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Temperature Dependent Protease Activity and Structural Properties of Human HtrA2 Protease

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Abstract—Human HtrA2 belongs to a new class of oligomeric serine protease, members of which are found in most organisms. Mature HtrA2 is released from mitochondria into the cytosol in response to apoptotic stimuli. In this report, the effect of temperature on proteolytic activity of HtrA2 and related structural properties were investigated. In the range from 25 to 55°C, the proteolytic activity of HtrA2 rapidly increased with temperature, and it drastically decreased at and over 60°C. Structural analysis using far-UV CD spectroscopy and gel filtration revealed no significant change in the secondary structure of HtrA2 from 25 to 70°C, or in the oligomeric size between 25 and 55°C. However, a significant change at the tertiary level, as examined using near-UV CD, was observed for HtrA2 in the range from 25 to 60°C. Differential scanning calorimetry indicated that HtrA2 exhibits a thermal transition beginning at around 61°C. The fluorescence intensity of ANS interacting with HtrA2 decreased with increasing temperature. HtrA2 was found to be able to complement DegP function at 44°C, indicating that HtrA2 could have protective functions in mitochondria.

Key words: HtrA2, protease, temperature, proteolytic activity, structural change

Almost all the cells in living organisms have to face problems caused by protein folding. A properly folded state is very important for a protein to carry out its function, because misfolded or damaged proteins can pose a serious hazard to the cells. Cells therefore have evolved a complex system to deal with this unfavorable condition. Molecular chaperones and proteases monitor the folding states through recognizing hydrophobic surfaces exposed by misfolded or damaged proteins. Molecular chaperones promote proper protein folding and prevent aggregation, while proteases eliminate irreversibly damaged proteins [1]. Among all the quality control factors for proteins, HtrA represents a new class of oligomeric serine proteases. The structural feature of HtrA family is that each member has a trypsin-like catalytic domain with at least one C-terminal PDZ domain [2]. The abbreviation PDZ derived from three eukaryotic proteins, Post-synaptic density protein, Disc large, and Zonula occludens [3].

Human serine protease HtrA2, also known as Omi, was initially identified as a stress-activated protease [3, 4]. It is expressed ubiquitously and the amount of the protein increases when cells are exposed to heat shock or treated with tunicamycin. Recent studies have shown that a truncated form of HtrA2 can play regulatory roles in apoptosis [5-9]. HtrA2 is synthesized as a 49-kD precursor carrying an amino terminal mitochondrial localization signal. After the full-length protein is imported into the mitochondria, the N-terminal 133 residues containing the transmembrane segment are removed, generating the mature 37-kD protein. Mature HtrA2 is released from the mitochondria to cytoplasm after an apoptotic stimulus. In the cytoplasm, HtrA2 interacts with XIAP and relieves its inhibition of caspase-9. However, HtrA2 can also promote apoptosis in a caspase independent manner as long as it retains protease activity. More recently, two papers have reported that IAPs are substrates for HtrA2 and their degradation could be a mechanism by which HtrA2 activates caspases and induces cell death [10, 11].

HtrA2 has extensive similarity to bacterial DegP/HtrA (High temperature requirement A) protease [3, 12], which is indispensable for cell survival at elevated temperatures [13]. DegP is a peripheral membrane protein localized on the periplasmic side of inner membrane [14]. It functions as a protease at high temperatures and as a chaperone at normal temperature [15]. The physiological role of DegP is to degrade and remove denatured

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or damaged proteins in the cellular envelope during heat shock and other stresses. Whether human HtrA2 has similar function is not clear although it is upregulated during heat shock and endoplasmic reticulum stress [4]. In this study, we systematically investigated the effects of temperature on the protease activity and the corresponding conformational changes of mature HtrA2. We also investigated the capacity of HtrA2 to suppress thermal sensitivity of the bacterial DegP/HtrA null mutant, and its physiological implication is discussed.

MATERIALS AND METHODS

Materials. 8-Anilinonaphthalene-1-sulfonate (ANS) and Ni-NTA agarose were purchased from Sigma (USA). Resorufin-labeled casein was obtained from Boehringer Mannheim (Germany). All other chemical reagents were of analytically pure grade.

