Altering Substrate Specificity of Bacillus sp. SAM1606 α-Glucosidase by Comparative Site-specific Mutagenesis*

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The Bacillus sp. SAM1606 α-glucosidase with a broad substrate specificity is the only known α-glucosidase that can hydrolyze α,α'-trehalose efficiently. The enzyme exhibits a very high sequence similarity to the oligo-1,6-glucosidases (O16G) of Bacillus thermoglucosidasius and Bacillus cereus which cannot act on trehalose. These three enzymes share 80% identical residues within the conserved regions (CR), which have been suggested to be located near or at the active site of the α-amylase family enzymes. To identify site-specific mutagenesis the critical residues that determine the broad substrate specificity of the SAM1606 enzyme we compared the CR sequences of these three glucosidases and selected five targets to be mutated in SAM1606 α-glucosidase, Met^{76}, Arg^{81}, Ala^{116}, Gly^{273}, and Thr^{342}. These residues have been specifically replaced by in vitro mutagenesis with Asn, Ser, Val, Pro, and Asn, respectively, as in the Bacillus O16G. The 12 mutant enzymes with single and multiple substitutions were expressed and characterized kinetically. These results showed that the 5-fold mutation virtually abolished the affinity of the enzyme for α,α'-trehalose, whereas the specificity constant for the hydrolysis of isomaltohexose, a good substrate for both the SAM1606 enzyme and O16G, remained essentially unchanged upon the mutation. This loss in affinity for trehalose was critically governed by a Gly^{273} → Pro substitution, whose effect was specifically enhanced by the Thr^{342} → Asn substitution in the 5-fold and quadruple mutants. These results provide evidence for the differential roles of the amino acid residues in the CR in determining the substrate specificity of the α-glucosidase.

It has been shown that α-amylases, α-glucosidases, glucoamylases, cyclodextrin glucanotransferases, and pullulases share several short conserved sequences (conserved regions, CR) (1–5) and have also been suggested to have a common (β/α)_{8}-barrel fold (6, 7). These enzymes are also proposed to share a common reaction mechanism (3, 8, 9). These characteristics suggest a strong evolutionary relationship in the origin of these enzymes, which have thus been categorized into a single protein family called the α-amylase family. X-ray crystallographic studies of several members of this family showed that the CR are located at or near the active site and contain putative catalytic carboxylates (5, 10–12). Recent structural elucidation of α-amylases and cyclodextrin glucanotransferase complexed with their substrates or inhibitors has shown that some amino acid residues in these regions indeed interact with the bound ligands (13–16). These observations indicate the significance of the CR sequences in maintaining catalytic activity and specificity of the enzyme.

α-Glucosidase (EC 3.2.1.20) catalyzes the hydrolysis of 1-O-α-D-glucopyranosides with a net retention of anomeric configuration. The substrate specificity of α-glucosidase differs greatly with the source of the enzyme (17). The majority of α-glucosidases preferentially hydrolyzes maltose, whereas another class of α-glucosidases, dextrin 6-α-glucanohydrolase (oligo-1,6-glucosidase, O16G; EC 3.2.1.10), acts exclusively on the 1,6-glucosidic linkage of isomaltooligosaccharides (18). We have recently found that a strain of thermophilic Bacillus, SAM1606, produced a novel thermostable α-glucosidase with a broad substrate specificity and high transglycosylation activity (19). The enzyme can hydrolyze efficiently a variety of 1-O-α-D-glucopyranosides such as α,α'-trehalose (trehalose), maltose, nigerose, isomaltohexose, sucrose, turanose, maltotriose, maltotetraose, and isomaltotriose. Indeed, it was the first such α-glucosidase that could hydrolyze trehalose efficiently. We cloned the SAM1606 α-glucosidase gene to determine its primary structure and expressed it in Escherichia coli (20). SAM1606 α-glucosidase exhibited sequence similarities to the enzymes of the α-amylase family and contained the CR (Fig. 1) as well as a suggested (β/α)_{8}-barrel fold. Thus, the enzyme was also assigned as a member of the α-amylase family. Unexpectedly, we found that the enzyme exhibits extremely high sequence similarities (62–65% identity along the entire polypeptide chain and 80% identity within the CR sequences) to the O16G of Bacillus cereus and Bacillus thermoglucosidasius (21–23). These O16G themselves are 72% (along the entire polypeptide chain) or 80% (along the CR sequences) identical to each other but, very interestingly, are distinct from SAM1606 α-glucosidase in substrate specificity; the O16G cannot hydrolyze trehalose, maltose, and sucrose (18, 24, 25). Thus, limited structural differences within the CR have been suggested to govern the significant differences in substrate specificity.

