Crystal Structure of the Protease Domain of a Heat-shock Protein HtrA from Thermotoga maritima*

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HtrA (high temperature requirement A), a periplasmic heat-shock protein, functions as a molecular chaperone at low temperatures, and its proteolytic activity is turned on at elevated temperatures. To investigate the mechanism of functional switch to protease, we determined the crystal structure of the NH2-terminal protease domain (PD) of HtrA from Thermotoga maritima, which was shown to retain both proteolytic and chaperone-like activities. Three subunits of HtrA PD compose a trimer, and multimerization architecture is similar to that found in the crystal structures of intact HtrA hexamer from Escherichia coli and human HtrA2 trimer. HtrA PD shares the same fold with chymotrypsin-like serine proteases, but it contains an additional lid that blocks access of the substrates to the active site. A corresponding lid found in E. coli HtrA is a long loop that also blocks the active site of another subunit. These results suggest that the activation of the proteolytic function of HtrA at elevated temperatures might occur by a conformational change, which includes the opening of the helical lid to expose the active site and subsequent rearrangement of a catalytic triad and an oxyanion hole.

Protein quality control, which is essential for cell viability, is tightly controlled by proteases and molecular chaperones (1). Especially under stress conditions such as high temperature, heat-shock proteins, which are mostly proteases or molecular chaperones, are induced to protect cells from toxic denatured proteins (2). Molecular chaperones bind to the hydrophobic patches on denatured proteins to prevent further aggregation and help them to fold back into their native states (3). The regulatory subunits of heat-shock proteases (4) recognize the hydrophobic surface on unfolded proteins and eliminate them (5). High temperature requirement A (HtrA, also called DegP or protease Ds) is a heat-shock protease localized in the periplasmic space of bacteria (6). It shows an ATP-independent proteolytic activity and plays an important role in the degradation of misfolded proteins accumulated by heat shock or other stresses (7). Therefore, its activity seems to be essential for bacterial thermostolerance and for cell survival at high temperatures (7). HtrA is also involved in pathogenesis of Gram-negative and Gram-positive bacteria by degrading damaged proteins that are produced by reactive oxygen species released from the host defense system (8). Therefore, HtrA is considered as a target for development of broad-spectrum antibiotics (8).

In addition to proteolytic activity, HtrA is known to have a molecular chaperone activity (9, 10). The chaperone function is dominant at low temperatures, whereas the proteolytic activity is turned on at elevated temperatures (9). This temperature-dependent functional switch is necessary for controlling protein stability as well as eliminating denatured proteins to maintain cellular viability (9). HtrA is a highly conserved protein found in species ranging from bacteria to humans. Two known human homologues of bacterial HtrA (HtrA1 and HtrA2) are also expected to be involved in mammalian stress response pathways (11, 12). However, because HtrA2 showed proteolytic activity even at room temperature, temperature-dependent activation of proteolytic activity seems to be absent from mammalian HtrAs (13).

HtrA is a serine protease with a catalytic triad in its active site. Recent crystal structure analyses revealed that Escherichia coli HtrA forms a hexameric complex composed of two trimers (14) and human HtrA2 forms a homotrimer (15). Each subunit is composed of one protease domain at the amino terminus and one or two PDZ (named after three proteins, PSD-95, Discs-large, and ZO-1) domains at the carboxyl terminus. The protease domain of E. coli HtrA fully retains the molecular chaperone activity, although the proteolytic activity is absent (9). The PDZ domains, also found in the Clp/Hsp100 family of heat-shock proteins, are known to play a role in substrate recognition (16). In the crystal structures of E. coli HtrA and human HtrA2, PDZ domains are proposed to mediate the initial binding of substrates (14) or to be involved in modulation of protease activity (15). However, PDZ domains do not participate in multimerization in both E. coli and human HtrAs (14, 15). Unlike other proteases of the Clp/Hsp100 family, HtrA does not have a regulatory component or an ATP binding domain because it is an ATP-independent heat-shock protease.

