was used for unit calculations. The concentrations of O16G and its mutants were determined by Bradford's method (*17*) with bovine serum albumin as the standard.

Molecular Modeling—The structure of the SAM1606 α-glucosidase was built with the coordinates from the X-ray structure of O16G of *B. cereus* ATCC7064 (*11*; PDB ID, 1UOK) as a template using INSIGHT II Homology software (Biosym Technologies, San Diego, CA, USA), and was subjected to limited energy refinement with the Insight II Discover 3 software (Biosym Technologies). Glycine 273 of the SAM1606 enzyme was mutated to isoleucine, methionine, serine, or valine, and then the resulting geometry of the model was optimized using the INSIGHT II Discover 3 software (Biosym Technologies). The 2.1-Å resolution coordinates of porcine α-amylase complexed with acarbose were superposed on the structure of the wild-type and mutant enzymes, yielding optimal docking of substrates into the active site of these enzymes.

RESULTS AND DISCUSSION

Acquired Broad Substrate Specificity of SAM1606 α-Glucosidase—Oligo-1,6-glucosidases, SAM1606 α-glucosidase, and dextran glucosidases (DexBs) may be phylogenetically categorized into a small subgroup, most of whose members are α-1,6-specific. This suggests that SAM1606 α-glucosidase could easily have "evolved," through a limited number of amino-acid substitutions including that at position 273*, from an α-1,6-specific ancestor (*i.e.*, O16G) to an enzyme with extended substrate specificity. To experimentally confirm the validity of this consideration, three mutants of *B. cereus*

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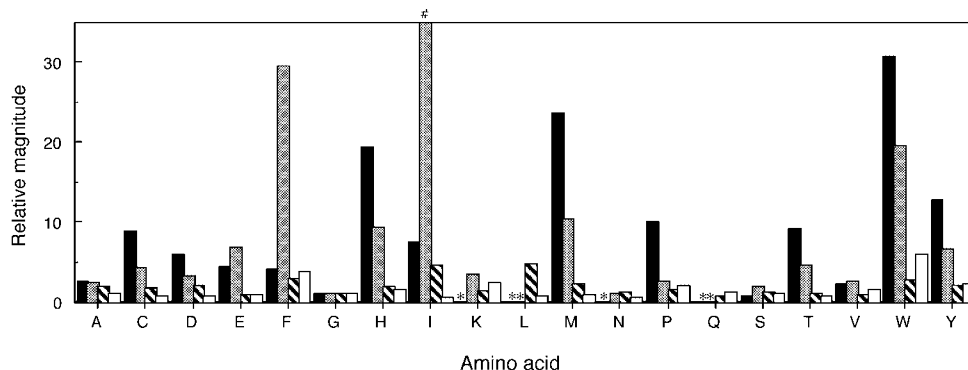

Fig. 3. **Effects of amino acid substitution at position 273 of the *Bacillus* sp. SAM1606 α-glucosidase on the K_m values for trehalose (black bars), maltose (gray bars), sucrose (diagonal-hatched bars), and isomaltose (white bars).** The relative magnitude of the alteration of the K_m values upon amino acid substitution (shown in a one-letter notation on the horizontal axis) is shown, the value for the wild-type enzyme (glycine at position 273) for each substrate being taken as 1.0. #, The K_m value was too large to be determined (400 mM ≪). *, The enzyme activities of these enzymes were too low to determine the K_m values for these substrates. The K_m values of the wild-type enzyme and some mutant enzymes are also presented in Table 2.

ATCC7064 O16G were prepared and kinetically characterized (Table 1). One of these mutants had the Pro^{273*}→Gly substitution, and another had both the Pro^{273*}→Gly and Asn^{342*}→Thr substitutions (see Fig. 1). The Asn^{342*}→Thr substitution was made with the aim of enhancing the effect of the Pro^{273*}→Gly substitution, because we previously observed that the amino acid residue at position 342 may enhance the effect of the Gly^{273}→Pro substitution in SAM1606 (reverse of the present Pro^{273*}→Gly substitution) but does not critically determine the specificity by itself (*10*). An O16G mutant that had the single Asn^{342*}→Thr substitution was also created. The wild-type O16G showed a very large K_m value (Table 1A) and very low relative catalytic efficiency as to trehalose (Table 1C), and the single Pro^{273*}→Gly and double Pro^{273*}→Gly/ Asn^{342*}→Thr substitutions introduced here resulted in significant decreases in the K_m value for trehalose (23- and 160-fold, respectively), increases in the K_m value for isomaltose, and decreases in the V_{max} values for both substrates. As the net results of these effects, the relative catalytic efficiencies of the Pro^{273*}→Gly mutant and Pro^{273*}→Gly/Asn^{342*}→Thr mutant as to trehalose were 13-fold and 192-fold higher than that of the wild-type O16G, respectively. These results, in turn, suggest the possibility that the substrate specificity of such an "evolved" α-glucosidase may be greatly modulated by the nature of the amino acid at position 273*, and this notion prompted us to examine the effect of amino acid substitution at position 273 on the substrate specificity of SAM1606 α-glucosidase.

