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Mutations on Aromatic Residues of the Active Site To Alter Selectivity of the *Sulfolobus solfataricus* Maltooligosyltrehalose Synthase

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Mutations Y290F, Y367F, F405Y, and Y409F located near subsite +1 were constructed in maltooligosyltrehalose synthase (MTSase) to alter the selectivity of the enzyme. These mutations were designed to evaluate the effects of hydrophobic interactions and/or hydrogen bondings on transglycosylation and side hydrolysis reactions. The catalytic efficiencies of Y290F MTSase for hydrolysis and transglycosylation reactions were only 6.6 and 5.6%, respectively, of those of wild-type MTSase, whereas the catalytic efficiencies of Y367F MTSase were decreased by about half. F405Y MTSase had similar catalytic efficiencies for transglycosylation and a somewhat lower catalytic efficiency for hydrolysis. Y409F MTSase had somewhat lower catalytic efficiencies for the transglycosylation and a similar catalytic efficiency for hydrolysis. Y290F and Y367F MTSases had large changes in $\Delta(\Delta G)$, suggesting that there are hydrogen bonds between the substrate and residues Y290 and Y367 of wild-type MTSase. Compared with wild-type MTSase, F405Y MTSase had decreased ratios of hydrolysis to transglycosylation, whereas Y290F, Y367F, and Y409F MTSases had increased ratios. These results suggest that use of F405Y MTSase might result in a higher yield of trehalose production from starch when it replaces wild-type MTSase.

KEYWORDS: Maltooligosyltrehalose synthase; selectivity; site-directed mutagenesis; trehalose; substrate specificity; *Sulfolobus*

INTRODUCTION

Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) is a nonreducing sugar that consists of an α-1,1 linkage between two glucose molecules. The sugar is gaining more applications in many different areas, such as a sweetener component, preservative or stabilizer for food, cosmetics, and medicines (*1*).

Two thermostable enzymes were involved in the production of trehalose from starch (*2*−*4*). One is maltooligosyltrehalose synthase (MTSase, also known as trehalosyl dextrin-forming enzyme or glycosyltrehalose-producing enzyme), which mainly catalyzes an intramolecular transglycosylation reaction to form maltooligosyltrehaloses from maltooligosaccharides by converting the α-1,4-glucosidic linkage at the reducing end to an α-1,1-glucosidic linkage. The other is maltooligosyltrehalose trehalohydrolase (MTHase, also known as trehalose-forming enzyme or glycosyltrehalose-hydrolyzing enzyme), which mainly cleaves the α-1,4-glucosidic linkage next to the α-1,1-glucosidic linkage of maltooligosyltrehalose to produce trehalose and the maltooligosaccharides with lower molecular weight. Both MTSase and MTHase also catalyze a side reaction to hydrolyze maltooligosaccharides to release glucose.

In a previous study, thermophilic MTSase and MTHase were combined with a debranching enzyme to produce trehalose from starch, and the yield of trehalose was limited to below 82% due to the side hydrolysis reactions catalyzed by MTSase and MTHase (*5*−*7*). The process of producing trehalose from starch is similar to those of producing other sugars, such as glucose. The glucose yield in saccharification can be further enhanced from ∼95 to 98% through the alterations of the substrate specificity of glucoamylase by site-directed mutagenesis (*8*−*10*). If the substrate specificities of both MTSase and MTHase, such as the selective reductions in the side hydrolysis reactions, can be altered, the yield of trehalose production from starch might be enhanced in a similar way to the production of glucose from starch by glucoamylase.

The substrate specificity of an enzyme is determined by its ability to form a stable complex with a ligand in the transition state, thus leading to the product. The stability of the enzyme−ligand complex is affected by steric constraints, hydrogen bonding, van der Waals forces, electrostatic forces, and hydrophobic contacts (*11*). Subsite mapping studies have indicated

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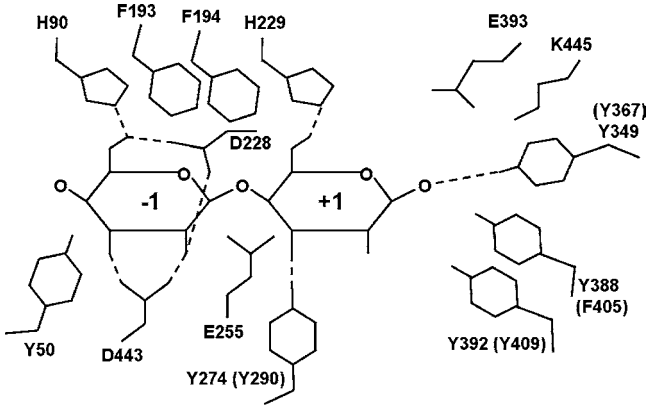