So far two crystal structures of HtrAs have been reported (14, 15), and they seem to differ in structural architecture, multimerization, and activation mechanism. For a better understanding of the dual role of HtrA and the activation mechanism of the proteolytic function, we have solved the crystal structure of the protease domain (PD, residues 24–262, Fig. 1) of Thermotoga maritima HtrA (Tm HtrA), which displays both...
molecular chaperone and proteolytic activities. The crystal structure indicates that the rearrangement of the active site of bacterial HtrA is necessary for the proteolytic activity and that oligomerization architecture of HtrA might vary depending on the presence of the lid covering the active site.

EXPERIMENTAL PROCEDURES

Protein Preparation and Crystallization—The protease domain of HtrA from T. maritima (PD, residues 24–262, Fig. 1) was cloned, purified, and crystallized as described elsewhere (18). The putative signal sequence residues (1–23) were deleted in the construct. For the translational start, a methionine residue was added in front of Asp24. Intact HtrA was also prepared in the same way as HtrA PD (18). HtrA PD was crystallized in the cubic space group P213, with the unit cell parameters a = b = c = 120.55 Å by the hanging drop vapor diffusion method at 22 °C from a reservoir solution containing 100 mM phosphate-citrate (pH 4.4), 110 mM Li2SO4, and 5% (v/v) PEG 1000 (18). There are two molecules in an asymmetric unit.

Light-scattering Measurement—The chaperone-like activities of HtrA and HtrA PD were measured as described previously (19) with some modifications using pig heart citrate synthase (CS) as the substrate (Sigma). CS (final 65 µM) was prepared by incubating 3 mg/ml protein in 5 mM Tris (pH 7.5) containing 10 mM dithiothreitol at 4 °C for 2 days. HtrA and HtrA PD were preincubated in the reaction buffer containing 5 mM Tris (pH 7.5) and 1.5 mM dithiothreitol at 25, 45, 65, and 85 °C. The reduced form of α-lactalbumin (Sigma) and 26 µg of HtrA PD or 45 µg of HtrA (at 1:2 molar ratio of protease to substrate) were incubated in 60 µl of reaction buffer containing 5 mM Tris (pH 7.5) and 1.5 mM dithiothreitol at 25, 45, 65, and 85 °C. The reduced form of α-lactalbumin was prepared by incubating 3 mg/ml protein in 5 mM Tris (pH 7.5) containing 10 mM dithiothreitol at 4 °C for 2 days. HtrA and HtrA PD were preincubated at each indicated temperature prior to the addition of the substrate protein. The reaction was performed in 100-µl tubes to minimize the evaporation effect. After incubation for 30 min at each temperature, 17 µl of 5X SDS-PAGE sample buffer was added to stop the reaction. Then, the samples were analyzed by 17% SDS-PAGE.

Structure Determination and Refinement—Multiwavelength anomalous diffraction data were collected from a frozen crystal of HtrA PD at the Pohang Accelerator Laboratory beamline 8B with a Mar345 203B area detector. Data collected at three wavelengths (edge, peak, and remote) were processed and integrated by DENZO and scaled by SCALEPACK using the HKL program suite (20). Native data of HtrA PD were also collected at the Pohang Accelerator Laboratory and processed using the HKL program suite (Table I). Two selenium sites (one methionine in one subunit) of PD were found and used for phase calculation in the program SOLVE (21). A relatively low figure of merit (0.25) is explained by the presence of only one selenium atom per 238 residues in the subunit.

Solvent flattening and 2-fold noncrystallographic symmetry (NCS) averaged by RESOLVE (22) resulted in a high quality electron density map sufficient for model building. Amino acids were assigned using the program O (23). Several cycles of rigid body refinement, positional refinement, and simulated annealing were performed at 3.0-Å resolution with CNS (24). The refinements were continued at 2.8-Å resolution using the data collected from the native HtrA PD. Successive refinement with temperature factors and addition of solvents resulted in an R-value of 22.2% and an Rfree value of 28.4%, with a bulk solvent correction and overall anisotropic thermal factor refinement. Rfree was calculated with 10% of the reflections. NCS restraints were enforced during the refinement except flexible regions (LA and L2), in which two subunits in an asymmetric unit showed different conformations.

