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Protein Engineering of *Sulfolobus solfataricus* Maltooligosyltrehalose Synthase To Alter Its Selectivity

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Maltooligosyltrehalose synthase (MTSase) is one of the key enzymes involved in trehalose production from starch and catalyzes an intramolecular transglycosylation reaction by converting the α-1,4- to α,α-1,1-glucosidic linkage. Mutations at residues F206, F207, and F405 were constructed to change the selectivity of the enzyme because the changes in selectivity could reduce the side hydrolysis reaction of releasing glucose and thus increase trehalose production from starch. As compared with wild-type MTSase, F405Y and F405M MTSases had decreased ratios of the initial rate of glucose formation to that of trehalose formation in starch digestion at 75 °C when wild-type and mutant MTSases were, respectively, used with isoamylase and maltooligosyltrehalose trehalohydrolase (MTHase). The highest trehalose yield from starch digestion was by the mutant MTSase having the lowest initial rate of glucose formation to trehalose formation, and this predicted high trehalose yield better than the ratio of catalytic efficiency for hydrolysis to that for transglycosylation.

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

INTRODUCTION

Maltooligosyltrehalose synthase (EC 5.4.99.15, MTSase, also known as glycosyltrehalose synthase, glycosyltrehalose-producing enzyme, and trehalosyl dextrin-forming enzyme) catalyzes an intramolecular transglycosylation reaction to produce a nonreducing maltooligosyltrehalose by converting the α-1,4-glucosidic linkage at the reducing end of maltooligosaccharide to an α,α-1,1-glucosidic linkage. Maltooligosyltrehalose can be further hydrolyzed by maltooligosyltrehalose trehalohydrolase (EC 3.2.1.141, MTHase, also known as glycosyltrehalose trehalohydrolase, glycosyltrehalose-hydrolyzing enzyme, and trehalose-forming enzyme) to produce trehalose by cleaving the α-1,4-glucosidic linkage next to the α,α-1,1-linked terminal disaccharide. However, both MTSase and MTHase catalyze a side reaction of hydrolyzing maltooligosaccharides to release glucose (G_1).

Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) is a nonreducing sugar that consists of an α,α-1,1-linkage between two G_1 molecules. The sugar protects proteins and cell membranes from dry, heat, and osmotic changes and is gaining more applications in many different areas, such as a sweetener component, preservative, or stabilizer for food, cosmetics, vaccines, and medicines (*1*). When thermophilic MTSase and MTHase were combined with a debranching enzyme to produce trehalose from starch, the yield of trehalose was limited to below 82% due to the side hydrolysis reactions catalyzed by MTSase and MTHase (*2*−*4*). This suggests that engineering the substrate specificities of both MTSase and MTHase to decrease the side hydrolysis reactions would increase the trehalose yield.

Previous kinetic studies have indicated that MTSase has 10 glucosyl binding subsites, numbered from +1 to −9, with the catalytic site that is located between subsites +1 and −1 (*5*). The MTSase catalytic mechanism involves three carboxyl groups, Asp228, Glu255, and Asp443, in *Sulfolobus acidocaldaricus* MTSase numbering (*6*). The intramolecular transglycosylation catalyzed by MTSase is considered to consist of two stages: (i) the cleavage of the α-1,4-glucosidic linkage and (ii) the reconnection of forming the α,α-1,1-glucosidic linkage (*6*). Asp443 first twists and deforms the G_1 ring located at the subsite −1 when the substrate is bounded to the active site. Glu255 and Asp228 may then function as the catalytic acid and base, respectively, to cleave the glucosidic bond between subsites −1 and +1. The cleaved G_1 unit was rotated in subsite +1 and then reconnected to form an α,α-1,1-glucosidic linkage (*6*). The hypothetical binding mode of carbohydrates in the active site pocket of MTSase, which was proposed by Kobayashi et al. (**Figure 1**) (*6*), has suggested that several amino acid residues located near subsites +1 and −1 of the active site may affect the rotation of the cleaved G_1 unit prior to the reconnection to form an α,α-1,1-linkage.

