Role of the Glutamate 332 Residue in the Transglycosylation Activity of *Thermus* Maltogenic Amylase†

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ABSTRACT: A sequence alignment shows that residue 332 is conserved as glutamate in maltogenic amylases (MAases) and in other related enzymes such as cyclodextrinase and neopullulanase, while the corresponding position is conserved as histidine in α-amylases. We analyzed the role of Glu332 in the hydrolysis and the transglycosylation activity of *Thermus* MAase (ThMA) by site-directed mutagenesis. Replacing Glu332 with histidine reduced transglycosylation activity significantly, but enhanced hydrolysis activity on α-(1,3)-, α-(1,4)-, and α-(1,6)-glycosidic bonds relative to the wild-type (WT) enzyme. The mutant Glu332Asp had catalytic properties similar to those of the WT enzyme, but the mutant Glu332Gln resulted in significantly decreased transglycosylation activity. These results suggest that an acidic side chain at position 332 of MAase plays an important role in the formation and accumulation of transfer products by modulating the relative rates of hydrolysis and transglycosylation. From the structure, we propose that an acidic side chain at position 332, which is located in a pocket, is involved in aligning the acceptor molecule to compete with water molecules in the nucleophilic attack of the glycosyl−enzyme intermediate.

For the past decade, we have studied new members of the α-amylase family, maltogenic amylases (MAases),[1] which possess properties distinct from those of other amylolytic enzymes (*1*−*3*). The enzymes hydrolyze both α-(1,4)- and α-(1,6)-glycosidic linkages of starch to yield maltose as the major product. Furthermore, MAases preferentially hydrolyze cyclodextrins (CDs) which are poor substrates for other known amylolytic enzymes. In addition to the hydrolysis activity, MAases catalyze the transglycosylation reaction in the presence of various acceptor molecules and accumulate branched oligosaccharide products. These catalytic properties are shared by cyclodextrinases and neopullulanases (*4*−*7*). Recently, we reported an interesting observation (*3*, *8*) that MAases could hydrolyze acarbose, a potent amylase inhibitor, to produce glucose and acarviosine-glucose (pseudotrisaccharide; PTS). Acarbose is a pseudotetrasaccharide containing a pseudo sugar ring linked to the nitrogen of 4-amino-4,6-dideoxy-D-glucopyranose, which is linked to a maltose unit by an α-(1,4)-glycosidic bond (*9*). The noncleavable bond of acarbose binds to the active site cleft of the target enzymes, which strongly inhibits the hydrolysis activity of various amylases, including glucoamylase (*10*), α-glucosidase (*11*), α-amylase (*12*), and CGTase (*13*). MAases not only hydrolyze acarbose but also transfer PTS to various acceptor molecules by forming an α-(1,3)-, α-(1,4)-, or α-(1,6)-glycosidic linkage (*3*, *8*).

Due to their catalytic versatility, especially transglycosylation activities, MAases and related enzymes have been subjects of intense investigation. Matsui et al. (*14*) reported that aromatic residues, conserved in the active center of *Saccharomycopsis* α-amylase, affect its transglycosylation. Cha et al. (*2*) and Kuriki et al. (*15*) showed that the hydrophobicity near the active sites was critical to the transglycosylation reaction of MAases. When the hydrophobicity near the active site was increased by site-directed mutagenesis, transglycosylation became dominant over hydrolysis in reactions catalyzed by an MAase (BSMA; *2*) and a neopullulanase (*14*) from *Bacillus stearothermophilus*. Recently, Saab-Rincón et al. (*16*) have tried to introduce transglycosylation activity in a liquefying α-amylase from *B. stearothermophilus*. In addition to hydrophobicity, however, transglycosylation activities are likely to be influenced by unknown structural determinants.

The structure of Taka-amylase A suggested that Glu230, Asp206, and Asp297, corresponding to Glu357, Asp328, and Asp424 of ThMA, respectively, are involved in acid−base catalysis while His122, Asp206, Lys209, His210, and

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[1] Abbreviations: MAase, maltogenic amylase; ThMA, *Thermus* maltogenic amylase; BSMA, *Bacillus stearothermophilus* maltogenic amylase; WT, wild-type; CD, cyclodextrin; CGTase, cyclodextrin glucanotransferase; α-MG, methyl α-D-glucopyranoside; PTS, pseudotrisaccharide (acarviosine-glucose); Ni−NTA, nickel−nitrilotriacetic acid; HPAEC, high-performance anion exchange chromatography.

