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Dissociation/association properties of a dodecameric cyclomaltodextrinase

Effects of pH and salt concentration on the oligomeric state

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As an effort to elucidate the quaternary structure of cyclomaltodextrinase I-5 (CDase I-5) as a function of pH and salt concentration, the dissociation/association processes of the enzyme were investigated under various pH and salt conditions. Previous crystallographic analysis of CDase I-5 indicated that it existed exclusively as a dodecamer at pH 7.0, forming an assembly of six 3D domain-swapped dimeric subunits. In the present study, analytical ultracentrifugation analysis suggested that CDase I-5 was present as a dimer in the pH range of 5.0–6.0, while the dodecameric form was predominant at pH values above 6.5. No dissociation of the dodecamer was observed at pH 7.0 and the above. Gel filtration chromatography showed that CDase I-5 dissociated into dimers at a rate of 8.58×10^{-2} h^{-1} at pH 6.0. A mutant enzyme with three histidine residues (H49, H89, and H539) substituted with valines dissociated into dimers faster than the wild-type enzyme at both pH 6.0 and 7.0. The tertiary structure indicated that the effect of pH on dissociation of the oligomer was mainly due to the protonation of H539. Unlike the pH-dependent process, the dissociation of wild-type CDase I-5 proceeded very fast at pH 7.0 in the presence of 0.2–1.0 M of KCl. Stopped-flow spectrophotometric analysis at various concentrations of KCl showed that the rate constants of dissociation (k_d) from dodecamers into dimers were 5.96 s^{-1} and 7.99 s^{-1} in the presence of 0.2 M and 1.0 M of KCl, respectively.

Enzymes in biological systems act not only as monomers but also associate to form dimers or higher order oligomers. Dimerization and oligomerization can provide enzymes with a number of functional advantages such as high stability and control over accessibility and specificity of active sites [1,2]. An example of this is the 3D domain-swapped maltogenic amylase from a *Thermus* strain (ThMA) that exhibits different binding preferences for various substrates by showing increased specificity via dimerization [3]. Recently, oligomeric states have been reported for the members of glycoside hydrolase family 13, especially cyclodextrin-/

Abbreviations
CD, circular dichroism; CDase, cyclomaltodextrinase; FRET, fluorescence resonance energy transfer; ITC, isothermal titration calorimetry; ThMA, maltogenic amylase from a *Thermus* strain.

pullulan-degrading enzymes such as cyclomaltodextrinase (CDase; EC 3.2.1.54), maltogenic amylase (MAase; EC 3.2.1.133), and neopullulanase (NPase, EC, 3.2.1.135) [4,5].

We demonstrated previously that CDase I-5 originated from an alkalophilic *Bacillus* sp. I-5 existed as a dodecamer, which was consisted of a hexamer of dimeric units, and that the formation of the supramolecular assembly resulted in an increase in the catalytic efficiency compared with that of the dimeric unit of the enzyme [6]. The monomeric structure of CDase I-5 contained a distinct N-domain in addition to a central $(\beta/\alpha)_8$-barrel domain and a C-domain. The N- (residues 1–123) and C- (residues 505–583) domains are composed exclusively of β-strands. Two CDase molecules form a domain-swapped dimer in which the N-domain of one molecule is involved in extensive interactions with the $(\beta/\alpha)_8$-barrel domain of the other molecule, as observed in the crystal structure of ThMA, which exists as a dimer in both the solution and crystal states [3]. The C-domain was, however, shown to be distinctly separated from the active site groove and was not involved in main-chain to main-chain hydrogen bonding with either the N- or the $(\beta/\alpha)_8$-barrel domain. Interestingly, the C-terminal domain was found to be critically involved in the supramolecular assembly of CDase [6].

In this study, we investigated the exogenous and endogenous factors affecting the supramolecular assembly of CDase I-5. Dissociation/association of the CDase I-5 dodecamer was found to be dependent on pH and salt concentration. At pH 6.0, the enzyme preferentially dissociated into its dimeric units, which were enzymatically active; at pH 7.0, the enzyme existed predominantly in the dodecameric form, which had higher catalytic activity than the dimeric form. Conversely, CDase I-5 rapidly dissociated into dimeric units in the presence of KCl at pH 7.0. The association/dissociation process of CDase I-5 was examined in various oligomeric states in order to identify the mechanism and forces that contribute to the supramolecular assembly and function of the enzyme. In addition, the role of histidine residues at the interfaces in the formation of the dodecamer was investigated by site-directed mutagenesis.

Results

pH-dependent dissociation/association of CDase I-5

To investigate the effect of pH on the dissociation of dodecameric CDase I-5, sedimentation equilibrium analysis was performed at pH 5.0–8.5. The apparent molecular mass of CDase I-5 determined using analytical ultracentrifugation was plotted as a function of pH (Fig. 1). The results indicated that CDase I-5 existed as a monomer/dimer in the pH range of 5.0–6.0, while dodecameric CDase I-5 was predominant at pH 6.5–8.5. Dimeric CDase I-5 began to associate with a transition midpoint of pH 6.2, forming dodecameric CDase I-5 as a major form at pH values higher than 6.5.

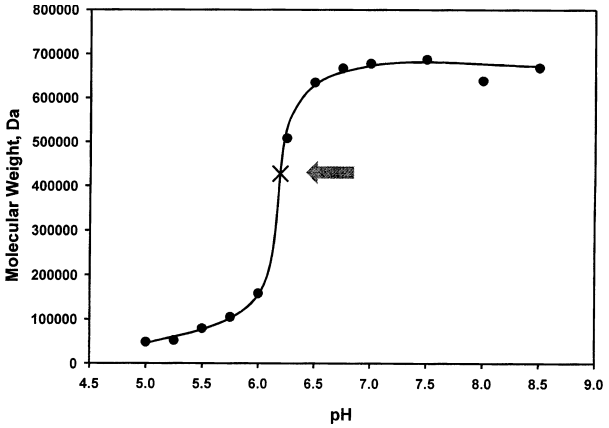

Fig. 1. Apparent molecular mass of CDase I-5 at various pH values determined by analytical ultracentrifugation analysis.

