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N- and C-terminal region mediated oligomerization of the cyclodextrin-/pullulan degrading enzymes

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Cyclodextrin-/pullulan (CD-/PUL)-degrading enzymes catalyze hydrolysis and transglycosylation reactions of various substrates such as starch, cyclodextrin, and pullulan. Recently, these enzymes have been proved to exist in equilibria of monomer-dimer and monomer-dimer-tetramer or -dodecamer. Two regions in the CD-/PUL-degrading enzymes were identified as being involved in oligomerization; one is close to the N-terminal and the other located near the C-terminal region. The three-dimensional structure and deletion mutagenesis analyses revealed that the N-terminal region affected the dimerization properties of monomeric *Thermus* maltogenic amylase (ThMA). On the other hand, both the N- and C-terminal regions were involved in dodecamerization of cyclodextrinase I-5 (CDase I-5) dimeric units. Oligomerization of these enzymes was also modulated by salt concentration and pH of the reaction buffer.

Key words: cyclodextrin degrading enzyme, dimer, dodecamer, oligomeric state.

Abbreviations: CD, cyclodextrin; CDase, cyclodextrinase; PUL, pullulan; ThMA, *Thermus* maltogenic amylase; TVAII, *Thermoactinomyces vulgaris* alpha-amylase II.

Introduction

The cyclodextrin-/pullulan (CD-/PUL)-degrading enzymes capable of hydrolyzing CDs, pullulan, and starch share similarity in substrate specificity and primary structure. They catalyze the cleavage of α-1,4- and α-1,6-glycosidic linkages in the substrate and can transfer the products to

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the sugar moiety of various acceptor molecules (MacGregor et al., 2001). These enzymes are often found as dimeric or dodecameric forms in solution. The crystal structures of *Thermus* maltogenic amylase (ThMA) (Kim et al., 1999) and pullulan-degrading *Thermoactinomyces vulgaris* alpha-amylase II (TVAII) (Kamitori et al., 1999) suggested that the N-terminal domain was involved in the dimerization of the enzymes. Recently, Park (2001) reported that cyclodextrinase (CDase) I-5 existed as a dodecamer.

However, it became obvious that our understanding of the relationship between quaternary structure and catalytic function of CD-/PUL-degrading enzymes was insufficient. The correlation between multisubstrate specificity and quaternary structure of this subgroup of enzymes is far more complex. Recently, however, new observations and evidence have accumulated and supported the possibility that each N- or C-terminal region contributes to the oligomerization of enzyme subunits (Park et al., 2000; Kim et al., 2001).

In general, the association constant of an oligomeric enzyme does not itself provide enough information about the precise molecular interactions between individual monomeric and dimeric subunits. Hence, introduction of some modifications to an enzyme, such as deletion and point mutations of amino acid(s), is necessary to determine the molecular interactions associated with the oligomerization of the enzyme. To investigate monomer-monomer/or dimer-dimer interactions in oligomeric enzymes, ThMA and CDase I-5 were modified by deletion and site-directed mutagenesis based on the information obtained from amino-acid sequence alignments and three-dimensional structures determined by X-ray crystallography.

In the present study, the oligomerization properties of *Thermus* maltogenic amylase (ThMA) and cyclodextrinase from *Bacillus* sp. I-5 (CDase I-5) representing the CD-/PUL-degrading enzymes were examined by mutagenesis of the amino-acid residues in the N- and the C-terminal regions, to illustrate the relationship between the quaternary structure and the substrate specificity of these enzymes.

Material and methods

Vectors and bacterial strains

E. coli MC1061[F^-, *araD*139, *recA*13, Δ(*araABleu*)1796, *galU*, *galK*, Δ *lacX*74, *rpsL*, *thi*, *hsdR*2, *merB*] carrying the pUCTJ3, p6xHThMA, or p6xHThMAΔ124 were used for gene manipulation and expression of wild-type CDase I-5, ThMA and its derivative, ThMAΔ124, respectively.