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Enzyme		Conserved regions I		II		III		IV		Ref.
MAase	*Thermus* strain (ThMA)	242 DAVFNH	324	GWRLDVANE	357	EIWH	419	LLGSHD		3
	Bacillus licheniformis (BLMA)	245 DAVFNH	324	GWRLDVANE	357	EIWH	419	LLDSHD		1
	B. stearothermophilus (BSMA)	242 DAVFNH	324	GWRLDVANE	357	EIWH	419	LLGSHD		2
Cyclodextrinase	alkalophilic *Bacillus*	238 DAVFNH	321	GWRLDVANE	354	EVWH	416	LLDSHD		4
TVA II	*Thermoactinomyces vulgaris*	239 DVVANH	321	GWRLDVANE	354	EIWH	292	LLDSHD		7
Neopullulanase	*B. stearothermophilus*	242 DAVFNH	324	GWRLDVANE	357	EIWH	419	LLGSHD		6
α-amylase	*Aspergillus oryzae*	117 DVVANH	202	GIRIDTVKH	230	EVLD	292	FVENHD		21
	B. stearothermophilus	101 DVVFDH	230	GFRLDGLKH	264	EYWS	326	FVDNHD		22
	barley	101 DIVINH	127	DGRLDWGPH	218	EVWD	299	FVDNHD		23
CGTase	*B. macerans*	135 DFAPNH	225	GIRFDAVKH	258	EWFL	324	FIDNHD		24
	B. stearothermophilus	131 DFAPNH	221	GIRMDAVKH	253	EWFL	319	FIDNHD		24
	Klebsiella pneumoniae	130 DYADNH	219	AIRIDAIKH	257	EWFG	328	FMDNHD		25
Pullulanase	*Klebsiella aerogenes*	590 DVVYNH	661	GFRFDLMGY	693	EGWD	817	YVSKHD		26

FIGURE 1: Comparison of amino acid residues in the conserved regions of maltogenic amylases and related enzymes. The glutamate conserved in MAases and the equivalent residues in other amylolytic enzymes are indicated with black highlight. Black circles (●) represent the residues constituting the catalytic site.

His296, corresponding to His247, Asp328, Asn331, Glu332, and His423 of ThMA, respectively, probably participate in substrate binding (*17*, *18*). Sequence alignments of α-amylases and related enzymes suggested a common $(\beta/\alpha)_8$-barrel as the catalytic core domain with four highly conserved regions (*19*, *20*). Sequence alignment of highly conserved regions revealed that residue 332 is glutamate in MAases and other related enzymes, while the corresponding position is conserved as histidine in typical α-amylases without potent transglycosylation activity (Figure 1). Also, we found that the Glu332 residue is located in a pocket, which we called the "extra sugar-binding space", from the recent X-ray structure of the ThMA dimer (*27*).

In this study, we investigated the role of Glu332 of ThMA in the transglycosylation activity. This report provides evidence, for the first time, that Glu332 of MAases is involved in the α-(1,3)-, α-(1,4)-, and α-(1,6)-linked transglycosylation. On the basis of these experimental results and the three-dimensional structure of ThMA (*27*), we propose a mechanism for transglycosylation reactions of MAases and related enzymes.

MATERIALS AND METHODS

Chemicals and Enzymes. Acarbose (*O*-4,6-dideoxy-4-{[4,5,6-trihydroxy-3-(hydroxymethyl)-2-cyclohexen-1-yl]-amino}-α-D-glucopyranosyl-(1,4)-*O*-α-D-glucopyranosyl-(1,4)-D-glucose) was obtained from Bayer (Leverkusen, Germany). Methyl α-D-glucopyranoside (α-MG) was purchased from Sigma Chemical Co. (St. Louis, MO), and other chemicals were purchased from Merck (Darmstadt, Germany) and Showa Chemicals, Inc. (Tokyo, Japan). Restriction enzymes and modifying enzymes was obtained from Boehringer Mannheim and New England Biolabs, Inc.

Bacterial Strain and Plasmid. Escherichia coli MC1061 [F$^-$, *araD*139, *recA*13, Δ(*araABC-leu*)7696, *gal*U, *gal*K, Δ*lac*X74, *rpsL*, *thi*, *hsdR*2, *mcrB*] was used as a host for DNA manipulation and transformation. *E. coli* transformants were grown in LB medium (1% bactotryptone, 0.5% yeast extract, and 0.5% NaCl) containing ampicillin (100 μg/mL) at 37 °C. An expression vector, p6xHis119, was constructed and used for overproduction of wild-type and mutant enzymes (*3*).

Site-Directed Mutagenesis. Site-directed mutagenesis to introduce an *Xba*I site was performed using a Quikchange site-directed mutagenesis kit (Stratagene) and a PE9600 thermal cycler (Perkin-Elmer). After PCR, the amplified DNA fragments were phosphorylated by T4 polynucleotide kinase and ligated by T4 DNA ligase. Transformation and screening the transformants were carried out by the calcium chloride (*28*) and iodine methods (*1*), respectively. Other mutations were introduced by cassette mutagenesis (*29*) using the *Xba*I and *Eco*RI sites. All mutations were confirmed by dideoxy chain termination sequencing (*30*) using an ABI377 PRISM DNA sequencer (Perkin-Elmer). Other genetic manipulation was carried out using the methods described by Sambrook et al. (*28*).

Purification and Assay of ThMA and Its Mutants. For efficient purification using an Ni−NTA column (Qiagen), six-His-tagged mutants or wild-type ThMA was used as described previously (*3*). The purity of the enzymes was confirmed by SDS−PAGE analysis, and all the six-His-tagged ThMA mutants were purified to apparent homogeneity by the same procedure. The enzyme activity was assayed at 60 °C in 50 mM sodium acetate buffer (pH 6.0) using 3,5-dinitrosalicylic acid (DNS) according to Miller (*31*) for β-CD, starch, and pullulan hydrolyzing activity and the glucose oxidase-peroxidase method (*32*) for acarbose hydrolyzing activity. The unit of hydrolyzing activity for β-CD (CU), pullulan (PU), starch (SU), and acarbose (AU) was defined as described previously (*2*). The protein concentration was measured by the Bradford method (*33*) using bovine serum albumin as a standard.