The MTSase from *Sulfolobus solfataricus* ATCC 35092, also known as P2, has been purified, characterized, and mutated in

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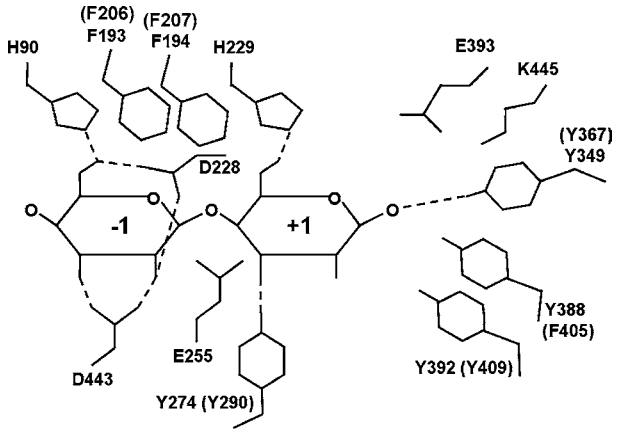

Figure 1. Schematic representation of the proposed binding model between the *S. acidocaldarius* MTSase and a substrate with an α-1,4-linkage (*6*, *8*). The subsites are numbered according to the general subsite-labeling scheme proposed for glycosyl hydrolases (*24*), in which the substrate reducing end is at position +1. The corresponding residues in *S. solfataricus* ATCC 35092 are shown in parentheses.

our previous study (*7*, *8*). Our previous results suggested that the decreases in the ratios of hydrolysis to transglycosylation might enhance the yield of trehalose production from starch (*7*). We also found that decreasing hydrophobic interactions between enzyme and substrate at positions near subsite +1 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 (*8*). We suggested that F405Y MTSase might result in a higher yield of trehalose production from starch when it replaces wild-type MTSase (*8*). In this study, site-directed mutagenesis was used to construct six mutations at positions near subsites +1 and −1. F405M, F405S, and F405W MTSases were designed to further decrease or increase the hydrophobic interactions between residue 405 and substrate as compared to F405Y MTSase. Additionally, F206W, F206Y, and F207Y MTSases were also constructed to study the hydrophobic interactions between enzyme and substrate at positions near subsite −1. In addition to investigating the kinetic parameters of the wild-type and mutant enzymes on both transglycosylation and hydrolysis reactions, we also carried out the production of trehalose from digestion of 10% (w/v) soluble starch at 75 °C by using wild-type and mutant MTSases, respectively, along with isoamylase and MTHase.

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). G_1, maltose (G_2), maltotriose (G_3), maltotetraose (G_4), maltopentaose (G_5), maltohexaose (G_6), maltoheptaose (G_7), glucoamylase, 3,5-dinitrosalicyclic acid (DNS), and bovine serum albumin (BSA) were supplied by Sigma (St. Louis, MO). Q-Sepharose, Sephacryl S-200 HR, and protein low molecular weight standards were from Amersham Pharmacia Biotech (Piscataway, NJ). Microcon Centrifugal Filter Unit was obtained from Millipore (Bedford, MA).

Site-Directed Mutagenesis. The MTSase gene in the previously constructed vector pET-15b-Δ*H-treY* (*7*) was mutated by polymerase chain reaction according to the instructions of QuickChange XL Site-Directed Mutagenesis Kit as previously described (*8*). The designed mutations were included in the primers as listed in **Table 1**.

Production and Purification of MTSase. Wild-type and mutant MTSases were produced by culturing pET-15b-Δ*H-treY*-transformed *E. coli* Rosetta (DE3) at 37 °C in terrific broth medium supplemented with 100 μg/mL ampicillin plus 34 μg/mL chloramphenicol as previously described (*7*). Wild-type and mutant MTSases were purified after cell disruption by using heat treatment, streptomycin sulfate precipitation, Q-Sepharose anion exchange column chromatography, and Sephacryl S-200 HR column chromatography as previously described (*8*). The protein concentration was quantitated by Bradford's method (*9*) with BSA as standards.

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

mutation	nucleotide sequence of the primer (5′ → 3′)
F206W	CAAATTATAGGAGA**TGG**TTCGCGGTAAATG
F206Y	CAAATTATAGGAGA**TAC**TTCGCGGTAAATG
F207Y	CAAATTATAGGAGATTC**TAC**GCGGTAAATGATTTG
F405M	CAGCAATC**ATG**GCTAAGGGCTATG
F405S	CATGCCAGCAATCT**CC**GCTAAGGGCTATG
F405W	CAGCAATC**TGG**GCTAAGGGCTATG

[a] Only the sequences of forward primers are shown. The sequence of reverse primer for each mutation is complementary to its forward primer. Nucleotides for the designed MTSase mutations are shown in bold. The silent mutation, decreasing the melting temperature of the primer hairpin, is underlined.