Figure 1. Schematic representation of the proposed hydrogen bonds between the *S. acidocaldarius* MTSase and a substrate with an α-1,4-linkage (*13*). The subsites are numbered according to the general subsite labeling scheme proposed for all glycosyl hydrolases (*28*), in which the substrate reducing end is at position +1. The corresponding residues in *S. solfataricus* ATCC 35092 are shown in parentheses.

that MTSase from *Sulfolobus solfataricus* KM1 has 10 glucosyl binding subsites with the catalytic site located between subsites −1 and +1 (*12*). The *Sulfolobus acidocaldaricus* MTSase catalytic domain contains two external segments and an incomplete $(\beta/\alpha)_8$-barrel structure, which is similar to the $(\beta/\alpha)_8$-barrel structure shared among α-amylase family enzymes (*13*). The carboxy edge of the central β sheet forms the bottom of the active-site cleft, and residues Asp228, Glu255, and Asp443—which correspond to the catalytic residues of the α-amylase family enzymes—are located conservatively at the bottom of the cleft (*13*). According to the information from CAZy, a World Wide Web resource on glycosyl hydrolases at http://afmb.cnrs-mrs.fr/CAZY/, these MTSases have been previously classified in family 13 of glycosyl hydrolases. Families 13, 70, and 77 in this classification constitute the α-amylase family, which contains functionally and structurally related enzymes (*14*). Homology analysis and understanding of the three-dimensional structure of MTSase make site-directed mutagenesis possible to alter MTSase selectivity with a reduced side hydrolysis reaction in order to meet the requirements for trehalose production from starch.

The MTSase from *S. solfataricus* ATCC 35092, also known as P2, has been purified and characterized in our previous study (*15*). Our previous results suggested that the hydrolysis reaction of MTSase was one of the major factors causing the decrease in the yield of trehalose production from starch (*15*). The decreases in the ratios of hydrolysis to transglycosylation may enhance the yield of trehalose production from starch (*15*). According to the hypothetical binding mode of carbohydrates in the active site pocket of *S. acidocaldaricus* MTSase proposed by Kobayashi et al. (**Figure 1**) (*13*), several amino acid residues located near subsite +1 of the active site may control the rotation of the cleaved glucose unit prior to the reconnection of the cleaved glucose unit and the remnant to form an α,α-1,1-linkage. In this study, site-directed mutagenesis was used to construct four mutations near subsite +1 of the active site of *S. solfataricus* ATCC 35092 MTSase in order to decrease the hydrolysis reaction, and we investigated the substrate specificities and kinetic parameters of the wild-type and mutant enzymes on both transglycosylation and hydrolysis reactions.

MATERIALS AND METHODS

Materials. *Escherichia coli* Rosetta (DE3) was from Novagen (Madison, WI). A QuickChange XL Site-Directed Mutagenesis Kit was purchased from Stratagene (La Jolla, CA). Glucose (G_1), maltose (G_2), maltotriose (G_3), maltotetraose (G_4), maltopentaose (G_5), maltohexaose (G_6), maltoheptaose (G_7), glucoamylase, 3,5-dinitrosalicylic acid (DNS), bovine serum albumin (BSA), isopropyl-β-D-thiogalactoside (IPTG), and benzamidine were supplied by Sigma (St. Louis, MO). Ultrafree-15 and Microcon centrifugal filter units were obtained from Millipore (Bedford, MA). Sephacryl S-200 HR and protein low molecular weight standards were from Amersham Pharmacia Biotech (Piscataway, NJ).

Table 1. Nucleotide Sequences of Primers Used in Site-Directed Mutagenesis[a]

mutation	nucleotide sequence of primer (5′→3′)
Y290F	F[b]: GGAAAGTAGATGGTACTACTGGA**TTT**GATTTCCTGAACTACG
	R[c]: CGTAGTTCAGGAAATC**AAA**TCCAGTAGTACCATCTACTTTCC
Y367F	F: CTAGCATGTATGAAAAAA**TTC**AGGACGTATTTACCATATGAGG
	R: CCTCATATGGTAAATACGTCCT**GAA**TTTTTTCATACATGCTAG
F405Y	F: CAATACATGCCAGCAATC**TAC**GCTAAGGGCTATGAGG
	R: CCTCATAGCCCTTAGC**GTA**GATTGCTGGCATGTATTG
Y409F	F: CTTCGCTAAGGGC**TTT**GAGGATACTACCC
	R: GGGTAGTATCCTC**AAA**GCCCTTAGCGAAG

[a] Nucleotides for the designed MTSase mutations are shown in bold. [b] Forward primer. [c] Reverse primer.