The final model includes residues 24–48 and 51–251 and 58 water molecules (Table I). Structural evaluation of the refined model using PROCHECK (25) reveals that the structure has good geometric parameters (Table I), and no residue falls in the disallowed region of the Ramachandran plot. Statistical analysis of B-factor distribution was performed by t test and the Wilcoxon rank sum test. p < 0.001 was considered to be significant. The figures in the article were drawn using the programs MOLSCRIPT (26) and GRASP (27). The final coordinates and structure factors have been deposited in the Protein Data Bank (PDB; accession number 1LLJ).

RESULTS

Biochemical Activities of Tm HtrA and Tm HtrA PD—Tm HtrA and Tm HtrA PD were overexpressed in E. coli and purified. The chaperone-like activities of both Tm HtrA and Tm HtrA PD were measured by their abilities to suppress the aggregation of CS, which has been widely used for molecular chaperone assays (19). The aggregation of CS was monitored by light scattering at 320 nm after chemically denatured CS was diluted in the refolding buffer. Tm HtrA or Tm HtrA PD suppressed CS aggregation, decreasing the initial velocity of aggregate formation (Fig. 2, A and B). By addition of 2- and 4-fold molar excesses of Tm HtrA to CS, the initial velocities of aggregation were decreased to 40.6 and 13.7%, respectively, compared with the initial velocity in the absence of HtrA or HtrA PD. It was decreased to 68.3% when a 4-fold molar excess of Tm HtrA PD was added, whereas 2-fold addition of Tm HtrA PD essentially did not change it (data not shown). An 8-fold excess of either Tm HtrA or Tm HtrA PD was enough to decrease the aggregation rate to about zero. Under the same assay conditions, the initial velocity of the reaction is not de-
creased when bovine serum albumin was used for the control protein (Fig. 2, A and B). These results indicate that Tm HtrA PD as well as the intact Tm HtrA have the chaperone-like activity to inhibit the aggregation of CS. However, because the enzyme activity of CS was not recovered (data not shown), it seems that Tm HtrA does not assist the refolding of CS, although the aggregation of denatured CS was completely suppressed by incubation with Tm HtrA. It can be inferred that Tm HtrA exhibited only chaperone-like activity against CS, as is observed for other heat-shock proteins such as α-crystallin (28).

The molar ratios of chaperones to substrates required for suppression of CS aggregation are different between intact HtrA and HtrA PD (Fig. 2), which is also observed for E. coli HtrA and E. coli HtrA PD. In the case of E. coli HtrA, a higher concentration of the protease domain was required for refolding of MalS protein than intact HtrA (9). Such a requirement for higher molar ratios of HtrA PD might be explained by the absence of PDZ domains, which are known to be involved in substrate recognition (14–16).

α-Lactalbumin, which is commonly used for the assays of heat-shock proteases such as 20 S proteosome (29) and E. coli HtrA (30), was employed as a substrate to investigate the proteolytic activity of Tm HtrA. Clearly, Tm HtrA displayed the proteolytic activity at elevated temperatures, and maximal activity was observed at 85 °C (Fig. 2C). As reported for E. coli HtrA (9, 31), the proteolytic activity of Tm HtrA increased with temperature, and Tm HtrA is autodegraded at high temperatures (Fig. 2C).
Compared with Tm HtrA, Tm HtrA PD showed a relatively weak proteolytic activity, although its activity also increased with temperature. The degradation products of Tm HtrA PD were long enough to be visible on 17% SDS-PAGE, whereas intact Tm HtrA completely degraded the substrate into short peptides that are not shown in the gel (Fig. 2C). Such a weak proteolytic activity of Tm HtrA can also be explained by the absence of a PDZ domain. In both *E. coli* HtrA and human HtrA2, PDZ domains play key roles in substrate binding and formation of the chamber near the active site (14, 15). Tm HtrA PD generates longer degradation products, because substrates are freely released from Tm HtrA PD after cleavage. In contrast, within the chamber of intact Tm HtrA, substrates are cleaved into small peptides simultaneously at adjacent active sites.