Enzyme Kinetics. The values of k_{cat} and K_M 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 0.1 K_M to 8 K_M as previously described (*8*). Values of k_{cat}, K_M, and k_{cat}/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 standard errors of these parameters were obtained from the fitting results. 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 (*10*).

Production of Trehalose from Starch. To produce trehalose from starch, purified thermophilic isoamylase, MTSase, and MTHase were used concurrently. The purified thermophilic isoamylase and MTHase were prepared according to the method described in our previous studies (*11*, *12*). The reaction was carried out at 75 °C with 10% (w/v) soluble starch in 50 mM citrate phosphate buffer at pH 5. The enzyme concentration was 0.041 μM for both wild-type and mutant MTSases. Aliquots of 800 U/g starch of isoamylase and 80 U/g starch of MTHase were also added into the reaction mixture. Samples were taken at various time intervals from 0 to 36 h to detect the produced G_1 and trehalose. The reactions were stopped by incubating the mixture in a boiling water bath for 10 min. For the detection of G_1, 0.4 volume of 4 M Tris-HCl buffer was added to the cooled mixture and the amount of G_1 was measured by a G_1 oxidase method (*13*). For the detection of trehalose, glucoamylase was added to the cooled mixture at a final concentration of 5 U/mL to hydrolyze the residual starch, maltooligosaccharides, and maltooligosyltrehaloses, and the reaction was carried out at 40 °C overnight. The reaction mixtures were then filtered through a Microcon Centrifugal Filter Unit with YM-3 membrane (MWCO 3000) to remove the enzyme. The filtrates were analyzed by a Vercopak Nucleosil 5 μm NH_2 column (4.6 mm × 250 mm) with a mobile phase consisting of 30% (v/v) H_2O and 70% (v/v) acetonitrile. Initial rates of trehalose and G_1 formation, respectively, were determined by fitting experimental data obtained within 1 h to the equation $c = At + B$, where c is the product concentration, t is the time, and A (the initial rate) and B are obtained by linear regression. The peak trehalose yield was the average of the three highest consecutive trehalose yield measurements.

RESULTS

Purity and Thermostability of Wild-Type and Mutant MTSases. The purity of the enzyme was analyzed by sodium dodecyl sulfate−polyacrylamide gel (SDS-PAGE; 10% minigel) according to the method of Laemmli (*14*). The purity of wild-

