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Changes in the Catalytic Properties of *Pyrococcus furiosus* Thermostable Amylase by Mutagenesis of the Substrate Binding Sites[▽]

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***Pyrococcus furiosus* thermostable amylase (TA) is a cyclodextrin (CD)-degrading enzyme with a high preference for CDs over maltooligosaccharides. In this study, we investigated the roles of four residues (His414, Gly415, Met439, and Asp440) in the function of *P. furiosus* TA by using site-directed mutagenesis and kinetic analysis. A variant form of *P. furiosus* TA containing two mutations (H414N and G415E) exhibited strongly enhanced α-(1,4)-transglycosylation activity, resulting in the production of a series of maltooligosaccharides that were longer than the initial substrates. In contrast, the variant enzymes with single mutations (H414N or G415E) showed a substrate preference similar to that of the wild-type enzyme. Other mutations (M439W and D440H) reversed the substrate preference of *P. furiosus* TA from CDs to maltooligosaccharides. Relative substrate preferences for maltoheptaose over β-CD, calculated by comparing k_{cat}/K_m ratios, of 1, 8, and 26 for wild-type *P. furiosus* TA, *P. furiosus* TA with D440H, and *P. furiosus* TA with M439W and D440H, respectively, were found. Our results suggest that His414, Gly415, Met439, and Asp440 play important roles in substrate recognition and transglycosylation. Therefore, this study provides information useful in engineering glycoside hydrolase family 13 enzymes.**

Glycoside hydrolase family 13 (GH-13) is the largest family of α-glycosidases characterized to date (7), and the members of this family display a variety of catalytic properties, including the hydrolysis of α-(1,4)-glycosidic linkages catalyzed by α-amylases (EC 3.2.1.1), the hydrolysis of α-(1,6)-glycosidic linkages by isoamylases (EC 3.2.1.68), the synthesis of α-(1,4)-linked cyclic maltooligosaccharides by cyclodextrin glucanotransferases (CGTases; EC 2.4.1.19), the synthesis of linear maltooligosaccharides by 4-α-glucanotransferases (α-GTases; EC 2.4.1.25), the synthesis of α-(1,6)-glycosidic linkages by branching enzymes (EC 2.4.1.18), and the catalysis of many more reactions (20). Therefore, many GH-13 glycosidases have been studied to identify key residues required for their function and to determine their mechanisms of action (23). Through mutagenesis and kinetic analyses of the variant enzymes of interest, these efforts have provided useful information required for the creation of valuable biocatalysts for various industrial applications.

Cyclodextrin (CD)-degrading enzymes belong to GH-13, but they have unique catalytic characteristics compared to other GH-13 enzymes (29). CD-degrading enzymes possess an extra domain at the N terminus of each enzyme that mediates the formation of domain-swapped homodimeric structures (8–10, 16). The narrow and deep active site created by the dimeric structure is responsible for the preference for small substrates, such as CDs and maltooligosaccharides, over relatively large molecules, such as starch and pullulan (12, 29). Most importantly, they catalyze the formation of predominantly α-(1,6)-transfer products rather than α-(1,3)- and α-(1,4)-linked products (28). To date, successful transglycosylations of various natural products have been accomplished by our group (1, 18). There are four subclasses of CD-degrading enzymes: cyclodextrinases (CDases; EC 3.2.1.54), maltogenic amylases (EC 3.2.1.133), neopullulanases (EC 3.2.1.135), and *Thermoactinomyces vulgaris* α-amylase II. Given their catalytic properties and structural similarities (8–10, 16), we have proposed that they should be combined into a single enzyme class (16). Our previous attempts to develop thermostable CD-degrading enzymes from hyperthermophiles, including members of the *Archaea*, revealed that these relatively ancient enzymes have unique properties compared to evolutionally differentiated mesophilic bacterial enzymes (11, 17, 34). Of the archaeal enzymes, *Pyrococcus furiosus* thermostable amylase (TA) has a distinguishable substrate preference for cyclic maltodextrins over linear maltooligosaccharides (34). Its unique catalytic properties are useful for producing high-value-added maltooligosaccharides with defined lengths, such as maltohexaose (G6), maltoheptaose (G7), and maltooctaose (G8), from commercially available α-, β-, and γ-CDs, respectively (33).

Here, we investigated the catalytic properties of several variant forms of *P. furiosus* TA modified at amino acids (His414, Gly415, Met439, and Asp440) located in the regions conserved among GH-13 enzymes (Fig. 1). Through kinetic and product analyses of *P. furiosus* TA mutant enzymes, we determined the roles of the residues in the function of *P. furiosus* TA during hydrolysis and transglycosylation.