In this study we have analyzed the broad substrate specificity of SAM1606 α-glucosidase by comparative site-specific mutagenesis. By comparing the CR sequences between the SAM1606 enzyme and the Bacillus O16G, we selected five targets to be mutated in the CR of SAM1606 α-glucosidase:

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‡ The abbreviations used are: CR, conserved region(s); O16G, oligo-1,6-glucosidase(s); trehalose, α,α'-trehalose; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis. For the nomenclature of mutant enzymes, see Table II.

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**Site-directed Mutagenesis and Expression**

A strategy for in vitro mutagenesis is depicted in Fig. 2A. Fragments I, II, and III, and IV of the SAM1606 α-glucosidase gene were amplified by PCR using a template plasmid pGBSU2 (Fig. 2B), and the PCR primers (see also Table I) which were so designed that the restriction enzyme sites were newly created at boundaries of neighboring fragments without a change in the amino acid sequences. Fragments I, II, III, and IV were ligated with each other, and the resultant fragment was substituted for the BamH1-AatII fragment of the pGBSU2 to obtain pGBSU5 (Fig. 2C). Replacement of the fragment(s) in the pGBSU5 with the mutated fragment(s), whose preparations are described below, allowed us to obtain α-glucosidase genes having various combinations of mutations. The mutations Met\(^{76}\) → Asn, Arg\(^{81}\) → Ser, Ala\(^{116}\) → Val, and their double and triple mutations were introduced on the amplified fragment II essentially as described by Kramer and Frits (27) using the mutagenesis primers M1, M2, and M3. For the mutation Gly\(^{273}\) → Pro, fragments IIIa and IIIb were amplified from pGBSU2 using PCR primers F-Ila-R-Ilaa and F-Ili-R-Ilib, respectively, which were so designed that the amplified fragments were to be ligated at a newly created Smal site, giving rise to the substitution of Pro for Gly\(^{273}\). The mutation Thr\(^{342}\) → Asn was introduced by PCR, which was directly performed on the pGBSU2 with PCR primers M5 (instead of F-IV) and R-IV. Individual mutations were verified by DNA sequencing.

Mutant and wild type α-glucosidase genes were expressed in E. coli W3110 transformant cells under the control of the icp promoter of the insecticidal protein gene from B. thuringiensis subsp. subito as described (20). E. coli transformant cells were grown to the stationary phase at 37 °C in 5 liters of L-broth containing 50 μg/ml ampicillin, and the cells were collected by centrifugation.