Based on these results, the reversibility of the association and dissociation processes of CDase I-5 was examined at pH 6.0 and 7.0. CDase I-5 was incubated in universal buffer (pH 6.0 or 7.0), and aliquots were taken at appropriate time intervals to determine the oligomeric state of the enzyme. Gel filtration chromatography was used to monitor the change of CDase I-5 from a dodecamer to a dimer. The corresponding relative molecular mass was estimated from the relative elution time of the standard proteins. At pH 6.0, the dodecameric enzyme dissociated into dimers, as determined by the relative elution times of dodecamers and dimers (Fig. 2A). The peak corresponding to the dodecameric form decreased, while that corresponding to the dimer increased as the incubation time proceeded. In 72 h of incubation at 4 °C, dodecameric CDase I-5 was fully converted into the dimeric form. On the other hand, if the pH of the enzyme solution was elevated to 7.0 after dissociation at pH 6.0, the reverse was observed. The peak corresponding to the dimeric form of the enzyme shifted towards that corresponding to the dodecamer (Fig. 2B). The association process by which dimeric enzymes fully recovered their dodecameric form was completed in 106 h at 4 °C (data not shown). These results indicated that separate dimers could form a dodecamer and that

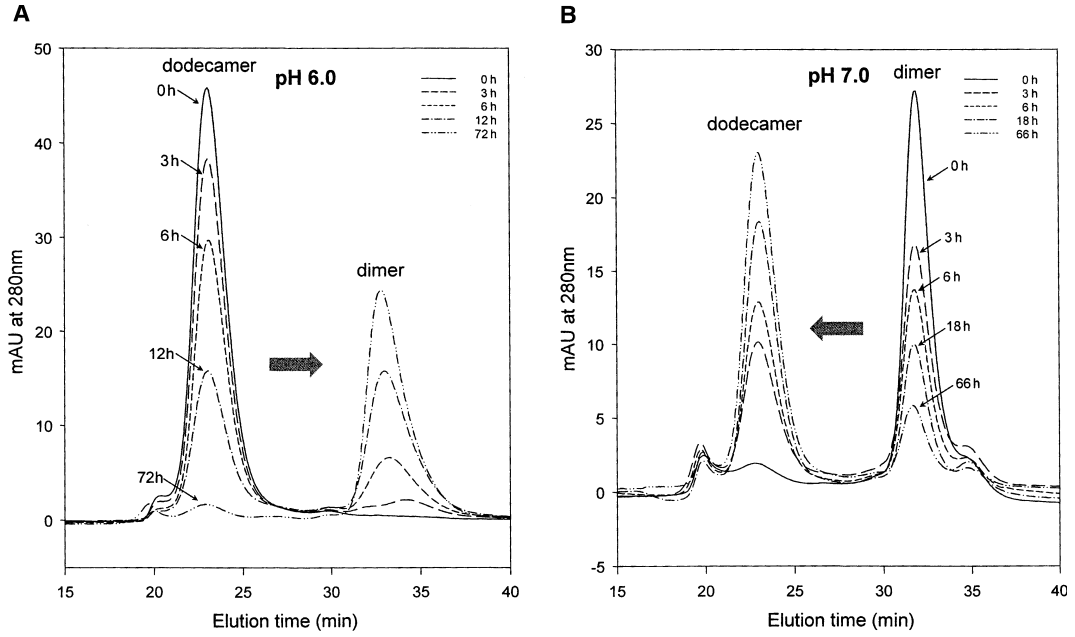

Fig. 2. Chromatographic separation of dimeric and dodecameric forms of CDase I-5. (A) Conversion of dodecamer to dimer. Dodecameric CDase I-5 at pH 7.0 was transferred to a buffer with pH 6.0 and incubated at 4 °C. (B) Conversion of dimer to dodecamer. Dimeric CDase I-5 at pH 6.0 was transferred to a buffer with pH 7.0 and incubated at 4 °C.

the dimer–dodecamer transition was a true association/dissociation equilibrium process.

The progress curve of the interconversion between dodecamer and dimer at pH 6.0 fitted a single exponential time course. Based on this observation, the kinetics of the dissociation process was analyzed in detail by calculating the peak area during the dissociation process. The rate of change in the peak area shown in Fig. 3A was estimated according to an equation of single exponential decay [7],

$$(\text{peak area})_t = Ae^{-kt} + B.$$

From the equation above, the slope of the exponential line in Fig. 3 was considered to be the rate constant, giving a rate constant of 8.58×10^{-2} h^{-1} for the dissociation of dodecamers to dimers (Table 1). The progress curve of the conversion of dimers to dodecamers at pH 7.0 also fitted a single exponential time course (Fig. 3B). From the above equation, the rate constant for the association of dimers to form dodecamers was determined as 1.09×10^{-1} h^{-1} (Table 1).

The kinetic parameters of CDase I-5 for β-cyclodextrin in either the dimeric or dodecameric state were compared by isothermal titration calorimetry at pH 6.0 and 7.0. The dodecameric form at pH 7.0 exhibited a k_{cat}/K_m value ~15 times larger than that of the dimeric form at pH 6.0 (Table 1).

Structural factors affecting dissociation/association of CDase I-5

Based on the information obtained about the 3D structure of CDase I-5, the quaternary state of CDase I-5 was likely to be maintained by the intrinsic capability of the N- and C-terminal regions of the enzyme to form a dodecamer at pH 7.0 and a dimer at pH 6.0. Crystallography of CDase I-5 has shown that a histidine residue in the C-terminal region (H539) and two of the four histidine residues in the N-terminal region (H49 and H89) are localized at the interfaces between dimeric units and are likely to be involved in the interaction between CDase I-5 molecules (Fig. 4A). The β-strand from K536 to L541 of a molecule is the major part contacting the adjacent β-strand from T50 to V54 of the other molecule in oligomerization. H539 is in the center of that contact region. The nitrogen (NE2) of the histidine residue forms a hydrogen bond to oxygen (OE1) in the side chain of Q516, of which the nitrogen (NE2) also forms hydrogen bond to side chain of D535. There are a total of six hydrogen bonds to support a sharp turn comprising from N533 to A537. Protonation of H539 may prevent the hydrogen bond to Q516 at a lower pH, thereby destabilizing the region hold tightly by the hydrogen bond network from K536-T540 and leading to conformational change at the interface of a dimer (Fig. 4B). There are

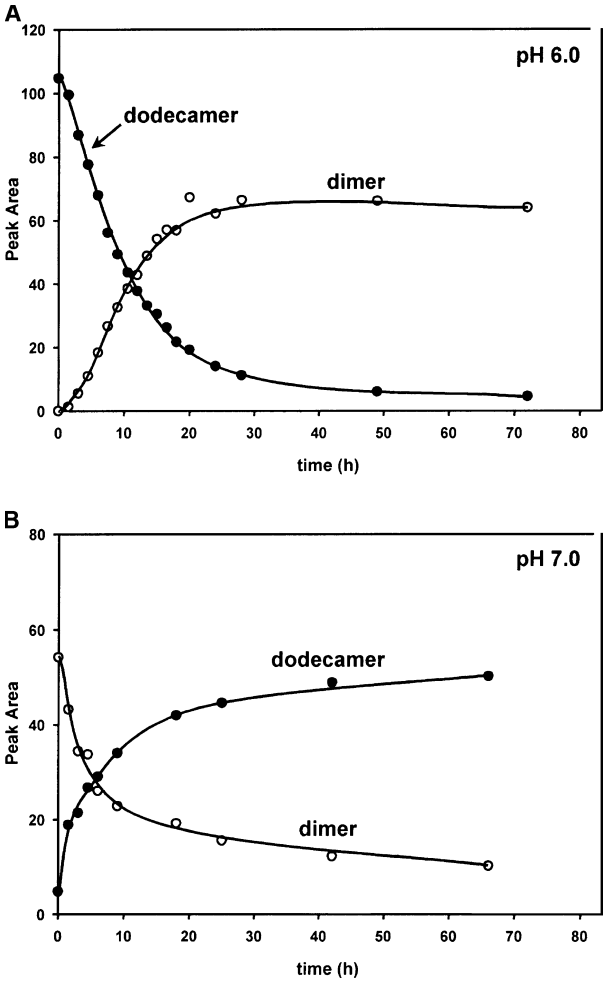

Fig. 3. The progress curves of the interconversion between dimer and dodecamer at pH 6.0 (A) and pH 7.0 (B). ●, dodecamer; ○, dimer.