Table 2. 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

	hydrolysis	transglycosylation			
MTSase form	G_3	G_4	G_5	G_6	G_7
wild-type[a]					
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
selectivity ratio[c] (× 10^2)		10.2	1.73	1.34	1.07
F206W					
k_{cat} (s^{-1})	17.0 ± 0.3			11.1 ± 0.1	
K_M (mM)	14.5 ± 0.8			1.93 ± 0.06	
k_{cat}/K_M (s^{-1} mM^{-1})	1.18 ± 0.05			5.78 ± 1.03	
selectivity ratio (× 10^2)				20.4	
$\Delta(\Delta G)$[d] (kJ/mol)	–0.13			7.24	
F206Y					
k_{cat} (s^{-1})	76.3 ± 1.7			6.11 ± 0.22	
K_M (mM)	48.5 ± 2.5			2.48 ± 0.23	
k_{cat}/K_M (s^{-1} mM^{-1})	1.57 ± 0.05			2.47 ± 0.15	
selectivity ratio (× 10^2)				63.6	
$\Delta(\Delta G)$ (kJ/mol)	–0.92			9.60	
F207Y					
k_{cat} (s^{-1})	3.08 ± 0.11			14.0 ± 0.3	
K_M (mM)	61.4 ± 4.7			2.92 ± 0.16	
k_{cat}/K_M (s^{-1} mM^{-1})	0.050 ± 0.002			4.80 ± 0.17	
selectivity ratio (× 10^2)				1.04	
$\Delta(\Delta G)$ (kJ/mol)	8.63			7.76	
F405M					
k_{cat} (s^{-1})	44.3 ± 2.0	196 ± 11	481 ± 9	414 ± 14	381 ± 21
K_M (mM)	56.1 ± 5.6	19.0 ± 2.0	6.60 ± 0.31	4.63 ± 0.46	3.38 ± 0.42
k_{cat}/K_M (s^{-1} mM^{-1})	0.79 ± 0.04	10.4 ± 0.6	72.9 ± 2.2	89.4 ± 6.0	113 ± 9
selectivity ratio (× 10^2)		7.60	1.08	0.88	0.70
$\Delta(\Delta G)$ (kJ/mol)	0.81	0.00	–0.48	–0.34	–0.37
F405S					
k_{cat} (s^{-1})	12.4 ± 0.4	61.0 ± 2.6	197 ± 10	169 ± 6	147 ± 4
K_M (mM)	73.9 ± 4.9	20.2 ± 1.6	9.25 ± 0.86	3.75 ± 0.40	2.84 ± 0.22
k_{cat}/K_M (s^{-1} mM^{-1})	0.17 ± 0.01	3.02 ± 0.13	21.3 ± 1.0	45.2 ± 3.3	51.9 ± 2.9
selectivity ratio (× 10^2)		5.63	0.80	0.38	0.33
$\Delta(\Delta G)$ (kJ/mol)	5.07	3.42	2.92	1.55	1.78
F405W					
k_{cat} (s^{-1})	72.4 ± 2.5	139 ± 7	410 ± 19	445 ± 11	429 ± 6
K_M (mM)	53.5 ± 4.4	20.6 ± 2.0	7.90 ± 0.79	5.90 ± 0.37	5.71 ± 0.16
k_{cat}/K_M (s^{-1} mM^{-1})	1.35 ± 0.07	6.73 ± 0.33	51.9 ± 2.9	75.6 ± 3.0	83.0 ± 1.6
selectivity ratio (× 10^2)		20.1	2.60	1.79	1.63
$\Delta(\Delta G)$ (kJ/mol)	–0.67	1.20	0.46	0.13	0.48
F405Y[a]					
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
selectivity ratio (× 10^2)		7.30	1.57	1.18	0.92
$\Delta(\Delta G)$ (kJ/mol)	0.53	–0.40	0.26	0.19	0.09

[a] Data from ref *8*. [b] Standard error. [c] Selectivity ratio: $[k_{cat}/K_M(G_3)]/[k_{cat}/K_M(G_n)]$. [d] Change of transition-state energy: $\Delta(\Delta G) = -RT\ln[(k_{cat}/K_M)_{mut}/(k_{cat}/K_M)_{wt}]$ (*10*).

type and mutant MTSases is greater than 95% as indicated by SDS-PAGE (data not shown). After a 2 h incubation at 80 °C, the wild-type and mutant MTSases retained virtually all of their activities (data not shown). The good thermostability of these enzymes suggested that the purified MTHases were well-folded, without long-range conformational changes that may be a consequence of mutation.

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 2**. F206W, F206Y, and F207Y MTSases were not very active on transglycosylation as compared with wild-type MTSase; therefore, only G_6 was used to measure the reaction rates for transglycosylation. Except F405S MTSase, all other MTSases exhibited a previously observed behavior (*8*), which k_{cat} values for transglycosylation increased rapidly with increasing DP from G_4 to G_5 then to a relatively constant value with longer substrates. Conversely, K_M values decreased rapidly with increasing DP from G_4 to G_5 and then dropped slowly beyond that point.

The catalytic efficiencies for hydrolysis and transglycosylation reactions of F206W MTSase were 110 and 7.3%, respectively, of those of wild-type MTSase, while those of F206Y MTSase were 150 and 3.1%, respectively, of those of wild-type MTSase. However, the catalytic efficiencies for hydrolysis and transglycosylation reactions of F207Y MTSase were only 4.7 and 6.1%, respectively, of those of wild-type MTSase.

By comparison with wild-type MTSase, F405M MTSase had slightly higher catalytic efficiencies for the transglycosylation of G_5−G_7 and a somewhat lower catalytic efficiency for the hydrolysis of G_3. The catalytic efficiencies of F405S MTSase for transglycosylation were 29−57% of those of wild-type MTSase, whereas the catalytic efficiency for hydrolysis was only 16% of those of wild-type MTSase. F405W MTSase had