Construction of mature and mutant HtrA2 plasmid. Complementary DNA fragments encoding human full-length HtrA2 was a generous gift from Dr. Changzhen Liu (Tsinghua University, China). A DNA fragment of 985-bp corresponding to amino acids 134 to 458 of human HtrA2 was amplified by polymerase chain reaction (PCR) using the full-length human HtrA2 cDNA as a template. To generate the enzyme with a N-terminal polyhistidine tag, a PCR reaction was conducted using the following two primers: forward (GTCCTCGCCCATATGGCCGTCCCT), and reverse (ATTACCGCTCGAGTCATTCTGTGACCTCA), with *Nde*I and *Xho*I restriction sites (underlined) incorporated, respectively. The PCR products were digested with *Nde*I and *Xho*I restriction enzymes and then were ligated into pET-15b predigested with the above-mentioned two enzymes. To construct mutant HtrA2S306A in which active site Ser306 was changed to alanine, overlap PCR was performed. The PCR products were ligated into pET-15b. All the resultant plasmids were verified by DNA sequencing.

Expression and purification of mature HtrA2 from *E. coli*. Protein expression from pET-15b carrying mature HtrA2 was performed following essentially the procedure described by Savopoulos et al. [16]. Cells were grown at 37°C in Luria–Bertani (LB) medium (plus 50 μg/ml of ampicillin) and then induced (at A_{600} of 0.8-1.0) with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside for 18 h at 20°C. After centrifugation at 5000 rpm for 15 min, the cell pellet was resuspended in buffer containing 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 20 mM imidazole, and 1 μg/ml pepstatin A. The cells were disrupted by sonication, and then the cell lysate was centrifuged for 50 min at 15,000 rpm. The supernatant was loaded onto a 4-ml Ni-NTA agarose column. The column was then washed with 100 ml buffer containing 10 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 20 mM imidazole. This was followed by the same volume of buffer containing 10 mM Tris-HCl, pH 8.0, 1000 mM NaCl, 20 mM imidazole. The protein concentration of fractions was determined and analyzed by SDS-PAGE.

Proteolytic cleavage assay for HtrA2. The activity of purified HtrA2 was assayed using resorufin-labeled casein. Fifty microliters of resorufin-labeled casein (0.4% in H_2O) were added to 150 μl of incubation buffer (200 mM Tris-HCl, pH 7.8) containing 7 μg of HtrA2. Samples were protected from light and incubated at temperatures between 25 and 70°C. The reaction was stopped by precipitation of casein with 480 μl of 5% trichloroacetic acid. After centrifugation for 5 min at 15,000 rpm, 400 μl of the supernatant was mixed with 600 μl of 0.5 M Tris-HCl (pH 8.8) to determine the absorbance at 574 nm.

Circular dichroism measurements. Far-UV and near-UV circular dichroism (CD) spectra of HtrA2 were measured on a Jasco J-715 (Japan) spectropolarimeter equipped with a constant temperature cell holder. The spectra were recorded using 2-mm path length cells. Protein concentrations of 0.5 and 3 mg/ml were used for far- and near-UV CD spectra measurements, respectively. All the spectra were cumulative averages of 10 repeated scans.

Differential scanning calorimetry. Calorimetric study was performed using a Microcal DSC-III MC-2D (Setaram, France) high-sensitivity differential scanning calorimeter. HtrA2 was performed in 10 mM Tris-HCl buffer (pH 8.0) at a concentration of 3 mg/ml. Calorimetric measurements were recorded from 20 to 80°C at a heating rate of 1°C/min.

Gel filtration. Gel filtration chromatography of HtrA2 was performed in an ACTA FPLC system using a XK 16/70 column packed with Superdex 200 (preparative grade) medium (all from Amersham Pharmacia Biotech, Sweden). The temperature of the chromatography process was controlled by connecting the thermostat jacket of the column to a water bath preheated to the indicated temperature. The protein sample was eluted in 10 mM Tris-HCl (pH 8.0, 0.1 M NaCl) and the column was calibrated at 25°C using high molecular mass standards (Bio-Rad, USA). The volume of 0.5 ml of 3 mg/ml sample was loaded onto the column.