Table 1. Physicochemical properties of wild-type CDase I-5 at pH 6 and 7.

	Wild-type CDase I-5	
Property	pH 6.0	pH 7.0
Transition to	Dissociation	Association
k (h^{-1})	$(8.58 \pm 0.23) \times 10^{-2}$	$(1.09 \pm 0.17) \times 10^{-1}$
Oligomeric state	Dimer	Dodecamer
k_{cat} (s^{-1}) [a]	8.5 ± 0.2	78.2 ± 0.4
K_m (mM) [a]	0.889 ± 0.045	0.454 ± 0.007
k_{cat}/K_m (s^{-1}·mM^{-1}) [a]	9.5 ± 0.5	172 ± 3

[a]Determined using β-cyclodextrin as a substrate.

two hydrogen bonds at G538 and T540 to the adjacent monomer, of which G538 forms a hydrogen bond to the carbonyl oxygen of M51. Two residues at the N-terminus (H49 and H89) of a subunit were located close to the C-domain of the other CDase I-5 subunit. The isoelectric point of CDase I-5 (pI 7.8) suggested that a decrease in pH from 7.0 to 6.0 would increase the number of positively charged residues at the C-terminal region, particularly those arising from protonation of the histidinyl groups. These might destabilize the dodecameric structure of CDase I-5 by electrostatic repulsion of positively charged residues at low pH, resulting in the dissociation of dodecamers to dimers.

Double and triple mutations at three histidine residues (H49, H89, and H539) were constructed in various combinations. All mutant CDases purified from *Escherichia coli* transformants carrying the mutant clones had specific activity toward β-cyclodextrin and optimal temperature and pH similar to those of wild-type CDase I-5 (data not shown). However, the dissociation rate constant was increased in all the mutants.

Dissociation of the CDase I-5 mutants at pH 6.0 and 7.0

To elucidate the role of histidine residues in the superassembly of CDase I-5, the dissociation rate constants of two mutants (H49V/H539V and H49V/H89/H539V) were determined. The dissociation process was analyzed in universal buffer (pH 6.0) by chromatography using a Superdex 200 HR 10/30 column. The peak area corresponding to the dodecamer diminished with incubation time. The progress curves representing the dissociation of dodecamers to dimers fitted the equation of a single exponential decay. The dissociation rate constants of all mutants were increased compared with that of wild-type CDase I-5. The dissociation rate constants for H49V/H539V and H49V/H89V/H539V were 6.80×10^{-1} h^{-1} and 1.36 h^{-1}, respectively (Table 2); the same constant for H49V/H89V/H539V was about 16 times larger than that of wild-type CDase I-5. The mutation of histidine to valine showed the same effect, even at pH 7 and above. These data indicated that the effect of pH on dissociation of the oligomer was mainly due to the protonation of a single residue rather than a global effect of pH on the protein. In agreement with the site-directed mutagenesis studies, H539 was most likely to be the target of this pH effect.

Wild-type and mutant CDases were stored in 50 mM sodium phosphate buffer (pH 7.0) at 4 °C, applied to a Superdex 200 HR 10/30 column on a Pharmacia Akta FPLC system, and eluted with 50 mM sodium phosphate butter (pH 7.0) at a flow rate of 0.4 mL·min^{-1}. The enzyme (100 μL) was applied to the column, and the absorbance of each eluent was measured at 280 nm. The proportion of dodecamers decreased as less protein was used. The dissociation constant (K_d) for the dodecamer was estimated as

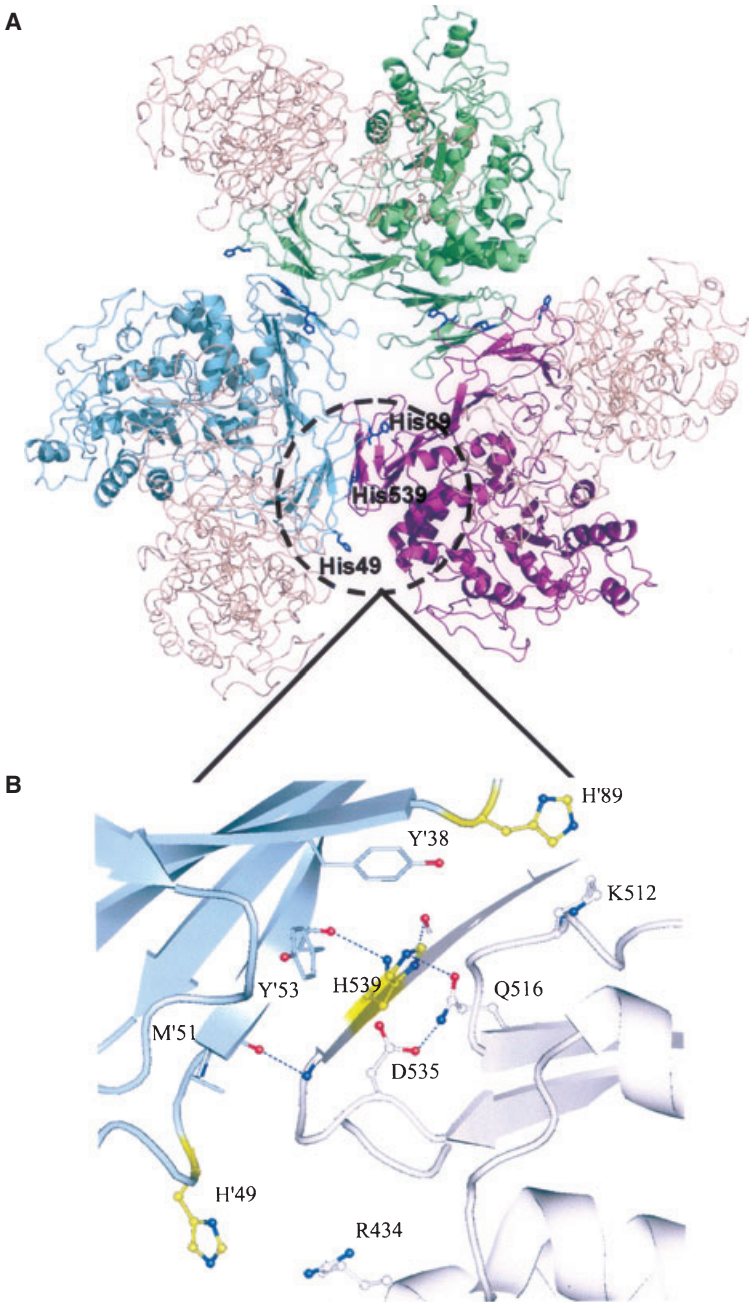

Fig. 4. The three histidine residues at the interface of two CDase I-5 subunits constituting a dodecamer (A). Close view of the interface shows that H539 is involved in various hydrogen bondages (B). Blue balls represent nitrogen, red balls oxygen, and yellow balls carbon of amino acids. Amino acid residues in one subunit are primed and those in the other subunit are not.