Measurement of Kinetic Parameters for Acarbose Hydrolysis. Hydrolysis of acarbose was carried out in 50 mM sodium acetate buffer (pH 6.0) at 60 °C, and the reaction was stopped by boiling for 5 min. Glucose released from acarbose was assessed using the glucose oxidase-peroxidase method (*32*). The initial velocities were obtained directly

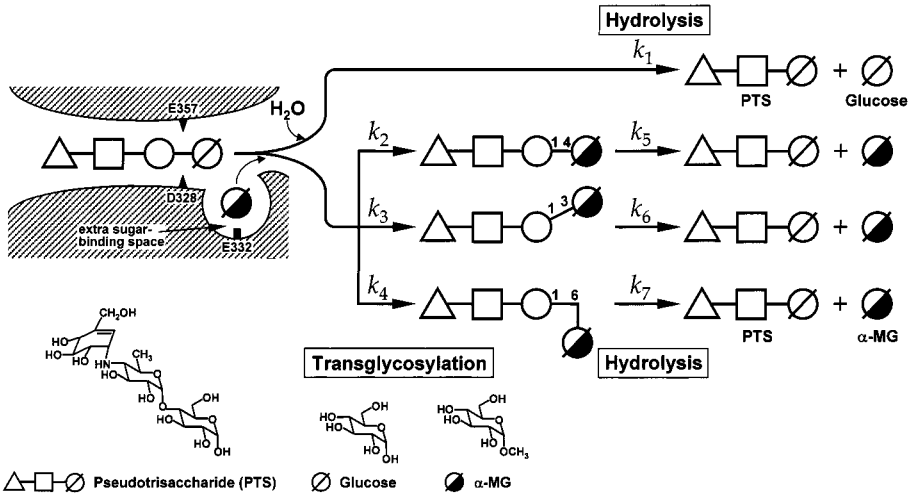

FIGURE 2: Action modes of ThMA in the hydrolysis and transglycosylation of acarbose with water or α-MG as an acceptor. Acarbose is hydrolyzed to PTS and glucose with water as an acceptor, which turned into acarbose transfer products with α-MG. From the equations, k_1 corresponds to the rate constant for acarbose (Acb) hydrolysis, k_2 that for α-(1,4)-transfer product formation, k_3 that for α-(1,3)-transfer product formation, k_4 that for α-(1,6)-transfer product formation, k_5 that for α-(1,4)-transfer product hydrolysis, k_6 that for α-(1,3)-transfer product hydrolysis, and k_7 that for α-(1,6)-transfer product hydrolysis.

from the time course plots of the reaction. The k_{cat} and K_m were calculated from the Lineweaver−Burk plot.

Analysis of Transglycosylation Products. ThMA (10 CU/mg of acarbose) was added to the reaction buffer [50 mM sodium acetate buffer (pH 6.0)] containing 77.4 mM (5%) acarbose (donor) and 514.9 mM (10%) α-MG (acceptor), and incubated at 60 °C for 48 h. The transfer products were analyzed by high-performance anion exchange chromatography (HPAEC) as described previously (*3*).

Formation and Isolation of Acarbose Transfer Products. ThMA (10 CU/mg of acarbose) was incubated with the reaction mixture that consisted of 77.4 mM acarbose and 514.9 mM α-MG in 5 mL of 50 mM sodium acetate buffer (pH 6.0) at 60 °C for the appropriate period of time. The reaction mixture was loaded onto a Bio-Gel P-2 (Bio-Rad) column (2 cm × 90 cm, Pharmacia Biotech) to remove glucose, PTS, and acceptors. The column was equilibrated with 50 mM NaCl and eluted with the same solution. After the fractions containing transfer products were desalted using the same gel column equilibrated with deionized water, desalted solutions were freeze-dried and resuspended in deionized and distilled water. The final mixture of transfer products was loaded onto a Whatman K6F TLC plate, which was irrigated with two gradients of isopropyl alcohol, ethyl acetate, and water (3:1:1, v/v/v) at room temperature. After the second gradient had been applied, the spots representing each transfer product on the TLC plate were cut out and the moisturized silica powder was scratched off from the plate and collected into a tube. Each sample was extracted with water shaking at room temperature for 2 h. The extract was centrifuged at 15000*g* for 15 min, and the supernatant was filtered through a 0.2 μm membrane filter (Micro Filtration System). Each product was freeze-dried and used to determine the hydrolyzing rate of each transfer product.