Analytical ultracentrifugation

Sedimentation equilibrium measurements were performed using a Beckman Optima XL-A analytical ultracentrifuge (Beckman Coulter, Inc., U.S.A.) equipped with a four-hole rotor with six-channel standard cells at a rotor speed of 5,000 or 10,000 rpm. (Zamyatnin, 1984).

Enzymatic activity measurements

The activity of ThMA and CDase was assayed in 50 mM sodium phosphate buffer using the copper-bicinchoninate method (Fox & Robyt, 1991) to determine the formation of reducing sugar from β-CD and soluble starch. One unit of enzyme activity was defined as the amount of enzyme that produced one μmol of maltose per min.

Results and discussion

Alignment and comparison of the amino-acid sequence in N-/C-terminal regions

The schematic alignment of the primary structures of CDase in comparison with those of Taka-amylase A and ThMA is shown in Figure 1. The secondary structure of the family 13 enzymes indicates that they share four common conserved regions located in the catalytic main domain. In addition, several other members of the family have a distinct domain (domain N), consisting of ∼130 amino-acid residues in the N-terminal part of the enzyme. The role of this domain has been suggested to be associated with formation of homodimer (Kim et al., 1999).

Some characteristics including hydrophobicity, the number of histidine residue, and pI of the enzymes are listed in Table 1. The C-terminal region of CDase I-5 contains four histidine residues while ThMA has one. The isoelectric point, pI, of the C-terminus of CDase I-5 is 7.75, much higher than the corresponding value (4.51) for the homologous region of ThMA. The higher pI for CDase I-5 is likely due to the high content of histidine.

Effects of salt and pH on the oligomeric state of the enzymes

The dependence of the oligomeric state of ThMA and CDase I-5 on the KCl concentration was investigated using analytical ultracentrifugation and gel permeation chromatography. The monomeric form of ThMA increased as the KCl concentration was increased from 0 to 1.0 M (Tab. 2). In the case of CDase I-5, the conversion of dodecamer to dimer was also facilitated greatly by KCl. CDase I-5 existed in a dimer/dodecamer mixture of 1:7

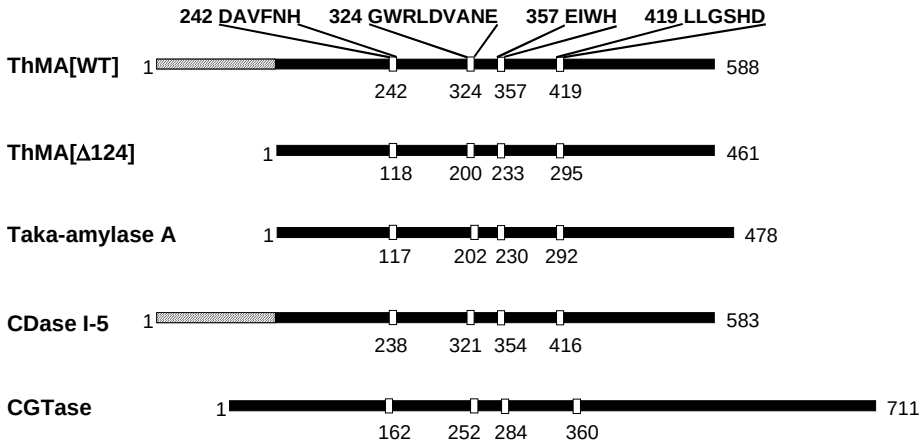


Fig. 1. Schematic alignment of the primary structure of ThMA, ThMAΔ124, and CDase I-5. The primary structures of ThMA, ThMAΔ124, and CDase I-5 are compared with those of Taka-amylase A, and *Bacillus stearothermophilus* cyclodextrin glucanotrasnferase (CHUNG et al., 1998). The N-terminal domain of ThMA and CDase I-5 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.

Table 1. Biochemical properties of N-/C-terminal regions of CD-/PUL-degrading enzymes.

	N-terminal				C-terminal				Total	
	No. of amino acid residues	Hydrophobicity[a]	His	pI[a]	No. of amino acid residues	Hydrophobicity	His	pI	His	pI
CDase	1–123	−0.69	4	5.75	505–583	−0.36	4	7.75	22	5.49
ThMA	1–124	−0.75	7	6.17	505–588	−0.04	1	4.51	22	5.56

[a] The values of pI and hydrophobicity were calculated by using the PROSIS program (v. 7.0, Hitachi Software Inc.).