MATERIALS AND METHODS

Site-directed mutagenesis. Variant forms of *P. furiosus* TA were constructed using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The recombinant plasmid pETPFTA-6h (34) carrying the *P. furiosus* TA gene (UniProt number Q8TZP8) was digested with NdeI and XhoI, and the DNA

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Enzyme	Conserved regions I	II	III	IV
PFTA	305 D G V F H H	407 G F R M D V A H G	437 E V M D	497 F L D N H D
ThMA	242 D A V F N H	324 G W R L D V A **N E**	357 E I **W H**	419 L L G S H D
BSMA	242 D A V F N H	324 G W R L D V A **N E**	357 E I **W H**	419 L L G S H D
CD I-5	238 D A V F N H	321 G W R L D V A **N E**	354 E V **W H**	416 L L D S H D
BsCD	240 D A V F N H	323 G W R L D V A **N E**	356 E I **M H**	L L G S H D
BsNPL	242 D A V F N H	324 G W R L D V A **N E**	357 E I **W H**	419 L L G S H D
TVAII	239 D A V F N H	321 G W R L D V A **N E**	354 E I **W H**	416 L L D S H D
	○	★ ○ ○	★ ○	○ ★
BSTA	135 D V V F D H	264 G F R L D A V **K H**	298 E Y **W S**	360 F V D N H D
BLTA	129 D V V I N H	256 G F R L D A V **K H**	290 E Y **W Q**	352 F V D N H D
TAA	138 D V V A N H	223 G L R I D T V **K H**	251 E V **L D**	313 F V E N H D
BcCGT	135 D F A P N H	225 G I R V D A V **K H**	257 E W **F L**	323 F I D N H D
BsCGT	131 D F A P N H	221 G I R F D A V **K H**	253 E W **F L**	319 F I D N H D
PfCGT	133 D Y V D N H	230 G F R I D A V **K H**	264 E Y **F A**	334 F L D S H D

FIG. 1. Comparison of amino acid residues in the conserved regions (I, II, III, and IV) of *P. furiosus* TA (PFTA) and various amylolytic enzymes. Invariant or highly conserved amino acids are shaded light gray. The amino acids corresponding to those investigated in this study are shown in bold. Invariant amino acids are shaded dark gray, and the residues in CD-hydrolyzing enzymes are boxed. For amylolytic enzymes, the catalytic amino acid residues are indicated by asterisks and substrate binding amino acids are indicated by circles. ThMA, *Thermus* sp. strain IM6501 maltogenic amylase (GenBank accession no. AAC15072); BSMA, *Bacillus stearothermophilus* maltogenic amylase (GenBank accession no. AAC46346); CD I-5, alkalophilic *Bacillus* sp. strain I-5 cyclodextrinase (GenBank accession no. AAA92925); BsCD, *Bacillus sphaericus* cyclodextrinase (GenBank accession no. CAA44454); BsNPL, *Bacillus stearothermophilus* neopullulanase (GenBank accession no. AAK15003); TVAII, *Thermoactinomyces vulgaris* R-47 α-amylase II (GenBank accession no. 1911217A); BSTA, *Bacillus stearothermophilus* α-amylase (GenBank accession no. 1713273A); BLTA, *Bacillus licheniformis* α-amylase (GenBank accession no. P06278); TAA, *Aspergillus oryzae* α-amylase (GenBank accession no. CAA31218); BcCGT, *Bacillus circulans* CGTase (GenBank accession no. CAA48401); BsCGT, *Bacillus stearothermophilus* CGTase (GenBank accession no. P31797); PfCGT, *P. furiosus* CGTase (GenBank accession no. AAL80602).

fragment containing the *P. furiosus* TA gene was then extracted from agarose gel and subcloned into pTKNd6xH (34). The resulting plasmid was designated pTKwtPA-6h and was used as the template for mutagenesis. The oligonucleotide sequences used for the mutagenesis reactions are summarized in Table 1. The mutations introduced into *P. furiosus* TA were confirmed using dideoxy chain termination sequencing with an ABI 3700 PRISM DNA sequencer (Perkin-Elmer, Wellesley, MA).