**Enzyme Purification**

The wild type and all of the mutant α-glucosidases were purified as described below. All steps were done at 4 °C unless otherwise stated. Sodium phosphate buffer (0.01 M, pH 7.0) was used as the standard buffer. Enzyme activity was routinely found by assay method I (see below). Cells (typically 20 g, wet weight) were ground with 40 g of aluminum oxide powder for 10 min in a mortar chilled on ice and suspended in 40 ml of standard buffer followed by centrifugation. Polyethyleneimine was added to the supernatant at a final concentration of 0.12% (w/w). After the mixture was left for 30 min, the precipitate was remixed and centrifuged. The supernatant was then kept at 60 °C for 30 min. After the heat treatment, the precipitate was removed by centrifugation. To the supernatant solution, solid ammonium sulfate was added slowly to 20% saturation. After the mixture was allowed to stand for 1 h, the insoluble material was removed by centrifugation. Sodium ammonium sulfate was then added to 50% saturation. The precipitate, 20–50% saturated fraction, was dissolved in the standard buffer. The enzyme activity was routinely found by assay method I (see below). Cells (typically 20 g, wet weight) were ground with 40 g of aluminum oxide powder for 10 min in a mortar chilled on ice and suspended in 40 ml of standard buffer followed by centrifugation. Polyethyleneimine was added to the supernatant at a final concentration of 0.12% (w/w). After the mixture was left for 30 min, the precipitate was remixed and centrifuged. The supernatant was then kept at 60 °C for 30 min. After the heat treatment, the precipitate was removed by centrifugation. To the supernatant solution, solid ammonium sulfate was added slowly to 20% saturation. After the mixture was allowed to stand for 1 h, the insoluble material was removed by centrifugation. Sodium ammonium sulfate was then added to 50% saturation. The precipitate, 20–50% saturated fraction, was dissolved in the standard buffer and dialyzed against the standard buffer. The insoluble material formed during the dialysis was removed by centrifugation. The enzyme solution was loaded on a DEAE-Sepharose CL-6B column (3.6 x 21 cm) equilibrated with the standard buffer. The enzyme was eluted with a linear gradient of NaCl (0–0.6 M) in the same buffer (400 ml each). Active fractions were analyzed for the subunit structure of the enzyme by native- and SDS-polyacrylamide gel electrophoresis (PAGE). It was found that at this step, a small fraction of the monomeric form of the enzyme could be separated from multimeric forms. Fractions containing >90% pure monomer were collected and concentrated with PM10 membrane in an Amicon 8200 ultrafiltration unit. The concentrate was put on a Sephacryl S200 HR column (2.6 x 67 cm) equilibrated with the standard buffer containing 0.1 M NaCl and eluted. The monomeric α-glucosidase fractions were combined, concentrated by ultrafiltration, dialyzed against the standard buffer and used for kinetic analyses.
the enzyme in a final volume of 1.0 ml. The mixture without the enzyme was brought to 55 °C. The reaction was started by the addition of enzyme, and changes in absorbance at 405 nm were recorded with a spectrophotometer (Shimadzu UV-160; kinetic mode) equipped with a temperature-controlled cell positioner (CPS240A). The extinction coefficient for \( p \)-nitrophenolate under these conditions was 13,400 cm\(^{-1}\) M\(^{-1}\) (19).

**Method II**—For the kinetic analysis for hydrolysis of trehalose, maltose, sucrose, and isomaltose, the reaction mixture contained varying amounts of sugar, 1.0 \( \mu \)mol of sodium phosphate buffer, pH 7.2, and the enzyme in a final volume of 100 \( \mu \)l. The mixture without the enzyme was brought to 55 °C. The reaction was started by the addition of enzyme. After incubation at 55 °C for 10 min, the reaction was stopped by heating at 100 °C for 3 min. Glucose that formed in the reaction mixture was determined by the method of Püttner and Becker (26) with a kit (Boehringer Mannheim). One unit of the enzyme is defined as the amount of enzyme which catalyzes the hydrolysis of 1 \( \mu \)mol of substrate/min at 55 °C. Km and

