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Modulation of the Multisubstrate Specificity of *Thermus* Maltogenic Amylase by Truncation of the N-Terminal Domain and by a Salt-Induced Shift of the Monomer/Dimer Equilibrium[†]

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ABSTRACT: The relation between the quaternary structure and the substrate specificity of *Thermus* maltogenic amylase (ThMA) has been investigated. Sedimentation diffusion equilibrium ultracentrifugation and gel filtration analyses, in combination with the crystal structure determined recently, have demonstrated that ThMA existed in a monomer/dimer equilibrium. The truncation of ThMA by removing the N-terminal domain, which is composed of 124 amino acid residues, resulted in the complete monomerization of the enzyme (ThMAΔ124) accompanied by a drastic decrease in the activity for β-cyclodextrin (β-CD) and a relatively smaller reduction of the activity for starch. Despite the overall low activity of ThMAΔ124, the activity was higher toward starch than β-CD, and the ratio of the specific activities toward these substrates was approximately 100 fold higher than that of wild-type ThMA. Furthermore, the addition of KCl to wild-type ThMA shifted the monomer/dimer equilibrium toward the monomer. In the presence of 1.0 M KCl, the relative activity of ThMA toward β-CD decreased to 74%, while that for soluble starch increased to 194% compared to the activities in the absence of KCl. Thus, the ThMA monomer and dimer are both inferred to be enzymatically active but with a somewhat different substrate preference. Kinetic parameters of the wild-type and truncated enzymes also are in accordance with the changes in their specific activities. We thus provide evidence in support of a model, which shows that the relative multisubstrate specificity of ThMA is influenced by the monomer/dimer equilibrium of the enzyme.

Maltogenic amylase from *Thermus* (ThMA), a member of the glycoside hydrolase family 13, shows multisubstrate specificity for cyclodextrins (CDs), pullulan, and soluble starch, which is distinctly different from the substrate specificity of α-amylases (*1*). The gene corresponding to ThMA has been cloned, sequenced, and overexpressed in *Escherichia coli* (*2*). It encodes a protein with a molecular mass of 68 kDa and an amino acid sequence highly homologous to those of other maltogenic amylases (MAases, EC 3.2.1.133; *3, 4*), cyclomaltodextrinases (CDases, EC 3.2.1.54; *5*), and neopullulanase (NPase, EC 3.2.1.135; *6*). This group of enzymes not only hydrolyzes a variety of oligo- and polysaccharides but also transglycosylates numerous sugar-acceptor molecules. The crystal structure of ThMA was determined at a 2.8 Å resolution (*7*) and found to contain three distinct domains: the N-terminal domain (residues 1−124) composed of 12 β strands, the central $(\beta/\alpha)_8$-barrel domain, and the C-terminal domain of 8 antiparallel β strands. The unique N-terminal domain is intimately involved in the formation of a homodimer. The dimerization of the enzyme modifies the wide and highly accessible active-site cleft, a characteristic of the monomer, by the N-terminal domain of one monomer covering the other to form a narrow and deep groove-shaped substrate-binding site (*7*).

Size exclusion chromatography performed previously proved that ThMA was present in a monomer/dimer equilibrium in aqueous solution (*7*). Similar oligomeric states have been reported for CDases (*8−10*) and TVA II amylase (*11*), which also hydrolyze CDs, pullulan, and starch, albeit with different relative activities (*12*). Although these enzymes exhibit closely related catalytic properties and possess a high

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degree of amino acid sequence identity, they are classified under different names: MAase, CDase, NPase, and EC numbers (*12*−*14*). Studies on the relationship between the multisubstrate specificity and the quaternary structure of the enzymes will provide information, which may improve and facilitate the subclassification of these glycoside hydrolase family 13 members.

In the present study, the role of the N-terminal domain of ThMA in the multisubstrate specificity and formation of a dimer was investigated through the truncation of the N-terminal domain and the salt-induced shift of the monomer/dimer equilibrium of the enzyme, respectively. In addition, a hypothetical model that correlates the dimerization of ThMA and the binding preference for different substrates is presented.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions. *E. coli* MC1061 [F$^-$, *araD*139, *recA*13, Δ*(araABC-leu)*7696, *galU*, *galK*, Δ*lacX*74, *rpsL*, *thi*, *hsdR*2, *mcr*B] was used as a host for the gene manipulation and expression of wild-type ThMA and its derivative, ThMAΔ124. Transformed *E. coli* cells were cultured in an LB medium (1% Bacto-tryptone, 0.5% yeast extract, and 0.5% NaCl) containing 100 μg/mL of ampicillin at 37 °C.

Construction of the N-Terminally Truncated ThMA Gene. The plasmid p6xHThMA carrying the gene encoding ThMA (*2*) was used to construct the gene for ThMA lacking the N-terminal domain. To delete the 124 amino acid residues corresponding to the N-terminal domain, two oligonucleotides (TMΔ124 and the universal primer, 5′-CATCGGGTCCATATGTTCCAAGCGCCGGACTGG-3′ and 5′-AGCGGATAACAATTTCACACAGGA-3′, respectively) were synthesized and used for polymerase chain reaction (PCR) amplification of the truncated ThMA gene with p6xHThMA as the template. The resulting DNA fragment was inserted into p6xHThMA at the *NdeI/Hin*dIII sites, replacing the wild-type gene to yield p6xHThMAΔ124. The entire insert was sequenced to verify that no error was introduced during PCR.

Isolation and Purification of the Truncated Enzyme. ThMAΔ124 was purified according to the ThMA purification procedure described previously (*2*). *E. coli* MC1061, harboring p6xHThMAΔ124, was grown in an LB medium supplemented with 100 μg/mL of ampicillin at 37 °C for 12 h with shaking (250 rpm) and harvested by centrifugation. The pellet was resuspended in a lysis buffer (300 mM NaCl, 10 mM imidazole, 50 mM Tris-HCl, pH = 7.0) and disrupted by sonication. The supernatant was filtered through a membrane with pores of 0.45 μm (Micro Filtration System Co., Dublin, CA) after centrifugation (10 000*g*, 15 min) and passed through a nickel−nitrilotriacetic acid (Ni−NTA) column. The column was washed with 50 mM Tris-HCl (pH = 7.0) containing 300 mM NaCl and 20 mM imidazole followed by the elution with 50 mM Tris-HCl (pH = 7.0) containing 300 mM NaCl and 250 mM imidazole. The purified enzyme was concentrated by ultrafiltration (Amicon Inc., Beverly, MA) after dialysis against 50 mM sodium acetate (pH = 6.0) and used for further investigation. The molecular mass of the truncated ThMAΔ124 was estimated by SDS−PAGE using a 10% gel (*15*).