Reaction Rate Constants for Hydrolysis and Transglycosylation Reactions. To understand the reaction mode of WT and mutated ThMAs precisely, the formation and degradation rate of each transfer product were determined. The whole reaction was divided into two stages, including the formation of the transfer products from acarbose (transglycosylation reactions) and the degradation of each transfer product (hydrolysis reactions). The rate constants for transglycosylation reactions were obtained under the assumption of a zero-order reaction since the concentration of acarbose was excessively high. To obtain the reaction rate constants for hydrolysis reactions, three kinds of transfer products with α-(1,3)-, α-(1,4)-, or α-(1,6)-linkages were purified from the reaction mixture and used for determining the reaction rate constants for WT and mutated enzymes. Under the assumption of zero-order kinetics with excess substrate concentration, the initial reaction rate constants obtained for different reaction times were evaluated by linear regression. For the hydrolysis rate of each transfer product, ThMA and its mutants were added to the reaction buffer [50 mM sodiumacetate buffer (pH 6.0)] containing each acarbose transfer product (22.7 mM) and incubated at 60 °C. For the transglycosylation rate of each transfer product, ThMA and its mutants were added to the same reaction buffer containing 77.4 mM acarbose and 514.9 mM α-MG and incubated at 60 °C. Each reaction aliquot taken at different reaction times was boiled for 5 min and analyzed by HPAEC, and the reaction rate constants for hydrolysis and transglycosylation reactions were determined by the assumption of a zero-order reaction. Catalytic reactions of ThMA are illustrated and specified in Figure 2.

Modeling of Acarbose Binding to ThMA Structure. Acarbose and α-MG molecules were modeled into the active site cleft of ThMA using the solid docking module in QUANTA (Molecular Simulations, Inc., San Diego, CA). The position of acarbose in the active site groove was determined on the basis of the β-CD−ThMA complex model established previously (*27*).

RESULTS

Construction and Expression of Mutant Enzymes. An *Xba*I enzyme site was introduced to p6×HThMA containing the ThMA gene tagged with six histidines at the amino terminus (*3*) via site-directed mutagenesis, and the resulting plasmid was designated as p6×HTMX. As shown in Figure 3, the complementary oligonucleotide primers containing specific

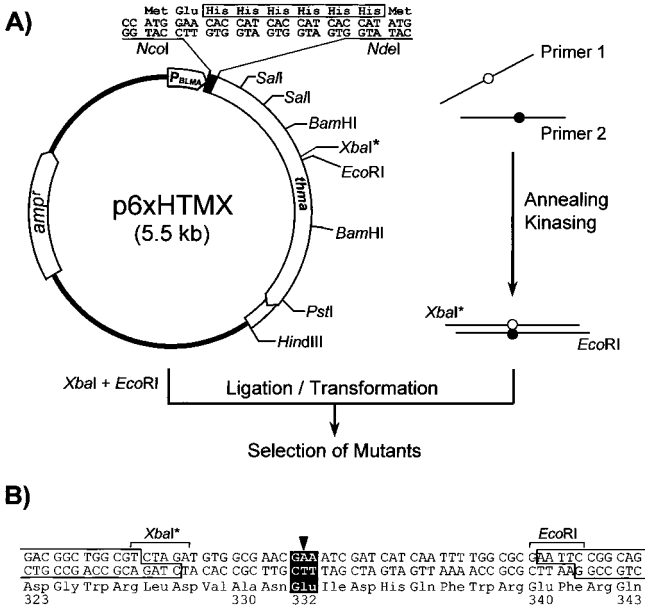

FIGURE 3: Construction and expression of the wild-type and mutant ThMA by cassette mutagenesis. Expression vector p6×His119 was used for the production of six-His-tagged ThMA (*3*). The *Xba*I restriction enzyme site labeled with an asterisk was generated via site-directed mutagenesis.

mutations were annealed and inserted into the cassette sequence of p6×HTMX between the *Xba*I and *Eco*RI sites. All mutations (E332H, E332D, and E332Q) were confirmed by DNA sequencing. From the resulting plasmids, six-histidine (six-His)-tagged WT and mutant ThMAs were successfully expressed in *E. coli*, and each enzyme was purified by using an Ni−NTA (nickel−nitrilotriacetic acid) column (*34*). The specific hydrolysis activity of purified six-His-tagged ThMA was identical to that of native ThMA (data not shown), indicating that the enzyme activity was not affected by the six extra histidine residues. Therefore, six-His-tagged WT and mutant enzymes were used throughout this study.

Table 1: Specific and Relative Activities of Wild-Type and Mutant ThMAs on Various Substrates

	specific activity (units/mg)			
ThMA	β-CD (CU)[a]	soluble starch (SU)[a]	pullulan (PU)[a]	acarbose (AU)[b]
WT	2035.71 (100.0%)	71.88 (100.0%)	125.60 (100.0%)	6.75 (100.0%)
E332D	534.31 (26.2%)	7.23 (10.1%)	10.73 (8.5%)	5.08 (75.3%)
E332Q	92.90 (4.6%)	1.61 (2.2%)	1.61 (1.3%)	3.83 (56.7%)
E332H	41.02 (2.0%)	1.32 (1.8%)	0.43 (0.3%)	2.02 (29.9%)

[a] Units for hydrolysis of β-CD (CU), soluble starch (SU), and pullulan (PU) were measured by the DNS method (*31*). [b] Acarbose hydrolyzing unit (AU) was measured by using the glucose oxidase-peroxidase method (*32*).