described in the Experimental procedures section. A very good fit to a line with a slope of 5.04 was obtained, and the K_d values for H49V/H89V/H539V and H49V/H539V were calculated as 1.79×10^{-30} and 4.63×10^{-32} M^5, respectively (Table 2). For wild-type CDase I-5, the enzyme was applied to a Superdex column at concentrations of up to 100 nM at pH 7.0, but no dissociation of the dodecameric enzyme was detected. The results indicated that the K_d value of wild-type CDase I-5 was much lower than those of the mutants. This result was confirmed by the sedimentation equilibrium and sedimentation velocity analytical ultracentrifugation analyses carried out at pH 7.0. In the sedimentation equilibrium analysis, the apparent molar masses of wild-type and mutant CDase were 736 and 491 kDa, respectively (Fig. 5A). The data from a series of scans (Fig. 5B) showed the common meniscus and the logical progression of the boundary and plateau regions. The sedimentation coefficient was calculated as described in the Experimental procedures

Table 2. Kinetic and equilibrium parameters of wild-type and mutant CDase I-5.

Parameter	pH	Wild-type	Mutants	
			H49V/H539V	H49V/H89V/H539V
Dissociation rate constant k_d (h^{-1})	6.0	$(8.58 \pm 0.23) \times 10^{-2}$	$(6.80 \pm 1.35) \times 10^{-1}$	1.36 ± 0.31
Equilibrium constant K_d ($\times 10^{-30}$)	7.0	0.0	0.046 ± 0.001	1.79 ± 0.10
Sedimentation coefficient (s)[a]	7.0	20	–[b]	20, 5

[a]Apparent weight average sedimentation coefficient in Svedbergs. [b]Not determined.

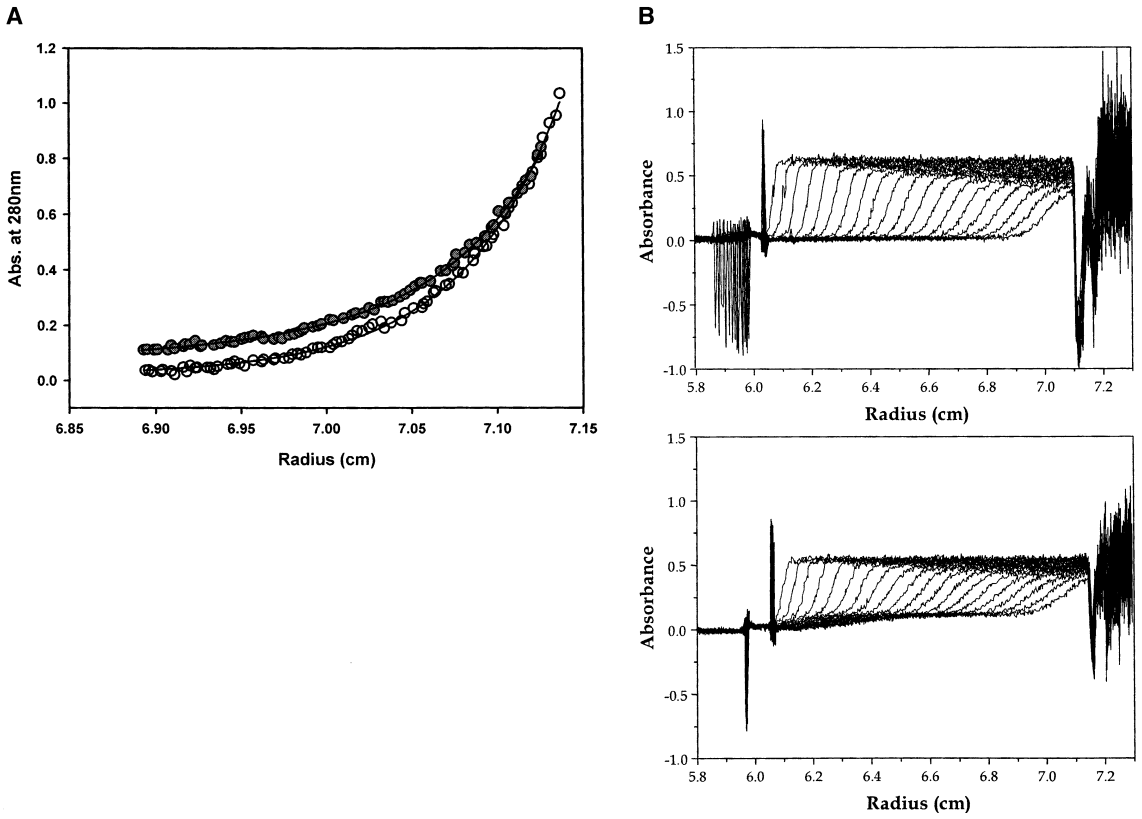

Fig. 5. (A) Sedimentation equilibrium analysis of wild-type CDase I-5 (open circles) and the CDase I-5 H49V/H89V/H539V mutant (closed circles). (B) Sedimentation velocity analytical ultracentrifugation of wild-type CDase I-5 and the CDase I-5 H49V/H89V/H539V mutant. Overlay plots represent the boundary sedimentation data of wild-type CDase I-5 (upper panel) and mutant CDase I-5 (lower panel).

section. The apparent weight average sedimentation coefficients were 20 for wild-type and 20 and 5 for H49V/H89V/H539V, respectively. These results implied that the CDase mutant existed in a dimer/dodecamer equilibrium at pH 7.0.

Effect of KCl on the quaternary structure of CDase I-5

To investigate the oligomeric state of CDase I-5 at pH 7.0, CDase I-5 was applied to a Superdex 200 HR 10/30 column. The apparent molecular mass of the enzyme, calculated by comparing the elution time with those of standard proteins [6], was 638 kDa, which was much larger than the molecular mass of the monomeric subunit (67.7 kDa). The result indicated that the major oligomeric state of CDase I-5 at pH 7.0 was dodecameric. However, the peak corresponding to dimer increased in the presence of 1 M KCl, while the area of the peak corresponding to dodecamer decreased, suggesting that the enzyme dissociated from dodecamers into dimers in the presence of salt [8].