Enzyme Assay. The copper-bicinchoninate method was used to measure the formation of reducing sugar using β-CD[1] (Sigma Co., St. Louis, MO) or soluble starch (Showa Chemical Inc., Tokyo, Japan) as a substrate and maltose as a standard (*16*). The enzymatic reactions were carried out as described previously except that the reaction mixtures were preincubated at 4 °C for 60 min to stabilize the monomer/dimer equilibrium. The specific activity on β-CD or starch was measured at a final enzyme concentration of 300 nM calculated as the total concentration of monomer present in both the monomer and dimer forms. All of the enzyme activities were determined by measuring the initial velocities of the reaction, which were calculated using a set of data obtained from appropriate time points with respect to zero-order reaction kinetics by showing linearity. Hydrolytic activities of the wild-type and truncated ThMAΔ124 were assayed using 0.5% (w/v) β-CD or soluble starch at their optimal temperatures, 60 and 45 °C, respectively, in 50 mM sodium acetate (pH = 6.0). One unit of enzyme activity was defined as the amount of enzyme which produces 1 μmol of maltose/min. The protein concentration was determined according to the method of Bradford (*17*) using bovine serum albumin (BSA) as a standard.

Kinetic Studies of Wild-Type ThMA and Truncated Mutant ThMAΔ124. The kinetic parameters of wild-type ThMA and ThMAΔ124 for β-CD and starch were determined as follows. The wild-type or mutant enzyme (0.1 mL) at an appropriate concentration was mixed with a substrate (0.9 mL) at various concentrations in a 50 mM sodium acetate buffer (pH = 6.0), and the reaction was performed as described previously. To analyze the kinetic properties of the enzyme, aliquots (0.2 mL) were taken from the wild-type and mutant enzyme reaction mixtures at every 30 sec and 10 min, respectively. An equal volume of 0.1 M NaOH was immediately added to stop the reaction. The copper-bicinchoninate method was then employed to measure the amount of reducing sugar. The kinetic parameters were determined using Lineweaver−Burk plots.

Analytical Ultracentrifugation. Sedimentation diffusion equilibrium measurements were performed using a Beckman Optima XL-A analytical ultracentrifuge (Beckman Coulter, Inc., Fullerton, CA) equipped with a four-hole rotor with two- or six-channel standard cells at a rotor speed of 10 000 rpm. The absorbance versus radius distributions, *A*(*r*), were recorded at 280 nm and evaluated using the nonlinear regression method in the SigmaPlot software (SPSS Science, Chicago, IL). The general equation used for fitting the *A*(*r*) data was

$$A(r) = \sum_i A_i(r) = \sum_i A_i(r_0)\exp[iM_1(1-\bar{\nu}\rho_0)\omega^2(r^2-r_0^{\,2})/2RT] \quad (1)$$

where i denotes the number of protomers per oligomer, A_i is the absorbance of the corresponding species, $\bar{\nu}$ is the partial specific volume of the protein [calculated as described by Zamyatnin (*18*) and assumed to be independent of the oligomerization state], ρ_0 is the solvent density, ω is the angular velocity of the rotor, and r_0 is the fixed radial

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[1] Abbreviations: β-CD, β-cyclodextrin; CDase, cyclomaltodextrinase; MAase, maltogenic amylase; NPase, neopullulanase; ThMA, *Thermus* maltogenic amylase; TVA II, *Thermoactinomyces vulgaris* amylase II.

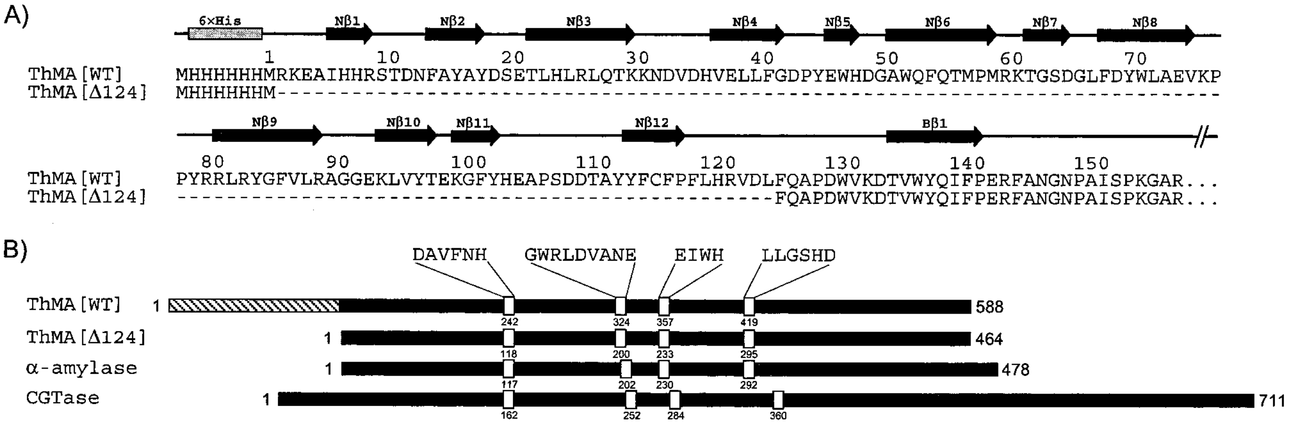

FIGURE 1: Schematics of the primary structure of ThMA and the truncated ThMAΔ124. (A) The sequences of ThMA and ThMAΔ124 are aligned with the secondary structure (*7*) marked above. The extra six histidine residues attached to facilitate protein purification are shown as a dotted box. (B) Schematic alignment of the primary structures of ThMA and ThMAΔ124 in comparison with those of Taka-amylase A (*19*) and CGTase (*20*). The N-terminal domain of ThMA is shown as a hatched box. Open boxes represented the four conserved regions. Their relative positions and the sequences of the conserved regions are also shown.

position. The experiments were conducted at 4, 20, and 30 °C. $K_{1,2}$ was calculated from the equation $K_{1,2} = [\mathrm{M}][\mathrm{M}]/[\mathrm{D}]$, where [M] and [D] stand for the molar concentrations of monomer and dimer, respectively, as determined by analytical ultracentrifugation using data fitting to eq 1. $K_{1,2}$ at 60 °C was obtained by extrapolation using the van't Hoff equation

$$\log \frac{K_2}{K_1} = \frac{\Delta H^\circ}{2.3R}\,\frac{T_2 - T_1}{T_1 T_2} \qquad (2)$$

where ΔH° is the standard enthalpy change, R is the gas constant, and K_1 and K_2 are the dissociation constants ($K_{1,2}$) at temperatures of T_1 and T_2, respectively.