Site-Directed Mutagenesis. The MTSase gene in the previously constructed vector pET-15b-ΔH-*treY* (*15*) was mutated by Polymerase Chain Reaction (PCR) according to the instructions of the QuickChange XL Site-Directed Mutagenesis Kit. The designed mutations were included in the forward/reverse primer pairs as listed in **Table 1**. By this method, *Dpn*I endonuclease—which is specific for methylated and hemimethylated DNA—is used to digest the parental DNA template and to select the PCR-amplified DNA (*16*).

Expression of MTSase. Wild-type and mutant pET-15b-ΔH-*treY* vectors were transformed into *E. coli* Rosetta (DE3) to express wild-type and mutant MTSases, respectively, as previously described (*15*).

Preparation of Cellfree Extract. Frozen cells (6 g) expressing wild-type and mutant MTSases were suspended in 18 mL of lysis buffer containing 20 mM Tris-HCl, pH 8.0, and 1 mM benzamidine. The suspended cells were disrupted using a French Press disruptor (Sim-Aminco, Rochester, NY) at 20000 psi. The cellfree extract was then prepared by removing the insoluble fractions from the supernatant of the above mixture by centrifugation at 10000*g* for 2 h.

Purification of MTSase. Heat treatment was first used to precipitate most of the undesired proteins by incubating the cellfree extract at 80 °C water bath for 1 h followed by centrifugation to remove the heat-labile proteins. A 10% (w/v) streptomycin sulfate stock solution was then added to a final concentration of 1% (w/v) to precipitate the nucleic acids. After centrifugation at 10000*g* for 1 h, the supernatant was dialyzed against 20 mM Tris-HCl buffer, pH 8.5, and was subsequently loaded onto a Q-Sepharose column (1.6 × 10 cm), which was pre-equilibrated with a buffer of the same composition as the above dialysis buffer. The column was first washed thoroughly with the same buffer until the absorbance of 260 nm became almost undetectable. Then, a linear gradient of 0−0.5 M NaCl in the above buffer was used to elute the bound proteins. The eluted fractions containing enzyme activity were collected and concentrated for further purification by gel filtration. The concentrated sample was loaded onto a Sephacryl S-200 HR column (1.6 × 60 cm) previously equilibrated with a buffer containing 20 mM Tris-HCl, pH 8.0, and 0.2 M NaCl. The eluted fractions containing enzyme activity were collected for the investigations of the substrate specificities and kinetic parameters.

Purity of Wild-Type and Mutant MTSases. The purity of the enzyme was analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE; 6−12% gradient minigel) according to the method of Laemmli (*17*). Phosphorylase (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (32 kDa) were used as molecular mass standards. The purity of wild-type and mutant MTSases is >95% as indicated by SDS-PAGE (**Figure 2**).

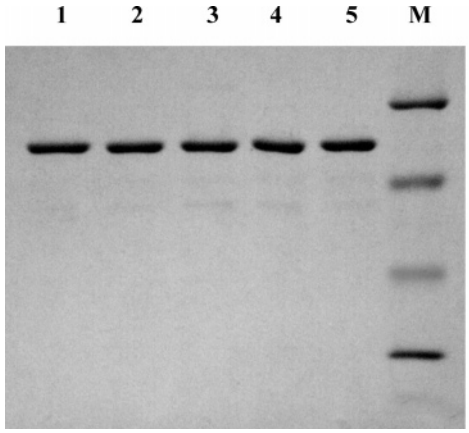

Figure 2. SDS-PAGE of purified wild-type and mutant MTSases. A 6–12% gradient minigel was stained with Coomassie Brilliant Blue R-250: lane M, molecular weight standards; lanes 1–5, wild-type, F405Y, Y290F, Y367F, and Y409F MTSases, respectively.

Transglycosylation Reaction Assay. The transglycosylation reaction assay was modified from the procedures described previously (*15*). Maltooligosaccharides with degrees of polymerization (DP) of 3−7 (14 mM) were incubated with MTSase in 50 mM citrate−phosphate buffer at pH 5 and 60 °C for 5 min. The amounts of residual reducing sugars, which were determined according to the DNS method (*18*) with maltooligosaccharides as standards, were used to determine the amounts of maltooligosyltrehaloses produced by MTSase because MTSase converts maltooligosaccharides, the compounds with reducing power, to maltooligosyltrehaloses, the compounds without reducing power.