Fluorescence measurements. Fluorescence spectra were measured with a Hitachi F-4000 (Japan) fluorescence spectrophotometer equipped with a constant temperature cell holder. For ANS fluorescence spectra, the excitation wavelength was set at 390 nm and the emission was scanned from 400 to 600 nm. The final concentrations for HtrA2 and ANS were 3.7 and 100 μM, respectively.

Complementation of DegP function at 44°C. Human HtrA2 and HtrA2S306A were amplified respectively by PCR from the plasmid pET-15b (as described above). The PCR products were then subcloned into pUC18 plasmid. The pUC18 derivatives were transformed into *degP* null mutant strain CLC198 (kindly provided by Dr.

Muller, University of Freiburg, Germany), respectively. For measuring growth curves in liquid medium, overnight cultures were diluted 1 : 100 into LB medium containing ampicillin (100 μg/ml) at 44°C, and A_{595} of the culture liquid was measured at various time intervals.

RESULTS

Effect of temperature on HtrA2 proteolytic activity. Previous studies showed that HtrA2 was upregulated in mammalian cells in response to heat-shock induced stress, and its proteolytic activity increased at the heat shock temperature [4]. Here we tested its proteolytic activity over a wide range of temperatures from 25 to 70°C. To monitor the activity, we incubated the enzyme with resorufin-labeled casein as a substrate, and measured the low molecular weight products of proteolysis. The results, presented in Fig. 1, showed that, in the range from 25 to 55°C, the proteolytic activity of HtrA2 rapidly increased with temperature, and it drastically decreased at and above 60°C.

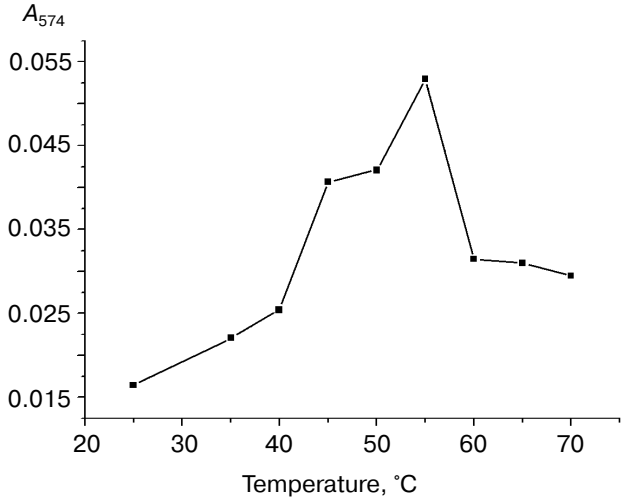

Fig. 1. Effect of temperature on HtrA2 proteolytic activity. Proteolytic activity was estimated as the ability to release resorufin-labeled peptides, soluble in 5% trichloroacetic acid, from resorufin-labeled casein at the temperatures indicated on the graph. The control reaction was without HtrA2 and the control values have been subtracted from the data plotted in the graph.

Effect of temperature on HtrA2 structure. What is the consequence of various temperatures in terms of HtrA2 protein structures? Far-UV CD and near-UV CD spectroscopies were utilized to examine the secondary and tertiary structure changes of heat-treated HtrA2. The far-UV CD spectroscopy results (Fig. 2) revealed that temperatures did not cause significant alteration of HtrA2 at the secondary structure level. HtrA2 was able to retain its secondary structure even at 70°C. The appearance of the spectral minimum at 25°C around 208 nm suggested a predominant presence of α-helix in the protein. Figure 3a shows the near-UV CD spectra of HtrA2 at various temperatures. The variation of the mean residual mass ellipticity at 262 nm with temperature is shown in Fig. 3b. The ellipticity decreased gradually on temperature increase from 25 to 50°C and fell steeply to below zero values by 60°C. This indicated that at 60°C HtrA2 lacked any rigid tertiary structural packing. Thus, our near-UV CD studies showed that HtrA2 lost its tertiary structure with temperature, and the loss is more pronounced at temperatures above 50°C.