Gel Permeation Chromatography (GPC). Two Superose-12 columns (300 × 10 mm) connected in tandem were used to estimate the apparent molecular weight of ThMA at room temperature. Protein samples (0.3 mg/mL) were applied to the column system equilibrated in a 50 mM sodium acetate buffer (pH = 6.0) containing 0, 0.1, 0.2, 0.4, 0.6, 0.8, or 1.0 M KCl and eluted with the same buffers at a flow rate of 0.2 mL/min. β-Amylase, alcohol dehydrogenase (ADH), BSA, and carbonic anhydrase were used for the calibration needed to estimate the apparent molecular weight of the enzyme.

RESULTS

Truncation of the N-Terminal Domain and Its Effect on the Quaternary Structure of ThMA. We previously proposed that the unique extra N-terminal domain of 120−130 amino acids present in different MAases was involved in the interaction between two enzyme monomers to form a homodimer (*7*). To verify this hypothesis, the 124 amino acid long N-terminal domain of ThMA was deleted to yield ThMAΔ124. The deleted part in ThMAΔ124 is shown in an alignment with wild-type ThMA (Figure 1). The molecular mass of ThMAΔ124 was predicted to be 54 kDa, which was confirmed by SDS−PAGE analysis (data not shown); the sequence of the truncated gene was verified by DNA sequencing.

ThMAΔ124 was found exclusively in a monomeric form and contained no dimer, as determined by analytical ultracentrifugation (Figure 2A). This result, together with the previously determined ThMA crystal structure, indicates that the N-terminal domain of ThMA is responsible for the formation of the homodimer and that it is possible to create a nonassociating monomeric ThMA by deleting the N-terminal domain. This result implies that the enzymatic properties of ThMAΔ124 can provide an important clue to uncovering the correlation between the monomer/dimer equilibrium and the enzymatic characteristics, particularly the multisubstrate specificity of ThMA and the related enzymes.

Substrate Specificity and Action Pattern of ThMAΔ124. Similarly to wild-type ThMA, the truncated enzyme, ThMAΔ124, has the capacity to hydrolyze both β-CD and soluble starch (Figure 3), even though, in comparison with wild-type ThMA, the optimal temperature was shifted from 60 to 45 °C and the specific activities were greatly reduced (Table 1). Differential scanning calorimetric analysis indicated that the thermostability of ThMA was also significantly reduced by the truncation of the N-terminal domain (data not shown). Most remarkably, the β-CD hydrolyzing activity of ThMAΔ124 was reduced by an additional 2 orders of magnitude compared to the reduction in soluble starch hydrolyzing activity, as illustrated by the ratio of the activities for β-CD and starch (Table 1). Furthermore, while maltotriose and larger hydrolysis products, such as maltopentaose and maltohexaose, accumulated during the starch hydrolysis as catalyzed by the truncated enzyme, these maltooligosaccharides were rapidly hydrolyzed to maltose and glucose by wild-type ThMA (Figure 3). These results convincingly showed that the specificity preference for starch relative to β-CD was achieved by the elimination of the N-terminal domain. This finding supports the hypothesis of a relationship between the dimerization of ThMA and the high substrate specificity for β-CD.

Effect of Ionic Strength on the Quaternary Structure of ThMA. For a thorough investigation in the relationship between dimerization and substrate specificity, the effect of the monomer/dimer equilibrium on the enzymatic properties

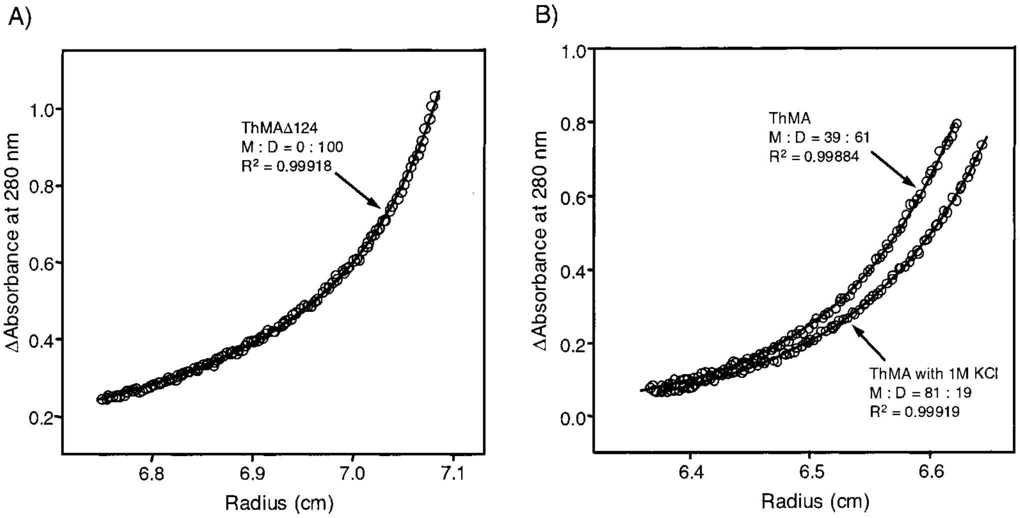

FIGURE 2: Sedimentation equilibrium analysis of (A) ThMAΔ124 and (B) ThMA in the absence and presence of 1 M KCl. The concentration of the enzymes was 0.117 mg mL^{-1} in 50 mM sodium acetate, pH = 6.0. Equilibrium was attained in 40 h. The concentration distribution of the protein as a function of the square of the radial position is also shown.