Purification of *P. furiosus* TA wild-type and mutant enzymes. *Escherichia coli* MC1061 [F^- *araD139 recA13* Δ(*araABC-leu*)*7696 galU galK* Δ*lacX74 rpsL thi hsdR2 mcrB*] was used as a host for the expression of wild-type *P. furiosus* TA and its variant forms. *E. coli* MC1061 transformants were grown in Luria-Bertani broth (1% [wt/vol] Bacto tryptone, 0.5% [wt/vol] yeast extract, and 0.5% [wt/vol] NaCl) supplemented with kanamycin (20 μg/ml) at 37°C for 20 h. The recombinant enzymes were purified by nickel-nitrotriacetate (Ni-NTA) affinity chromatography with a system from QIAGEN (Hilden, Germany) as previously described (34). The eluted fractions were dialyzed against 50 mM Tris-HCl buffer (pH 7.0) containing 150 mM NaCl. The purity of the enzymes was estimated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The protein concentrations were determined using the Bradford method (3), with bovine serum albumin as a standard.

Enzyme assay for β-CD hydrolysis. The activities of the wild-type and mutant enzymes in β-CD hydrolysis were assayed at 85°C in 50 mM sodium acetate buffer (pH 4.5) with 3,5-dinitrosalicylic acid as described by Miller (22). One hundred fifty microliters of 1% (wt/vol) β-CD and 130 μl of 50 mM sodium acetate buffer were mixed and preincubated at 85°C. Twenty microliters of the enzyme was added to the preheated solution, and then the reaction mixture was incubated for 10 min. The reaction was stopped by adding 900 μl of 3,5-dinitrosalicylic acid solution and then boiling for 5 min and cooling with iced water. The absorbance of the reaction mixture at 570 nm was measured using a spectrophotometer (UV-160A; Shimadzu Co., Tokyo, Japan). One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of reducing sugars from the substrate per min.

TABLE 1. Oligonucleotides used for site-directed mutagenesis

Oligonucleotide	DNA sequence (5′ to 3′)[a]
H414N-f	TGGATGTTGCTAATGGGGTTCCTCCAGAA
H414N-r	TTCTGGAGGAACCCCATTAGCAACATCCA
G415E-f	TGGATGTTGCTCATGAGGTTCCTCCAGAA
G415E-r	TTCTGGAGGAACCTCATGAGCAACATCCA
H414N/G415E-f	ATGGATGTTGCTAATGAGGTTCCTCCAGAA
H414N/G415E-r	TTCTGGAGGAACCTCATTAGCAACATCCAT
M439W-f	AATTGGAGAAGTTTGGGATGATGCAAGGT
M439W-r	ACCTTGCATCATCCCAAACTTCTCCAATT
D440H-f	AATTGGAGAAGTTATGCATGATGCAAGGT
D440H-r	ACCTTGCATCATGCATAACTTCTCCAATT
M439W/D440H-f	AATTGGAGAAGTTTGGCATGATGCAAGGT
M439W/D440H-r	ACCTTGCATCATGCCAAACTTCTCCAATT

[a] Mutated codons are underlined.

Hydrolytic patterns of *P. furiosus* TA. To examine the hydrolytic patterns of *P. furiosus* TA wild-type and mutant enzymes, 0.5 ml of the purified enzyme (1.5 U of β-CD hydrolysis activity) was incubated with 0.5 ml of 1% (wt/vol) substrate in 50 mM sodium acetate buffer (pH 4.5) at 75°C for 1 h. The substrates used were CDs (α-, β-, and γ-CDs), maltooligosaccharides (maltose [G2] to G7), and soluble starch. The reaction products were analyzed on K5F silica gel plates (Whatman, Maidstone, United Kingdom) by thin-layer chromatography (TLC) with isopropyl alcohol-ethyl acetate-water (3:1:1, vol/vol/vol) as the solvent system. After two elutions, each TLC plate was dried and visualized by dipping it into 0.3% (wt/vol) *N*-(1-naphthyl)-ethylenediamine and 5% (vol/vol) H_2SO_4 in methanol followed by charring it for 10 min at 110°C (28).

HPAEC. The reaction mixture was analyzed by high-performance anion-exchange chromatography (HPAEC) using a pulsed amperometric detector (ED40; Dionex, Sunnyvale, CA). The system was equipped with a CarboPac PA-100 column (4 by 250 mm; Dionex) and run with a gradient of 0 to 0.4 M sodium acetate in 0.15 M NaOH with a flow rate of 1 ml/min.