Table 2. Effect of KCl on Monomer/Dimer/Dodecamer equilibrium of ThMA and CDase monitored by analytical ultracentrifugation.

KCl (M)	ThMA		ThMAΔ124		CDase I-5	
	Monomer (WT %)	Dimer (WT %)	Monomer (WT %)	Dimer (WT %)	Dimer (WT %)	Dodecamer (WT %)
0.0	5.0	95.0	100.0	0.0	12.7	87.3
0.1	–	–			23.4	76.6
0.2	8.5	91.5			–	–
0.4	16.7	83.3			22.2	77.8
0.6	22.9	77.1			32.7	67.3
0.8	33.9	66.1			25.4	74.7
1.0	74.8	25.2			33.3	66.7
2.0	–				72.3	27.7

without the addition of salt, while dimer was predominant (72%) in the presence of 2.0 M KCl.

To investigate the change of association/dissociation equilibrium at various pHs for CDase I-5, sedimentation equilibrium analysis was performed in the range of pH 5.5–7.5. The apparent molecular

weight of CDase I-5 was determined by sedimentation equilibrium ultracentrifugation as a function of pH (Tab. 3). As shown in Table 3, CDase I-5 exists as a mixture of monomer/dimer in the range of pH less than 6.0 while the dodecameric form is predominant at pH higher than 6.5. To observe the oligomeric transition between dimer and dodecamer, the pH of the enzyme solution of CDase I-5 was shifted from pH 7.0 to pH 6.0, and the enzyme samples were taken at appropriate time intervals to determine its oligomeric state by gel permeation chromatography. At pH 6.0 the dodecameric enzyme dissociated to dimers as deduced from the unique elution profiles of dodecamers and dimers on gel permeation chromatography. The peak corresponding to the dodecameric form decreased while the peak for the mixture of monomer/dimer increased (data not shown). At pH 7.0, however, the opposite result was observed; the peak for dodecamer increased while that corresponding to the monomer-dimer form decreased. The results indicate that the dimer-dodecamer transition is a true association/dissociation equilibrium process. Analysis of the specific activity of each dimeric or dodecameric enzyme fraction toward CD and starch suggested that both enzyme forms were enzymatically active.

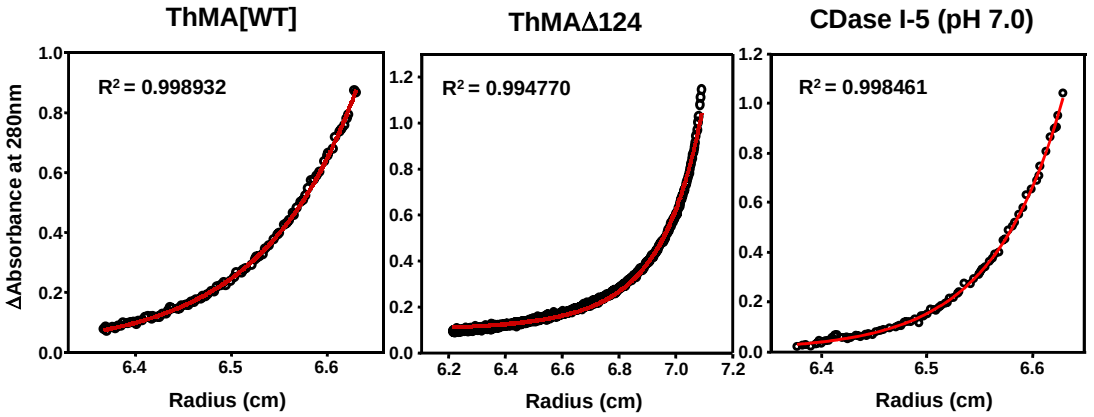
Table 3. Apparent molecular weight and major oligomeric state of CDase I-5 according to pH.

pH	Apparent molecular weight	Major oligomeric state
5.5	78,000	Monomer
6.0	158,000	Dimer
6.5	635,000	Dodecamer
7.0	678,000	Dodecamer
7.5	668,000	Dodecamer

We have determined the effect of pH on ThMA in the range of 5.0–8.0 where the ThMA is catalytically active. No significant shift of equilibrium between monomer and dimer was observed in the solution.