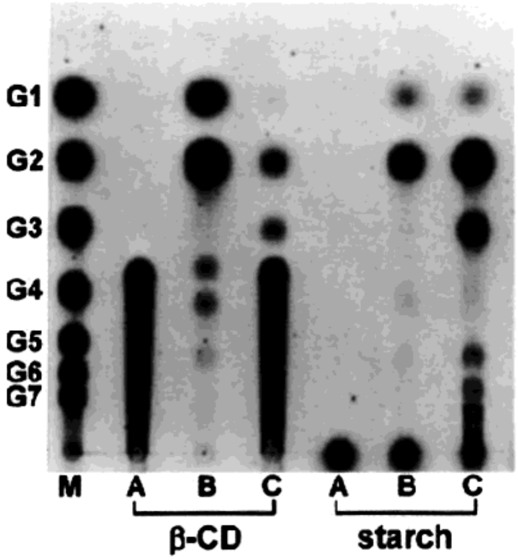

FIGURE 3: TLC analysis of substrate hydrolysis. ThMA (B lanes) and ThMAΔ124 (C lanes) were reacted with one of the substrates (0.5% w/v), β-CD or starch, at 45 °C for 12 h. The A lanes represented controls (i.e., the substrates without enzyme treatment). Mixtures of G1−7 (glucose to maltoheptaose; M lane) were used as standards.

of ThMA was examined when modulated by the varying salt concentration. First, the role of KCl in the dissociation of the dimeric ThMA was investigated by analytical ultracentrifugation (Table 2 and Figure 2B). The equilibrium constant $K_{1,2}$ = [M][M]/[D], where M stands for monomer and D for dimer, was found to increase as the KCl concentration was increased from 0 to 1.0 M. Thus, at 1.0 M KCl, the monomeric form of ThMA was predominant (81%; Figure 2B). To assess the quaternary state of ThMA at the standard reaction temperature of 60 °C, the dependence of the monomer/dimer equilibrium constant on the temperature was described at 4, 20, and 30 °C by analyzing the effect of KCl on the monomer/dimer ratio using analytical ultracentrifugation (Table 2). The dissociation constants at 60 °C and different concentrations of KCl (Table 1) have been estimated by extrapolating the van't Hoff plot made from the data listed in Table 2. As the $K_{1,2}$ values decreased with increasing temperature, the percentage of dimer also increased with increasing temperature because of a significant albeit modest shift of the monomer/dimer equilibrium toward the dimer in the studied temperature range.

The dependence of the dimeric state of ThMA on the KCl concentration was also investigated using GPC at room temperature. ThMA eluted as a single symmetrical peak in the range of 94−112 kDa at different salt concentrations (Figure 4). This behavior reflects that the association/dissociation reactions at equilibrium proceed very fast at room temperature, as described earlier by other research groups for different proteins (*21*−*24*). The elution position with increasing concentration of KCl presumably represented the weight-average molecular mass, using the increasing monomer/dimer ratio as described by Thoma et al. (*25*). The estimation of the weight-average molecular mass through the comparison of the elution positions of standard proteins allowed for the calculation of the corresponding monomer/dimer ratios of ThMA. While ThMA existed in a monomer/dimer ratio of 48:52 in the absence of KCl, the monomer dominated in 1.0 M KCl at room temperature, yielding a ratio of 78:22 (data not shown). The corresponding monomer/dimer ratios obtained by ultracentrifugation for ThMA at 20 °C were calculated to be 39:61 and 81:19 in 0 and 1.0 M KCl, respectively. Although the values of the monomer/dimer ratios obtained by analytical ultracentrifugation and GPC were not identical, the overall tendency for the salt on the monomer/dimer ratio was consistent for the two methods. A higher enzyme concentration was used for GPC (0.3 mg/mL) than for ultracentrifugation (0.12 mg/mL), but obviously the precise concentration of the enzyme in the GPC column is not known. The individual difference at a low KCl concentration between ultracentrifugation and GPC could not be explained by the different enzyme concentrations used in the two procedures; a higher amount of monomer at a low salt concentration was obtained by GPC, whereas a slightly higher amount of monomer at a high salt concentration was obtained by ultracentrifugation conducted using the lower amount of the protein concentrations. This may be related to the actual protein concentration in GPC not being

Table 1: Specific Activities for Two Substrates and Dissociation Constants of Wild-Type and Truncated ThMA

			specific activity[b] (μmol/min/mg)[c]			oligomeric state[d]	
ThMA	KCl (M)	$K_{1,2}$[a] (M)	β-CD	starch	ratio (β-CD/starch)	monomer (%)[e]	dimer (%)
WT	0.0	$(9.2 \pm 0.8) \times 10^{-8}$	278.0 ± 8.6	14.2 ± 0.7	19.6	48.8	51.2
	0.2	$(1.8 \pm 0.1) \times 10^{-7}$	257.8 ± 6.2	21.1 ± 1.2	12.2	58.9	41.1
	0.4	$(3.5 \pm 0.6) \times 10^{-7}$	242.9 ± 7.2	24.0 ± 0.8	10.1	68.9	31.1
	0.6	$(8.8 \pm 0.8) \times 10^{-7}$	227.2 ± 5.3	26.0 ± 1.2	8.7	81.1	18.9
	0.8	$(1.6 \pm 0.2) \times 10^{-6}$	210.0 ± 5.6	26.3 ± 2.0	8.0	87.3	12.7
	1.0	$(2.5 \pm 0.3) \times 10^{-6}$	206.8 ± 5.9	27.6 ± 1.1	7.5	90.9	9.1
Δ124	0.0	*f*	$(1.0 \pm 0.1) \times 10^{-3}$	$(5.0 \pm 0.2) \times 10^{-3}$	0.2	100.0	0.0

[a] $K_{1,2}$ at 60 °C was calculated by the van't Hoff equation using data obtained through analytical ultracentrifugation at different temperatures (see Experimental Procedures and Table 2). [b] Activity of the wild-type and mutant enzymes was measured at their respective temperature optima of 60 and 45 °C, respectively; moreover, ThMAΔ124 is unstable at 60 °C. [c] μmol of maltose equivalent. [d] Ratios were calculated based on a ThMA concentration of 300 nM and the $K_{1,2}$ values obtained at 60 °C. [e] Molar ratio. [f] Exists exclusively in monomeric form.