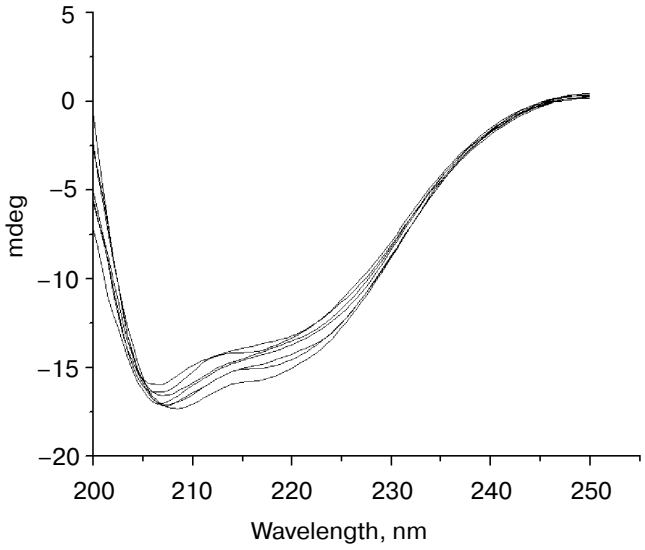

Fig. 2. Far UV-CD spectra of HtrA2. Curves from top to bottom represent the CD spectra of HtrA2 measured at 70, 65, 60, 55, 45, 37, and 25°C, respectively.

Differential scanning calorimetry (DSC) was also used to characterize the conformational transitions of HtrA2. The DSC scan of HtrA2 revealed a sharp thermal transition at 62.4°C with the onset of transition at about 61°C (Fig. 4). The total calorimetric enthalpy was −283 kJ/mol, indicating that the protein began to aggregate at the transition temperature due to protein denaturation. We did not observe any other transitions from 20 to 80°C, so it was very possible that the thermal denaturation and aggregation occurred at almost the same temperature.

Since HtrA2 is a homotrimer in solution, and formation of a homotrimer is a prerequisite for its function [17], it would be important to find out whether the quaternary structure is perturbed with temperature. The oligomeric size of the HtrA2 protein treated at different temperatures was examined using size-exclusion chromatograph. Results from Fig. 5 showed that the elution position of HtrA2 at 25°C was similar to that at 55°C, indicating that the increased proteolytic activity of HtrA2 was not due to the change of oligomeric structure.

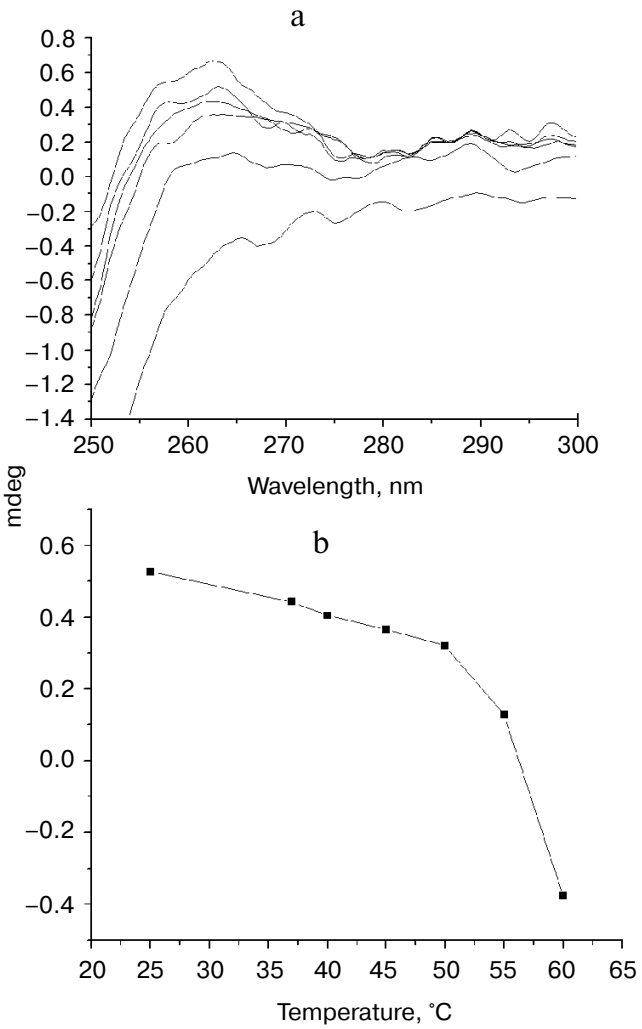

Fig. 3. Near UV-CD spectra of HtrA2: a) curves from top to bottom represent the CD spectra measured at 25, 37, 45, 50, 55, and 60°C, respectively; b) plot of the mean residue mass ellipticity of HtrA2 at 262 nm at different temperatures.