Hydrolysis Activity of the Mutant Enzymes. Hydrolysis activities of WT and the three mutant ThMAs were determined using starch, β-CD, pullulan, and acarbose as substrates. The hydrolysis activities of E332H and E332Q mutant enzymes on β-CD, starch, and pullulan were significantly decreased compared with that of the WT enzyme. However, mutant ThMAs retained about 30−75% of the acarbose-hydrolyzing activity of WT ThMA (Table 1). Hydrolysis products from β-CD, soluble starch, pullulan, and acarbose were analyzed by TLC and HPAEC, but there was no significant difference between WT and mutant enzymes (data

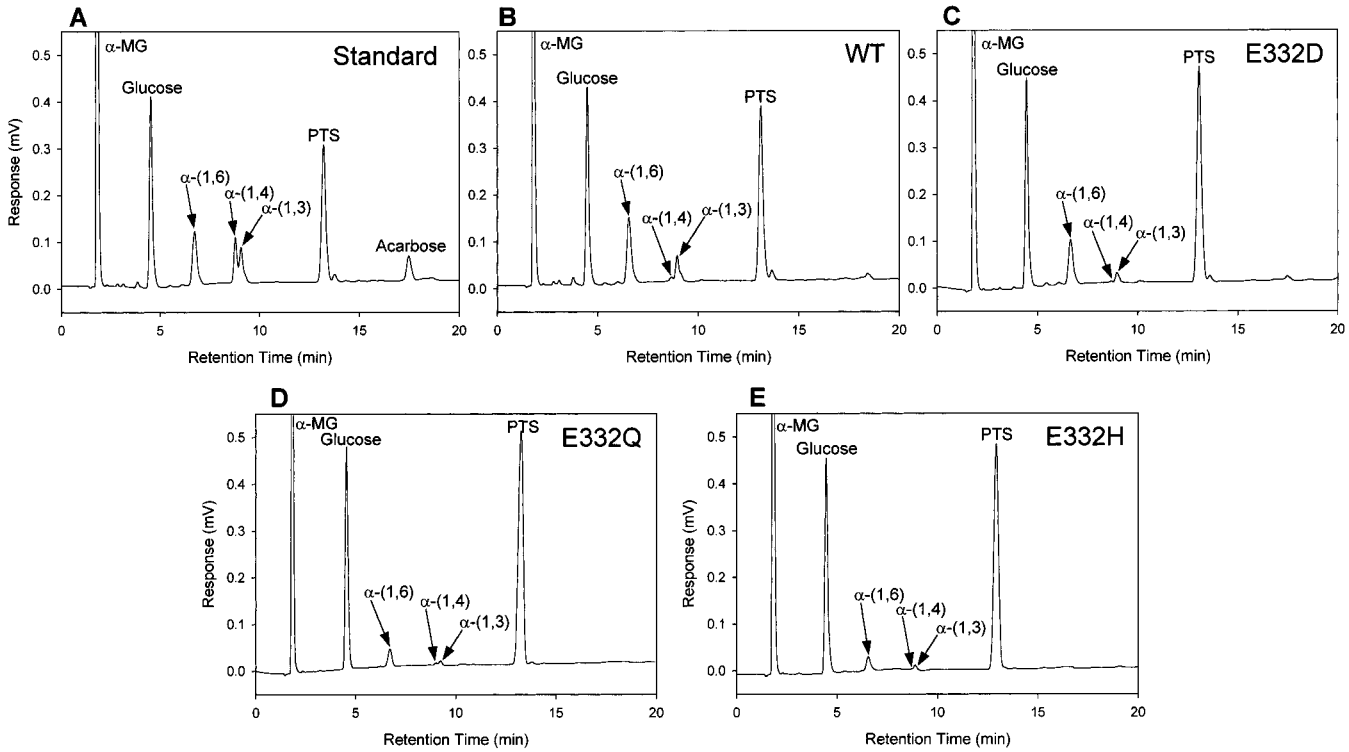

FIGURE 4: Analysis of acarbose transfer products by HPAEC. ThMA transferred PTS resulting from the hydrolysis of acarbose to α-MG. Transglycosylation reaction mixtures with WT and mutant ThMA were analyzed by HPAEC after 48 h. The standard corresponded to the reaction mixture with the WT enzyme analyzed after 6 h. The resulting transfer products were designated as α-(1,3), α-(1,4), and α-(1,6), respectively.

Table 2: Kinetic Parameters of Acarbose Hydrolysis by WT and Mutant ThMA[a]

ThMA	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
WT	0.426 ± 0.013	67.83 ± 0.57	159.23
E332D	0.545 ± 0.013	45.11 ± 0.29	82.77
E332Q	1.00 ± 0.06	41.35 ± 0.74	41.35
E332H	1.08 ± 0.06	18.68 ± 0.30	17.30

[a] Acarbose hydrolyzing activities were measured by the glucose oxidase-peroxidase method (*32*).

not shown). This result implied that the substitution of a histidine for the glutamate residue at position 332 affected the hydrolysis activity and substrate specificities of ThMA. Kinetic parameters of acarbose hydrolysis, K_m and k_{cat}, were also determined by measuring the amount of glucose produced from acarbose (Table 2). The mutant enzymes exhibited a relatively lower affinity on binding of acarbose molecule than that of WT ThMA and the reduced hydrolysis activity of them. The result suggested that Glu332 affects acarbose binding of ThMA.