Transglycosylation patterns of *P. furiosus* TA and the *P. furiosus* TA(H414N/G415E) mutant. To examine the transglycosylation patterns of wild-type *P. furiosus* TA and the mutant enzyme with amino acid changes H414N and G415E [*P. furiosus* TA(H414N/G415E)], 0.5 ml of each purified enzyme (3 U of β-CD hydrolysis activity) was incubated with 0.5 ml of 1% (wt/vol) substrate in 50 mM sodium acetate buffer (pH 4.5) at 75°C for 2 h. The substrates used were CDs (α-, β-, and γ-CDs), maltooligosaccharides (G2 to G7), and soluble starch. Two microliters of each reaction product was added to a Whatman K5F silica gel TLC plate and eluted twice using acetonitrile-ethyl acetate-isopropyl alcohol-water (85:20:50:70, vol/vol/vol/vol) as the solvent system. The reaction products were visualized using the same methods described above for hydrolytic pattern analyses. To verify the structures of the reaction products corresponding to *P. furiosus* TA(H414N/G415E), β-amylase (Sigma Co.) was added directly to the reaction mixture and the treated mixture was then incubated overnight at 37°C. The reaction mixture and the β-amylase-treated mixture were analyzed using HPAEC. The maltooligosaccharide standard was prepared from the reaction of isoamylase (Hayashibara Biochemical Laboratories, Inc., Okayama, Japan) with

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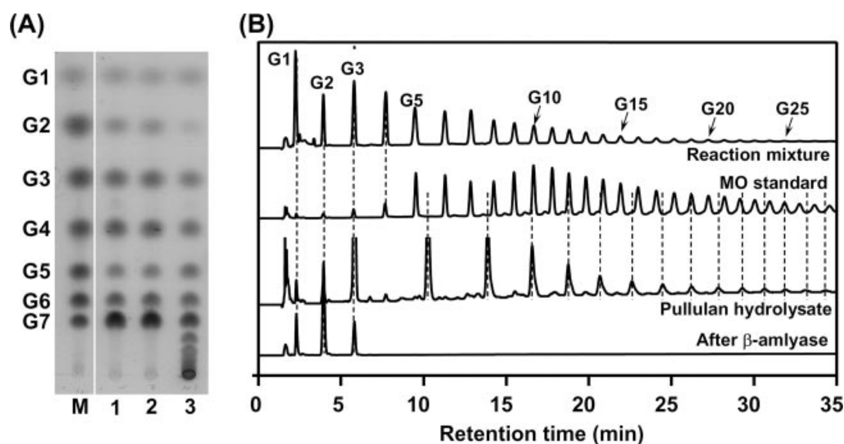

FIG. 2. Analyses of the reaction products formed from β-CD by *P. furiosus* TA mutant enzymes with amino acid changes H414N, G415E, and H414N and G415E. Each enzyme (0.7 U) was reacted with 0.5% (wt/vol) β-CD in 50 mM sodium acetate buffer (pH 4.5) at 75°C for 1 h. (A) TLC analyses of the reaction mixtures containing TA(H414N), TA(G415E), and TA(H414N/G415E). Lane M, maltooligosaccharide standards; lane 1, reaction mixture with TA(H414N); lane 2, reaction mixture with TA(G415E); lane 3, reaction mixture with TA(H414N/G415E). (B) HPAEC analysis of the reaction mixture containing *P. furiosus* TA(H414N/G415E). MO standard, maltooligosaccharide standard; pullulan hydrolysate, products of the partial hydrolysis of pullulan by a pullulanase; after β-amylase, β-amylase-treated reaction mixture with TA(H414N/G415E).

0.5% (wt/vol) amylopectin (Sigma Co., St. Louis, MO) in 50 mM sodium citrate buffer (pH 4.3) for 60 h at 60°C. The standard of branched maltooligosaccharides was prepared from the reaction of pullulanase (Promozyme; Novo Nordisk, Copenhagen, Denmark) with 0.5% (wt/vol) pullulan (Sigma Co.) in 50 mM sodium citrate buffer (pH 4.3) for 10 min at 60°C.

Measurement of kinetic parameters for hydrolysis by *P. furiosus* TA and its derivatives. The substrate concentrations for the kinetic analyses ranged from 0.3 to 8 mM for β-CD and 0.2 to 8 mM for G7 in 50 mM sodium acetate buffer (pH 4.5). The reactions were carried out at 85°C. Aliquots (50 μl) of the mixtures were taken at 90-s intervals, and the reactions were immediately stopped by transferring each mixture into 50 μl of 0.1 M HCl and placing the mixture on ice. After neutralizing the mixture by adding 50 μl of 0.1 N NaOH, the amount of hydrolyzed substrate was analyzed quantitatively using HPAEC. GraFit version 4.0 (Erithacus Software, United Kingdom) was used to calculate kinetic parameters by the direct fitting of initial rates.