Hydrolysis Reaction Assay. Maltooligosaccharides of DP 3−7 (14 mM) were incubated with MTSase in 50 mM citrate−phosphate buffer at pH 5 and 60 °C for 1 h. The reactions were stopped by adding 0.4 volume of 4 M Tris-HCl buffer at pH 8.0 and incubating the mixture in the boiling-water bath for 10 min. The released glucose was measured by the glucose oxidase method (*19*).

Enzyme Kinetics. The initial rates for hydrolysis of G_3 and transglycosylation of G_4−G_7 were determined at 60 °C in 50 mM citrate−phosphate buffer at pH 5 by using 8−10 substrate concentrations ranging from $0.1K_M$ to $8K_M$. Samples taken at five different time intervals were stopped by adding 4 M Tris-HCl buffer, pH 8.0 (used for hydrolysis of G_3) or DNS (used for transglycosylation of G_4−G_7). The released glucose and the molar concentrations of the converted maltooligosyltrehaloses were determined as described in the methods of hydrolysis and transglycosylation reaction assays, respectively. Values of k_{cat} and K_M were calculated by fitting the initial rates as a function of substrate concentration to the Michaelis−Menten equation using Enzfitter software (Elsevier-Biosoft). The change of transition-state binding energy [$\Delta(\Delta G)$] for substrate hydrolysis caused by the mutation was used to estimate the binding strength of the substrate in the transition-state complex and was calculated by the equation $\Delta(\Delta G) = -RT \ln[(k_{cat}/K_M)_{mut}/(k_{cat}/K_M)_{wt}]$, where the subscripts mut and wt denote mutant and wild-type enzymes, respectively (*20*).

Protein Concentration Measurement. Protein concentration was quantitated according to Bradford's method (*21*) with BSA as standard.

Circular Dichroism (CD) Measurements. CD spectra were measured from 200 to 260 nm at room temperature by a J810 CD spectrometer (Jacso, Tokyo, Japan). The concentration of wild-type or mutant MTSase was 0.2 mg/mL in 20 mM Tris-HCl, pH 8.0.

RESULTS

Mutation Design and Comparison of Amino Acid Sequences of Several MTSases from the *Sulfolobus* Genus. On the basis of the hypothetical binding mode of carbohydrates in the active site pocket of *S. acidocaldarius* MTSase proposed by Kobayashi et al., several amino acid residues located near subsite +1 of the active site are expected to control the rotation of the cleaved glucose unit prior to the reconnection of the

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                                                    *
S. solfataricus ATCC35092 279 R D D W K V D G T T G Y D F L N Y V N M 298
S. solfataricus MT4       279 R D D W K V D G T T G Y D F L N Y V N M 298
S. solfataricus KM1       279 R D D W K V D G T T G Y D F L N Y V N M 298
S. shibatae               279 R D D W K V D G T T G Y D F L N Y V N M 298
S. acidocaldarius         266 K L N . . S D G T T G Y D F L N Y S N L 282
S. tokodaii               257 R W D F . I D G T T G Y D F L N Y S N L 275

                                                  *
S. solfataricus ATCC35092 359 D F L A C M K K Y R T Y L P Y E D I N G 373
S. solfataricus MT4       359 D F L A C M K K Y R T Y L P Y E D I N G 373
S. solfataricus KM1       359 D F L A C M K K Y R T Y L P Y E D I N G 373
S. shibatae               359 D F L A C M K K Y R T Y L P F E D I N G 373
S. acidocaldarius         341 D Y L S C I D V Y R T Y A N Q . . I . . 356
S. tokodaii               332 E F L S C L K V Y R T Y I T E N D F R D 346