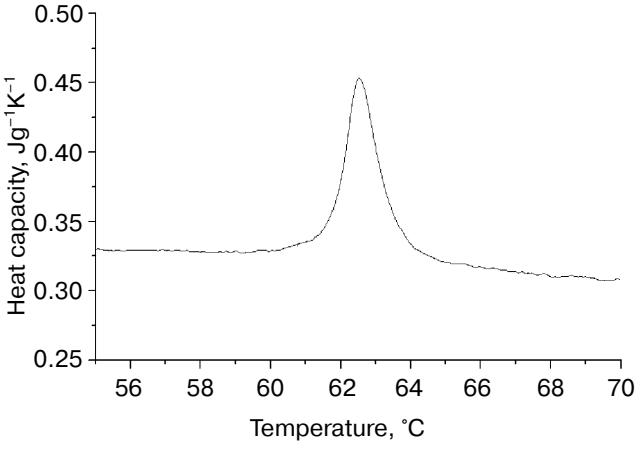

Fig. 4. DSC heating curve of HtrA2. The scan rate was 1°C/min, and HtrA2 concentration was 3 mg/ml.

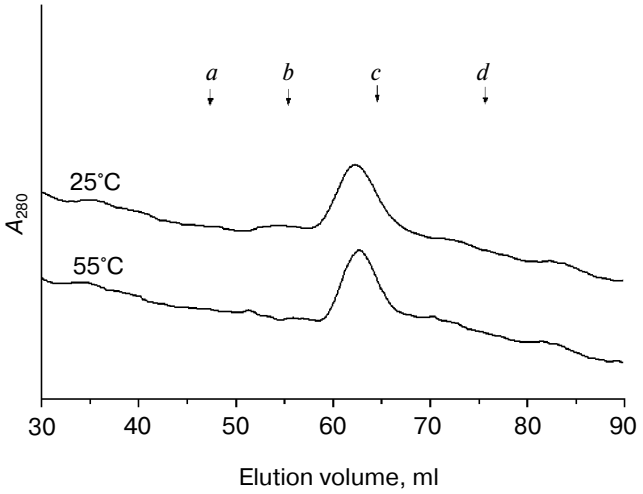

Fig. 5. Size determination of the heat-treated HtrA2 protein using gel filtration chromatography. Curves shown here represent the elution profile of HtrA2 at 25 and 55°C, respectively. Markers *a*, *b*, *c*, and *d* correspond to the molecular masses of 660 kD (thyroglobulin), 440 kD (ferritin), 68 kD (BSA), and 45 kD (egg ovalbumin), respectively.

ANS fluorescence spectra of heat-treated HtrA2. ANS has been widely used as a fluorescence probe to demonstrate the presence of hydrophobic patches on protein surfaces. The fluorescence intensity usually increases when ANS binds to hydrophobic protein surfaces [18, 19]. It has been reported that both HtrA2 and DegP substrate binding sites are remarkably hydrophobic [2], and increased proteolytic activity of DegP at elevated temperatures correlates with an increased amount of hydrophobic surface [20]. Are the rapidly enhanced proteolytic activities of HtrA2 at increased measuring temperatures accompanied by exposure of hydrophobic surfaces? ANS was thus used to probe the exposure of such hydrophobic surfaces on HtrA2 during heating treatment. The results, shown in Fig. 6, demonstrated that the fluorescence intensity of ANS interacting with HtrA2 decreased with increasing temperature, indicating much lower surface hydrophobicities of HtrA2 at higher temperatures. We also found that with increasing temperature the maximal emission wavelength of ANS bound to HtrA2 was red shifted.