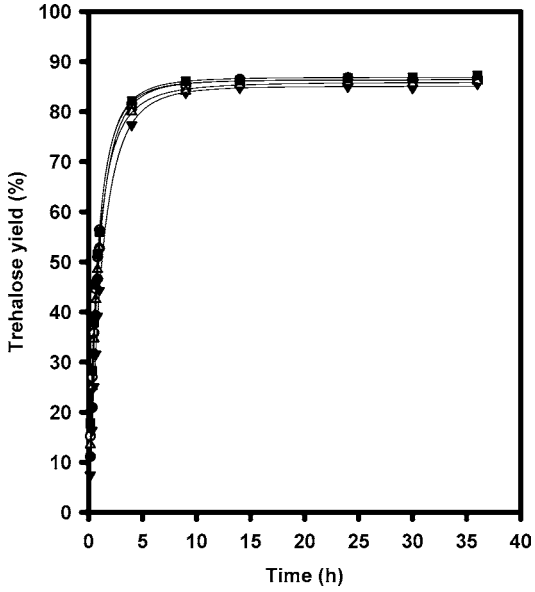

Figure 2. Formation of trehalose during the digestion of 10% (w/v) soluble starch at 75 °C in 50 mM citrate phosphate buffer (pH 5) using wild-type and mutant MTSases along with isoamylase and MTHase. Wild-type (●), F405M (○), F405S (▼), F405W (△), and F405Y (■).

Table 3. Initial Rates of Trehalose Formation and G_1 Formation and the Ratios of These Two Initial Rates in the Digestion of 10% (w/v) Soluble Starch Using Wild-Type and Mutant MTSases along with Isoamylase and MTHase at 75 °C

MTSase form	initial rate (μM/min)		initial rate G_1/ initial rate trehalose
	G_1	trehalose	
wild-type	114 ± 13[a]	556 ± 25	0.21
F405M	96 ± 3	479 ± 32	0.20
F405S	114 ± 10	474 ± 24	0.24
F405W	120 ± 10	563 ± 34	0.21
F405Y	106 ± 5	541 ± 27	0.19

[a] Standard error.

catalytic efficiencies in general slightly lower than those of wild-type MTSase except for G_3 hydrolysis.

Production of Trehalose from Starch. The digestion of 10% (w/v) soluble starch was used to study the yield of trehalose production by wild-type and mutant MTSases along with isoamylase and MTHase at both a high reaction temperature (75 °C) and a high substrate concentration. All of the reactions reached more than 80% trehalose yield in 10 h (**Figure 2**). The productivities of trehalose for wild-type and F405M, F405S, F405W, and F405Y MTSases calculated from the initial rates of trehalose production were 11.8 ± 0.5, 10.2 ± 0.7, 10.1 ± 0.5, 12.0 ± 0.7, and 11.5 ± 0.6 g/L/h, respectively. Because both MTSase and MTHase catalyze a side reaction to hydrolyze maltooligosaccharides to release G_1, the G_1 formation in the digestion of starch was also determined. The initial rates of trehalose and G_1 formation in the digestion of soluble starch and their ratios are given in **Table 3**. Ratios of the initial rates of G_1 formation to trehalose formation were calculated to evaluate the selectivity for G_1 formation vs trehalose formation. F405W and F405Y MTSases had similar initial rates for trehalose formation, while F405M and F405S MTSases had slightly decreased initial rates. F405 M and F405Y MTSases had slightly lower initial rates for G_1 formation as compared with that of wild-type MTSase, while F405S and F405W MTSases had similar initial rates. This led to the slightly lower ratios of initial rates of G_1 formation to those of trehalose formation for F405M and F405Y MTSases as compared with that of wild-type MTSase. The relationship between peak trehalose yield (the average of the three highest consecutive trehalose yield measurements from **Figure 2**) and the ratios of initial rates of G_1 formation to those of trehalose formation is plotted in **Figure 3**. Peak trehalose yields and ratios of initial rates of G_1 formation to those of trehalose formation were inversely correlated.

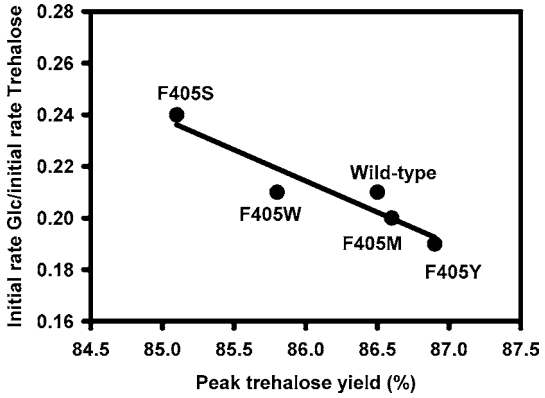

Figure 3. Relationship between peak trehalose yields and initial rate of G_1 formation/initial rate of trehalose formation during the digestion of 10% (w/v) soluble starch at 75 °C in 50 mM citrate phosphate buffer (pH 5) using wild-type and mutant MTSases along with isoamylase and MTHase.