Transglycosylation Activity of the Mutant Enzymes. Transglycosylation reactions of WT, E332H, E332Q, and E332D ThMA were investigated using α-MG as an acceptor and acarbose as a donor. As shown in Figure 4, transfer products were detected in WT ThMA by HPAEC analysis after reaction for 48 h. On the contrary, there were dramatic decreases in the amounts of both transfer products using E332H and E332Q. HPAEC analysis revealed that not only was the amount of the α-(1,6)-linked transfer product diminished but also the α-(1,3)- and α-(1,4)-linked transfer products disappeared in E332H (Figure 4E). This result suggested that Glu332 was critical to the transglycosylation activity of ThMA and replacing Glu332 with histidine, which is a conserved amino acid in α-amylases, resulted in smaller amounts of transfer products linked by α-(1,3)-, α-(1,4)-, and α-(1,6)-glycosidic linkages compared with WT ThMA. Although less product was detected, E332D ThMA produced α-(1,3)-, α-(1,4)-, and α-(1,6)-linked transfer products equivalent to WT ThMA (Figure 4C). However, the E332Q enzyme exhibited a transglycosylation pattern very similar to that of E332H ThMA (Figure 4D). These results indicated that replacing an acidic amino acid with an uncharged or positively charged amino acid (glutamine or histidine) at position 332 of ThMA results in a harmful effect on the transglycosylation activity.

Role of Glu332 in Hydrolysis and Transglycosylation. To investigate the mode of the transglycosylation reaction of WT and mutant ThMAs in detail, the amounts of transfer products in the reaction mixture were monitored for 48 h using HPAEC analysis (Figure 5). In the case of WT and E332D ThMAs, the α-(1,4)-linked transglycosylation product was predominant at the beginning of the reaction. However, the resulting product was rapidly hydrolyzed for the rest of the reaction. On the other hand, the amount of α-(1,3)- and α-(1,6)-linked transglycosylation products was increased during the first 5 h of the reaction, and steadily maintained during the rest of the reaction. Although the tendencies of the transglycosylation and hydrolysis of E332H and E332Q were similar to those of WT and E332D enzymes, the extent of further hydrolysis of each transfer product was increased remarkably. Rate constants for the formation and degradation of each transglycosylation product were determined under

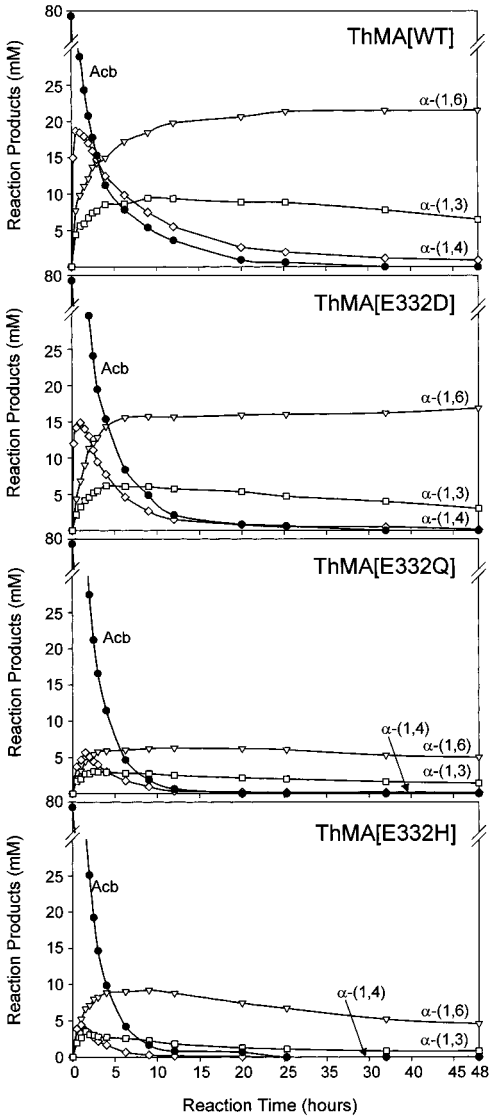

FIGURE 5: Composition of reaction products as a function of reaction times. WT or mutant ThMAs were reacted with 5% acarbose (w/v) and 10% α-MG (w/v) at 60 °C for 48 h. The graph shows the hydrolysis of acarbose (●) and the formation of α-(1,6)-linked products (▽), α-(1,4)-linked products (◇), and α-(1,3)-linked products (□).

the assumption of zero-order kinetics with an excess substrate concentration (Table 3). The rate constants for the formation of transglycosylation products decreased in E332H and E332Q ThMAs compared to those of WT and E332D ThMAs, as expected. In fact, rate constants for the formation of α-(1,6)-, α-(1,4)-, and α-(1,3)-linked transglycosylation products of E332H ThMA were reduced to 18, 17, and 15% of that of WT ThMA, respectively. The influence of mutation on the rate constants for the degradation of the transfer products was also significant. The rate constants for the degradation of α-(1,6)- and α-(1,3)-linked transfer products were enhanced by 1.8- and 4.1-fold, respectively, as compared with those of WT ThMA. In particular, while α-(1,6)-linked products formed via the transglycosylation reaction of the WT enzyme remained stable throughout the 48 h reaction, the same products produced by E332H ThMA were degraded slowly during the same period (Figure 5). As shown in Table 3, the ratios of hydrolysis to transglycosylation ([H]/[T]) represented total accumulation of each transfer product