Kinetic Studies of Site-specific Mutants of SAM1606 α-Glucosidase—Amino acid substitutions were made at position 273 of SAM1606 α-glucosidase, and the K_m values of the resultant 19 mutants for trehalose, maltose, sucrose, and isomaltose were first determined using partially purified preparations of these mutants. Among the site-directed mutants created, all except for the Gly^{273}→Arg one showed enzyme activity, and were stable at 60°C and pH 7.0 for prolonged periods of time (more than 1 h). It was revealed that substitution of the amino acid at position 273 caused quite different effects on the enzyme's affinity to substrates, and these effects also varied with the substrate used for evaluation. Figure 3 shows the relative magnitude of the alteration of K_m values upon amino acid substitution, the value of the wild-type enzyme for each substrate being taken as 1.0. The enzyme's affinities to trehalose and maltose were most severely affected by the nature of the amino acid at position 273—substitution at this position by bulky residues tends to result in large increases in the K_m values for these substrates. The affinity to sucrose was affected to a lesser extent, whereas the affinity to isomaltose was only minimally affected by an amino acid substitution at this position. These observations were consistent with the notion that the specificity of SAM1606 α-glucosidase should inherently be α-1,6-specific, as mentioned above, to which the ability of this enzyme to act on trehalose and maltose should be added.

The most important effects observed here were large increases in the K_m value for trehalose upon substitution at position 273 by tryptophan, methionine, histidine, and tyrosine, and that for maltose upon substitution by isoleucine, phenylalanine, and tryptophan (Fig. 3). Among the mutants with these single substitutions, the substrate specificities of the Gly^{273}→Ile and Gly^{273}→Met mutants were expected to be significantly different from that of the wild type, because the K_m values for isolmaltose of these mutants were even smaller than that of the wild type. Thus, these two mutants were purified to homogeneity and their kinetic properties were analyzed in more detail (Table 2). It was found that, contrary to the large increases in the K_m values for trehalose and maltose of these mutants, their V_{max} values for these two substrates remained at 14–62% of that of the wild type, although the value for maltose of the Gly^{273}→Ile mutant could not be determined due to its very large K_m value (400 mM ≪). A comparison of the relative catalytic efficiencies between these mutants and the wild-type enzyme (Table 2D) revealed that maltose and trehalose were virtually inert as substrates for these mutants, and that the relative reactivity of sucrose was also decreased with these substitutions. Thus, SAM1606 α-glucosidase could be converted into highly specific O16Gs through single amino-acid substitutions.

The other important observation that can be made from Fig. 3 is that the smallest set of K_m values for these four substrates could be obtained when the amino acid at this position was glycine (Fig. 3), the wild-type amino acid residue, indicating the specific importance of this residue at this position for broad substrate specificity of

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Table 2. **Kinetic properties of the wild-type SAM1606 α-glucosidase and selected mutants.**

