Cyclomaltodextrinase, Neopullulanase, and Maltogenic Amylase Are Nearly Indistinguishable from Each Other*

Hee-Seob Lee‡‡, Min-Sung Kim§§, Hyun-Soo Cho¶¶, Jung-In Kim††, Tae-Jip Kim‡‡, Ji-Hye Choi‡‡, Cheonseok Park**, Heung-Soo Lee‡‡, Byung-Ha Oh††††, and Kwan-Hwa Park‡‡

From the §National Creative Research Initiative Center for Biomolecular Recognition, Department of Life Science, Pohang University of Science and Technology, and the §§Pohang Accelerator Laboratory, Pohang, Kyungbuk 790-784, Korea, the ¶¶Research Center for New Bio-Materials in Agriculture and the Department of Food Science and Technology, Seoul National University, Suwon 441-744, Korea, and the **Department of Food Science and Technology, Kyung Hee University, Suwon 449-701, Korea

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Over 20 enzymes denoted as cyclomaltodextrinase, maltogenic amyrase, or neopullulanase that share 40–86% sequence identity with each other are found in public databases. These enzymes are distinguished from typical α-amylases by containing a novel N-terminal domain and exhibiting preferential substrate specificities for cyclomaltodextrins (CDs) over starch. In this research field, a great deal of confusion exists regarding the features distinguishing the three groups of enzymes from one another. Although a different enzyme code has been assigned to each of the three different enzyme names, even a single differentiating enzymatic property has not been documented in the literature. On the other hand, an outstanding question related to this issue concerns the structural basis for the preference of these enzymes for CDs. To clarify the confusion and to address this question, we have determined the structures of two enzymes, one from alkalophilic Bacillus sp. 1-5 and named cyclomaltodextrinase and the other from a Thermus species and named maltogenic amyrase. The structure of the Bacillus enzyme reveals a dodecameric assembly composed of six copies of the dimer, which is the structural and functional unit of the Thermus enzyme and an enzyme named neopullulanase. The structure of the Thermus enzyme in complex with β-CD led to the conclusion that Trp47, a well conserved N-terminal domain residue, contributes greatly to the preference for β-CD. The common dimer formation through the novel N-terminal domain, which contributes to the preference for CDs by lining the active-site cavity, convincingly indicates that the three groups of enzymes are not different enough to preserve the different names and enzyme codes.

Three groups of enzymes known as cyclomaltodextrinases (CDases; EC 3.2.1.54), maltogenic amylases (MAases; EC 3.2.1.133), and neopullulanases (NPases; EC 3.2.1.135) efficiently hydrolyze cyclomaltodextrins (CDs),¹ which are cyclic oligomers of glucose linked by α-(1,4)-glycosidic bonds (1). Comparatively, these enzymes hydrolyze starch and pullulan inefficiently. These enzyme activities are distinguished from those of typical smaller α-amylases, e.g. TAKA amyrase A of Aspergillus oryzae, which virtually cannot hydrolyze CDs. The three groups of enzymes contain ~130 residues at the N-terminus that are absent in the smaller α-amylases. The central and C-terminal regions of these enzymes exhibit a noticeable sequence homology to the entire sequence of the smaller α-amylases. The central region contains three critical catalytic residues that are invariant throughout α-amylase family members. Therefore, the N-terminal region had been speculated to contribute primarily to their catalytic property. The crystal structure of MAase from a Thermus species (ThMA) (2) revealed that the N-terminal domain, together with the central (α/β)₅-barrel domain, is involved in domain-swapped homodimer formation. The interaction between the N-terminal domain of one molecule and the central domain of the other molecule shapes a narrow and deep active-site groove, which is distinctively different from the wide and shallow active-site groove of the smaller α-amylases. The width of the active-site cleft of ThMA appears to be optimal for binding CDs, but not for binding the linear substrates starch and pullulan.