Table 2: Effect of KCl and Temperature on the Monomer/Dimer Equilibrium Constant of Wild-Type ThMA

	$K_{1,2}$ (M)[a]			
KCl (M)	4 °C	20 °C	30 °C	$\Delta H°$ (kcal/mol)[b]
0.0	$(4.7 \pm 0.5) \times 10^{-7}$	$(2.6 \pm 0.2) \times 10^{-7}$	$(2.1 \pm 0.3) \times 10^{-7}$	-5.3 ± 0.4
0.2	$(6.8 \pm 0.5) \times 10^{-7}$	$(4.3 \pm 0.2) \times 10^{-7}$	$(3.4 \pm 0.5) \times 10^{-7}$	-4.4 ± 0.1
0.4	$(1.2 \pm 0.1) \times 10^{-6}$	$(7.4 \pm 0.5) \times 10^{-7}$	$(6.5 \pm 0.7) \times 10^{-7}$	-3.9 ± 0.6
0.6	$(2.4 \pm 0.1) \times 10^{-6}$	$(1.8 \pm 0.1) \times 10^{-6}$	$(1.4 \pm 0.1) \times 10^{-6}$	-3.3 ± 0.3
0.8	$(4.0 \pm 0.2) \times 10^{-6}$	$(3.1 \pm 0.2) \times 10^{-6}$	$(2.5 \pm 0.1) \times 10^{-6}$	-3.1 ± 0.4
1.0	$(6.1 \pm 0.3) \times 10^{-6}$	$(4.8 \pm 0.1) \times 10^{-6}$	$(3.8 \pm 0.2) \times 10^{-6}$	-3.0 ± 0.4

[a] $K_{1,2}$ was determined by analytical ultracentrifugation at 4, 20, and 30 °C, respectively. [b] Calculated by plotting $-\log K$ versus $1/T$ based on the van't Hoff equation.

known as well as to the GPC applying a weight-average molecular weight. The reason for this slight inconsistency is not understood. Both analyses, however, revealed that the ratio of monomer to dimer increased as the salt concentration increased. Likewise, ThMA was present mainly as a monomer in 0.8 M sodium phosphate (i.e., at a high ionic strength, with $K_{1,2} = 5.7 \times 10^{-6}$ M (data not shown), corresponding to a monomer content of 95%). These various results all supported that ThMA existed as a mixture of monomer and dimer forms, depending on the ionic strength and temperature of the aqueous solution.

Effect of Ionic Strength on the Activity of ThMA in a Monomer/Dimer Equilibrium. To assess the impact of the dimeric state on the specificity of ThMA for different substrates (i.e., CDs and starch), the enzyme was preincubated at varying concentrations of KCl at 4 °C for 60 min to stabilize the monomer/dimer equilibrium. The specific activities of the enzymes toward β-CD or soluble starch remained constant after preincubation at each of the KCl concentrations. Whereas the activity toward β-CD decreased by 26% with increasing KCl concentration from 0 to 1.0 M, the activity toward soluble starch almost doubled to give 194%. In contrast however, the relative specific activities of ThMAΔ124 for β-CD and soluble starch did not change significantly (Figure 5).

The effect of salts on the activity of ThMA was correlated with the dissociation of the dimeric enzyme (Table 1 and Figure 5). As the degree of ThMA dimerization decreased to 9.1% in 1.0 M KCl, the activity for soluble starch hydrolysis thus increased by 94% (Figure 5), while the activity toward β-CD decreased concomitantly with the decrease in the dimer content caused by the increased concentration of KCl. As a consequence, the preference for β-CD over starch decreased from an activity ratio of 20 to a ratio of 8 with an increasing KCl concentration from 0 to 1.0 M (Table 1). The specific activity of ThMAΔ124 toward β-CD and soluble starch remained almost constant from 0 to 1.0 M KCl because the truncated enzyme was present exclusively in the monomeric form. A very similar effect was observed when the activity of ThMA was investigated at varying ionic strengths of sodium phosphate used for the activity assay (data not shown). Increasing the sodium phosphate concentration from 0.05 to 0.7 M thus enhanced the activity of ThMA toward starch to 150%, while the activity toward β-CD was reduced to 79%. All of these results indicated that the enzymatic activity of ThMA on various substrates was affected differently by the monomer/dimer ratio of ThMA in the reaction mixture. Because a higher ionic strength favored the monomeric state in the monomer/dimer equilibrium of the enzyme, the increased activity toward starch at a higher ionic strength indicated that monomeric ThMA preferred starch to β-CD as a substrate.

To eliminate the possibility that the effect on activity originated from a conformational change of the substrate due to a high salt concentration, the hydrolysis of soluble starch by α- and β-amylases in 1.0 M KCl was found to lead only to an activity increase of less than 10%. Furthermore, starch dissolved in 1.0 M KCl had an absorbance maximum of I^-/I_2 shifting very little from 570 to 550 nm, indicating that the helical conformation of soluble starch was not changed significantly by KCl. These experiments support that the effect of KCl on starch hydrolysis by ThMA stemmed from the influence of the ionic strength on the monomer/dimer equilibrium of the enzyme and not from the modification of the substrate conformation.

Effect of Ionic Strength on the Kinetic Parameters of ThMA and ThMAΔ124. The kinetic parameters of the wild-type ThMA and ThMAΔ124 were determined using starch and