RESULTS

Identification of residues critical for substrate binding. GH-13 enzymes have four conserved regions. The catalytically important residues, including three catalytic residues and five key residues for substrate binding, are indicated in Fig. 1. According to the structures of GH-13 enzymes bound to their substrates, the last two residues of the second conserved region (Lys and His in α-amylases and CGTases and Asn and Glu in CD-degrading enzymes) and a hydrophobic residue in the third conserved region (Trp in α-amylases and CD-degrading enzymes and Phe in CG-Tases) play important roles in substrate binding to the +1 and +2 subsites (16, 25). As shown in Fig. 1, three residues are strictly conserved among the bacterial CD-degrading enzymes, Asn331, Glu332, and Trp359 (*Thermus* sp. strain IM6501 maltogenic amylase numbering). In addition, the last residue in the third conserved region of α-amylases and CGTases is relatively variable, while the corresponding residue in CD-degrading enzymes is a strictly conserved His (Fig. 1). However, *P. furiosus* TA has different amino acids at the positions listed above, and these variant residues (H414, G415, M439, and D440) are predicted to determine the substrate recognition and catalytic properties of the enzyme. For this reason, these four residues in the second and third conserved regions of *P. furiosus* TA were mutated to the corresponding residues (Asp, Glu, Trp, and His, respectively) typical in CD-degrading enzymes and the catalytic properties of the variant enzymes were investigated.

Role of H414 and G415 in the active site of *P. furiosus* TA. The role of His414 and Gly415 in determining the catalytic properties of *P. furiosus* TA was investigated by analyzing three variant enzymes modified at these residues, TA(H414N), TA(G415E), and TA(H414N/G415E). Each mutant enzyme was expressed in *E. coli* and purified to apparent homogeneity by Ni-NTA affinity chromatography. The hydrolysis of β-CD by TA(H414N), TA(G415E), and TA(H414N/G415E) decreased 8-fold (21 U/mg), 10-fold (17 U/mg), and 80-fold (2 U/mg), respectively, compared to that by the parent enzyme (170 U/mg). The substrate preferences of TA(H414N) and TA(G415E) were roughly the same as that of the wild-type enzyme; all three enzymes hydrolyzed β-CD into G7 as the major product plus several small maltooligosaccharides (Fig. 2A, lanes 1 and 2). However, the double mutant form [TA(H414N/G415E)] yielded a series of oligosaccharides that were longer than the maltooligosaccharides generated from the β-CD ring-opening reactions (Fig. 2A, lane 3). As CD-degrading enzymes produce predominantly α-(1,6)-transfer products, we expected the longer products to be oligosaccharides with multiple α-(1,6)-glycosidic linkages. Unexpectedly, when the reaction mixture containing *P. furiosus* TA(H414N/G415E) was compared to that containing maltooligosaccharide standards by HPAEC (see Materials and Methods), the transfer products had retention times identical to those of α-(1,4)-linked linear maltooligosaccharides (Fig. 2B). In contrast, the retention times of the transfer products were different from those of the products of the partial hydrolysis of pullulan by a pullulanase (Fig. 2B). To confirm their structures, the products synthesized by TA(H414N/G415E) were treated with β-amylase (Sigma, St. Louis, MO). β-Amylase completely hydrolyzed all transfer products into glucose, maltose, and maltotriose with neither unhydrolyzed long oligosaccharides nor small α-(1,6)-linked oligosaccharides (Fig. 2B). These results indicate that the transfer products had α-(1,4)-glycosidic linkages only. To further investi-

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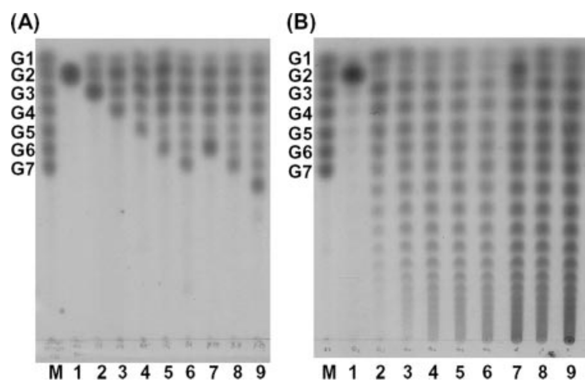

FIG. 3. TLC analyses of the reaction products formed from various substrates by wild-type *P. furiosus* TA (A) and *P. furiosus* TA(H414N/G415E) (B). Lanes M, maltooligosaccharide standards (glucose to maltoheptaose); lanes 1, maltose; lanes 2, maltotriose; lanes 3, maltotetraose; lanes 4, maltopentaose; lanes 5, maltohexaose; lanes 6, maltoheptaose, lanes 7, α-CD; lanes 8, β-CD; lanes 9, γ-CD. Each enzyme (1.5 U) was reacted with each substrate at a concentration of 0.5% (wt/vol) in 50 mM sodium acetate buffer (pH 4.5) at 75°C for 2 h.