Many enzymes denoted as CDase have been isolated from various microbial sources, including Bacillus coagulans (3), Clostridium thermohydrosulfuricum 39E (4), Flavobacterium sp. (5), and Bacillus sphaericus E-244 (6). We isolated and cloned a CDase gene from alkalophilic Bacillus sp. 1-5. Like ThMA, the enzyme hydrolyzes β-CD 13 times faster than it hydrolyzes starch and is able to degrade acarbose, a potent pseudo-tetrasaccharide inhibitor of glucoamylases (7). We designated it as CDase on the basis of its about two times higher specific activity of hydrolyzing β-CD compared with ThMA and the highest sequence homology (59% sequence identity) to CDase from B. sphaericus (gi1168855).

We sought to find a structural basis, if any, for distinguishing CDase from MAase. Herein, we report the structure of CDase from Bacillus sp. 1-5 (referred to as CDase hereafter) along with structure-inspired biochemical studies. The enzyme forms a dodecamer that is a hexamer of the dimeric form observed for ThMA and other related enzymes. We demonstrate that the formation of the supramolecular assembly results in an ~10-fold increase in catalytic efficiency compared

¹ The abbreviations used are: CD, cyclomaltodextrin; CDase, cyclomaltodextrinase; MAase, maltogenic amyrase; NPase, neopullulanase; ThMA, Thermus maltogenic amyrase.
with the dimeric unit of the enzyme. In addition, we determined the structure of an active-site mutant of ThMA in complex with β-CD, which led to the identification of Trp 47 as an important residue in imparting the preference of the enzyme for β-CD over starch. The two structures and the recently reported structure of NPase (8) provide enough information that CDase, NPase, and MAase are similar enough to be classified under the same name.

MATERIALS AND METHODS
Protein Purification and Crystallization—Gene cloning and overproduction of CDase were carried out as described (9). Escherichia coli MC1061 cells carrying the CDase gene on plasmid pUC18 were grown in a 5-liter fermentor jar and harvested at a late log phase. Purification of the protein required fractionation by ammonium sulfate precipitation and the use of a Q-Sepharose column (Sigma), a Mono-Q column (Amersham Biosciences), and a DEAE-SHR column (Waters). The CDase crystals were obtained by vapor diffusion from droplets containing 2 µl of protein solution (10 mg/ml in 10 mM HEPES (pH 7.5) containing 1 mM β-mercaptoethanol) plus 2 µl of precipitant solution containing 50% polyethylene glycol 200 and 0.1 M HEPES (pH 7.0), which were equilibrated against 1 ml of the same precipitant solution at 22 °C. The crystals belonged to the space group F23 and contained two molecules of CDase in the asymmetric unit.

The E257L mutant ThMA gene was constructed by the site-directed mutagenesis method using a Muta-gene kit (Promega). The vector construction resulted in the mutant enzyme fused to a His6 tag at the N terminus. The purification and crystallization of the mutant enzyme were carried out as described previously for the wild-type enzyme (2). E. coli BL21(DE3) cells carrying the mutant ThMA gene on the pUC119 plasmid were grown in flasks. The enzyme was purified using a nickel-nitrilotriacetic acid column (QIAGEN Inc.) and a Q-Sepharose column. The crystals of the mutant enzyme were isomorphous to those of the wild-type enzyme. They belonged to the space group P62, and contained two molecules of ThMA in the asymmetric unit. The crystals were soaked in the precipitant solution containing 5 mM β-CD for 36 h before data collection.

X-ray Data Collection, Structure Determination, and Refinement—The X-ray diffraction data for CDase were collected from a flash-cooled crystal using synchrotron radiation. The cryoprotectant solution was the same as the precipitant solution. Despite the large size of the crystals (typically 0.3 × 0.2 × 0.2 mm), maximum resolution of the data was 2.3Å, which can be attributed to the large size of the unit cell (Table I). Data reduction, merging, and scaling were accomplished with the program HKL10. By checking the data at the twin server (11), we found that the crystal was merohedrally twinned with a twin fraction of 0.2488 and the twin operator (h, k, −l). The data were detwinned according to the algorithm described by Yeates (11), removing the twin components of the reflections. The structure was determined by the molecular replacement method with the CCP4 version of AmoRe (12) using the monomeric structure of ThMA (Protein Data Bank code 1SMA) as a search model. The resulting model was refined in conjunction with model rebuilding using the programs CNS (13) and O (14), respectively. The 2-fold non-crystallographic symmetry restraints were maintained until the last refinement. From the beginning of the refinement, 5% of total reflections from the native data set were set aside for monitoring Rfree values. The final model containing 1166 amino acids were refined to an R value of 21.4% and an Rfree value of 25.6% at 3.2Å resolution.