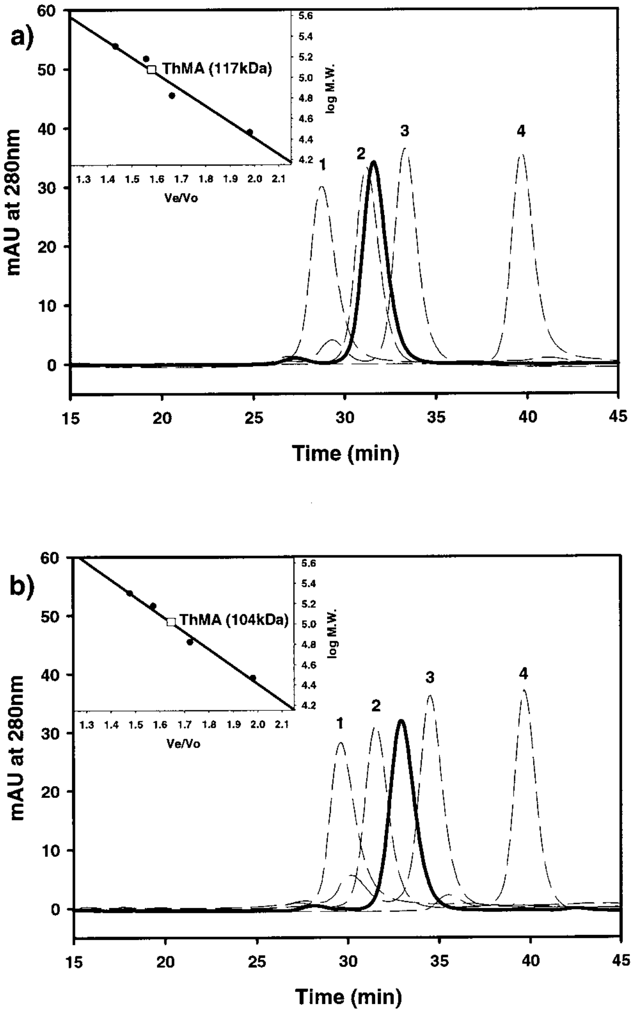

FIGURE 4: Gel permeation chromatography of ThMA. Elution profiles of ThMA using two Superose-12 columns in tandem at room temperature (a) without KCl or (b) with 0.5 M KCl. The standards (dashed lines) comprised β-amylase (200 kDa, peak 1), alcohol dehydrogenase (ADH; 150 kDa, peak 2), bovine serum albumin (BSA; 66 kDa, peak 3), and carbonic anhydrase (29 kDa, peak 4). The elution profiles for ThMA are shown as solid lines and the standards as dashed lines. The ratio of monomer to dimer was deduced to be (a) 48:52 in the absence of KCl and (b) 67:33 in the presence of 0.5 M KCl.

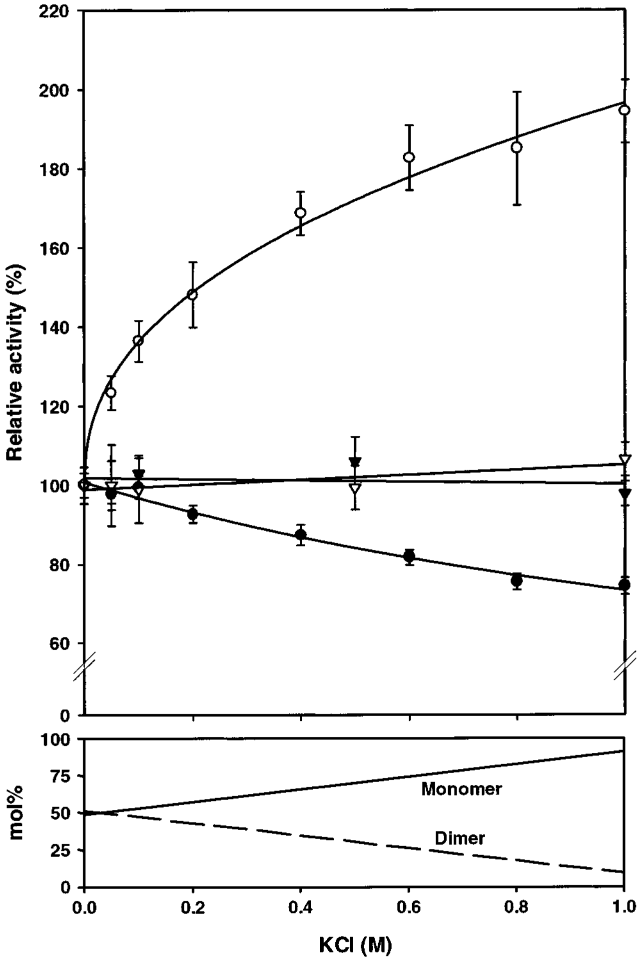

FIGURE 5: Effect of KCl on the hydrolysis of a substrate. Relative activities of ThMA toward soluble starch (open circles) and β-CD (closed circles) and of ThMAΔ124 toward soluble starch (open triangles) and β-CD (closed triangles) at various concentrations of KCl are shown. The bottom panel describes the monomer/dimer ratio of wild-type ThMA at various KCl concentrations. The activity of ThMA and ThMAΔ124 was measured at the respective temperature optima of 60 and 45 °C because ThMAΔ124 is unstable at 60 °C.

β-CD (Table 3). A small but significant decrease in k_{cat} of wild-type ThMA was observed for β-CD upon the addition of 1.0 M KCl, while k_{cat} of starch hydrolysis was increased significantly. In contrast, the k_{cat} values of ThMAΔ124 for β-CD and starch changed only slightly by the addition of 1.0 M KCl (Table 3). These results are in accordance with the ultracentrifugation data (Figure 2 and Table 1), showing that ThMAΔ124 exists only as a monomeric form.

K_m of ThMAΔ124 for β-CD in the absence of KCl was 175% of that of wild-type ThMA. In contrast, K_m of ThMAΔ124 for starch in the absence of KCl was 9% of that of ThMA. k_{cat}/K_m values of wild-type ThMA for β-CD in the absence and presence of 1.0 M KCl were 636 and 408 s^{-1} mM^{-1}, while the values for starch were 4.9 and 9.2 s^{-1} mL mg^{-1}, respectively. These results implied that, of the two substrates, starch was hydrolyzed more readily by monomeric ThMA (i.e., in KCl) and ThMAΔ124 than by dimeric ThMA. β-CD, however, was preferentially hydrolyzed to a higher degree by the ThMA dimer than by the monomer. Thus, the dimeric form predominant in wild-type ThMA at a low ionic strength hydrolyzed β-CD most efficiently. In contrast, the monomeric form, which either was greatly enriched in the equilibrium mixture at an elevated ionic strength for wild-type ThMA or was the only form present in ThMAΔ124, preferred polysaccharide substrates such as starch. This preference for the high-molecular-weight substrate correlated with the finding that the substrate-binding site is more accessible in the monomeric than in the dimeric ThMA.

Effect of ThMA Concentration on Monomer/Dimer Equilibrium and Specific Activity. The changes in the monomer/dimer ratios and substrate hydrolysis as a function of ThMA concentration were examined in the range of 25−520 nM (Figure 6). As the enzyme concentration was increased, the relative activity of ThMA toward β-CD increased by 45%, while the activity toward starch decreased by 27%. The monomer/dimer ratio at each enzyme concentration was calculated using the $K_{1,2}$ value for ThMA, $(9.2 \pm 0.8) \times 10^{-8}$, obtained in the absence of KCl and at 60 °C (Table