In order to investigate the relationship between the oligomeric state of the enzyme and the salt

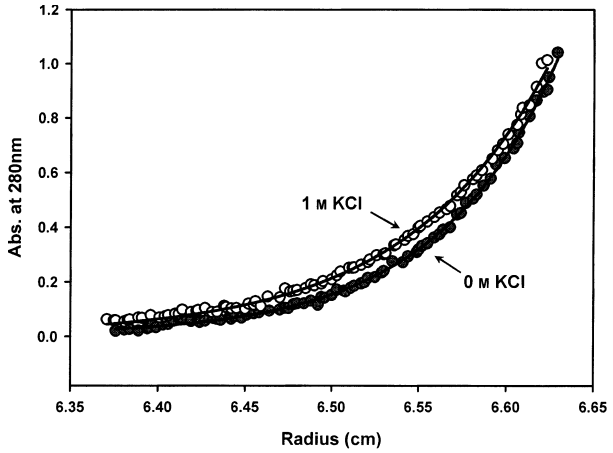

Fig. 6. Sedimentation equilibrium analytical ultracentrifugation analysis of CDase I-5 in the presence or absence of KCl. ●, CDase I-5 with no KCl added; ○, enzyme in 1.0 M KCl.

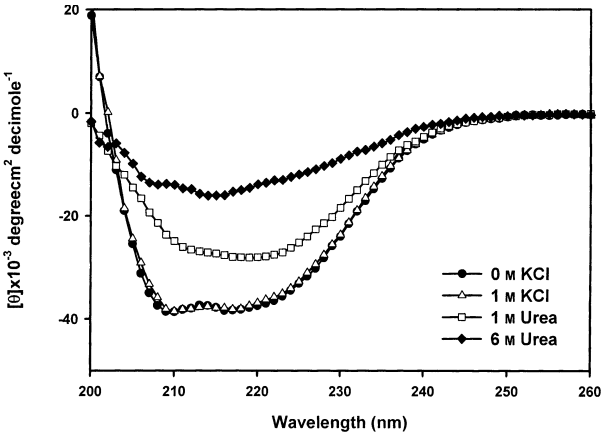

Fig. 7. Far UV-CD spectra of CDase I-5 at various concentrations of KCl and urea. The spectrum shown in closed circles represents the spectrum of CDase with no KCl; △, with 1.0 M KCl; □, with 1.0 M urea; ◆, with 6.0 M urea.

concentration, the effect of the dimer/dodecamer equilibrium on the enzymatic properties of CDase I-5 was examined at various concentrations of KCl. First, the role of KCl in dissociation of CDase I-5 was investigated by analytical ultracentrifugation. As the KCl concentration was increased from 0 to 1.0 M, the apparent molecular weight of the enzyme decreased and the amount of dimeric CDase I-5 increased (Fig. 6). In the presence of 1.0 M KCl, the dodecamerization degree of CDase I-5 decreased to 69% [8].

In order to determine whether any change occurred in the secondary structure of CDase I-5, far-UV circular dichroism (CD) analysis was carried out. When the enzyme was treated with 1.0 M KCl, there was no significant change in the CD spectrum, while treatment with 1.0 M or 6.0 M urea produced significant changes (Fig. 7). The results indicated that the secondary structure of CDase I-5 was not altered by KCl at concentrations of up to 1.0 M. Likewise, the ellipticity also showed that 1.0 M KCl did not affect the secondary structure of the enzyme, while urea and guanidine hydrochloride exerted a great influence. We concluded that the secondary structure and peptide backbone of native CDase I-5 were stable and rigid at pH 7.0 in the absence or presence of KCl at concentrations up to 1.0 M.

Kinetic study of rapid dissociation of CDase I-5

To characterize the changes in the quaternary structure of CDase I-5, the intrinsic fluorescence of CDase I-5 was measured at various concentrations of KCl and denaturants. In general, the intrinsic fluorescence results mainly from tryptophan residues, which show an emission maximum at around 340 nm when dissolved in water (Fig. 8). Tryptophan covered by the protein matrix in the aqueous phase causes a blue shift. When excited at 295 nm, dodecameric CDase I-5 had an emission maximum at 335 nm. Upon the addition of KCl to a final concentration of 1.0 M, the dodecamer should be dissociated into dimeric units, and the aromatic amino acid residues buried by dodecamerization would become exposed. The aromatic amino acid residue, tryptophan, would then contribute to an increase in the intrinsic fluorescence.

Based on the crystal structure analysis of CDase I-5, the tryptophan residues of CDase I-5 at the 68, 68′, 93, and 93′ positions were possible candidates contributing to increased fluorescence intensity through dissociation upon exposure to solvent. The fluorescence intensity of CDase I-5 increased as the dodecameric enzyme dissociated into dimers upon the addition of 1.0 M KCl (Fig. 8A). Conversely, upon denaturation and unfolding of the protein by chemical modification, nonpolar interior groups became exposed to the polar exterior phase, and the quenching of fluorescence was accompanied by a red shift and a decrease in intensity [9]. The intensity of fluorescence of CDase I-5 treated with 1.0 or 6.0 M urea at 25 °C was weak, and the wavelength of the spectral maximum was shifted to 355 nm (Fig. 8B).

To investigate the dissociation process of CDase I-5, changes in fluorescence intensity of the reaction mixture were monitored using an SFM-4 stopped-flow apparatus at different KCl concentrations (0–1.0 M KCl). The fluorescence intensity of CDase I-5 increased as the concentration of KCl increased (Fig. 9A). For a pseudo-first-order reaction, the rate

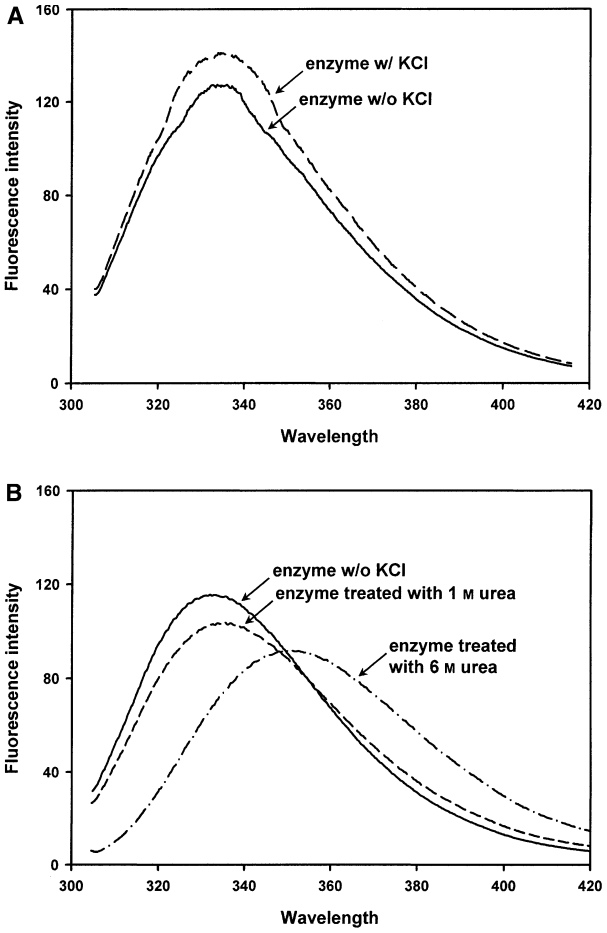

Fig. 8. Fluorescence spectra of CDase I-5. (A) - - - -, the intensity of fluorescence of CDase I-5 treated with 1 M KCl; ——, native CDase I-5. (B) The curve shown by —— represents the fluorescence intensity of native CDase I-5; - - - -, CDase I-5 denatured with 1.0 M urea; –·–·–, CDase I-5 denatured with 6.0 M urea at 25 °C.