*Overall Structure of Tm HtrA PD*—The crystal structure of Tm HtrA PD (residues 24–262) has been solved by multiwavelength anomalous diffraction at 3.0 Å resolution and refined to 2.8 Å resolution (Table I). The experimental electron density map calculated with multiwavelength anomalous diffraction phases and improved by solvent flattening and NCS averaging was of sufficient quality to locate most main chains and some side chains. Tm HtrA PD is composed of two β-barrel domains connected by a long loop between β6 and β7 (Figs. 3 and 4A). Residues in the catalytic triad, Asp127-His97-Ser206, are located in the cleft of two β-barrels. The structural comparison by DALI server (32) reveals that its topology is similar to proteases in the chymotrypsin family (33). Among the members of the chymotrypsin family, *γ*-lytic protease (PDB accession number 1qq4; Ref. 34) can be superimposed on Tm HtrA PD with the lowest r.m.s.d. of 2.5 Å for 161 Cα atoms of 198 Cα atoms of *γ*-lytic protease (Figs. 4B and 5). However, its fold is different from those of proteolytic cores of ATP-dependent proteases such as ClpP (35) and HslV (36).

It is notable, however, that several structural differences exist between Tm HtrA PD and *γ*-lytic protease. The most significant of them is the length of LA (loop A connecting β1 and β2, according to the nomenclature in Ref. 33) in two proteins (Figs. 4 and 5). LA of Tm HtrA (residues 47–76), including an amphipathic helical lid (α2, residues 55–66) and α3, is located on top of the catalytic residue Ser206 (Figs. 4A and 6A). Interestingly, the residues corresponding to the helical lid of Tm HtrA PD are found only in bacterial HtrAs, not in human homologues (Fig. 1), and this lid is expected to have the impor-

| Data collection and phasing | PD | PD-SeMet |
|-----------------------------|----|----------|
| Wavelength (Å)              | 0.9717 | 0.9791 |
| Resolution (Å)              | 20.0–2.8 (2.9–2.8) | 30.0–2.0 (3.11–3.30) |
| Complementary (%)*          | 86.0 (27.0) | 8.0 (32.5) |
| No. of unique reflections   | 13,843 (1376) | 10,379 (1047) |
| Redundancy                  | 2.43 | 3.20 |
| Rmerge (%)                  | 0.25 | 15.4 |

Overall figure of merit
Before density modification
After density modification

| Refinement                  | PD-SeMet |
|-----------------------------|----------|
| Resolution (Å)              | 20.0–2.8 |
| Rmerge (%)                  | 0.25 |
| Mean B factors (Å²)         | 54.8 |
| r.m.s.d. in bonds (Å)       | 0.008 |
| r.m.s.d. in angles (°)      | 1.53 |

* Rmerge = Σ|Fo| - <Fo>|ΣFo|

* Rmerge = Σ|Fo| - <Fo>|ΣFo|/|Fo|

**Fig. 3. Structure of Tm HtrA PD.** A stereo Cα trace of Tm HtrA PD. Every 20th residue and NH$_2$- and COOH-terminal residues are indicated as black balls and labeled. Overall structure of Tm HtrA PD is similar to the structure of proteases in the chymotrypsin family.
tant functional or structural roles in bacterial HtrAs. There are differences in several other loops connecting \( \beta \)-strands (Fig. 5). Among them, structural changes near the loop containing the putative oxyanion hole and the catalytic residue Ser-206 (residues 202–206) seem to be significant for explaining the functional differences of Tm HtrA and \( \alpha \)-lytic protease (Figs. 5 and 6). Three conserved disulfide bonds found in almost all members of the chymotrypsin family of serine proteases are absent from all known HtrAs (Fig. 4).

**Tm HtrA Trimer and Structural Comparison with Other HtrAs**—Overall structure of Tm HtrA PD turns out to be similar with the protease domains of \( E. coli \) HtrA and human HtrA2 with an r.m.s.d. of 2.2 Å for 179 Ca atoms of 215 Ca atoms of \( E. coli \) HtrA and 1.9 Å for 181 Ca atoms of 196 Ca atoms of human HtrA2, respectively (Figs. 4 and 5). The large r.m.s.d. among HtrAs are mostly caused by the structural difference near the active site and loop LA (Fig. 5). Interestingly, Tm HtrA shows more structural resemblance with human HtrA than \( E. coli \) HtrA.