MTSase Selectivity toward Hydrolysis vs Transglycosylation. Selectivity ratios were calculated from the kinetic parameters of wild-type and mutant MTSases for hydrolysis of G_3 and transglycosylation of G_4−G_7 as given in **Table 2**. The selectivity ratio is expressed as the percentage of catalytic efficiency of G_3 hydrolysis over that of G_4−G_7 transglycosylation. F206W and F206Y had significantly increased selectivity ratios as compared to that of wild-type MTSase, while F405M, F405S, and F405Y MTSases had decreased selectivity ratios.

The relationships between peak trehalose yield from starch digestion and the selectivity ratios from kinetic studies for wild-type and F405M, F405S, F405W, and F405Y MTSases are plotted in **Figure 4**. When F405S MTSase was not included, peak trehalose yields and selectivity ratios were inversely correlated.

Mutations on residue 405 were designed to further alter the hydrophobic interactions between residue 405 and substrate. The hydrophobic scale for the side chains of tryptophan, phenylalanine, methionine, tyrosine, and serine were 2.9, 2.3, 2.3, 1.6, and 0.2 kcal/mol, respectively, according to Karplus (*15*), and those were 3.06, 2.43, 1.67, 1.31, and −0.05 kcal/mol, respectively, according to Fauchere and Pliska (*16*). The relationships between hydrophobicity of residue 405 and the selectivity ratios from kinetic studies for wild-type and F405M, F405S, F405W, and F405Y MTSases are plotted in **Figure 5**. Except for the hydrophobicity of methionine obtained from Karplus (*15*), hydrophobicities of residue 405 and selectivity ratios were linearly correlated. In addition, peak trehalose yields and hydrophobicities of residue 405 were inversely correlated except for F405S MTSase (**Figure 6**).

DISCUSSION

In addition to enzyme kinetics, we investigated the effects of MTSase mutations on trehalose yield by using 10% (w/v) soluble starch as substrate and combined isoamylase and MTHase in the reaction mixture. We have previously suggested that mutant F405Y MTSase with a decreased ratio of hydrolysis

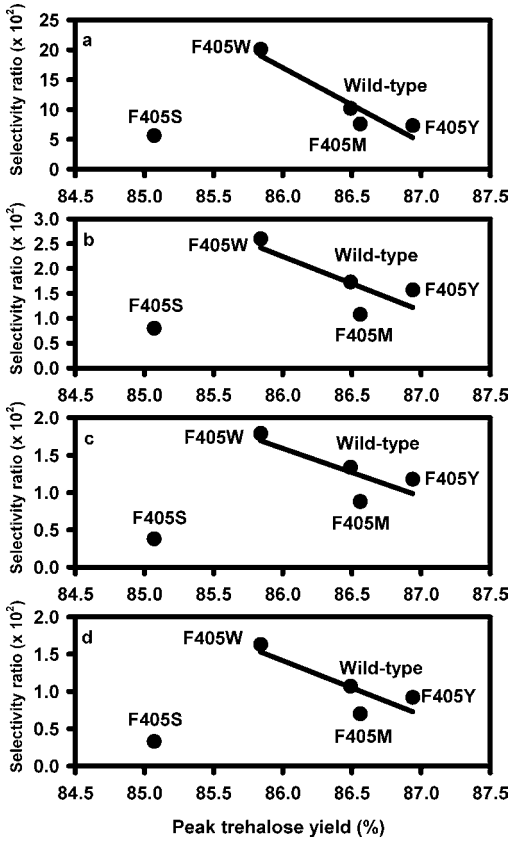

Figure 4. Relationships between peak trehalose yields from starch digestion and the selectivity ratios from kinetic studies by wild-type and mutant MTSases: (**a**) selectivity ratios for G_3 hydrolysis over G_4 transglycosylation, (**b**) selectivity ratios for G_3 hydrolysis over G_5 transglycosylation, (**c**) selectivity ratios for G_3 hydrolysis over G_6 transglycosylation, and (**d**) selectivity ratios for G_3 hydrolysis over G_7 transglycosylation.