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**Fig. 2. Strategy for in vitro mutagenesis.** Panel A, a black rectangle indicates a BamHI-HindIII fragment encoding the full-length SAM1606 \( \alpha \)-glucosidase gene with white circles shown with numerals (in italics) indicating sites of amino acid substitutions (see Fig. 1). White rectangles with Roman numerals indicate amplified DNA fragments of the SAM1606 \( \alpha \)-glucosidase gene by PCR using the PCR primers (arrows) shown above the fragments. Restriction enzyme sites (vertical lines), except for the terminal BamHI and HindIII sites, are those to be newly created by PCR. Horizontal lines (M1-M3) and an arrow (M5) shown below the fragments are the mutagenesis primers for amino acid substitutions. For further details, see “Results” and Table I. Panel B, a restriction enzyme map for plasmid pGBSU2, a template for the amplification of fragments, containing the full-length coding region for the wild type SAM1606 \( \alpha \)-glucosidase gene (black arrow) in which the initiation codon was changed from TTG to ATG (see Ref. 20). icp indicates the 0.15-kilobase icp promoter region of the insecticidal crystal protein gene from *B. thuringensis* subsp. *sotto*, and a gray arrow indicates an ampicillin resistance (Amp') gene. Numbers in parentheses indicate distances in kilobase pairs from the first nucleotide of the icp promoter region. It should be noted that for the construction of pGBSU5, a unique HindIII site of pGBSU2 was inactivated by digestion of the plasmid with HindIII and then blunt with T4 DNA polymerase followed by blunt end ligation with SalI adopter molecules. Panel C, a general restriction enzyme map for pGBSU5 and its derivatives, which were obtained by replacing the fragment(s) in the pGBSU5 with mutated one(s). Only the plasmids with the Gly^{273} \rightarrow Pro substitution have an SmaI site, which is shown in a square brackets.
**Specificity Determinants of an α-Glucosidase**

$V_{\text{max}}$ values and their standard errors were estimated by fitting the initial velocity data to the Michaelis-Menten equation by nonlinear regression methods (29). The absorption coefficient of the purified SAM1606 α-glucosidase, $A_{280}^\text{max} = 25.5$, which was calculated from the amino acid sequence (20), was used for unit calculations.

**pH Activity Profiles**

Enzymatic hydrolyses of trehalose, maltose, sucrose, and isomaltose were assayed by method II except that the reaction mixtures contained 5.0 μmol of substrate and 1.0 μmol of either sodium acetate, pH 2.0–6.0, or sodium phosphate, pH 6.0–8.0.

**Analytical Methods**

Native-PAGE and SDS-PAGE were done with a 10% gel by the procedures of Davis (30) and Laemmli (31), respectively. Proteins were stained with Coomassie Brilliant Blue R-250 and destained in a destaining solution (a 2:1:7 mixture of methanol, acetic acid, and water). The blots were probed with a 1:400 dilution of the primary anti-SAM1606 antibody raised against the wild type enzyme, as determined by Western blotting analysis. Amino-terminal amino acid sequence analyses up to 10 cycles of the wild type and all of the mutant enzymes confirmed the expected primary structure Ser-Thr-Ala-Leu-Thr-Gln-Thr-Ser-Thr-A.

The steady-state kinetic parameters for hydrolysis of four different substrates, trehalose, maltose, sucrose, and isomaltose, were determined for the wild type and mutant enzymes at pH 6.0 and are given in Table III. Among these substrates, trehalose is known to be a very poor substrate for most known α-glucosidases including the *Bacillus* O16G but has been shown to be hydrolyzed effectively by SAM1606 α-glucosidase. Maltose and sucrose were also substrates for which distinct differences in reactivity have been established between the wild type and O16G, whereas isomaltose can serve as an excellent substrate for both enzymes.

None of the single and multiple mutations caused a significant reduction in $V_{\text{max}}$ for all of the substrates tested; all mutant enzymes had $V_{\text{max}}$ values that were more than 20% of those of the wild type enzyme for all substrates. Some mutant enzymes exhibited even higher specific activities than that of the wild type enzyme. These are consistent with the fact that the mutagenic targets selected in this study did not contain the putative catalytic residues.

No significant variation in $K_m$ was detected with maltose, sucrose, and isomaltose upon each mutation. For trehalose, however, Gly273 → Pro as well as multiple changes containing the Gly273 → Pro mutation had distinct effects on $K_m$ from those obtained by the other mutations; only these mutations caused appreciable increases in the $K_m$ value for this substrate. In contrast, a quadruple mutant without the Gly273 → Pro substitution (i.e. $Q^4$) showed a $K_m$ value for trehalose.