The diffraction data for the ThMA/β-CD complex were collected with a crystal mounted on a capillary tube at room temperature on a Rigaku Raxis IV+ area detector system with a CuKα X-ray generated by rotating the anode generator operated at 100 mA and 50 kV. A short exposure time was employed to collect rather complete data at the sacrifice of the resolution due to a rapid radiation-decay of the crystal. When data were collected with a cryostream-cooled crystal mounted on a loop, the large unit cell dimensions of the crystal (see Table I) and difficulties in crystal alignment caused too many overlaps of the reflections. The structure of the complex was solved by direct refinement of the structure of wild-type ThMA protein Data Bank code 1SMA against the diffraction data using the program CNS (13). The bound β-CD was placed into the fairly strong donut-shaped electron density.

The statistics for the diffraction data and the final refined structures are given in Table I.

Enzyme Assay—The copper-bicinchoninate method was employed to measure the concentration of reducing sugars produced from the reaction of CDase or ThMA with β-CD (Sigma) or soluble starch (Showa Chemical Inc.) as a substrate (15). The mixture of enzyme and substrate was not agitated during the reaction, except for brief mixing at the start of the reaction. One unit of enzyme activity is defined as the amount of enzyme that produces 1 μmol of maltose equivalent.

Gel Permeation Chromatography—Size-exclusion column chromatography was employed to separate the dodecameric, dimer, and monomeric forms of CDase at different pH values. To investigate the association of the enzyme into a dodecamer, the enzyme solution was incubated in universal buffer (14.3 mM citric acid, 14.3 mM KH2PO4, 14.3 mM boric acid, and 14.3 mM diethylbarbituric acid) for 3 days at pH 6.0 and then transferred to the buffer solution with an adjusted pH of 7.0 at 4 °C. At appropriate time intervals, an aliquot of this solution was injected onto a Superdex 200 HR 10/30 column (Amersham Biosciences) that was equilibrated with the same buffer. The dissociation of the enzyme was similarly investigated with a pH shift from 7.0 to 6.0.

Analytical Centrifugation—A Beckman Optima XL-A analytical ultracentrifuge equipped with a four-hole rotor with standard six-channel cells at a rotor speed of 5000 rpm. The absorbance versus radius distribution of the protein were recorded at 280 nm and evaluated using the nonlinear regression method of the SigmaPlot software (SPSS Inc.).

RESULTS
Dimeric Structure of CDase—The monomeric structure of CDase contains a distinct N-terminal domain in addition to a central (α/β)8-barrel domain and a C-terminal domain. The N-terminal (residues 1–123) and C-terminal (residues 505–583) domains are composed exclusively of β-strands (Fig. 1a).

Two molecules of CDase form a domain-swapped dimer in which the N-terminal domain of one molecule is involved in extensive interactions with the (α/β)8-barrel domain of the adjacent molecule (Fig. 1b), as is observed in the structure of ThMA, which is dimeric in both solution and crystals (2). In the dimeric structures, the C-terminal domain is distinctively separated from the active-site groove and is not involved in main chain-to-main chain hydrogen bonds with either the N-terminal or (α/β)8-barrel domain. Instead, the interface between the (α/β)8-barrel domain and an adjacent molecule comprises extensively interactions with the (α/β)8-barrel domain. Instead, the interface between the (α/β)8-barrel domain and a C-terminal domain. The interaction with the (α/β)8-barrel domain of the adjacent molecule (Fig. 1b), as is observed in the structure of ThMA, which is dimeric in both solution and crystals (2). In the dimeric structures, the C-terminal domain is distinctively separated from the active-site groove and is not involved in main chain-to-main chain hydrogen bonds with either the N-terminal or (α/β)8-barrel domain. Instead, the interface between the (α/β)8-barrel domain and an adjacent molecule comprises extensively interactions with the (α/β)8-barrel domain.