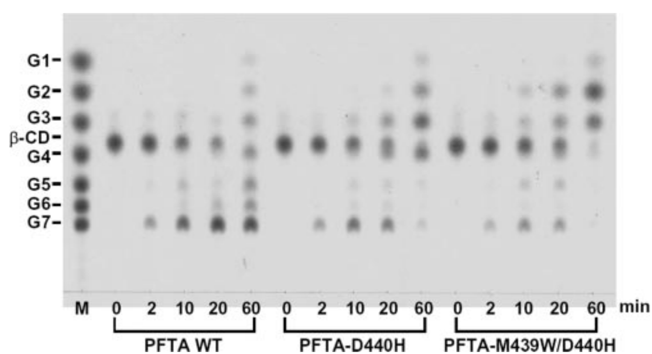

FIG. 4. TLC analyses of the reaction products of wild-type *P. furiosus* TA (PFTA WT), *P. furiosus* TA(D440H) (PFTA-D440H), and *P. furiosus* TA(M439W/D440H) (PFTA-M439W/D440H). Lane M contains maltooligosaccharide standards (glucose to maltoheptaose). Each aliquot was taken at different reaction times of 0, 2, 10, 20, and 60 min. Each enzyme (0.5 U) was reacted with β-CD at a concentration of 0.5% (wt/vol) in 50 mM sodium acetate buffer (pH 4.5) at 75°C.

gate the substrate specificity of TA(H414N/G415E) for the disproportion activity, the enzyme was incubated with 0.5% (wt/vol) maltooligosaccharides (G2 to G7) and CDs (α-, β-, and γ-CDs) and each reaction mixture was analyzed by TLC. Wild-type *P. furiosus* TA only hydrolyzed the substrates (Fig. 3A), whereas TA(H414N/G415E) successfully carried out disproportion reactions using maltotriose (G3) and longer maltooligosaccharides (Fig. 3B). Large amounts (10 to 20 U) of wild-type TA were unsuccessful at yielding any transfer products (data not shown).

The role of Met439 and Asp440 in the active site of *P. furiosus* TA. To clarify the role of Met439 and Asp440 in the catalytic activity of *P. furiosus* TA, these residues were mutated to Trp and His, respectively. Recombinant TA(M439W) could not be produced in a soluble form in *E. coli* (data not shown); however, the variant enzymes TA(D440H) and TA(M439W/D440H) were successfully produced in a soluble form and purified to apparent homogeneity by Ni-NTA affinity chromatography. By using 0.5% (wt/vol) β-CD as the substrate, *P. furiosus* TA(D440H) and TA(M439W/D440H) were shown to have 50% [86 U/mg of TA(D440H)] and 13% [22 U/mg of TA(M439W/D440H)] of the hydrolyzing activity of wild-type TA (170 U/mg). To explore the catalytic properties of the variant forms, we compared the hydrolysis patterns of the wild-type and variant enzymes by using 0.5% (wt/vol) β-CD as the substrate with equal numbers of enzyme units (0.1 U of enzyme per 1 mg of substrate). Time course analysis of the reaction mixture showed that wild-type *P. furiosus* TA simply opened the rings of the β-CD without significantly degrading the resulting G7 products (Fig. 4). The same result was obtained in our previous experiments (33). In contrast, TA(D440H) and TA(M439W/D440H) liberated various small maltooligosaccharides from the CDs (Fig. 4). In addition, the double mutant TA(M439W/D440H) hydrolyzed G7 faster than TA(D440H). These results imply that M439 and D440 each contribute to the preference of wild-type *P. furiosus* TA for cyclic over linear substrates.

To verify the effects of the mutations on substrate specificity, kinetic analyses of the hydrolysis of β-CD and G7 were conducted using the wild-type and mutant enzymes. The kinetic parameters of *P. furiosus* TA(D440H) for G7 were not greatly altered compared to those of wild-type TA (Table 2); however, the catalytic efficiency of the enzyme (k_{cat}/K_m ratio) for β-CD was reduced from 100 to 16 s^{-1} mM^{-1} due to a dramatic decrease in the k_{cat}. Interestingly, the introduction of an additional mutation (M439W) into TA(D440H) led to enhanced hydrolysis of G7 without significantly changing the catalytic efficiency of the enzyme for β-CD (Table 2). Overall, replacing M439 and D440 in *P. furiosus* TA with Trp and His increased the ratio of the catalytic efficiency for G7 to that for β-CD [(k_{cat}/K_m ratio for G7)/(k_{cat}/K_m ratio for β-CD)] by 26-fold relative to the ratio of the catalytic efficiencies of wild-type TA for these substrates. These results suggest that M439 and D440 are critical for the recognition and hydrolysis of linear and cyclic maltodextrins, respectively, by *P. furiosus* TA.