                                                      *       *
S. solfataricus ATCC35092 394 I M R L Q Q Y M P A I F A K G Y E D T T 413
S. solfataricus MT4       394 I M R L Q Q Y M P A I F A K G Y E D T T 413
S. solfataricus KM1       394 I M R L Q Q Y M P A I F A K G Y E D T T 413
S. shibatae               394 I M R L Q Q Y M P A I F A K G Y E D T T 413
S. acidocaldarius         377 Y T K L Q Q Y M P A V Y A K A Y E D T F 396
S. tokodaii               367 F M K L Q Q Y M P A V F A K A Y E D T V 386
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Figure 3. Comparison of partial amino acid sequences near four aromatic residues mutated in this study. The four aromatic residues mutated in this study are marked with asterisks (*). Identical residues among MTSases from six different strains are shown in boldface. The deduced amino acid sequence of MTSase from *S. solfataricus* MT4 was from de Pascale et al. (*29*); the others were obtained from the TrEMBL protein database on the website http://pir.georgetown.edu/pirwww/search/pirnref.shtml under the following NREF IDs: NF00193302 for *S. solfataricus* ATCC35092, NF00193391 for *S. solfataricus* KM1, NF00190809 for *S. shibatae*, NF00190642 for *S. acidocaldarius*, and NF00781958 for *S. tokodaii*.

cleaved glucose unit and the remnant to form an α,α-1,1-linkage (**Figure 1**) (*13*). We chose four aromatic amino acid residues, Y274, Y349, Y388, and Y392, near subsite +1 as the targets for site-directed mutagenesis to decrease the side hydrolysis reaction. On the basis of the binding model, Y274 and Y349 could form a hydrogen bond with O1 and O3, respectively, of the glucose unit on the reducing end of the bound carbohydrate. The tyrosine residue of position 388 in *S. acidocaldarius* MTSase was replaced by phenylalanine in other MTSases from the *Sulfolobus* genus (**Figure 3**). Y274, Y349, Y388, and Y392 of *S. acidocaldarius* MTSase correspond to Y290, Y367, F405, and Y409 of *S. solfataricus* ATCC 35092 MTSase, respectively (**Figure 3**). Mutations Y290F and Y367F were designed to verify the existence of hydrogen bonds between substrate and residues Y290 and Y367 and also to evaluate the effects of both hydrogen bonds and hydrophobic interactions on transglycosylation and side hydrolysis reactions. Mutations F405Y and Y409F were designed to evaluate the effects of hydrophobic interactions on transglycosylation and side hydrolysis reactions.

Secondary Structure Analysis. CD spectra of wild-type and mutant MTSases were essentially identical (**Figure 4**), suggesting the absence of long-range conformational changes as a consequence of mutation.

Substrate Specificities of Wild-Type and Mutant MTSases. A comparison of the substrate specificities among DP 3−7 maltooligosaccharides for wild-type and mutant MTSases is shown in **Table 2**. For both transglycosylation and hydrolysis reactions, the maltooligosyltrehalose and glucose formation rates of mutant MTSases were in general lower than those of wild-type MTSase (**Table 2**). The maltooligosyltrehalose formation rates of mutant MTSases generally became higher when the DP of the substrates increased, which is in a way similar to those of wild-type MTSase. Additionally, the glucose formation rates of mutant MTSases generally became higher when the DP of the substrates decreased, which is also in a way similar

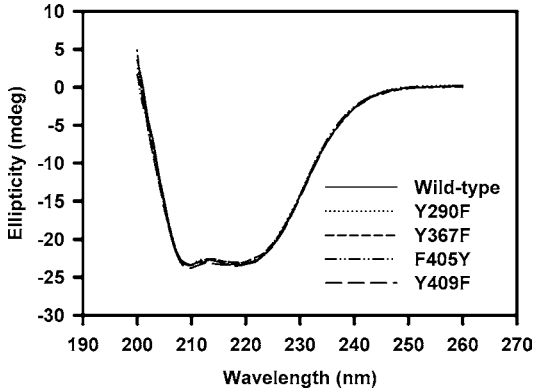

Figure 4. CD spectra of purified wild-type and mutant MTSases. CD spectra were measured from 200 to 260 nm at room temperature. The concentration of MTSase used was 0.2 mg/mL in 20 mM Tris-HCl, pH 8.0.

Table 2. Substrate Specificities of Wild-Type and Mutant MTSases for Hydrolysis and Transglycosylation of G_3–G_7 at 60 °C in 50 mM Citrate Phosphate Buffer, pH 5