Hydrophobic interactions were observed primarily between the β-strands of the dimerizing α-helices. The hydrophobic interactions are predominantly within hydrophobic residues. We noticed that a mutant CDase lacking the C-terminal domain was expressed as an insoluble protein in E. coli, probably due to the exposure of an otherwise shielded hydrophobic surface. The C-terminal domain is found in the structures of all α-amylase family enzymes. Binding of raw starch is known to be the functional role of this domain in

### Table I

| Source | CDase | ThMA - β-CD |
|--------|-------|-------------|
| Wavelength (Å) | 1.0000 | 1.5148 |
| Resolution range (Å) | 20.0 to 3.2 | 20.0 to 3.3 |
| Total/unique reflections | 671,314/5,030 | 271,781/33,889 |
| Rfree (%) | 63.3 (17.8) | 10.7 (26.0) |
| Completeness (%) | 96.8 (85.8) | 89.3 (81.4) |
| Space group | P23 | P62 |
| Cell parameters (Å) | a = b = c | a = b = 119.09 |
| Angles | 1.226 ° | 1.481 ° |
| R-factor/Rfree (%) | 21.4/25.6 | 19.243/ |

The numbers in parentheses are statistics from the highest resolution shell. r.m.s., root mean square.

- Disallowed region a
- Average deviation b
- Average deviation c

- Rfree was calculated with 9% of the data.
some amylases such as cyclomaltodextrin glucanotransferase (16) and barley a-amylase (17, 18). In all the known structures of a-amylase family enzymes in complex with an oligosaccharide at the active site, the bound sugar molecule is not in contact with the C-terminal domain; and therefore, it plays no direct role in the hydrolysis of substrate. Interestingly, the C-terminal domain is critically involved in the supramolecular assembly of CDase as described below.

**Dodecameric Structure of CDase**—The crystal packing of CDase revealed an assembly composed of six copies of the dimeric units (Fig. 1b). The dimeric units are related by the crystallographic 2- and 3-fold symmetry axes of the cubic cell, resulting in a tightly packed hexameric assembly of the dimer (Fig. 1c). The predominant intermolecular interactions between the dimers are mediated by the C-terminal domain of one molecule and the N-terminal domain of an adjacent molecule. A solvent-accessible surface area of 564 Å² of one monomer is excluded by the interactions of the C-terminal domain. The observation led us to probe the oligomerization state of CDase in solution by size-exclusion column chromatography and equilibrium centrifugation analysis at pH 7.5. The apparent molecular mass of the enzyme was estimated as 638 kDa by the chromatographic method (Fig. 2a) and 687 kDa by centrifugation analysis (data not shown), which is ~10 times larger than the calculated molecular mass of CDase (67,690.12 Da). These results and the crystallographic analysis demonstrate that CDase forms a dodecamer (or a hexamer of the dimer) at pH 7.5. The huge molecular size and the shape of the dodecamer are probably responsible for the underestimated molecular masses of the assembly in solution by the two methods. Although the oligomerization states of CDase and ThMA are different, a superposition of the C-a atoms of the dimeric or monomeric unit of CDase and of ThMA shows that the relative orientations of the C- and N-terminal domains with respect to the central domain are very similar in the two structures (Fig. 1d). The observation indicates that the dimeric structure is highly conserved in the two proteins, which share 58% sequence identity.

**pH-dependent Association/Dissociation of CDase**—Unexpectedly, at pH 6.0, both the size-exclusion column chromatography and the analytical centrifugation indicated that CDase exists as a dimer in solution with a minor fraction of a monomer. We found that the dissociation of the dodecamer and the association of the dimer/monomer were slow enough at 4 °C to allow us to separate the dimer/monomer from the dodecamer and vice versa by size-exclusion column chromatography after pH shifts. By virtue of the slow process, the elution profile was used to monitor the interconversion between the two forms. The dodecameric form of the enzyme took ~72 h of incubation at pH 6.0 and 4 °C to fully dissociate into the dimeric form (Fig. 2). It is a reversible process, and a complete dimer-to-dodecamer conversion at pH 7.0 required less time, with the apparent association rate being about two times faster than the apparent dissociation rate (data not shown). The conversions in both directions became faster at higher temperature, and the separation of the two forms was impossible at room temperature by column chromatography.