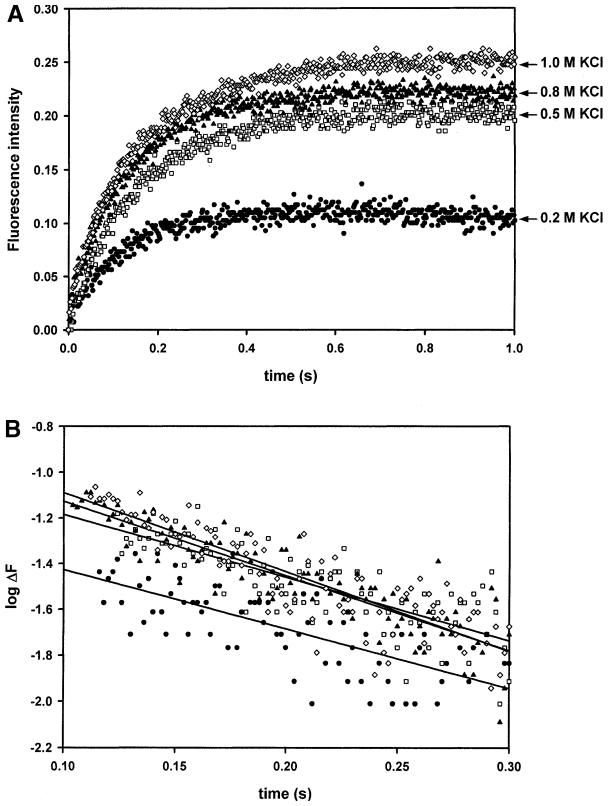

Fig. 9. (A) Time course fluorescence spectra of CDase I-5 dissociation at various concentrations of KCl at pH 7 and 25 °C. (B) Plot of log ΔF versus time by the Guggenheim method. ●, dissociation of CDase I-5 in the presence of 0.2 M KCl; □, 0.5 M KCl; ▲, 0.8 M KCl; ◇, 1.0 M KCl.

constant of dissociation (k_d) from dodecamer into dimer was estimated at various concentrations of KCl using the Guggenheim method [10]. The k_d values in the presence of 0.25 M and 1.0 M KCl were 5.96 and 7.99 s^{-1}, respectively (Fig. 9B and Table 3). The rate constants increased as the pH was lowered or the concentration of KCl was increased. The results suggested that the effect of salts on the oligomeric state of CDase I-5 correlated with the dissociation of the dodecameric form of the enzyme.

Table 3. Salt-induced dissociation rate constants (k_d)[a] of CDase I-5 determined by fast kinetic measurements.

	Dissociation rate constant (s^{-1})			
pH	0.2 M KCl	0.5 M KCl	0.8 M KCl	1.0 M KCl
7.0	5.96 ± 0.0	6.36 ± 0.16	7.53 ± 0.12	7.99 ± 0.13
6.9	6.30 ± 0.11	7.03 ± 0.10	7.64 ± 0.06	8.79 ± 0.07
6.7	9.15 ± 0.17	10.46 ± 0.11	–[b]	11.81 ± 0.21
6.5	15.18 ± 0.13	18.38 ± 0.16	20.92 ± 0.15	21.92 ± 0.29
6.0	–[b]	–[b]	–[b]	0.99 ± 0.01

[a]Values for k_d were determined according to the Guggenheim method. Final concentrations after mixing were [CDase I-5] = 10 σ′M and [KCl] = 0.2–1.0 M. [b]Not determined.

Discussion

An earlier study on the CDase I-5 crystal structure demonstrated that this enzyme adopts a dodecameric form in solutions with a pH above 7 [6]. To the authors' knowledge, the dodecamerization of CDase I-5 is by far the highest order oligomerization observed for an amylolytic enzyme. To understand the role of the oligomerization of CDase I-5, its dissociation/association properties were investigated at low and high pHs and in the presence of KCl.

Considering also the 3D structure of CDase I-5, the analysis of the quaternary state of CDase I-5 revealed

the intrinsic capability of the N- and C-terminal regions of the enzyme to form dodecamers at pH 7.0 and dimers at pH 6.0. The observed isoelectric point of CDase I-5 (pI 7.8) in the C-terminal domain (amino acid residues 505–583) was much higher than those of other maltogenic amylases that exist in a monomer–dimer equilibrium [8]. CDase I-5 has four histidine residues (H539, H547, H552, and H563) in the C-terminal region that were thought to have pK_a values within the range of 5.0–7.0; thus, modifying the structure of CDase I-5 by protonation and deprotonation might allow these residues to interact with the charged groups of other residues. The isoelectric point of CDase I-5 (pI 7.8) suggests that a decrease in pH from 7.0 to 6.0 would increase the number of positively charged residues in the C-terminal region, particularly those arising from protonation of histidinyl groups. The results indicated that the electrical charge of the amino acid residues was involved in a self-association process leading to the formation of dodecamers. The force driving the dissociation process was very likely to be the destabilizing effect of electrostatic repulsion between positively charged residues in the C-terminal domain at low pHs. Thus, H539 that is in the center of the C-terminal region plays an important role in determining the quaternary structure of the dodecamer. Four histidine residues are present in the C-terminal region of CDase I-5, while only one histidine residue is found in the corresponding region of ThMA, which is mostly present in the dimeric form. Oligomerization states of certain proteins have been reported to be pH dependent [7,11,12]. For example, bovine F_1-ATPase inhibitor protein, IF_1, forms tetramers at pH 8.0, while the protein is predominantly in the dimeric form below pH 6.5 [11,12]. The protonation of histidine residues appears to modify the structure of IF_1 and play an important role in the interconversion between dimers and tetramers, given that the mutation of this residue to lysine abolishes the pH-dependent oligomerization without an alteration of enzyme activity [11]. A 10-kDa light chain subunit of the cytoplasmic dynein complex LC8 shows a reversible monomer–dimer equilibrium at pH 7.0, but the dimers dissociate into monomers at lower pHs, with a transition midpoint at pH 4.8 [13]. This was explained by the titration of a histidine pair at the interface of the dimer. D-amino acid transaminase undergoes a reversible process of dissociation/association that is pH-dependent [7], but this occurs at rates much slower than those of CDase I-5.