A: K_m (mM)[a]					
Substrate	Wild-type	Gly[273]→Ile	Gly[273]→Met	Gly[273]→Val	Gly[273]→Ser
Trehalose	20	150	460	42	12
Maltose	10	–[b]	110	25	20
Sucrose	13	59	29	10	16
Isomaltose	2.4	1.2	2.0	3.6	2.5
B: V_{max} (U mg^{-1})[a]					
Substrate	Wild-type	Gly[273]→Ile	Gly[273]→Met	Gly[273]→Val	Gly[273]→Ser
Trehalose	21	2.9	11	31	25
Maltose	15	–[b]	9.1	35	26
Sucrose	47	34	55	69	52
Isomaltose	38	11	28	37	31
C: V_{max}/K_m (U mg^{-1} mM^{-1})					
Substrate	Wild-type	Gly[273]→Ile	Gly[273]→Met	Gly[273]→Val	Gly[273]→Ser
Trehalose	1.1	0.019	0.024	0.74	2.1
Maltose	1.5	0.010[b]	0.083	1.4	1.3
Sucrose	3.6	0.58	1.9	6.9	3.3
Isomaltose	16	9.2	14	10	12
D: Relative catalytic efficiency (%)					
Substrate	Wild-type	Gly[273]→Ile	Gly[273]→Met	Gly[273]→Val	Gly[273]→Ser
Trehalose	6.3	0.21	0.17	7.4	18
Maltose	9.4	0.11	0.59	14	11
Sucrose	23	6.3	14	69	28
Isomaltose	100	100	100	100	100

[a]The standard errors of kinetic parameters were within ± 20%. [b]A linear relationship between initial velocity (v) and substrate concentration [S] was obtained in the range of [S] examined (up to 400 mM), suggesting that the K_m value of this mutant for maltose should be significantly larger than 400 mM, because the enzyme-catalyzed reactions proceeds with first-order kinetics under the conditions of [S] $\ll K_m$. Therefore, only the V_{max}/K_m value was determined from slope of v *versus* [S] plots.

this α-glucosidase. However, the Gly[273]→Ser and Gly[273]→Val mutants were also expected to show substrate specificities similar to that of the wild type (see Fig. 3). Thus, detailed kinetic characterization of these mutants was also carried out using homogeneous preparations of them (Table 2). It was found that the V_{max} values of these mutants for trehalose, maltose, and sucrose were even higher than those of the wild-type enzyme. When the substrate specificities of these mutants were evaluated on the basis of their relative catalytic efficiency, they were indeed found to be as broad as that of the wild-type enzyme (Table 2D).

Modeling Studies to Elucidate the Mechanism of Specificity Determination by the Amino Acid at Position 273—To further clarify the mechanism of specificity determination by the amino-acid residue at position 273 of SAM1606 α-glucosidase, we constructed three-dimensional models of enzyme-substrate complexes of SAM1606 α-glucosidase and its mutants (Figs. 4–6). These models predict that the amino acid residue at position 273 is located near substites +1and +2 of these enzymes. In the Gly[273]→Ile and Gly[273]→Met mutants (Fig. 4), the amino acid residues at this position somewhat protrude into the space of these subsites, whereas in the wild-type and the Gly[273]→Ser mutant (Fig. 5), the glycine and serine residues at the same position can provide a wider space. Importantly, the side chains at position 273 of the Gly[273]→Ile and Gly[273]→Met mutants do not fully occupy subsite +1, which, therefore, still appears to be able to accommodate a glucose residue of the substrate. However, it is predicted that optimal docking of the substrates into the active site of the Gly[273]→Ile and Gly[273]→Met mutants gives rise to different conformations of the bound substrates, depending on their α-glucosidic linkage types (α-1,1-, α-1,4-, or α-1,6-), due to steric hindrance by the protuberant side chain at subsite +1 (Fig. 4; A, B, and C). The conformations of trehalose and maltose for their optimal binding to these mutants are predicted to be significantly different from (and energetically less favorable than) those predicted for their binding to the wild-type enzyme to avoid their collision with the bulky side chains at position 273 of these mutants (Fig. 4, A and B). In striking contrast, binding of isomaltose to these mutants appears to occur with conformations which were very similar to that predicted for the complex with the wild-type enzyme (Fig. 4C). On the other hand, the conformations of trehalose (Fig. 5) and maltose (not shown) that are bound to the wild-type enzyme, the Gly[273]→Ser mutant (Fig. 5), and the Gly[273]→Val mutant (not shown) are predicted to be similar to each other because of the absence or negligible degree of hindrance around subsite +1 of these enzymes. Thus, these predictions consistently explain the observations that substitution at this position by bulky residues tends to result in large increases in the K_m values for these substrates, whereas the affinity to isomaltose was only minimally affected by an amino acid substitution at this position. It must be mentioned, however, that the size of the proline residue that was identified at position 273* of some O16Gs (Fig. 1) was comparable with those of serine and valine residues, and that the previously observed large increase in K_m for trehalose upon the