Table 3: Reaction Rate Constants of Acarbose Hydrolysis and Transglycosylation by WT and Mutant ThMA

	reaction rate constants (s^{-1})[a]							[H]/[T] ratio		
	hydrolysis [H]				transglycosylation [T]			α-(1,4)	α-(1,3)	α-(1,6)
ThMA	k_1	k_5	k_6	k_7	k_2	k_3	k_4	k_5/k_2	k_6/k_3	k_7/k_4
WT	67.83 ± 0.57	37.2 ± 2.6	2.25 ± 0.31	0.421 ± 0.032	12.9 ± 2.2	1.20 ± 0.10	2.12 ± 0.35	2.88	1.88	0.199
E332D	45.11 ± 0.29	54.9 ± 3.7	2.22 ± 0.14	0.432 ± 0.051	9.39 ± 0.75	0.435 ± 0.051	0.843 ± 0.065	5.85	5.10	0.512
E332Q	41.35 ± 0.74	37.6 ± 3.1	8.64 ± 0.42	0.738 ± 0.084	2.44 ± 0.18	0.181 ± 0.011	0.387 ± 0.033	15.4	47.7	1.91
E332H	18.68 ± 0.30	31.8 ± 2.4	9.22 ± 0.29	0.778 ± 0.056	2.18 ± 0.20	0.179 ± 0.015	0.381 ± 0.028	14.6	51.5	2.04

[a] Reaction rate constants were expressed as k_0/[E].

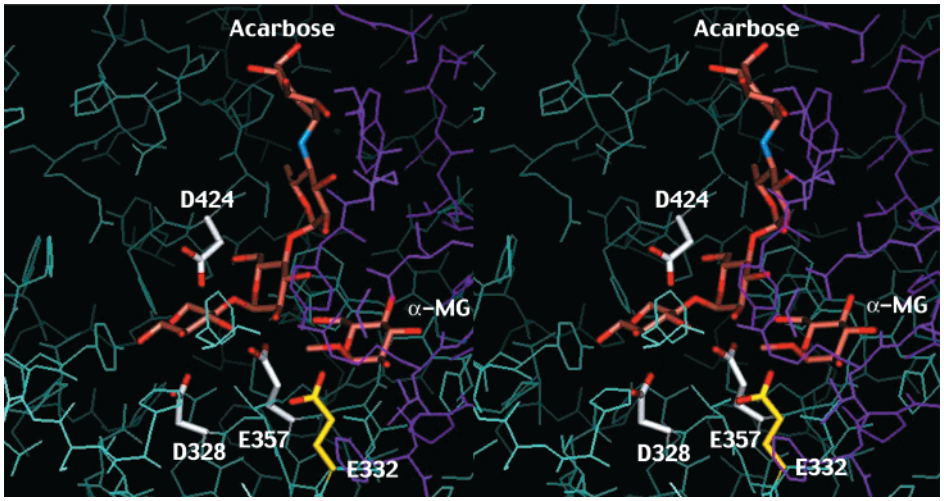

FIGURE 6: Stereoview of the ThMA active site with hypothetical binding modes of acarbose and an α-MG (in pink) as a substrate and an acceptor, respectively, in extra sugar-binding space. The residues constituting the active site (Asp328, Glu357, and Asp424) and Glu332, which was identified as important for transglycosylation in this study, are labeled in white and yellow, respectively. Residues from the N-domain of the opposite monomer are colored in violet. All oxygen atoms are in red.

by WT and mutant enzymes. As expected from lower [H]/[T] values, α-(1,6)-linked product accumulated to a greater extent than α-(1,4)- or α-(1,3)-linked products in the reaction mixture. These results indicated that accumulation of transfer products depended on the relative reaction rate between the transglycosylation and the hydrolysis. According to the results, the hydrolysis of the transfer products was presumed to be dependent on the molecular structure of the linkages formed in the transfer products. In addition, the lower [H]/[T] values in WT and Glu332Asp compared to those in Glu332His and Glu332Gln indicated that the acidic amino acid at position 332 preferred transglycosylation to the hydrolysis reaction. The acidic amino acid residue at position 332 in ThMA is involved not only in the formation of α-(1,3)-, α-(1,4)-, and α-(1,6)-transfer products but also in the further hydrolysis of each product, especially, α-(1,6)- and α-(1,3)-transfer products. Therefore, the replacement of glutamate with another amino acid (glutamine or histidine) shifted the reaction in favor of hydrolysis of transfer products over the accumulation of them. Considering that α-amylases containing histidine at position 332 catalyzed mainly the hydrolysis of α-(1,4)-glycosidic linkage, it is interesting that the change of glutamate at 332 position to histidine significantly increased the rate constants for the hydrolysis of transglycosylation products.

Critical reduction of the transglycosylation activities of E332H ThMA was observed also when other carbohydrate derivatives such as glucose, maltose, and cellobiose were used as acceptors (data not shown). Transglycosylation products accumulated to a lower extent, when the E332H mutation was introduced into other MAases such as BLMA and BSMA (data not shown). From the results, the observation that the conserved Glu332 in MAases modulates the catalytic specificity between the hydrolysis and transglycosylation activities is generally applicable to most MAases and various acceptors.