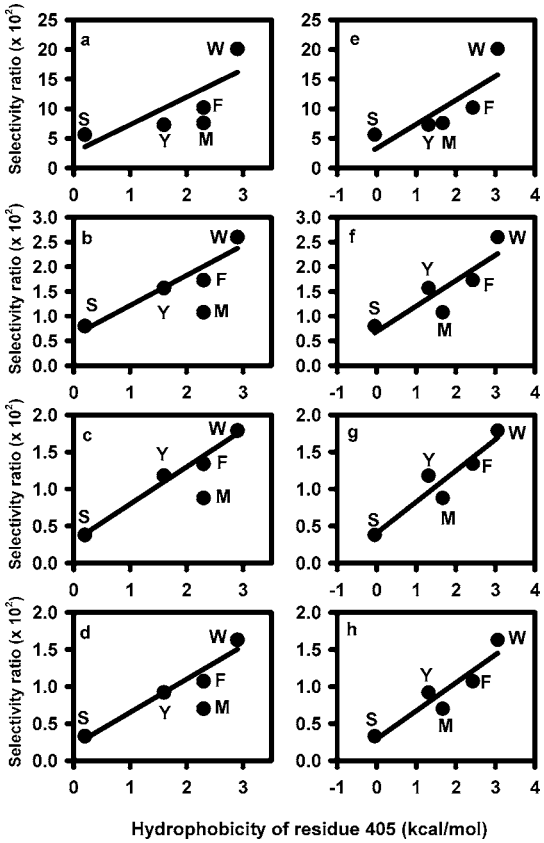

Figure 5. Relationships between hydrophobicities of residue 405 and the selectivity ratios from kinetic studies for wild-type and mutant MTSases: (**a** and **e**) selectivity ratios for G_3 hydrolysis over G_4 transglycosylation, (**b** and **f**) selectivity ratios for G_3 hydrolysis over G_5 transglycosylation, (**c** and **g**) selectivity ratios for G_3 hydrolysis over G_6 transglycosylation, and (**d** and **h**) selectivity ratios for G_3 hydrolysis over G_7 transglycosylation, where the hydrophobicity values of the left and the right panels were based on the estimations of Karplus (*15*) and Fauchere and Pliska (*16*), respectively. F, wild-type; M, F405M; S, F405S; W, F405W; and Y, F405Y.

to transglycosylation might result in a higher yield of trehalose production from starch when it replaces wild-type MTSase. This hypothesis, however, has not been tested directly. In this study, we found that mutant MTSases with decreased selectivity ratios for hydrolysis over transglycosylation indeed had a higher peak trehalose yield except for F405S MTSase. However, the ratios of the initial rate of G_1 formation to that of trehalose formation from the digestion of soluble starch provide a better prediction for high trehalose yield.

Residues 206 and 207 were mutated to study how the hydrophobic interactions between enzyme and substrate at positions near subsite −1 affect MTSase selectivity. The mutations on the above residues including F206W, F206Y, and F207Y are all very conservative mutations. Mutation F206W was designed to have an increased hydrophobic interaction between MTSase and substrate, while F206Y and F207Y were designed to have a decreased hydrophobic interaction. Decreased hydrophobic interactions between residue F207 and substrate had decreased selectivity ratio of hydrolysis over transglycosylation. However, either decreased or increased hydrophobic interactions between residue F206 and substrate had a significantly increased selectivity ratio of hydrolysis over transglycosylation.

In an effort to examine whether the alterations in hydrophobicity would change the bindings between enzyme and substrate, we also 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, while 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 (*17*, *18*).