Trimeric interactions found in \( E. coli \) HtrA hexamer or human HtrA2 trimer seem to be conserved in Tm HtrA PD (Fig. 5). A stereo Ca trace of Tm HtrA PD (green), \( E. coli \) HtrA PD (blue), human HtrA2 PD (red), and \( \alpha \)-lytic protease (magenta). Three residues of the catalytic triad of Tm HtrA PD are drawn as black stick models. Most structures in the core region containing the central \( \beta \)-barrel and \( \alpha \)-helices are well conserved in the four proteins, whereas loop regions display differences. The loop LA of each HtrA, where a large conformational movement is observed, is labeled and emphasized by a thicker line.
7), in which three subunits of Tm HtrA PD related by 3-fold crystallographic rotation symmetry are tightly packed by hydrophobic interactions. A hydrophobic patch composed of the residues near H9251, H9252, and H92511 is involved in the hydrophobic packing in a trimer (Figs. 4 and 7A). Those hydrophobic residues are quite well conserved in most other HtrAs (Fig. 1), implying that hydrophobic packing in a trimer is a general feature of HtrAs. By trimerization, the $6700\,\text{Å}^2$ surface area of Tm HtrA PD is buried, which is comparable with the $6044\,\text{Å}^2$ in human HtrA2 (15). Taken together, it appears that the Tm HtrA forms a trimer by hydrophobic interaction mediated by the protease domain, as observed in $E.\ coli$ HtrA and human HtrA2 (14, 15). Analytical ultracentrifugation and gel filtration experiments also support the existence of Tm HtrA PD as a trimer in solution (data not shown).

The main differences among HtrAs might be the size and conformation of LA (Figs. 1, 4, and 5). $E.\ coli$ HtrA has a long loop reaching the active site of the opposite subunit (Figs. 4C, 6C, and 7B). In addition, $\beta_1$ and $\beta_2$ in $E.\ coli$ HtrA is long enough to make a $\beta$-sheet with two other $\beta$-strands from the trimer in the other side, leading to a hexamer structure (Fig. 7B) (14). In contrast, LA in Tm HtrA PD is mainly composed of a helix ($\alpha_2$) covering the active site of the same subunit in the current structure (Figs. 4A, 6A, and 7A) and is shorter than its...
Fig. 7. Trimeric packing of Tm HtrA PD and comparison with other HtrA PDs. Bottom and side views of Tm HtrA PD (A), E. coli HtrA PD (B), and human HtrA2 PD (C) trimers were drawn in ribbon diagrams with the same color schemes used in Fig. 4. LA, LA*, β1, β2, and NH2- and COOH-terminal residues in one subunit are labeled. In E. coli HtrA PD trimer, three LA loops (LA*) protruding into the active sites from the opposite trimer are drawn. The NH2-terminal helix and COOH-terminal barrel domain are involved in trimer formation by hydrophobic packing. LA and neighboring strands, β1 and β2, show the most different conformations in three HtrAs. D, surface charge distribution of Tm HtrA PD trimer. The red and blue areas represent negatively and positively charged surfaces, respectively. White patch, representing the hydrophobic surface, is not found, implying the substrate binding region necessary for chaperone activity is not observed in the current structure. Ser206 residues are not visible in this figure because they are buried by LA. However, their positions are indicated by black arrowheads.
Crystal Structure of the *T. maritima* HtrA Protease Domain

**TABLE II**

|                  | N^O(His)-O^2 (Ser) | N^O(His)-O^2 (Ser or Ala) | N^O(His)-OD1 (Asp) |
|------------------|---------------------|--------------------------|-------------------|
| Tm HtrA PD1      | 3.40                | 4.03                     | 2.75              |
| Tm HtrA PD2      | 3.69                | 4.69                     | 2.64              |
| *E. coli* HtrA   | 5.67                | 3.41                     |                  |
| Human HtrA       | 4.20                | 2.84                     |                  |
| α-Lytic protease | 2.96                | 3.29                     | 2.80              |

The counterpart in *E. coli* HtrA (Figs. 1 and 7). Interestingly, human HtrA2 has a very short LA, which is not involved in the dimerization of trimers or the covering of the active site (Figs. 4D, 6D, and 7C).

Two molecules of HtrA PD in an asymmetric unit related by 2-fold NCS are associated by the minimal hydrophobic interactions among a few residues (Tyr^25^, Pro^28^, Val^32^, and Ala^35^; figure not shown) in the NH2-terminal helix (a1). Therefore, the presence of two molecules in the asymmetric unit seems to have no biological relevance. The two molecules in the asymmetric unit show identical conformations except the regions near LA and L2 (loop 2 connecting β11 and β12), suggesting those regions are relatively flexible.