Proposed Structure of the ThMA−Acarbose Complex with α-MG. Kim et al. (*27*) reported that there is an extra sugar-binding space at the bottom of the catalytic site in ThMA, not found in other typical α-amylases which can be occupied by an acceptor molecule. To determine the role of Glu332 in transglycosylation, acarbose and α-MG molecules were docked computationally into the active site cleft of ThMA using the solid docking module in QUANTA (Molecular Simulations, Inc.). The favorable docking did not require a reorientation of amino acid side chains and a change in torsion angles of glycosidic bonds in acarbose. The cleft is well-occupied by an acarbose molecule, and many residues from both the $(\beta/\alpha)_8$-barrel and N-terminal domains of dimeric model are involved in favorable interactions with the acarbose as shown in a closeup view of the docking model (Figure 6). From the model, Glu332 in the extra space is located within hydrogen bonding distance of the α-MG molecule. An acceptor molecule such as α-MG occupies the space to compete with a water molecule for attacking the enzyme−PTS intermediate.

DISCUSSION

Although MAases are classified as members of the α-amylase family (glycoside hydrolase family 13; *35*, *36*), they exhibit multisubstrate specificities and catalytic versatil-

ity, especially, transglycosylation activity. To investigate the role of Glu332 in the transglycosylation activity of ThMA, the acarbose transfer reaction with an α-MG acceptor was used as a model system. While the activities of typical α-amylases are strongly inhibited by acarbose, MAases not only hydrolyze acarbose but also transfer the resulting PTS to acceptor molecules. α-MG used as the acceptor in this study possesses several advantages over other acceptors. (1) The acarbose transfer reaction occurrs efficiently enough to yield various transfer products. (2) The resulting transfer products are easily separated and monitored by TLC and HPAEC analyses. (3) As the structure of α-MG is similar to that of glucose, all transfer reactions occur with glucose and α-MG.

Generally, tranglycosylation reactions carried out by α-amylases are unfavorable under normal conditions and often require extremely high concentrations of substrate and a nonaqueous environment. Brzozowski et al. (*18*) determined the three-dimensional structure of *Aspergillus oryzae* α-amylase complexed with acarbose by X-ray crystallography. The tetrasaccharide inhibitor was present, however, as a hexasaccharide in the complex. The extended oligosaccharide probably resulted from a simple transglycosylation reaction. Also, the crystal of porcine pancreatic α-amylase in its complex with acarbose (*37*) exhibited a pseudohexasaccharide, two acarviosine units separated by a glucose molecule bound to the active site of the enzyme. In each case, formation of the observed ligands could result from the transfer of the first three subunits of acarbose to another acarbose molecule after the initial hydrolysis of glucose from the acarbose. The transglycosylation reaction accomplished by MAase could be similar to the formation of the pseudohexasaccharide observed in the crystal structure of α-amylases (*18*, *37*). At first, acarbose occupies the +1 to −3 subsites of the active site in MAase so that the enzyme can cleave the first α-(1,4)-glycosidic bond of acarbose to generate a glucose and a PTS−enzyme intermediate. Then, the acceptor molecule enters the +1 subsite, and the enzyme catalyzes the formation of α-(1,3)-, α-(1,4)-, and α-(1,6)-linked transfer products.

Recently, Cha et al. (*2*) showed that an increase in the hydrophobicity between the third and fourth conserved regions improved the transglycosylation activity of MAase. Also, Kuriki et al. (*15*) reported that transglycosylation activity of neopullulanase was affected by hydrophobicity around the fourth conserved region. However, we focused on the differences between MAases and typical α-amylases, because the previous investigations assumed an active monomer. The crystal structure of ThMA revealed a homodimer and an extra sugar-binding space in the active site cleft formed by adjacent N-terminal domains of each subunit. The space could provide room for an acceptor molecule in such a way as to compete with a water molecule (*27*). From the docking model (Figure 6), acceptor molecules can compete with water molecules to act on the PTS−enzyme intermediate complex. Glu332 located in the second conserved region of ThMA and at the extra sugar-binding space may be involved in binding small oligosaccharide acceptors. Upon binding the acceptor molecule (α-MG in this study), the carboxylate group of Glu332 may position the acceptor molecule in a proper orientation, allowing the formation of an α-(1,3)-, α-(1,4)-, or α-(1,6)-linkage between α-MG and PTS.

Between α-MG and PTS, the molecular docking was carried out without changing the positions of protein atoms. In a real situation, a minor conformational change of protein side chains may allow binding of an acceptor molecule in several different modes that would result in transglycosylation at three different carbon positions.

In this study, substitutions of Glu332 in ThMA with histidine or glutamine resulted in a substantial decrease in the extent of formation of transfer products with increased hydrolysis activities. The differences of transglycosylation activities in mutants are probably due to the difference in the interaction of charged and neutral amino acid residues at position 332 with the acceptor molecules. With respect to accumulation of transfer products, ThMA was changed to an α-amylase type enzyme by modulating Glu332. In conclusion, these results imply that the acidic side chain of glutamate at position 332 of ThMA is important in the formation and accumulation of transglycosylation products. The results obtained in this study will be helpful in elucidating the mechanism of hydrolysis and transglycosylation of MAases and related enzymes and may improve their catalytic properties.

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