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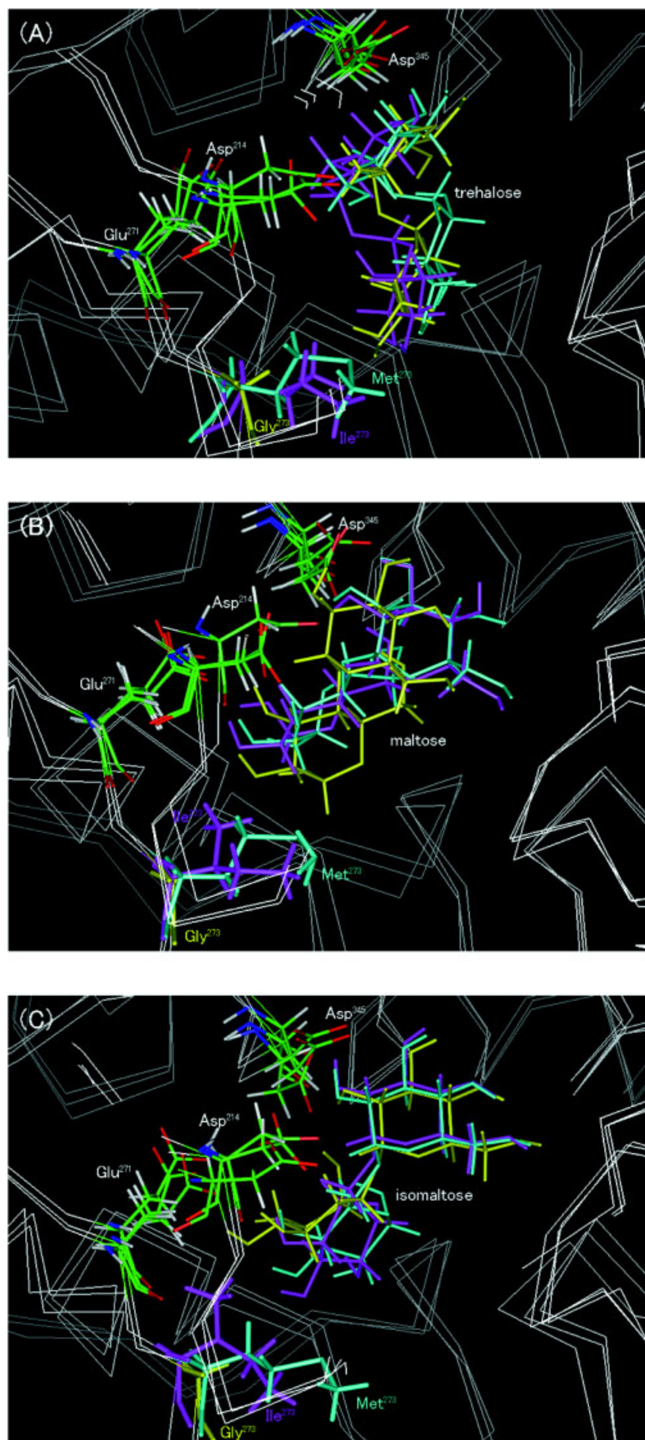

Fig. 4. **Close-up view of the active sites (superimposed) of SAM1606 α-glucosidase and mutants complexed with trehalose (A), maltose (B), and isomaltose (C).** Three-dimensional structures of the enzyme-substrate complexes were built as described under "MATERIALS AND METHODS." Putative catalytic residues (Asp^{214}, Glu^{271}, and Asp^{345}) are shown in green. Gly^{273} of the wild-type enzyme is shown in yellow. The Ile^{273} and Met^{273} residues of the Gly^{273}→Ile and Gly^{273}→Met mutants are shown in magenta and light blue, respectively. Sugars that are bound to the wild-type enzyme, the Gly^{273}→Ile mutant, and the Gly^{273}→Met mutant are also shown in yellow, magenta, and light blue, respectively.