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| TABLE I | Synthetic oligonucleotides used in the mutagenesis of SAM1606 α-glucosidase |
|---------|--------------------------------------------------------------------------------|
| F-I     | CAGATCCCAGCAGATTCAGCG |
| F-II    | TTGAGGATCTAGCATCGAGAG |
| F-IIIa  | ATCAGCTAGCATCGATAGAAC |
| F-IIIb  | TGGTCAGTCACCCGAGG |
| R-I     | TGGTCAGTCACCCGAGG |
| R-II    | TGGTCAGTCACCCGAGG |
| R-IIIa  | TGGTCAGTCACCCGAGG |
| R-IIIb  | TGGTCAGTCACCCGAGG |
| R-IV    | TGGTCAGTCACCCGAGG |
| R-V     | TGGTCAGTCACCCGAGG |
| M1      | GAAATGACGACATGCTAGAC |
| M2      | GTTACGATCATGACATGCTA |
| M3      | GCGACTGTGTCACCACATC |
| M4      | GTTACGATCATGACATGCTA |

| TABLE II | Nomenclature of mutant SAM1606 α-glucosidases |
|----------|---------------------------------------------|
| Name     | Mutation |
| S1       | Met$^{16}$ → Asn |
| S2       | Arg$^{115}$ → Ser |
| S3       | Ala$^{116}$ → Val |
| S4       | Gly$^{273}$ → Pro |
| S5       | Thr$^{142}$ → Asn |
| D4/5     | Gly$^{273}$ → Pro/Thr$^{142}$ → Asn |
| Q1       | Arg$^{115}$ → Ser/Ala$^{116}$ → Val/Gly$^{273}$ → Pro/Thr$^{142}$ → Asn |
| Q2       | Met$^{16}$ → Asn/Ala$^{116}$ → Val/Gly$^{273}$ → Pro/Thr$^{142}$ → Asn |
| Q3       | Met$^{16}$ → Asn/Arg$^{115}$$^{*}$ → Ser/Ala$^{116}$ → Val/Gly$^{273}$ → Pro/Thr$^{142}$ → Asn |
| Q4       | Met$^{16}$ → Asn/Arg$^{115}$ → Ser/Ala$^{116}$ → Val/Gly$^{273}$ → Pro/Thr$^{142}$ → Asn |
| Q5       | Met$^{16}$ → Asn/Arg$^{115}$ → Ser/Ala$^{116}$ → Val/Gly$^{273}$ → Pro/Thr$^{142}$ → Asn |
| F        | Met$^{16}$ → Asn/Arg$^{115}$ → Ser/Ala$^{116}$ → Val/Gly$^{273}$ → Pro/Thr$^{142}$ → Asn |
which was similar to that of the wild type enzyme. These results indicate that the increase in $K_m$ for trehalose is critically governed by the Gly273 → Pro substitution, which solely caused a 10-fold greater increase in the $K_m$ value than those of mutants without it, as shown by comparison of $K_m$ values for trehalose of S4 and the other single mutants. In addition, the results with mutants with four and five alterations also indicate that the effect of the Gly273 → Pro substitution was enhanced further by a Thr242 → Asn substitution in these mutants; the presence of Asn342 in these cases (i.e. Q1', Q2', Q3', and F) caused more than 10-fold additional increases in $K_m$ for trehalose than with the Gly273 → Pro mutants without Thr242 → Asn substitution (i.e. S4 and Q5', Table III). Interestingly, however, such an enhancement was not observed in the D4/5 mutant where the Thr242 → Asn substitution was only introduced into the single Gly273 → Pro mutant (S4). It should be emphasized that, judging from the $K_m$ values, Q1', Q2', Q3', and F cannot bind trehalose under the assay conditions that have been employed routinely with a relatively low substrate concentration (i.e. 5 mM; Ref. 19), albeit their $K_m$ values for isomaltose were almost unchanged (Table III).