**Hydrophobic Patches on the Surface of HtrA PD**—Most molecular chaperones have hydrophobic substrate binding sites on their surfaces to recognize and bind to the exposed hydrophobic patches of substrates (3). However, in Tm HtrA PD trimer, most of the hydrophobic surface near the active site is buried and no noticeable hydrophobic region is exposed (Fig. 7D). Therefore, certain conformational changes might occur to expose the hydrophobic substrate binding site when Tm HtrA or Tm HtrA PD shows the chaperone-like activity.

**Proteolytic Active Site of HtrA PD**—Most hydrophobic residues in the helical lid of LA form wide contacts by hydrophobic interactions with Leu^69^ in β2, Pro^103^ and Leu^10^ in LD (loop D connecting β7 and β8), Pro^203^ and Gly^204^ in L1 (loop 1 connecting β9 and β10), and Ala^223^ and Ile^224^ in L2 (Fig. 6A). Because LA^a^ of *E. coli* HtrA makes intimate contact with L1 and L2 (the asterisk denotes the loops in the neighboring subunit, see Figs. 4C, 6C, and 7B; Ref. 14), the hydrophobic interactions between the loops near the active site and lid seem to be common in bacterial HtrAs. However, in other HtrAs residues interacting with LA appear to vary depending on the size of the lid (Fig. 1).

Possible substrate binding sites of Tm HtrA (S3, S2, S1, S1′, S2′, and S3′) defined in Ref. 33) are completely blocked by the lid and inaccessible to the solvent (Fig. 6A). Because the average temperature factor of the residues in LA is relatively higher (64.9 Å) than that for the whole protein (54.8 Å), it appears that the lid is rather flexible and its interaction with the loops is not tight. B-factor difference between LA and other regions is significant (p < 0.001). Flexibility of the lid is also inferred from different conformations of the lids of two molecules in an asymmetric unit. When the molecules in the asymmetric unit are superposed, 28 Ca atoms in LA (residues 47, 48, and 51–76) give a r.m.s.d. of 1.52 Å, whereas other Ca atoms (except another flexible region at residues 225–232) give 0.45 Å (Fig. 5). We also suspect that residues 49 and 50 do not show electron density because of the flexibility of LA. Structural flexibility of LA found both in Tm and *E. coli* HtrAs (14) suggests that this loop could undergo a conformational change in bacterial HtrAs.

In addition to the fact that the active site is blocked by the helical lid, several residues that are essential for proteolytic activity are positioned differently from those in the α-lytic protease (Fig. 6, A and B). For hydrolytic cleavage of a peptide bond, the residues in the catalytic triad need to be aligned close enough for electron transfer from Asp to Ser through His. However, in the current crystal structure of Tm HtrA PD, distances between the N^2^ atom of His^97^ and the O^1^ atom of Ser^206^ of each molecule in the asymmetric unit are 3.69 and 3.40 Å (Fig. 6A, Table II), whereas the N^2^ atom of His^96^ and the O^1^ atom of Ser^143^ in the α-lytic protease are hydrogen-bonded at a distance of 2.96 Å (Fig. 6B, Table II). Consequently, His^97^ is not expected to function as a general base to remove the proton from Ser^206^. In the crystal structures of *E. coli* and human HtrAs, the distance between the N^2^ atom of His and the O^1^ atom of Ser in the catalytic triad cannot be measured because Ser mutated to Ala (Fig. 6, C and D) (14, 15). However, the distance between the N^2^ atom of His and the O^1^ atom of the mutated Ala in catalytic triads of *E. coli* HtrAs is too long compared with the corresponding distance in the α-lytic protease (Fig. 6, Table II), although a little conformational change in the Ala mutant is expected. Therefore, it is obvious that both Tm and *E. coli* HtrAs are inactive because of the distortion of the loops near the active site, whereas α-lytic protease is in an active state.