DISCUSSION

Recent developments in bioinformatics have enabled us to predict the roles of specific amino acids in enzymatic catalysis; however, based on multiple sequence alignments alone, identifying the key residues that determine the desirable properties of novel biocatalysts remains a challenge. In this study, we analyzed the substrate binding residues of the archaeal CD-degrading enzyme *P. furiosus* TA, which belongs to GH-13, to elucidate its catalytic mechanism.

The double mutation of *P. furiosus* TA (H414N and G415E) not only reduced the hydrolysis of CD by 80-fold relative to that by the wild-type enzyme but also enhanced the formation of a series of maltooligosaccharides with exclusive regioselectivity toward α-(1,4)-glycosidic linkages (Fig. 2 and 3). Naturally occurring α-GTases catalyze such transglycosylation reactions (32). To date, all known α-GTases have been classified into GH-13, GH-57, or GH-77 (7). Those enzymes grouped into GH-57 or GH-77 utilize maltotriose and longer maltooligosaccharides as sugar donors; furthermore, they are involved in the degradation of polysaccharides such as starch and glycogen (4, 32), as well as in the elongation of short maltooligo-

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TABLE 2. Kinetic parameters of wild-type *P. furiosus* TA and its variant forms[a]

Form of TA	β-Cyclodextrin			Maltoheptaose		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
Wild type	610	6	100	160	14	11
D440H variant	64	4	16	170	13	13
M439W/D440H variant	23	2	11	55	1.8	31

[a] Kinetic parameters were determined by using 50 mM sodium acetate buffer (pH 4.5) at 85°C. Errors for the data in this table range from 5 to 15%.

saccharides to yield longer maltooligosaccharides that serve as substrates for maltodextrin phosphorylase (2).

Only two GH-13 α-GTases have been described. Both enzymes were isolated from *Thermotoga maritima*, and they display catalytic properties that distinguish them from the other α-GTases (i.e., those of GH-57 and GH-77), including a preference for G4 donors and sugar transfers (19, 21). Similarly, *T. maritima* maltosyltransferase (TMMT) will transfer only maltosyl residues (21), and *T. maritima* α-GTase (TMGT) has certain substrate restrictions; maltotetraose is the smallest possible donor, and glucose cannot serve as an acceptor (19).

P. furiosus TA(H414N/G415E), which was derived from a hydrolytic GH-13 enzyme, exhibited a mixed pattern of catalysis resembling that of TMMT or TMGT. The smallest donor for TA(H414N/G415E) was maltotriose, which is the same as that for TMMT and other known α-GTases, except TMGT (maltotetraose). The amount of maltose was significantly smaller than that of any other maltooligosaccharide in the reaction mixtures catalyzed by TA(H414N/G415E) (Fig. 2 and 3B), whereas we could not see such a difference in the reaction mixtures catalyzed by the wild-type enzyme (Fig. 3A); this implied that TA(H414N/G415E) transferred predominantly maltosyl moieties. However, the transfer specificity of the TA mutant enzyme was not as strong as that of TMMT, and the products were obtained as a series of maltooligosaccharides that differed in length by a single glucose unit, as observed for TMGT and other α-GTases. Unlike other α-GTases, *P. furiosus* TA(H414N/G415E) did not catalyze intramolecular transglycosylation via cycloamylose formation, which is a general feature of α-GTases (32). Unfortunately, we were unable to clarify the mechanism of transglycosylation by TA(H414N/G415E) based on our homologous model structure (data not shown).