To find the net changes in substrate preference of the enzyme upon mutations, we compared the relative specificity constants using isomaltose as the reference substrate (Fig. 3); isomaltose showed the least variation in the specificity constant upon all mutations (Table III), consistent with the fact that this sugar serves as a good substrate for both the SAM1606 enzyme and O16G. The largest changes in the substrate specificity were detected with mutant enzymes exhibiting exclusive diminutions in the relative specificity constant for trehalose.

We also examined the apparent pH activity profiles for hydrolysis of these substrates with six representative mutants (S1, S2, S3, S4, S5, and F) to address the possibility that the observed change in the substrate specificity is due to a change in the pH dependence of the hydrolysis reaction upon mutations. Optimum pH values for hydrolysis of trehalose, maltose, and sucrose were at 5.5 for all six mutant enzymes and were unchanged from those of the wild type enzyme, although some variations in the optimum pH were observed with isomaltose. Optimum pH values were at 4.7 for the wild type enzyme, S1, S2, and S3, and at 3.5 for F; but S3 and S4 showed a broad optimum pH ranging from 4.5 to 6.0. Thus, the change in the specificity was not due to a specific shift in the pH optimum for trehalose hydrolysis.

### Table III

| Substrate and enzyme | $K_m$ | $V_{max}$ | $V_{max}/K_m$ |
|----------------------|-------|-----------|---------------|
| **Trehalose**        |       |           |               |
| Wild type            | 9.3 ± 2.4 | 16.5 ± 1.4 | 1.77          |
| S1                   | 4.7 ± 1.0 | 16.5 ± 0.9 | 3.51          |
| S2                   | 12.1 ± 1.1 | 17.9 ± 0.8 | 1.48          |
| S3                   | 6.8 ± 1.3 | 15.4 ± 0.7 | 2.26          |
| S4                   | 113 ± 24 | 12.0 ± 1.5 | 0.11          |
| S5                   | 9.8 ± 1.1 | 20.6 ± 0.7 | 2.10          |
| D4/5                 | 88.7 ± 11 | 4.2 ± 0.5  | 0.05          |
| Q1'                  | 1103 ± 267 | 4.8 ± 0.9  | 0.004         |
| Q2'                  | 1001 ± 338 | 6.5 ± 1.7  | 0.006         |
| Q3'                  | 1210 ± 439 | 7.1 ± 2.2  | 0.006         |
| Q4'                  | 4.9 ± 1.6 | 6.6 ± 0.3  | 1.35          |
| Q5'                  | 117 ± 36  | 4.6 ± 1.3  | 0.04          |
| F                    | 1183 ± 401 | 16.7 ± 4.6 | 0.014         |
| **Maltose**          |       |           |               |
| Wild type            | 7.5 ± 2.6 | 14.7 ± 1.5 | 1.96          |
| S1                   | 4.4 ± 1.5 | 18.8 ± 1.6 | 4.27          |
| S2                   | 5.8 ± 1.6 | 10.5 ± 0.8 | 1.81          |
| S3                   | 13.9 ± 2.2 | 29.1 ± 1.5 | 2.09          |
| S4                   | 11.5 ± 4.4 | 5.7 ± 0.6  | 0.50          |
| S5                   | 2.4 ± 0.6 | 18.9 ± 1.0 | 7.88          |
| Q4'                  | 1.9 ± 0.3 | 14.1 ± 1.2 | 7.42          |
| F                    | 56.2 ± 11 | 9.6 ± 1.3  | 0.26          |
| **Sucrose**          |       |           |               |
| Wild type            | 6.9 ± 1.6 | 56.2 ± 4.3 | 8.14          |
| S1                   | 7.2 ± 1.2 | 46.5 ± 2.1 | 6.46          |
| S2                   | 7.2 ± 1.3 | 77.4 ± 2.7 | 10.88         |
| S3                   | 8.5 ± 2.3 | 39.1 ± 2.8 | 4.60          |
| S4                   | 9.1 ± 2.5 | 26.6 ± 2.2 | 2.92          |
| S5                   | 6.8 ± 1.6 | 35.7 ± 2.3 | 5.25          |
| Q4'                  | 16.4 ± 6.9 | 12.8 ± 1.8 | 0.78          |
| F                    | 10.5 ± 3.4 | 11.9 ± 1.0 | 1.13          |
| **Isomaltose**       |       |           |               |
| Wild type            | 3.5 ± 0.9 | 34.0 ± 2.3 | 9.71          |
| S1                   | 4.0 ± 1.1 | 39.5 ± 2.8 | 9.88          |
| S2                   | 3.7 ± 0.7 | 34.0 ± 1.5 | 9.19          |
| S3                   | 1.7 ± 0.8 | 37.0 ± 3.4 | 21.8          |
| S4                   | 5.8 ± 2.2 | 35.3 ± 4.2 | 6.09          |
| S5                   | 3.8 ± 1.0 | 50.8 ± 3.2 | 13.4          |
| D4/5                 | 1.4 ± 0.2 | 10.6 ± 3.3 | 7.57          |
| Q1'                  | 9.0 ± 3.0 | 18.1 ± 2.2 | 2.01          |
| Q2'                  | 16.0 ± 2.7 | 30.0 ± 2.9 | 1.88          |
| Q3'                  | 13.9 ± 4.0 | 31.5 ± 3.2 | 2.27          |
| Q4'                  | 9.9 ± 4.5 | 44.3 ± 7.3 | 4.47          |
| Q5'                  | 161 ± 2.5 | 37.9 ± 1.1 | 2.35          |
| F                    | 7.6 ± 1.1 | 37.4 ± 1.8 | 4.92          |