MTSase form substrate (14 mM)	hydrolysis, glucose formation rate (mol/mol of MTSase·s)	transglycosylation, maltooligosyltrehalose formation rate (mol/mol of MTSase·s)	ratio of H/T[a] (%)
wild-type			
G_3	9.31 ± 0.53[b]	45.6 ± 2.6	20.4
G_4	2.22 ± 0.06	116 ± 2	1.92
G_5	1.85 ± 0.14	259 ± 5	0.71
G_6	0.94 ± 0.04	242 ± 10	0.39
G_7	0.94 ± 0.09	293 ± 6	0.32
Y290F			
G_3	0.45 ± 0.12	1.89± 0.21	23.8
G_4	0.36 ± 0.01	8.07 ± 2.52	4.5
G_5	0.51 ± 0.10	57.1 ± 4.2	0.89
G_6	0.13 ± 0.01	34.0 ± 6.9	0.38
G_7	0.15 ± 0.02	46.0 ± 6.8	0.33
Y367F			
G_3	3.94 ± 0.26	12.5 ± 0.9	31.6
G_4	2.21 ± 0.04	43.5 ± 6.4	5.08
G_5	2.31 ± 0.19	177 ± 20	1.30
G_6	1.25 ± 0.04	147 ± 15	0.85
G_7	1.31 ± 0.03	219 ± 17	0.60
F405Y			
G_3	7.23 ± 0.14	36.0 ± 0.8	20.1
G_4	1.59 ± 0.12	91.3 ± 4.5	1.74
G_5	1.31 ± 0.10	210 ± 10	0.63
G_6	0.59 ± 0.06	202 ± 5	0.29
G_7	0.65 ± 0.08	239 ± 8	0.27
Y409F			
G_3	8.07 ± 0.65	35.7 ± 5.4	22.6
G_4	2.07 ± 0.10	94.3 ± 5.0	2.20
G_5	1.56 ± 0.07	210 ± 8	0.74
G_6	0.92 ± 0.05	220 ± 9	0.42
G_7	0.99 ± 0.02	292 ± 4	0.34

[a] Ratio of hydrolysis to transglycosylation. [b] Mean ± SD from triple experiments.

to those of wild-type MTSase. The ratios of hydrolysis to transglycosylation of G_3−G_7 for F405Y MTSase were lower than those of wild-type MTSase; however, the ratios of hydrolysis to transglycosylation of G_3−G_7 for Y290F, Y367F, and Y409F MTSases were higher than those of wild-type MTSase.

Enzyme Kinetics. Kinetic parameters (k_{cat} and K_M) for the hydrolysis of G_3 and transglycosylation of G_4−G_7 at 60 °C and pH 5 are given in **Table 3**. Y290F and Y367F MTSases were not very active compared with wild-type MTSase; therefore, only the reaction rates for the hydrolysis of G_3 and transglycosylation of G_6 were measured. Wild-type, F405Y, and Y409F MTSases had k_{cat} values for transglycosylation increasing rapidly with increasing DP from G_4 to G_5 to a relatively constant value with longer substrates. Conversely, K_M values decreased rapidly with increasing DP from G_4 to G_5, falling only slowly beyond that point.

Y290F MTSase had a significantly lower k_{cat} value and a significantly higher K_M value for the hydrolysis of G_3 than those of wild-type MTSase. In addition, Y290F MTSase had a significantly lower k_{cat} value and a slightly higher K_M value for the transglycosylation of G_6 than those of wild-type MTSase. As a result, the catalytic efficiencies for hydrolysis and transglycosylation reactions were only 6.6 and 5.6%, respectively, of those of wild-type MTSase. $\Delta(\Delta G)$ values were 7.52 and 7.97 kJ/mol for the hydrolysis of G_3 and transglycosylation of G_6, respectively.

Y367F MTSase had a somewhat lower k_{cat} value and a significantly higher K_M value than those of wild-type MTSase for the hydrolysis of G_3 and a significantly lower k_{cat} value and a slightly lower K_M value for the transglycosylation of G_6. The catalytic efficiencies for these two reactions were generally about half of those of wild-type MTSase. $\Delta(\Delta G)$ values were 2.08 and 2.11 kJ/mol for the hydrolysis of G_3 and the transglycosylation of G_6, respectively.

F405Y MTSase had k_{cat} values and K_M values similar to those of wild-type MTSase, except that the K_M value for the hydrolysis of G_3 was somewhat higher than those of wild-type MTSase. This led to similar catalytic efficiencies for the transglycosylation of G_4−G_7 and a somewhat lower catalytic efficiency for the hydrolysis of G_3. $\Delta(\Delta G)$ values were slightly negative for the transglycosylation of G_4 and between 0.09 and 0.53 kJ/mol otherwise.

Y409F MTSase had k_{cat} values for the transglycosylation of G_4−G_7 slightly lower than those of wild-type MTSase and K_M values similar to those of wild-type MTSase. The k_{cat} and K_M values for the hydrolysis of G_3 were similar to those of wild-type MTSase. This led to somewhat lower catalytic efficiencies for the transglycosylation of G_4−G_7 and a similar catalytic efficiency for the hydrolysis of G_3. $\Delta(\Delta G)$ values ranged from 0.03 and 1.13 kJ/mol.