In 1.0 M KCl solution, the dodecamerization degree of CDase I-5 decreased to 29% and the activity on β-cyclodextrin decreased to 66% in parallel with the concentration of the dodecamer [8]. We have previously shown that the dodecameric form of the enzyme exhibited a catalytic efficiency for β-cyclodextrin that was ~10 times higher than that of the dimeric form [3]. These results correlated with the data shown in Table 1. Furthermore, the far-UV CD spectra of CDase I-5 were similar in the absence or presence of 1.0 M KCl (Fig. 7), indicating that the conformational changes were negligible in terms of secondary structure.

Unlike the pH-dependent process that was slow enough to enable monitoring by gel filtration chromatography of the interconversion of CDase I-5 between dodecamers and dimers, the dissociation process of the enzyme was very fast in the presence of KCl at pH 7.0. Therefore, the salt-induced dissociation of CDase was investigated using a stopped-flow apparatus. The rate constant of dissociation (k_d) from dodecamers into dimers was 7.99 s^{-1}, and the dissociation process was completed within seconds. Stevens *et al.* [14] reported that class Sigma glutathione S-transferase lost 60% of its catalytic activity and a single tryptophan residue per subunit became partly exposed when NaCl was added at concentrations up to 2 M. They reported that no significant change was detected either in the secondary structure of the protein according to far-UV CD data or in the size of the protein determined by size-exclusion HPLC. They suggested that the change might occur either at or near the active site. However, in the case of CDase I-5, when the protein dissociated from dodecamers to dimers as shown by gel filtration chromatography, the activity on β-cyclodextrin decreased to 66%, but the activity on soluble starch increased by 160% (data not shown). Large substrates such as soluble starch seemed to be able to access dimeric CDase more easily than the dodecameric form owing to less steric hindrance. These results suggested that the effect of salts on the oligomeric state of CDase I-5 correlated with the dissociation of the dodecameric form of the enzyme.

In conclusion, dimerization or oligomerization is a physical property common to proteins. The assembly of supramolecules is an alternative mechanism for the formation of a large and stable dynamic structure without increasing genome size in biological systems [1]. CDase I-5 existed as dodecamer formed from two hexamers of 3D domain-swapped dimeric units. The results obtained in this study show that the association/dissociation process of dodecameric CDase I-5 was modulated by pH and salt concentration. Dissociation of wild-type CDase I-5 into dimers rarely happened at pH 7, but it could be promoted by KCl. The

mutagenesis studies of the enzyme revealed that the dodecamerization of dimeric CDase I-5 was mediated by the protonation of H539 at the C-terminus. Dodecamerization would expand the opportunities for the regulation of an enzyme by providing a number of functional advantages, such as high stability and control over the accessibility and specificity of active sites. The evolutionary role of supramolecular assembly is likely to be associated with the adaptation of proteins to a harsh alkaline environment by the formation of stable and dynamic structures.

Experimental procedures

Protein purification

Gene cloning and overproduction of CDase I-5 were carried out as described previously [15]. *E. coli* MC1061 carrying the CDase I-5 gene on pUC18 was cultured in a 5-L fermentor jar (KF-5 L, Korea Fermentor Co. Ltd) at 37 °C in Luria-Bertani broth containing ampicillin and was harvested in the late log phase. The enzyme was purified by ammonium sulfate precipitation followed by chromatography using a Q-Sepharose column (Amersham Pharmacia Biotechnology, Uppsala, Sweden) and a DEAE-Toyopearl 650 M column (Tosoh Corporation, Tokyo, Japan).

Enzyme assay

Hydrolytic activity of CDase I-5 was measured as described before [16] with some modifications. A solution of substrate was prepared in 50 mM sodium phosphate buffer (pH 7.5). Enzyme digest was composed of 250 μL of 1% (w/v) β-cyclodextrin (Sigma Chemical Co., St. Louis, MO, USA) or soluble starch (Showa Chemical Inc., Tokyo, Japan) solution as substrates, 200 μL of reaction buffer, and 50 μL of properly diluted enzyme solution. Reaction mixture was prewarmed at 50 °C for 5 min, then diluted enzyme solution was added and the mixture incubated for 10 min. The reaction was stopped by adding 0.5 μL of 100 mM NaOH solution. Aliquots (200 μL) of the enzyme digest were taken and added to 200 mL of copper-bicinchoninate working reagent [17]. One unit (U) of enzyme activity was defined as the amount of enzyme that produced one micromole of maltose equivalent.

Site-directed mutagenesis

Site-directed mutagenesis was carried out to replace a histidine residue with valine using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and a PE9600 thermal cycler (Perkin-Elmer, Norwalk, CT, USA). Mutants were made by altering His49 to Val49, His89 to Val89, and His539 to Val539 using the following primers: for the H49V mutant, 5′-AGTACATGTGGGACGTCAC CATGGAGTATGTCCC-3′ (forward) and 5′-GGGACAT ACTC CATGGTGACGTCCCACATGTACT-3′ (reverse); for the H89V mutant, 5′-TCTGCTGCAGCA GGGTGTT GAGAAGCGCTGGATG-3′ (forward) and 5′-CATCCAG CGCTTCTCAACACCCT GCTGCAGCAGA-3′ (reverse); for the H539V mutant, 5′-CGACAAGGCGGGCGTC ACGTTA ACGCTGCCTGTCC-3′ (forward) and 5′-GG ACAGGCAGCGTTAACGTGACGCCCGCCTTGTCG-3′ (reverse). PCR was performed under the following conditions: denaturation at 95 °C for 30 s followed by 18 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min, and extension at 68 °C for 2 min. After digestion with *Dpn*I, the amplified DNA fragments were phosphorylated and ligated with T4 DNA ligase. Transformation and the screening of the resulting transformants were carried out by the calcium chloride [18] and iodine methods [19], respectively. All mutations were confirmed by sequence analysis using the dideoxy chain termination method and an ABI377 PRISM DNA sequencer (Perkin-Elmer, Norwalk, CT, USA).

Gel filtration chromatography

Chromatography using a Superdex 200 H 10/30 column (Amersham Pharmacia Biotech., Uppsala, Sweden) was carried out to separate the dodecameric and dimeric forms of CDase I-5 at different pH values or in 1 M KCl. Sample (100 μL) was applied to the column equilibrated with an appropriate buffer and eluted at a flow rate of 0.4 $mL \cdot min^{-1}$. For determination of dissociation rate constant at pH 6, 3–6 μM (0.2–0.4 $mg \cdot mL^{-1}$) of CDase I-5 were used. For determination of equilibrium constant at pH 7, various amounts of wild-type and mutant CDase I-5 were used in the range of 0.72–11.9 μM. Thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (ADH; 150 kDa), bovine serum albumin (BSA; 66 kDa), and carbonic anhydrase (29 kDa) were used to estimate the apparent molecular weight of the enzyme.