Table 3: Enzyme Activity and Kinetic Parameters of Wild-Type and Truncated ThMA

		k_{cat} (s^{-1})		K_m (mM; mg mL^{-1})		k_{cat}/K_m (s^{-1} mM^{-1}; s^{-1} mL mg^{-1})	
ThMA	substrate	no KCl	1 M KCl	no KCl	1 M KCl	no KCl	1 M KCl
WT	β-CD[a]	167 ± 4	126 ± 6	0.263 ± 0.014	0.308 ± 0.031	636	408
	starch[b]	301 ± 12	457 ± 29	61.0 ± 3.5	49.7 ± 5.0	4.94	9.19
Δ124	β-CD[a]	$(2.06 \pm 0.81) \times 10^{-4}$	$(2.20 \pm 0.33) \times 10^{-4}$	0.461 ± 0.302	0.360 ± 0.189	4.47×10^{-4}	6.11×10^{-4}
	starch[b]	$(1.47 \pm 0.33) \times 10^{-2}$	$(1.99 \pm 0.71) \times 10^{-2}$	5.34 ± 0.28	7.72 ± 0.57	2.75×10^{-3}	2.58×10^{-3}

[a] K_m and k_{cat}/K_m for β-CD were expressed in mM and s^{-1} mM^{-1}, respectively. [b] K_m and k_{cat}/K_m for starch were expressed in mg mL^{-1} and s^{-1} mL mg^{-1}, respectively.

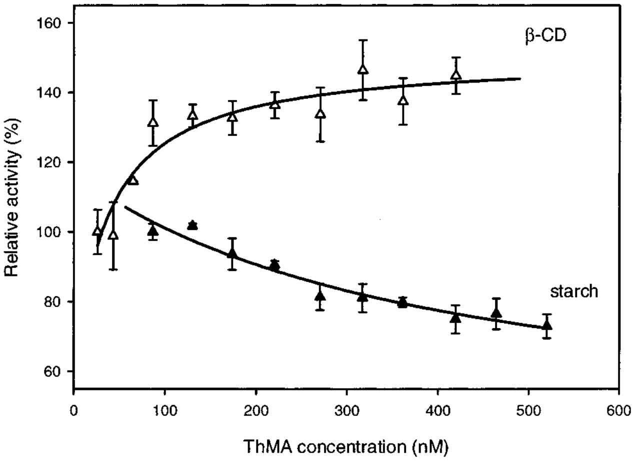

FIGURE 6: Effect of the ThMA concentration on the substrate hydrolysis. ThMA was diluted and incubated for 2 h at 4 °C in order to reach a monomer/dimer equilibrium. After 2 h of preincubation, the enzyme activities for β-CD and soluble starch were measured.

Table 4. Specific Activities of ThMA Monomer and Dimer toward β-CD and Soluble Starch

	specific activity (U/mg)		ratio
substrate	monomer	dimer	(dimer/monomer)
β-CD	194.1 (100%)[a]	342.7 (100%)	1.8
soluble starch	30.5 (15.7%)	5.4 (1.6%)	0.2

[a] Values in parentheses are the relative activities for each substrate. The activity for β-CD is assigned to 100%.

1). The predicted monomer/dimer ratio changed from 84:16 to 41:59 as the enzyme concentration increased from 25 to 520 nM.

The relative activity ratio of the monomer and dimer toward β-CD or starch was calculated from the monomer/dimer ratio and the total activity by fitting the data to the equation $A_T = A_M(\%M/100) + A_D(\%D/100)$, where A_T was the total activity, A_M was the relative activity of the monomer, A_D was the relative activity of the dimer, and %M and %D were the percentages of the monomer and dimer in solution, respectively. The specific activities of the monomer and dimer for β-CD were 194.1 and 342.7 U/mg, and those for soluble starch were 30.5 and 5.4 U/mg, respectively, implying that the dimer hydrolyzed β-CD faster than the monomer and that the latter hydrolyzed starch faster than the former (Table 4). The results strongly supported the hypothesis that the monomer/dimer equilibrium of ThMA was closely related to the multisubstrate specificity of the enzyme and also proved that the equilibrium can be modulated not only by ionic strength but also by the enzyme concentration in the reaction mixture.

DISCUSSION

The crystal structure of ThMA shows that this enzyme is a homodimer. The unique N-terminal domain of one monomer is critically involved in dimerization by partly covering the top of the active-site cleft of the other monomer (*7*). In solution, however, ThMA exists as a monomer/dimer equilibrium mixture, with a dissociation constant ranging from approximately 10^{-7} to 10^{-6} M depending on the ionic strength and temperature. This finding is further illustrated by the fact that the truncation of the N-terminal domain resulted in the monomerization of the enzyme. Moreover, as evidenced through ultracentrifugation analyses, the ThMA dimer dissociated to the monomer state with increasing KCl concentration until complete dissociation was achieved in 2 M KCl at 20 °C (data not shown). In a similar system reported for formyl transferase, it was not possible to determine whether a dimeric and a tetrameric formyl transferase were active because the dissociation of the tetramer into dimer occurred at a very fast rate (*26*). In the present study, however, the monomeric form of ThMA was shown to be enzymatically active as revealed for ThMAΔ124, which existed only in a monomeric form, exhibiting very low albeit significant activity coupled with the characteristic relative activities for β-CD and starch. This is compatible with the finding that the monomer hydrolyzed starch most readily. Furthermore, a clear correlation was also demonstrated between the monomer/dimer ratio of ThMA and the enzymatic activity toward different substrates. The substrate preferences of monomeric and dimeric ThMA were remarkably different. The ratio of the β-CD/starch hydrolyzing activity of wild-type ThMA present as a mixture of 51% dimer and 49% monomer was thus approximately 100 times higher than that of the truncated enzyme present only in the monomeric form. The dimer hydrolyzes β-CD much faster than the monomer, whereas the latter shows a relatively higher activity for starch. Hence, the hydrolytic activity of ThMA toward various substrates was modulated by the ionic strength of the reaction mixture through its influence on the monomer/dimer equilibrium of the enzyme.