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**Fig. 1. Structure of CDase.** a, monomeric structure. The N-terminal, central (a/b)₆-barrel, and C-terminal domains are in magenta, coral, and green, respectively. The numbers indicate the starting residue positions of the central and C-terminal domains. b, dimeric structure. The structure is colored as described for a, with one monomer in a lighter tone. c, dodecameric structure. The three dimers in front are in different colors, except for the N-terminal and C-terminal domains. The other three dimers in back are in the same color and in a lighter tone for clarity. d, superposition of the C-a traces of the monomeric structures of CDase and ThMA. The root mean square deviation of the C-a atoms after superposition is 2.28 Å.
The Supramolecular Assembly Increases the Rate of Substrate Hydrolysis—All 12 active sites are outwardly located on the dodecameric assembly. The hexamer formation does not shield any of the active sites from the access of the bulk solvent and therefore from the access of a substrate molecule. Notably, three active sites related by the crystallographic 3-fold axis are close to each other, thereby forming four identical clusters of three active sites according to the cubic symmetry of the supramolecular assembly. Although the two active sites on the dimer are 180° away from each other, facing the opposite direction, the clustered active sites face each other (Fig. 3a). The spatial arrangement of the active sites implied that the supramolecular assembly could confer a better enzyme activity than the dimeric form of ThMA and CDase because a hydrolyzed product released from one active site on the assembly would be readily accepted into the other active sites of a cluster (Fig. 3b). To test this possibility, we carried out the following experiments. The first experiment was to compare the specific activities of CDase and ThMA in the hydrolysis of β-CD, both under their optimal experimental conditions (pH 7.5 and 50 °C for CDase and pH 6.0 and 60 °C for ThMA). We found that the specific activity of CDase (320.5 ± 40.1 units/mg) is about two times higher than that of ThMA (159.6 ± 18.7 units/mg). The second experiment was to compare the $K_m$ and $k_{cat}$ values of CDase in the dimeric and dodecameric states for the hydrolysis of β-CD. In one experiment, the enzyme was equilibrated with a buffer solution at pH 6.0 for 3 days at 4 °C. An aliquot of the solution of the enzyme, which must be in the dimeric state, was reacted with the substrate at pH 6.0 and 50 °C. An aliquot of the solution of the enzyme, which must be in the dimeric state, was reacted with the substrate at pH 6.0 and 50 °C. In a parallel experiment, equilibration and reaction of the enzyme at pH 7.0 were performed to measure the reactivity of the enzyme in the dodecameric state. The dodecameric form of the enzyme at pH 7.0 exhibited a $k_{cat}/K_m$ value 10 times higher than the dimeric form at pH 7.0 (Table II). Conceivably, $k_{cat}$ values of the two oligomeric forms should be similar, but they differ by 5-fold (Table II). We speculate that the difference, despite the identical active site in the two forms, is probably due to different hydrolysis rates for β-CD, maltohexaose, and maltotetraose. To determine whether the different enzyme activities may arise from chemical effects, i.e. changes in the ionization
states of the active-site residues, we measured the enzyme activities of the dodecameric form at two different pH values. We first generated the dodecameric form of the enzyme by equilibrating it with the buffer solution at pH 7.0 for 3 days. Each of the same aliquots of the enzyme solution was reacted with the substrate at pH 6.0 and 7.0, respectively. Nearly the same enzyme activities were observed for the dodecameric form at pH 7 and 6 (data not shown). This control experiment indicates that the 10 times higher catalytic efficiency of the dodecameric form at pH 7 compared with that of the dimeric form at pH 6 arises from the physical difference, i.e. the difference in the oligomeric states. It represents a novel example of how oligomerization of an enzyme increases catalytic efficiency in hydrolyzing a polymeric substrate that requires multiple entries into the active site for complete degradation.