Effects of mutations on the association/dissociation of oligomers

In order to investigate the role of the N-terminal domain, the 124 amino-acid-long N-terminal domain of ThMA was deleted by PCR. The se-



Monomer (Wt%)	5.0%	100.0%	
Dimer (Wt%)	95.0%	0.0%	12.7%
Dodecamer (Wt%)			87.3%

Fig. 2. Sedimentation equilibrium analysis of ThMA, ThMAΔ124, and CDase I-5 in the absence of 1 M KCl. Concentration of the enzymes was 0.117 mg/mL in 50 mM sodium acetate, pH 6.0. Equilibrium was attained in 40 h. Concentration distribution of the protein as a function of the square of the radial position is shown. The bottom panel represents the monomer/dimmer/dodecamer ration of the enzymes in the absence of KCl.

Table 4. Physicochemical properties of ThMA and CDase I-5 at various oligomeric states.

Enzyme		Major interaction	Specific activity for β-CD[a]	pH	Thermostability[b]
ThMA		N-terminal	+++	independent	stable
			+	independent	less stable
CDase I-5		N-/C-terminal	+++++	pH > 7.0	more stable
		N-terminal	+++	pH < 6.0	stable

[a] Unpublished results.
[b] Thermostability was determined on the basis of melting point of the enzymes by differential scanning calorimetry (unpublished results).

quences of ThMA and the truncated enzyme, ThMAΔ124, are aligned with the secondary structure (Fig. 1). The truncated ThMA was exclusively in a monomeric form when analyzed by analytical ultracentrifugation (Fig. 2). This result indicated that the N-terminal domain of ThMA was responsible for the formation of dimer.

Among the residues at the dimer-dimer interface which could titrate between pH 6.0 and 7.0, His547 and 539 are the most probable residues that may be involved in the dodecamerization of CDase I-5. An analysis, with molecular graphics computer techniques, of CDase I-5 showed that His539 is a favorable site for dimer-dimer interaction since His539, among the four histidine residues, occupies the key position at the dimer interface. Hence, the mutated enzyme, H539V, has been created by site-directed mutagenesis and analyzed for its oligomerization characteristics. Indeed, its association was clearly weakened as compared to the wild-type enzyme at pH 6.0 (data not shown).

In general, protonation of histidine residues may cause dissociation of oligomer by eliminating a favorable interaction between residues, or by introducing an unfavorable interaction. Weaker interaction between the dimers is explained by reduction in the number of possible hydrogen bond interactions. Likewise, protonation of His539 could also weaken the intermolecular interaction with the hydrogen-bonding partner at the N-terminal region of the other dimer.

The oligomerization states of certain proteins have been known to be pH-dependent. Bovine F_1-ATPase inhibitor protein, IF_1, forms a tetramer at pH 8.0, while the protein is predominantly in dimeric form below pH 6.5 (Cabezon et al., 2000; Gordon-Smith et al., 2001). The protonation of histidine residues has been proposed to modify the structure of IF_1 and play an important role in interconversion between dimer and tetramer. Also, D-amino-acid transaminase undergoes a reversible process of dissociation/association that is pH-dependent (Kishimoto et al., 2000) but seems to occur at rates much slower than CDase I-5.

The N-terminal region of glucose-fructose oxidoreductase is essential for tetramer formation (Lott et al., 1998). The dimer of this enzyme is formed by stacking of the open concave faces of the β-sheets of two monomers. In contrast, the N-terminal region of CD-degrading amylases is involved in dimerization or dodecamerization by covering the top parts of the active sites in the other subunit (Kim et al., 1999).

Some physicochemical properties of ThMA and CDase I-5 related to oligomeric state are summarized in Table 4 and extend the scope of our crystallographic findings that the dimer or dodecamer is the principal structural intermediate in the dissociation and association of the CD-/PUL-degrading enzymes.

Acknowledgements

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