DISCUSSION

Residues 290, 367, 405, and 409 were mutated to study how the hydrogen bonds and/or hydrophobic interactions between enzyme and substrate affect MTSase selectivity. The mutations on the above residues including Y290F, Y367F, F405Y, and Y409F are all very conservative mutations.

Substitutions of phenylalanine at positions 290 and 367 were designed to remove the proposed hydrogen bonds between *S. solfataricus* ATCC35092 MTSase and substrate. We calculated the transition-state energy to estimate the binding strength of the enzyme−substrate complex in the transition state. As some earlier studies pointed out, the change of transition-state energy, $\Delta(\Delta G)$, associated with the loss of a hydrogen bond between uncharged groups on the substrate and enzyme is between 2.1 and 6.3 kJ/mol, whereas that for loss of a hydrogen bond between an uncharged group on the substrate and a charged group on the enzyme is between 14.6 and 18.8 kJ/mol (*8*, *11*).

In this study, the large changes in $\Delta(\Delta G)$ for G_3 hydrolysis and for G_6 transglycosylation by Y290F MTSase, 7.52 and 7.97 kJ/mol, respectively, and Y367F MTSase, 2.08 and 2.11 kJ/mol, respectively (**Table 3**), are close to the loss of an uncharged hydrogen bond between MTSase and the substrate in the transition state. These results suggested that there are hydrogen bonds between substrate and residues Y290 and Y367 on the

Table 3. Kinetic Parameters of Wild-Type and Mutant MTSases for Hydrolysis of G_3 and Transglycosylation of G_4–G_7 at 60 °C in 50 mM Citrate Phosphate Buffer, pH 5[a]

	hydrolysis	transglycosylation			
MTSase form	G_3	G_4	G_5	G_6	G_7
wild-type					
k_{cat} (s^{-1})	44.0 ± 1.3[b]	138 ± 4	364 ± 7	359 ± 13	383 ± 14
K_M (mM)	41.3 ± 3.0	13.2 ± 0.9	5.94 ± 0.27	4.53 ± 0.41	3.88 ± 0.38
k_{cat}/K_M (s^{-1} mM^{-1})	1.06 ± 0.05	10.4 ± 0.4	61.2 ± 0.8	79.2 ± 4.7	98.8 ± 6.6
Y290F					
k_{cat} (s^{-1})	9.48± 0.75			26.5 ± 1.4	
K_M (mM)	134 ± 17			6.00 ± 0.76	
k_{cat}/K_M (s^{-1} mM^{-1})	0.070 ± 0.007			4.45 ± 0.36	
$\Delta(\Delta G)$[c] (kJ/mol)	7.52			7.97	
Y367F					
k_{cat} (s^{-1})	35.9 ± 1.3			139 ± 5	
K_M (mM)	71.8 ± 5.2			3.78 ± 0.35	
k_{cat}/K_M (s^{-1} mM^{-1})	0.500 ± 0.020			36.9 ± 2.4	
$\Delta(\Delta G)$ (kJ/mol)	2.08			2.11	
F405Y					
k_{cat} (s^{-1})	47.4 ± 1.9	139 ± 13	354 ± 9	362 ± 9	363 ± 13
K_M (mM)	54.1 ± 4.9	11.6 ± 2.0	6.34 ± 0.45	4.90 ± 0.31	3.80 ± 0.37
k_{cat}/K_M (s^{-1} mM^{-1})	0.876 ± 0.050	12.0 ± 1.1	55.8± 2.6	74.0 ± 3.1	95.6 ± 6.3
$\Delta(\Delta G)$ (kJ/mol)	0.53	–0.40	0.26	0.19	0.09
Y409F					
k_{cat} (s^{-1})	44.9 ± 2.1	114 ± 6	267 ± 8	330 ± 10	282± 9
K_M (mM)	42.8 ± 4.9	16.1 ± 1.7	5.85 ± 0.42	6.26 ± 0.48	3.8 ± 0.4
k_{cat}/K_M (s^{-1} mM^{-1})	1.05 ± 0.08	7.07 ± 0.40	45.5 ± 2.1	52.7 ± 2.7	74.5 ± 4.9
$\Delta(\Delta G)$ (kJ/mol)	0.03	1.07	0.82	1.13	0.78

[a] Y290F and Y367F MTSases were not very active compared with wild-type MTSase; therefore, only the reaction rates for the hydrolysis of G_3 and the transglycosylation of G_6 were measured. [b] Standard error. [c] Change of transition-state energy: $\Delta(\Delta G) = -RT \ln[(k_{cat}/K_M)_{mut}/(k_{cat}/K_M)_{wt}]$ (*20*).

wild-type MTSase of *S. solfataricus* ATCC 35092. However, mutations F405Y and Y409F showed small $\Delta(\Delta G)$ values, from −0.4 to 1.13 kJ/mol, indicating that these two mutations had only minor effects on transition-state substrate binding.