The H414N and G415E mutations in *P. furiosus* TA likely cause enhanced transglycosylation activity, leading to the formation of new α-(1,4)-glycosidic linkages. In addition, the significantly reduced hydrolytic activity caused by the mutations should have retarded the degradation of the transfer products, resulting in the accumulation of long maltooligosaccharides (Fig. 2 and 3). Feng et al. and Osanjo et al. developed novel variant forms of glycosidase with high levels of transglycosylation activity through directed evolution based on the modulation of hydrolysis and transglycosylation (6, 27). The regioselectivity of the *P. furiosus* TA mutant enzyme toward α-(1,4)-glycosidic linkages was unexpected because generally α-(1,6)-linked products are the major transfer products produced by CD-degrading enzymes with Asn and Glu residues at the positions corresponding to H414 and G415 of *P. furiosus* TA (1, 14, 28). Indeed, the α-(1,6)-regioselectivity of CD-degrading enzymes is a consequence of the relatively high level of resistance of α-(1,6)-glycosidic linkages to enzymatic hydrolysis compared to that of α-(1,4)-linked transfer products, which are produced more rapidly in the early stage of the reaction than α-(1,6)-linked products (13). Therefore, the rate of formation of α-(1,4)-glycosidic linkages via transglycosylation by CD-degrading enzymes is generally higher than that of their α-(1,6)-linked counterparts. It is unclear why no α-(1,6)-linked products were detected in the mixtures containing the mutant *P. furiosus* TA; however, this observation suggests that these two residues in the second conserved region of GH-13 enzymes play an important role in both hydrolysis and transglycosylation. The control of these activities and the modulation of the transglycosylation patterns of CD-degrading enzymes, and potentially those of other GH-13 enzymes, may therefore be achieved by mutagenizing these residues.

Parallel-stacking interactions (i.e., CH-π interactions) between the hydrophobic faces of carbohydrates and aromatic amino acids (His, Phe, Trp, and Tyr) are involved in the ligand recognition of such enzymes as glycosidases, glycosyltransferases, and carbohydrate binding modules (5, 15, 30). In the case of CD-degrading enzymes, the role of a conserved aromatic amino acid (Trp359 in *Thermus* sp. maltogenic amylase, Trp356 in *Thermoactinomyces vulgaris* R-47 α-amylase II, and Phe255 in *Bacillus stearothermophilus* CGTase) (Fig. 1) in the third conserved region has been highlighted by solving the structures of the enzymes bound to their various ligands, which demonstrated the multiple substrate specificities of these enzymes (10, 26, 31). Crystal structures of *Thermoactinomyces vulgaris* R-47 α-amylase II in complexes with linear maltooligosaccharides, such as maltotetraose and maltohexaose, suggested that the strong stacking interaction between the Trp residue in the third region and the glucose unit in the +2 subsite plays a major role in stabilizing the conformation of the substrates during catalysis (26, 31). However, the archaeal CD-degrading enzyme *P. furiosus* TA has a Met residue at the corresponding position (Fig. 1) and shows a strong preference for cyclic substrates (β-CD) rather than linear maltooligosaccharides (34). The replacement of Met439 in *P. furiosus* TA with a Trp residue, in combination with the D440H mutation, significantly altered the recognition and hydrolysis of both cyclic and linear substrates (Fig. 4). *P. furiosus* TA(M439W/D440H), which has the same amino acids as those of bacterial CD-degrading enzymes, showed a 26-fold greater preference for linear over cyclic substrates [(k_{cat}/K_m for G7)/(k_{cat}/K_m for β-CD)] than the wild-type TA (Table 2).

This pattern of substrate specificity is common to most bacterial CD-degrading enzymes (29). The kinetic parameters of the single mutant TA(D440H) clarified the role of each residue. Interestingly, each residue is responsible for the substrate preference of *P. furiosus* TA through a different mechanism. Asp440 promotes the hydrolysis of cyclic maltodextrins without affecting the use of linear maltooligosaccharides (Table 2). In

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contrast, the substitution of a Trp residue for Met439 in TA(D440H) increased the catalytic efficiency of the enzyme for G7 by twofold relative to that of TA(D440H) without significantly changing its catalytic activity for β-CD. Therefore, Met439 in *P. furiosus* TA plays a critical role in the hydrolysis of linear maltooligosaccharides rather than cyclic substrates, resulting in the unique substrate preference of the enzyme. Indeed, our expectations for the role of M439 are consistent with the findings of a kinetic study of a CDase from *Bacillus sphaericus* having Met358 and Asp359 at the positions corresponding to Met439 and His440 in *P. furiosus* TA (Fig. 1). The CDase displayed a threefold greater V_{max}/K_m value for G7 than for β-CD (24).

In conclusion, the roles of substrate binding residues in the archaeal CD-degrading enzyme *P. furiosus* TA were investigated through site-directed mutagenesis and analysis of the catalytic performance of the variant enzymes. The results obtained in this study suggest that the residues (His414, Gly415, Met439, and Asp440) in the +1 and +2 subsites play important roles in substrate recognition and transglycosylation. These results provide useful information for rational protein engineering of GH-13 enzymes.

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