*Abbreviations are as in Table II.*

![Fig. 3](image-url)
DISCUSSION

The strategy we have taken in this study to probe amino acid residues responsible for the uniquely broad substrate specificity of the SAM1606 α-glucosidase can be called comparative site-specific mutagenesis; the sites and amino acids chosen for replacement were selected by comparing the CR sequences with those of reference enzymes that show high sequence similarities but have distinct and narrower substrate specificities. This strategy is based on the recent reports that the CR sequences are at or near active and substrate binding sites of the enzyme and are suggested to be important in determining the specificity of the enzyme (5, 10–16). The O16G of *B. thermoglucosidasius* and *B. cereus* were very good reference enzymes for the SAM1606 enzyme for this purpose because they are 80% identical to the SAM1606 enzyme in the CR but are very different in terms of substrate specificity from the SAM1606 enzyme (19, 20). Five nonconserved amino acids, Met<sup>165</sup>, Arg<sup>281</sup>, Ala<sup>116</sup>, Gly<sup>273</sup>, and Thr<sup>342</sup>, were identified and selected for mutagenesis and were replaced with Asn, Ser, Val, Pro, and Asp, respectively. Enzymes with all possible combinations of one through five mutations could be constructed easily and expressed in our established system.

In this study, examination of 12 of the possible mutant enzymes successfully led us to find that replacing Gly<sup>273</sup> with Pro caused a significant and specific diminution of the affinity of an enzyme for trehalose without a significant decrease in *V<sub>max</sub>* and thus permitted us to identify Gly<sup>273</sup> as a critical determinant for differential reactivity to trehalose between the SAM1606 enzyme and O16G. The present studies also established the role of other amino acid residues in the CR in determining the specificity of the enzyme; the Thr<sup>342</sup> → Asn substitution in the mutants with four and five alterations is important in enhancing the effect of the Gly<sup>273</sup> → Pro substitution. Thus, the specificity of the α-glucosidase for trehalose arises from two distinct types of effects of amino acid residues. One of them determines critically the specificity, and the other enhances the effect of the Gly<sup>273</sup> without any critical effect by itself. The latter effect by the Thr<sup>342</sup> → Asn substitution was observed in the four- and five-substitution mutants, but not in the double mutant (D4/S), suggesting that the enhancement effect of the Thr<sup>342</sup> → Asn substitution emerged in the enzymes with the CR sequences that are more similar to those of O16G than to that of the SAM1606 enzyme. Although the SAM1606 enzyme is the only known α-glucosidase that can efficiently act on trehalose (19, 20), these results suggest that this uniqueness is simply ascribed to the exclusive ability of the SAM1606 enzyme to bind this sugar efficiently. This implies that O16G and probably the other α-glucosidases of the same family lack the ability to bind trehalose because of their different CR sequences; however, they might be engineered genetically to hydrolyze trehalose by enabling them to bind trehalose through appropriate substitution of amino acid residues in their CR sequences, because these α-glucosidases are proposed to share a common reaction mechanism for cleaving the α-glucosidic linkages (3, 8, 9) and thus potentially allow hydrolysis of the α-1,1-linkage.