Complementation of DegP function at 44°C. DegP has been reported to be essential for survival of bacteria at temperatures above 42°C [13]. It degrades heat-denatured *E. coli* proteins both *in vivo* and *in vitro* [21]. To test whether HtrA2 has the capacity to complement DegP function, we transformed HtrA2, HtrA2S306A, and pUC18 alone into a *degP* null mutant strain CLC198, respectively, and monitored the bacterial growth at 44°C. Results from Fig. 7 show that CLC198 strains transformed with pUC18 alone and pUC18 containing HtrA2S306A were both very sensitive to high tempera-

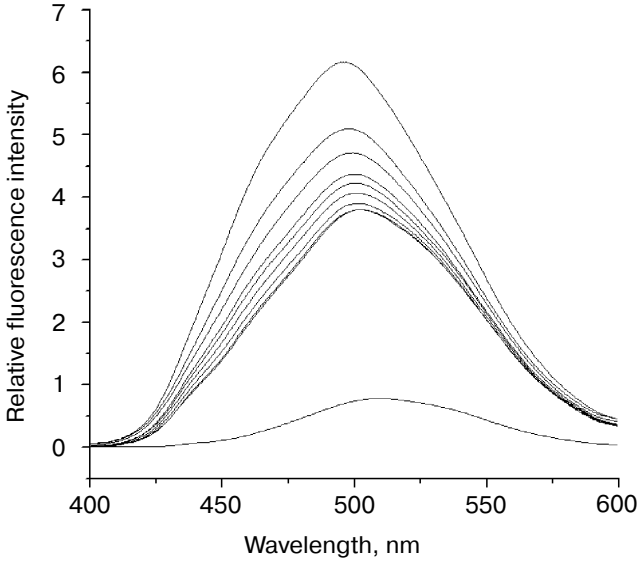

Fig. 6. Fluorescence spectra of ANS bound to heat-treated HtrA2. Curves from top to bottom represent the ANS-binding fluorescence spectra recorded at 25, 37, 40, 45, 50, 55, 60, 65, and 70°C, respectively.

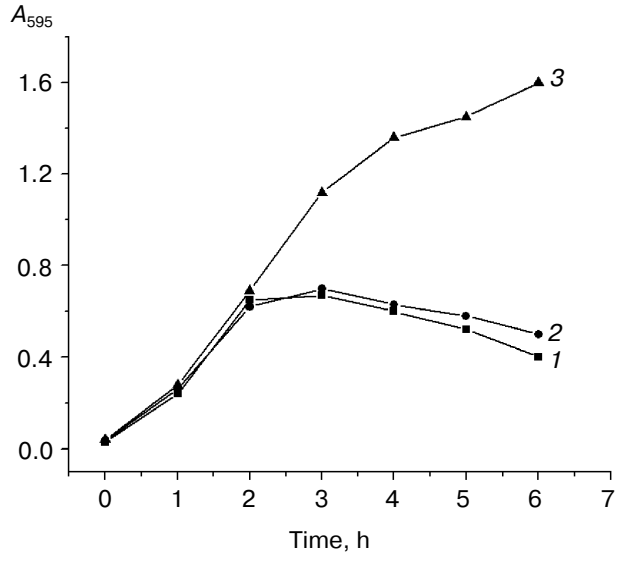

Fig. 7. Complementation of DegP function by HtrA2 at 44°C. Curves *1*, *2*, and *3* represent the growth curves of *degP* null mutant strain CLC198 transformed with pUC18 plasmid, pUC18 containing *htrA2S306A*, and pUC18 containing *htrA2*, respectively.

ture. However, CLC198 strain transformed with pUC18 containing HtrA2 was able to grow at 44°C, indicating that HtrA2 had the capacity to complement DegP function at high temperature, and the proteolytic activity of HtrA2 was essential for the survival of cells at elevated temperatures.

DISCUSSION

As a bacterial heat shock protease, DegP was found to experience a significant increase in proteolytic activity when the temperature increased from 37 to 55°C [22]. Here we systematically investigated the effects of temperatures (from 25 to 70°C) on human HtrA2 and revealed that increase in proteolytic activity can be observed from 25 to 55°C, with the highest value at 55°C (Fig. 1).