**ThMA in Complex with β-CD**—To understand the structural basis for the preference of ThMA and other related enzymes, we determined the structure of a catalytically inactive mutant ThMA (E357L) in complex with β-CD at 3.3Å resolution. The crystals of this dimeric enzyme were easier to work with than those of CDase. The substrate is bound to the deep active-site groove and interacts with various residues of both the (α/β)_{n}-barrel and N-terminal domains (Fig. 4). Although the central domain residues interact with the substrate at the bottom part of the active site, the N-terminal domain residues Tyr45, Trp47, and Asp110 interact with the substrate at the upper part of the active site. The aromatic ring of Phe289 interacts with the inner part of the ring structure of the bound β-CD, whereas Trp17 provides a stacking interaction with one of the sugar rings located at the entrance of the active site. Tyr45 provides a rather remote interaction with the substrate compared with the two residues. In the 20 enzymes present in the public data bases with the annotation of CDase, MAase, or NPase, Tyr45 is conserved or substituted with phenylalanine in two entries. Trp17 is also conserved or substituted with phenylalanine (two entries), tyrosine (one entry), or proline (two entries) (Fig. 5). Asp110 is close to one of the hydroxyl groups of the bound β-CD and could be involved in a hydrogen-bonding interaction. However, this residue is not a conserved residue, as it is substituted with valine, leucine, or methionine in three entries (Fig. 5). A Y45A mutant exhibited only a slightly decreased reaction rate (<2-fold) in hydrolyzing both β-CD and soluble starch (data not shown). In contrast, the W47A substitution resulted in differential decreases in enzyme activity toward the two substrates, as shown in Table III. Markedly, the mutation increased the $K_m$ by >10-fold for β-CD and by <2-fold for soluble starch. Therefore, Trp17 is an important residue in imparting the preference of the enzyme for β-CD over starch. The structure of the complex suggests that Trp17, located at the entrance to the active site, may not be reached by the linear starch chain, unless it is highly bent at the active site to assume a ring-like conformation that mimics the CD ring.

**DISCUSSION**

The crystal structure and the solution study demonstrated that CDase adopts a dodecameric form in solution at pH > 7. As far as we know, the dodecamer is by far the highest oligomeric state observed for an amylolytic enzyme. In the oligomer formation, the C-terminal domain plays a central role in providing a large contact surface between the dimeric units. At least in this enzyme and probably in other related enzymes of alkaloiphilic organisms, the function of this domain may lie in the oligomer formation. We investigated whether the dodecamer formation imparts a better thermostability by using differential scanning calorimetry. Although the onset temperature of denaturation of CDase was 64.4°C at pH 7.0, it decreased to 61.0°C at pH 6.0, indicating a marginal enhancement in the thermostability by forming the dodecameric assembly (data not shown). The most salient feature of the dodecamer is that the oligomerization brings the active sites close together in space, forming four clusters of the active sites on the supramolecular assembly. The deposition of the active sites on the assembly strongly indicated that the dodecamer is not a random assembly, but a molecular design that allows more efficient hydrolysis of the substrate, which is converted finally to maltose by repeated enzymatic cleavages. We demonstrated that the dodecameric form of the enzyme exhibits >10 times higher catalytic efficiency than the dimeric form toward β-CD. We also noted that the dodecameric form of CDase is able to hydrolyze β-CD two times faster than the dimeric enzyme ThMA despite the lower reaction temperature employed for CDase. These data suggest that the physical clustering of the active sites results in the enhanced catalytic activity of the enzyme. A degradation product released from one active site could have a high chance to enter into a nearby active site on the same
of ThMA and CDase (8). The presence of the novel N-terminal domain is the most salient feature distinguishing these enzymes from other amylolytic enzymes. With a mutant ThMA containing the deletion of the N-terminal domain, we previously demonstrated that the domain is important for the dimerization, stability, and substrate preference of the enzyme for β-CD (19). We suggested that the narrow and deep active-site cleft shaped by the N-terminal domain that partly covers the otherwise wide and shallow cleft would physically restrict the easy access of the linear starch chain, whereas it allows efficient binding of CDs (1, 2). Herein, we demonstrated that the N-terminal domain residue Trp47 provides a catalytically important interaction with CDs, whereas it provides a mere interaction with soluble starch. We suggest that the physical shape of the active site, as well as the favorable interaction provided by Trp47, allows efficient binding of CDs to the active site of these enzymes. In addition to Trp47, the central domain residue Phe289 in ThMA appears to provide catalytically more important interactions with β-CD than with soluble starch.