Another crucial factor for the proteolysis by chymotrypsin-like proteases is the stabilization of a negative charge of carbonyl oxygen on the reaction intermediate (oxyanion hole) by hydrogen bonds from the amide nitrogen atoms of two peptide bonds in the backbone. In Tm HtrA PD, nitrogen atoms of Ser^206^ and Gly^204^ are assumed to be the hydrogen donors to the putative oxyanion hole. However, the NH group of Gly^204^ in Tm HtrA PD and its counterpart in the α-lytic protease (the NH group of Gly^143^) point to opposite directions (Fig. 6, A and B). In this conformation it is impossible for Tm HtrA PD to form an oxyanion hole. This unique conformation of the loop near the oxyanion hole is also found in two other HtrAs (Fig. 6, C and D). Similarly, triacylglycerol lipase and *Staphylococcus aureus* epidermolytic toxin A do not have pre-formed oxyanion holes (37, 38), leading to inactive states, as seen in HtrA.

**DISCUSSION**

Considering the helical lid, catalytic triad, and oxyanion hole in the crystal structure of Tm HtrA PD, it can be referred that the current crystal structure determined at room temperature represents an inactive conformation of Tm HtrA PD (Figs. 4A and 6A). This is also true in *E. coli* HtrA, in which the distortion of the active site loops and the intervening LA are assumed to prevent the proper position of the catalytic triad and the formation of an oxyanion hole, resulting in an inactive conformation of the protease domain (Figs. 4C and 6C). However, because Tm HtrA PD shows protease activity at high temperatures (Fig. 2C), a structural change should occur to activate its proteolytic activity. LA appears to be flexible because of the high temperature factors in the crystal structure and different conformations in two molecules in the asymmetric unit; therefore, it is tempting to propose that LA becomes more flexible and flips up at elevated temperatures. By these plausible conformational changes, Tm HtrA becomes ready for proteolysis by exposing the substrate binding site and rearranging the residues in the active site. A similar temperature-dependent activation mechanism involving conformational changes was suggested based on the biochemical properties of *E. coli* HtrA (9). In addition, a conformational change of the loop covering the catalytic site of HtrA is also expected from the
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In support of our assumptions, an infrared spectroscopic study (31) and 1-anilino-8-naphthalenesulfonate binding experiment (30) of E. coli HtrA suggest that a conformational change and exposure of the hydrophobic region could occur when HtrA is activated. In human HtrA, which does not have the helical lid (Figs. 1 and 6D), proteolytic activity is evident even at room temperatures because of the exposed active site (13, 15). However, human HtrA might also experience some conformational changes near the active site by binding to the substrate, because its active site also seems to be imperfection formed in the crystal structure (Fig. 6D) (15). The proposed activation mechanism of HtrA is reminiscent of that found in lipase, whose hydrolytic action is achieved by activation at an oil-water interface (37). Although triggered by different factors, both lipase and HtrA may undergo quite similar conformational changes: the opening of the lid and rearrangement of the hydrophobic groove necessary for chaperone function is exposed in E. coli HtrA PD (Fig. 7D). To display the chaperone-like activity seen for the chaperone activity of Tm HtrA PD, because no remarkable substrate binding sites are found on the surface of Tm HtrA PD (Fig. 7D). To display the chaperone-like activity seen under the experimental conditions (Fig. 2A), we propose that a hydrophobic groove necessary for chaperone function is exposed by certain conformational changes. The most important finding in the crystal structure of the protease domain of Tm HtrA is the presence of the helical lid covering the active site; this lid is expected to open at high temperatures for proteolytic action. However, we cannot rule out the possibility that LA may not have the same conformation in intact HtrA as the current crystal structure of protease domain, in which LA extends to the opposite subunit instead of covering its own active site and contributes to the formation of hexamer in solution. Similarly, in E. coli HtrA, LA* blocks the active site of the subunit in the opposite trimer (Figs. 6C and 7B) (14). Considering the common hydrophobic patches composed of the loops near the active site in bacterial HtrAs, however, the active site of Tm HtrA could be blocked by either LA in the same subunit or LA* in the other subunit, and the active site is moved away for proteolytic activity. Therefore, a temperature-dependent activation mechanism might be a general feature of bacterial HtrAs. To explore this possible conformational change of LA and to confirm the proposed activation, the crystal structure of intact Tm HtrA and its biochemical character-ization will be required.

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