To understand the cause of such proteolytic activity change induced by heating treatment, the secondary, tertiary, and quaternary structures of HtrA2 protein at various temperatures were examined. Our results did not show any significant change on the secondary structure of HtrA2, nor in the oligomeric size between 25 and 55°C (Figs. 2 and 5). However, a significant change at the tertiary level, as examined using near-UV CD, was observed for HtrA2 in the range from 25 to 60°C (as shown in Fig. 3). So, the change in tertiary structure should be considered as the main reason for increased proteolytic activity.

What is the nature of this structural change? The HtrA family shares a modular architecture composed of an N-terminal segment believed to have regulatory functions, a conserved trypsin-like protease domain, and one or two PDZ domains [2]. PDZ domains are protein modules defined by a unique sequence of 80-100 amino acids able to recognize specific C-terminal sequences in target proteins [23, 24]. Although the protease domains form a rigid part of the funnel-like trimer, the PDZ domains are relatively mobile [2]. The crystal structure of HtrA2 revealed that the PDZ domain may play a regulatory role in proteolytic activity [17]. In HtrA2 structure, Li et al. proposed that the inhibitory interaction produced by the peptide-binding groove in the PDZ domain with the loop between β_5 and β_6 in the protease domain makes this groove unavailable for interaction with other proteins. Thus, the binding of HtrA2 PDZ domain by target proteins will involve a large conformational change [17]. Based on the above analysis, we propose that high temperature may break the interaction and cause the PDZ domain to adopt an active orientation, thus making the peptide-binding groove free for interaction with substrate, resulting in the increased proteolytic activity of HtrA2 with temperature.

Our differential scanning calorimetry (DSC) study revealed a thermal transition temperature of 62.4°C with the onset of transition at approximately 61°C. This coincides with the drastic decrease in HtrA2 activity, observed at and over 60°C (Fig. 1), confirming that incubation at 60°C inactivated HtrA2 protease. Interestingly, DegP was also found to have an initial phase of protein denaturation at around 60°C, as revealed by studies using FT-IR spectroscopy [22], and denaturation and aggregation occurred concomitantly in the degP sample [14], which is consistent with our observations reported here.

Hydrophobic interaction has long been considered to be of great importance in understanding protein–pro-

tein interaction. It is generally believed that HtrA proteins interact with their substrates hydrophobically [2, 17, 25]. Contrary to its bacterial homolog DegP, which demonstrated a correlation between the proteolytic activity and exposure of hydrophobic surfaces [20], the significantly enhanced proteolytic activity of HtrA2 accompanied a decreased fluorescence intensity of the bound ANS (Fig. 6), indicating that exposure of hydrophobic surface is not the sole determinant of the proteolytic activity of HtrA2. This inconsistency was also observed in other proteins such as small heat shock proteins Hsp16.3 and α-crystallin [26, 27]. Both proteins exhibited lower chaperone-like activity and stronger surface hydrophobicity at lower temperatures than at higher temperatures. Another explanation for this inconsistency is that the reorientation of the PDZ domain triggered by increasing temperature (as discussed above) may cause the hydrophobic surface of HtrA2 to become more exposed to polar liquid environment, thus leading to the observed decrease in ANS fluorescence intensity and red shift.

HtrA2 is an evolutionarily conserved protease. By analogy to HtrA2, *E. coli* DegP is necessary for bacterial thermal, oxidative, and osmotic tolerance [12]. Our finding that HtrA2 can complement DegP function at 44°C (Fig. 7) is in agreement with the fact that the proteolytic activity increases with high temperature (Fig. 1) and HtrA2 is upregulated in cellular stress such as heat shock [4]. This strongly suggests that in addition to promoting cell death in response to apoptotic stimuli, HtrA2 could also have protective functions in mitochondria. HtrA2 may protect mitochondria against cellular stress through recognizing unfolded or damaged proteins in the intermembrane space and helping to degrade them. Identification of the physiological substrates of HtrA2 in mitochondria will provide insight into the function of this protein.

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