A hypothetical model of β-CD docked into the active site of ThMA (*7*) indicated that β-CD would interact with many residues from the $(\beta/\alpha)_8$ barrel of one molecule and from the N-terminal domain of the other molecule in the dimeric form of the enzyme, respectively. The model showed that Phe289 in the $(\beta/\alpha)_8$ barrel of one monomer and Tyr45 from the N-terminal domain of the other monomer participated in hydrophobic interactions with the inner face of β-CD. This β-CD binding model supported our results that β-CD was a better substrate for the dimeric state of the enzyme than for the monomer. This enzyme−substrate complex was obtained by docking without a reorientation of the protein side chains

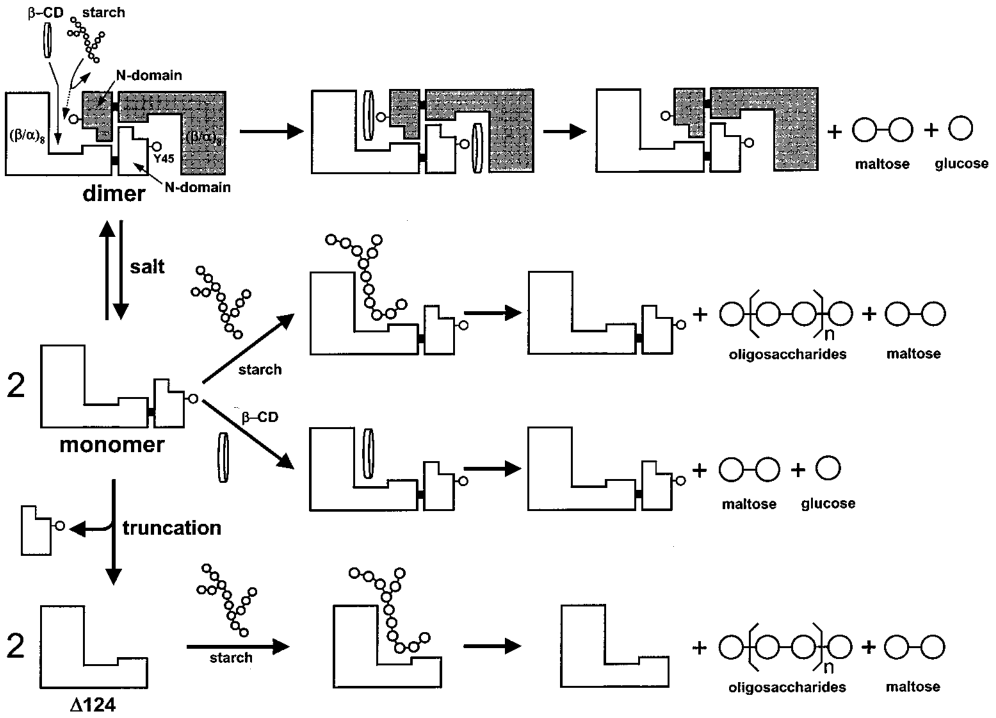

FIGURE 7: Proposed model of the ThMA monomer/dimer equilibrium in relation to its substrate specificity. One subunit of ThMA is shown in gray boxes and the other in empty boxes, each having the N-terminal domain linked by a short segment to the rest of the molecules. β-CD and soluble starch are shown as a disk and a chain of circles, respectively. When ThMA forms a dimer, the active site becomes narrow and deep, thereby readily accommodating β-CD and hydrolyzing it mainly to maltose. On the other hand, when ThMA turns into a monomer either through increased ionic strength or truncation of the N-terminal domain, the active site becomes wide and shallow, allowing for the ready access of the bulky starch molecule.

(*7*, *27*). Earlier, we proposed that the N-terminal domain played an important role in β-CD hydrolysis by modulating the shape of the active site and allowing hydrophobic interaction with the substrate (*7*). The substrate specificity was thus interpreted in relation to the geometry of the active site in the homodimer; it was proposed that the monomer/dimer equilibrium could control the multisubstrate specificity in relation to the molecular size and shape of the substrate. The effects of the salt and the N-terminal domain truncation of ThMA on the dissociation and substrate specificities are schematically summarized in Figure 7. Large substrates such as soluble starch can access the wide and shallow active site in the monomer, while small compact substrates such as CDs fit into the deep, narrow groove found in the dimer with more stabilizing contacts at the catalytic site. Furthermore, the monomer/dimer dissociation constant $K_{1,2}$ at 60 °C was calculated to be 9.2×10^{-8} M in the absence of KCl and 2.5×10^{-6} M in 1.0 M KCl (Table 1). The results indicate that ThMA predominantly exists in a dimeric form at normal conditions for hydrolysis and predominantly in the monomer form in 1 M KCl.

A dilution-dependent loss of enzyme activity was observed for mutant and wild-type enzymes of another $(\beta/\alpha)_8$-barrel protein, human triosephosphate isomerase, which also formed a dimer (*28*). These authors proposed that the monomeric form of the enzyme was catalytically inactive and that the enzyme activity was associated only with the dimeric form. Interestingly, unlike human triosephosphate isomerase, both the monomeric and the full-length dimeric ThMA forms were active, although they revealed different activities and substrate specificities. ThMA, in addition, exhibited a concentration-dependent multisubstrate specificity in which the starch hydrolyzing activity increased while the β-CD hydrolysis decreased as the enzyme was diluted (Figure 6). The created monomeric mutant, ThMAΔ124, lacking the N-terminal domain was also enzymatically active, but the specific activity was reduced significantly as compared to the wild-type ThMA monomer (i.e., in KCl). Remarkably, however, the catalytic properties of ThMAΔ124 were different from those of the dimeric wild-type ThMA as mentioned previously and shown in Table 1. In addition, maltotriose and larger hydrolysis products such as maltopentaose and maltohexaose were accumulated during starch hydrolysis by ThMAΔ124, while these maltooligosaccharides were rapidly hydrolyzed into maltose and glucose by wild-type ThMA, again indicating that the dimeric enzyme hydrolyzes smaller substrates more efficiently. This agrees with the finding that the active site provides a superior stabilization of such compounds as revealed by the high k_{cat}/K_m value for β-CD.

In glycoside hydrolase family 13, MAase, CDase, NPase, and TVA II amylase are closely related in their catalytic properties and amino acid sequences, although they are classified under separate EC numbers (*29*). MAase and CDase were reported to hydrolyze CDs much faster than pullulan and soluble starch, while NPase degrades pullulan efficiently and information is lacking on its CD-hydrolyzing activity (*12*). In contrast, TVA II showed an apparently higher hydrolytic activity toward starch than CDs (*12*). In the present study, we clearly demonstrated that the substrate specificity of MAase is highly related to the quaternary structure of the enzyme and that this finding can be extended to the other enzymes in glycoside hydrolase family 13 mentioned to share similar multisubstrate activity. Hence, comparative studies on both the multisubstrate specificities and the quaternary structure, including the monomer/dimer equilibrium characteristics among CDases, NPases, and TVA II amylase, will clarify the subclassification of these glycoside hydrolases of family 13. Combined with their three-dimensional structures, such studies will address the question as to whether these enzymes can be classified under one enzyme category with the same name and EC number.

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