The loss of the hydrogen bonds between substrate and residues Y290 and Y367 might contribute to the seriously decreased catalytic efficiencies of Y290F and Y367F MTSases (**Table 3**). Compared to wild-type MTSase, Y290F and Y367F MTSases also had increased ratios of hydrolysis to transglycosylation for substrates G_3−G_7 (**Table 2**). Substituting phenylalanine at residues Y290 and Y367 not only removed the hydrogen bonds between enzyme and substrate but also provided slightly increased hydrophobic interactions between enzyme and substrate.

Residue F405 in *S. solfataricus* ATCC35092 MTSase is equivalent to residue Y388 in *S. acidocaldarius* MTSase (**Figures 1** and **2**). Mutation F405Y was designed to create a slightly decreased hydrophobic interaction between MTSase and substrate. Indeed, F405Y MTSase showed decreased ratios of hydrolysis to transglycosylation for substrates G_3−G_7 (**Table 2**).

On the other hand, mutation Y409F was designed to have a slightly increased hydrophobic interaction between MTSase and substrate. Y409F MTSase showed increased ratios of hydrolysis to transglycosylation for substrates G_3−G_7 (**Table 2**).

According to the model suggested by Kobayashi et al., several amino acid residues located near subsite +1 of the active site may control the rotation of the cleaved glucose unit prior to the formation of an α,α-1,1-linkage (**Figure 1**) (*13*). Y290F, Y367F, and Y409F MTSases had slightly increased hydrophobic interactions between enzyme and substrate compared to that of wild-type MTSase, and they also had increased ratios of hydrolysis to transglycosylation. F405Y MTSase had slightly decreased hydrophobic interactions between enzyme and substrate compared to that of wild-type MTSase, and this mutant also had decreased ratios of hydrolysis to transglycosylation. Making mutations near the substrate-binding site could dramatically increase the success rate to change the substrate specificity compared to those of distant ones (*22*). Several studies demonstrated that active site hydrophobicity could affect the catalytic activity and/or substrate binding of an enzyme (*23*−*27*). Our results also suggested that manipulating the strengths of hydrophobic interactions near subsite +1 of MTSase active site might play a role in changing the ratios of hydrolysis to transglycosylation for substrates G_3−G_7. For mutations Y290F and Y367F, the loss of hydrogen bonds might also contribute to the increased ratios of hydrolysis to transglycosylation for substrates G_3−G_7.

In conclusion, in this study we have confirmed the existence of two hydrogen bonds between substrate and enzyme at positions Y290 and Y367. MTSase selectivity could be changed by altering hydrogen bonding and/or hydrophobic interactions between substrate and enzyme at positions near subsite +1 of the enzyme−substrate complex. Decreasing hydrophobic interactions between substrate and the enzyme led to the decreased ratios of hydrolysis to transglycosylation for F405Y MTSase, whereas increasing hydrophobic interactions and/or removing hydrogen bonding resulted in the increased ratios of hydrolysis to transglycosylation for Y290F, Y367F, and Y409F MTSases. In addition, F405Y MTSase has similar catalytic efficiencies for the transglycosylation of G_4−G_7 and a somewhat lower catalytic efficiency for the hydrolysis of G_3. F405Y MTSase showed a potential to replace wild-type MTSase to be used in combination with other enzymes for the production of trehalose from starch with a higher yield.

ABBREVIATIONS USED

MTSase, maltooligosyltrehalose synthase; MTHase, maltooligosyltrehalose trehalohydrolase; G_1, glucose; G_2, maltose; G_3, maltotriose; G_4, maltotetraose; G_5, maltopentaose; G_6, maltohexaose; G_7, maltoheptaose; DNS, 3,5-dinitrosalicylic acid;

BSA, bovine serum albumin; IPTG, isopropyl-β-D-thiogalactoside; DP, degree of polymerization; $\Delta(\Delta G)$, change of transition-state energy; CD, circular dichroism; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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