It should be pointed out that the effects of mutations on kinetic parameters varied with the substrates; contrary to the results obtained with trehalose, less variation in kinetic parameters was observed with maltose, sucrose, and isomaltose by mutations introduced in this study. Although distinct differences in reactivity have been established for maltose and sucrose between the SAM1606 and O16G enzymes, these differences could not be explained fully in terms of amino acid substitutions within the CR sequences. The current results indicate that replacement of the amino acid residues within the CR causes distinct effects on the reactivity of each substrate and suggest that critical amino acid residue(s) determining the reactivity to individual substrates may vary with the substrate. These conclusions are consistent with observations from x-ray crystallographic studies of several α-amylase family enzymes complexed with substrates and inhibitors (13–15). (i) Binding of a ligand to the enzyme is maintained through many polar and nonpolar protein-ligand interactions, including a hydrogen bonding network, which is in many cases engaged in interactions with the solvent water. (ii) Binding of a different ligand produces a different set of interactions. Thus, the five-substitution mutation may disrupt the interactions necessary for trehalose binding but may not essentially affect those interactions necessary for binding and subsequent catalytic steps in the hydrolysis of maltose, sucrose, and isomaltose, although it does somewhat perturb the pH dependence of isomaltose hydrolysis. Reactivity to sucrose and maltose of the SAM1606 enzyme may be governed by amino acid residue(s) other than the sites selected for mutagenesis in this study. Knowledge of the interactions between substrate and enzyme in the stereostructure of the SAM1606 α-glucosidase-substrate complex will be necessary to elucidate further the broad substrate specificity of this enzyme.

Gly<sup>273</sup> and Thr<sup>342</sup> are located near the putative catalytic residues of SAM1606 α-glucosidase, Gly<sup>271</sup> and Asp<sup>345</sup>, and are positioned at less conserved sites in the CR of α-amylase family enzymes, implying their potential roles in defining the specificity. It is very interesting to find amino acid residues at positions in the CR corresponding to those of the Gly<sup>273</sup> and Thr<sup>342</sup> of the SAM1606 enzyme in the other enzymes of α-amylase family and their interactions with bound ligand in the reported stereostuctures of the enzyme-inhibitor complexes. This is exemplified by the recent x-ray crystallographic studies of the porcine pancreatic α-amylase complexed with acarbose, a pseudosaccharidial inhibitor (13), and that with Temdamistat, a proteineous inhibitor (14). In this α-amylase, Ile<sup>235</sup> and Asp<sup>297</sup>, respectively, correspond to the Gly<sup>273</sup> and Thr<sup>342</sup> of the SAM1606 enzyme (Fig. 1) and are located near the bound inhibitors. Particularly, Ile<sup>235</sup> in the porcine pancreatic α-amylase appears to be in a close, though indirect, contact with the bound inhibitors. These observations corroborate the importance of Gly<sup>273</sup> in substrate binding and the remarkable effect of its replacement with Pro.

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Specificity Determinants of an \(\alpha\)-Glucosidase

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