Sedimentation equilibrium and velocity analytical ultracentrifugation

Sedimentation equilibrium analytical ultracentrifugation was performed using a Beckman Optima XL-A analytical ultracentrifuge (Beckman Coulter Inc., Fullerton, CA, USA) equipped with a four-hole rotor with standard six-channel cells at a rotor speed of 5000 r.p.m. The absorbance-versus-radius distributions, $A(r)$, were recorded at 280 nm. These were evaluated using the nonlinear regression method provided by the SIGMAPLOT software (SPSS Science, Chicago, IL, USA). The general equation used for fitting the $A(r)$ data was

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

where i denotes the number of protomers per oligomer; A_i, the absorbance of the corresponding species; $\bar{\nu}$, the partial specific volume of the protein (calculated as described by Zamyatnin [20] and assumed to be independent of the state of oligomerization); ρ_o, the solvent density; ω, the angular velocity of the rotor; r_o, the fixed radial position; R the gas constant; and T the temperature.

Sedimentation velocity analytical ultracentrifugation was performed using a Beckman Optima XL-A analytical ultracentrifuge (Beckman Coulter Inc., Fullerton, CA, USA) equipped with a four-hole rotor with standard two-channel cells at a rotor speed of 25000 r.p.m. Radial scans at 280 nm were taken every 5 min, and the sedimentation coefficient was calculated from the movement of the sedimentation boundary using the SLAVEL program (Beckman Coulter Inc.).

Evaluation of K_d values

The dodecamer dissociation constant, k_d, was estimated as follows [21]. If the maximal amount of CDase I-5 dodecamer is [Max] and the concentrations of dodecameric and dimeric species are [Dod] and [Di], respectively, so that percentageDod = 100[Dod]/[max], it follows that:

$$\begin{aligned} K_d &= [\mathrm{Di}]^6/[\mathrm{Dod}] = 6^6([\mathrm{max}] - [\mathrm{Dod}])^6/[\mathrm{Dod}] \\ &= 6^6(100)^{-5}[\mathrm{max}]^5(100 - \%\mathrm{Dod})^6/\%\mathrm{Dod} \\ &= 4.6656 \times 10^{-6}[\mathrm{max}]^5(100 - \%\mathrm{Dod})^6/\%\mathrm{Dod}. \end{aligned}$$

Hence,

$$\begin{aligned} \mathrm{Log}(K_d) = {} & 5 \times \mathrm{Log}[\mathrm{max}] \\ & [-\log(\%\mathrm{Dod}/4.6656 \times 10^{-6}(100 - \%\mathrm{Dod})^6) \end{aligned}$$

Thus, a plot of $\log(\%\mathrm{Dod}/4.6656 \times 10^{-6}\ (100 - \%\mathrm{Dod})^6)$ with respect to log[Max] will yield a straight line with a slope of 5. When $\log(\%\mathrm{Dod}/4.6656 \times 10^{-6}\ (100 - \%\mathrm{Dod})^6) = 0$, $k_d = [\mathrm{max}]^5$.

Isothermal titration calorimetric analysis

Calorimetric assays were carried out using VP-ITC instruments (MicroCal Inc., USA) as described by Todd *et al.* [22]. Reaction cells (1.4428 mL) were filled with degassed solutions and equilibrated at 37 °C. Stirring speed and reference power was 310 r.p.m. and 15 $\mu\mathrm{Cal}\cdot\mathrm{s}^{-1}$, respectively. Once thermal equilibrium was reached, CDase I-5 (2.5 nM) incubated with increasing amount of β-CD was injected every 3 min and a decrease in instrumental thermal power was observed following each injection. The change in instrumental thermal power after an injection was completed in several minutes. Data collection at each substrate concentration was truncated in 3 min and another injection was made. The thermal power obtained was averaged for 30 s prior to the subsequent injection to obtain the most accurate power measurements. These rates were corrected for ΔH_{app} and the data were fitted to the Michaelis-Menten equation using nonlinear least-square regression to give the kinetic constants.

Fluorescence emission spectrophotometry

The protein fluorescence emission spectrum was monitored at 25 °C in an F-4500 fluorescence spectrophotometer (Hitachi Ltd, Tokyo, Japan) using a 1-cm path length quartz cuvette. The enzyme solutions were prepared in 50 mM sodium phosphate buffer (pH 7.0). When the final protein concentration was 15 μM, intrinsic fluorescence measurement of the protein solution was carried out with excitation at 295 nm and emission scanning in the range of 290–450 nm according to the KCl concentration [9]. The excitation and emission bandwidths were 5 nm, and the scan speed was 1200 $\mathrm{nm}\cdot\mathrm{min}^{-1}$.

CD measurements

CD spectra of CDase I-5 in different concentrations of KCl were obtained using a Jasco J-715 spectropolarimeter (Jasco Inc., Tokyo, Japan). The secondary structure of the enzyme at a concentration of 200 $\mu\mathrm{g}\cdot\mathrm{mL}^{-1}$ in 300 μL of 50 mM sodium phosphate buffer (pH 7.0) was determined by CD spectroscopy in the far-UV spectral region (190–250 nm) using a cell with a 0.1 cm path length, at 25 °C [23]. The width of the spectral band was 2 nm, and the time constant was 2 s at 25 °C. The data were expressed as molar ellipticity, θ (mdeg). The ellipticity at 222 nm was examined to calculate the α-helix content by the method of Chen [24].

Stopped-flow spectrophotometry

Exposed tryptophan residues were detected by measuring the amount of FRET using an SFM-4 stopped-flow apparatus (Bio-Logic, Claix, France) [25,26]. Stopped-flow experiments were carried out mixing two or more solutions rapidly and making the mixture reach an optical observation point as quickly as possible. A photomultiplier tube cut-off filter (324 nm) and an FC-20 cuvette were used. The voltage limit in ADC was ± 10. All experiments were carried out in 50 mM sodium phosphate buffer (pH 7.0) at 25 °C. The enzyme concentration and the volume after mixing were 50 μM and 320 μL, respectively.

Guggenheim plot method

The Guggenheim method was used to compare the shape of the exponential curve at one time (t) with that at another time (t + Δt). 'A' is the amount of reactant at time t;

ΔA is the amount of A at time (t + Δt); and Δt is a constant and arbitrary time interval that was approximately 1/3–1/2 of the period over which the reaction was studied.

Given that A – ΔA = (constant) exp(– *k*t) and taking logarithms to base e on each side produces the following:

$$\ln(A - \Delta A) = (\text{constant}) - kt, \text{ or } \ln(A - \Delta A) = -kt + \text{constant}.$$

Therefore, the graph of ln(A – ΔA) versus time (t) yields a straight line with a slope equal to $-k$.

For a pseudo-first-order reaction, a plot of logA vs. time should be linear with a slope of – *k*t/2.303 based on the following equation:

$$\log \Delta A = (-k/2.303)t + \text{constant},$$

where k is the pseudo-first-order rate constant. Thus, rate constants can be calculated from the gradient of the plot [10].

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