This is because the substitution of Phe289 with alanine in Thermococcus vulgaris R-47 α-amylase II, which is structurally similar to ThMA, results in more significant reduction of the $k_{cat}$ of the enzyme for β-CD (63-fold) than for starch (2-fold) (20). However, the mutation causes similar increases in the $K_m$ values for β-CD (3.6-fold) and starch (5.2-fold), indicating that the residue is important for chemical steps in the catalytic reaction with β-CD. In the structure of ThMA in complex with β-CD, Phe289, which corresponds to Phe 286 in Bacillus stearothermophilus α-amylase II, provides prominent interactions with an inner part of the β-CD ring (Fig. 4). The interactions could be important for precise positioning of the scissile bond of CDs with respect to the catalytic residues. It should be noted that Phe289 is invariant in the three groups of enzymes, but is substituted with aspartic acid in TAKA amylase A (Fig. 5), which is a nearly invariant residue in the typical smaller α-amylases.

In retrospect, different investigators have independently coined each of the three names mainly based on their observation of the preference for CDs over other starch materials such as amylose and pullulan (CDase), the hydrolysis of pullulan to produce panose (NPase), or the production of maltose as the main product from CDs and starch (MAase) by an enzyme under study. ThMA and CDase display all of these catalytic activities, and other related enzymes are expected to have the catalytic property in common. We propose that these enzymes should be classified under the same name and enzyme code to avoid confusion. Cyclomaltodextrinase is probably the best name of choice for two reasons. First, CDs are the most preferred substrates for these enzymes, although some variations may exist in the relative hydrolytic activity of these enzymes toward starch or pullulan, the less preferred substrates. Second, the open reading frames for CDase, cyclomaltodextrin glucanotransferase, and the CD uptake system are commonly found as a gene cluster on the chromosomal DNA in several organisms, including Klebsiella oxytoca (21), Thermococcus kodakaraensis KOD1 (22), and Thermococcus sp. strain B1001 (23). Cyclomaltodextrin glucanotransferase is an extracellular enzyme, whereas CDase (MAase and NPase) is an intracellular enzyme. Therefore, it is reasonable to assume that these enzymes have been evolved for intracellular degradation of CDs generated extracellularly by cyclomaltodextrin glucanotransferase and transported into the cell by a specific uptake system. We suggest that the presence of the N-terminal domain is the primary criterion and that the conservation of hydrophobic residues corresponding to Trp47 and Phe289 in ThMA is a secondary criterion that could be used for distinguishing CDases.

### Table III

| Substrate | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
|-----------|----------|-------|---------------|
| β-CD⁺     |          |       |               |
| WT ThMA   | 126 ± 11 | 0.17 ± 0.04 | 726 ± 9.0      |
| W47A ThMA | 180 ± 8  | 2.03 ± 0.13  | 88.7 ± 4.3     |
| Starch     |          |       |               |
| WT ThMA   | 249 ± 31 | 73.5 ± 14.6 | 3.40 ± 0.80    |
| W47A ThMA | 335 ± 30 | 131 ± 15.0  | 2.55 ± 0.37    |

* $K_m$ and $k_{cat}/K_m$ values for β-CD are expressed in mm and s⁻¹ mm⁻¹, respectively.
* $K_m$ and $k_{cat}/K_m$ values for starch are expressed in mg ml⁻¹ and s⁻¹ mg⁻¹, respectively.
from other amylolytic enzymes. In this context, nine entries in the data bases containing no N-terminal domain but denoted as CDase (three entries), MAase (one entry), and NPase (five entries) are incorrectly annotated. Also, in this context, *T. vulgaris* α-amylase II is considered as a novel variant of CDase in that it hydrolyzes starch as well as CDs efficiently and exhibits similar $K_m$ values for CDs and starch on a weight/volume basis (20), in contrast with ThMA, which exhibits an ~43-fold lower $K_m$ for β-CD compared with that for starch. The substitution of Trp$^{47}$ with serine in *T. vulgaris* α-amylase II is likely to be primarily responsible for the difference. In conclusion, the data regarding CDase, NPase, and MAase provided by this study and by others reported in the literature convincingly suggest that they are nearly the same enzymes in terms of their structures and catalytic property and that they can be rather easily distinguished from other amylases on the basis of the presence of the N-terminal domain and the conservation of Trp$^{47}$ and Phe$^{289}$.

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