In this study, the large changes in $\Delta(\Delta G)$ for G_3 hydrolysis and for G_6 transglycosylation by F207Y MTSase, 8.63 and 7.76 kJ/mol, respectively, and for G_6 transglycosylation by F206W and F206Y MTSases, 7.24 and 9.60 kJ/mol, respectively (**Table 2**), are larger than the loss of an uncharged hydrogen bond between the enzyme and the substrate in the transition state. However, $\Delta(\Delta G)$ values for G_3 hydrolysis by F206W and F206Y MTSase were only −0.13 and −0.92 kJ/mol, respectively. Because residues F206 and F207 have no abilities of forming hydrogen bonds, the mutations presumably caused losses of uncharged hydrogen bonds between the enzyme and the specified substrate in the neighborhood of mutated residues. In addition, the losses of hydrogen bonds caused by mutations on F206 and F207 were different. The losses of hydrogen bonds caused by mutation on F207 occurred between subsites +1 to −2 and were observed for the large changes in $\Delta(\Delta G)$for G_3 hydrolysis and for G_6 transglycosylation, whereas the losses of hydrogen bonds caused by mutations on F206 occurred between subsites −3 to −5 and were observed for the large changes in $\Delta(\Delta G)$ only for G_6 transglycosylation, not for G_3 hydrolysis.

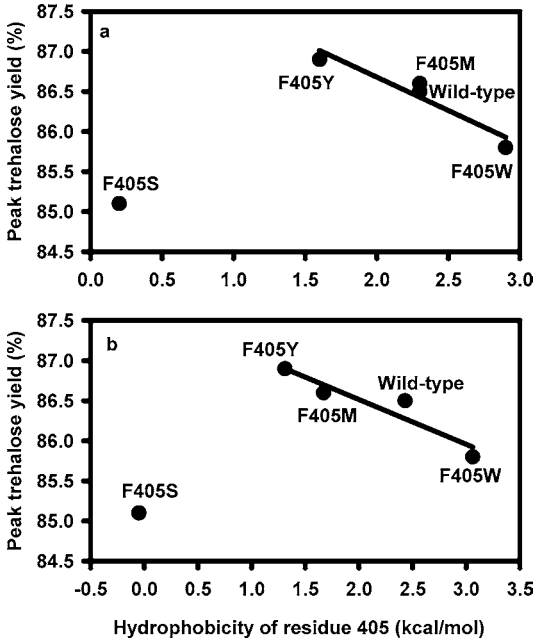

Figure 6. Relationships between hydrophobicities of residue 405 and peak trehalose yields from starch digestion for wild-type and mutant MTSases: (**a**) hydrophobicity values based on the estimations of Karplus (*15*) and (**b**) hydrophobicity values based on the estimations of Fauchere and Pliska (*16*).

F405S MTSase also showed a large change in $\Delta(\Delta G)$ for G_3 hydrolysis, while the value for transglycosylation decreased with increasing DP from G_4 to G_6. These values are close to the loss of an uncharged hydrogen bond between the enzyme and the substrate in the transition state. The losses of hydrogen bonds caused by mutation F405S occurred between subsites +1 to −2 and might only occur when G_3−G_5 are used as substrates. On the other hand, mutations F405M and F405W showed small $\Delta(\Delta G)$ values, from −0.67 to 1.20 kJ/mol, indicating that these mutations had only minor effects on transition-state substrate binding.

Altering the hydrophobicity of an active site could affect the catalytic activity and/or substrate binding of an enzyme (*19*−*23*). Several mutations near subsite +1 of MTSase, including Y290F, Y367F, F405Y, and Y409F, had been constructed in our previous study (*8*). Our previous results showed that decreasing hydrophobic interactions between the 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 (*8*). In this study, residue F405 was mutated to methionine, serine, and tryptophan, which have different degrees of hydrophobicities, in an effort to further evaluate the relationship between hydrophobic interactions and selectivity. Indeed, the hydrophobicities of residue 405 and selectivity ratios were linearly correlated in general (**Figure 5**), and the peak trehalose yields and hydrophobicities of residue 405 were inversely correlated except for F405S MTSase (**Figure 6**). Because F405Y MTSase has a higher peak trehalose yield and similar trehalose productivity as compared to those of wild-type, this mutant might be able to replace wild-type enzyme in the production of trehalose from starch.

In conclusion, the decreased hydrophobicities near subsite +1 of MTSase could lead to decreased selectivity ratios for hydrolysis over transglycosylation and may result in increased trehalose yield from the digestion of soluble starch when MTSase was combined with isoamylase and MTHase. Altering hydrophobic interactions near subsite −1 of the enzyme−substrate complex could also change MTSase selectivity; however, either decreased or increased hydrophobic interactions led to significantly increased selectivity ratios. In addition, the ratios of the initial rate of G_1 formation to that of trehalose formation from the digestion of soluble starch provide a better prediction for high trehalose yield than do the selectivity ratios.

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; $\Delta(\Delta G)$, change of transition-state energy; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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