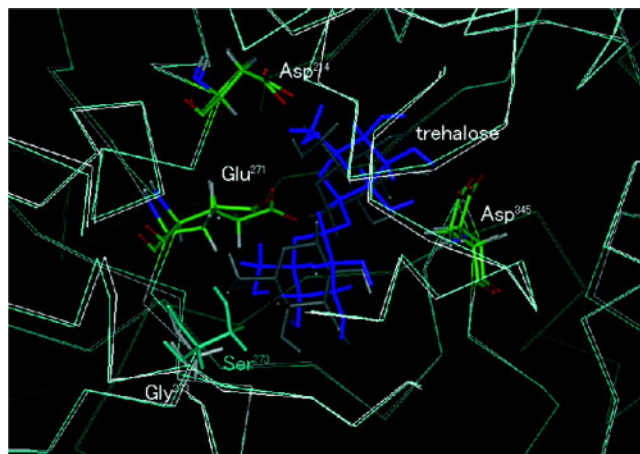

Fig. 5. **Close-up view of the active sites (superimposed) of SAM1606 α-glucosidase and its Gly^{273}→Ser mutant complexed with trehalose.** Three-dimensional structures of the enzyme-substrate complexes were built as described under "MATERIALS AND METHODS." Putative catalytic residues (Asp^{214}, Glu^{271}, and Asp^{345}) are shown in green. Gly^{273} of the wild-type enzyme is shown in silver, and Ser^{273} of the mutant is shown in light blue. Trehalose molecules that are bound to the wild-type and mutant enzymes are shown in gray and blue, respectively.

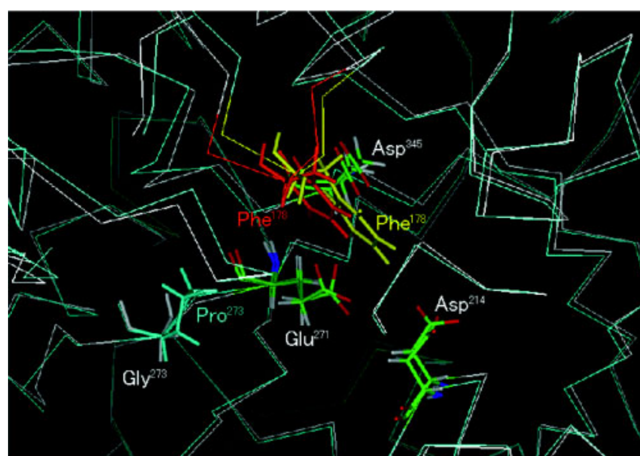

Fig. 6. **Close-up view of the active sites (superimposed) of SAM1606 α-glucosidase and Gly^{273}→Ile mutant.** Three-dimensional structures of the enzyme-substrate complexes were built as described under "MATERIALS AND METHODS." Putative catalytic residues (Asp^{214}, Glu^{271}, and Asp^{345}) are shown in green. The Gly^{273} and Phe^{178} residues of the wild-type enzyme are shown in silver and red, respectively. The Pro^{273} and Phe^{178} residues of the Gly^{273}→Pro mutant are shown in light blue and yellow, respectively.

Gly^{273}→Pro substitution (*10*) cannot simply be explained in terms of steric hindrance by proline at subsite +1. In an attempt to find an alternative mechanism that accounts for the previous observation, we also constructed a three-dimensional structural model of the Gly^{273}→Pro mutant, and compared it with those of the wild-type and mutant enzymes (Fig. 6). It was predicted that, in the Gly^{273}→Pro mutant, Phe^{178} protrudes more toward the space near subsite +1 than in the wild-type and other mutants. The "enzyme-substrate" docking studies predicted that, because of this protrusion, trehalose could bind to the mutant enzyme only in its energet-

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ically unfavorable conformation, whereas isomaltose could bind in its energetically more favorable one.

It must be mentioned that, although the O16G_BACSP enzyme (Fig. 1) has been designated as "oligo1,6-glucosidase" on the basis of its sequence similarity to O16Gs, specificity analyses revealed that this enzyme is not highly specific for isomaltose but can also act on maltotriose (relative activity to isomaltose, 67%) and maltose (8%) (*18*). This ability to efficiently act on maltose and maltotriose may be, at least in part, explained in terms of the occurrence of the glycine residue at position 273* of this enzyme.

CONCLUSION

Bacillus sp. SAM1606 α-glucosidase is a variant of O16G with increased ability to act on trehalose and maltose. It is proposed that this ability could have been attained through substitution at position 273* by glycine to eliminate steric hindrance around subsites +1 and +2 that might have originally occurred in the parental O16G, and that this effect can be enhanced by amino acid substitution at position 342*.

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