PDZ Domains Facilitate Binding of High Temperature Requirement Protease A (HtrA) and Tail-specific Protease (Tsp) to Heterologous Substrates through Recognition of the Small Stable RNA A (ssrA)-encoded Peptide*

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The *Escherichia coli* protease HtrA has two PDZ domains, and sequence alignments predict that the *E. coli* protease Tsp has a single PDZ domain. PDZ domains are composed of short sequences (80–100 amino acids) that have been implicated in a range of protein–protein interactions. The PDZ-like domain of Tsp may be involved in binding to the extreme COOH-terminal sequence of its substrate, whereas the HtrA PDZ domains are involved in substrate assembly and are predicted to be responsible for substrate binding and subsequent translocation into the active site. *E. coli* has a system of protein quality control surveillance mediated by the *ssrA*-encoded peptide tagging system. This system tags misfolded proteins or protein fragments with an 11-amino acid peptide tag that is recognized by a battery of cytoplasmic and periplasmic proteases as a degradation signal. Here we show that both HtrA and Tsp are able to recognize the *ssrA*-encoded peptide tag with apparent *K_D* values of ~5 and 390 nM, respectively, and that their PDZ-like domains mediate this recognition. Fusion of the *ssrA*-encoded peptide tag to the COOH terminus of a heterologous protein (glutathione S-transferase) renders it sensitive to digestion by Tsp but not HtrA. These observations support the prediction that the HtrA PDZ domains facilitate substrate binding and the differential proteolytic responses of HtrA and Tsp to *ssrA*-tagged glutathione S-transferase are interpreted in terms of the structure of HtrA.

*Escherichia coli* has a system of quality control surveillance monitoring proteins located in the cytoplasm and periplasm. This system uses a series of proteases to target misfolded or damaged proteins by recognition of an 11-amino acid peptide tag, which is added to the COOH terminus by a mechanism that requires the *ssrA*-encoded RNA. The *ssrA* RNA has 363 nucleotides and can form a tRNA-like structure that is chargeable with alanine. In a model proposed by Keiler et al. (1), SsrA charged with alanine can bind to stalled ribosomes. After the contribution of the *ssrA*-encoded alanine, translation switches to a short open reading frame in *ssrA* that encodes the COOH-terminal peptide tag degradation signal. This system provides a general quality control mechanism to dispose of incomplete protein fragments and avoid the build-up of ribosomes stalled on defective mRNA molecules (2–7). Cytoplasmic proteases that respond to this system are largely ATP-dependent (e.g. the ClpXP and ClpAP proteases) whereas SsrA-tagged proteins with signal sequences are directed to the periplasm and degraded there by ATP-independent proteases.

Tsp is a periplasmic serine protease of *E. coli* that was purified on the basis of its ability to degrade a variant of the NH₂-terminal domain of the bacteriophage lambda repressor. The wild-type repressor domain, which is not degraded, contains the polar COOH-terminal sequence Arg-Ser-Glu-Tyr-Glu, whereas the variant repressor protein contains the apolar sequence Trp-Val-Ala-Ala-Ala, and it is this that allows degradation by Tsp (1, 8, 9). Tsp preferentially degrades substrates that are not stably folded, by digestion at several sites that have broad primary sequence specificity (1). Tsp is thought to bind to the COOH terminus of the protein in question, with no proteolysis occurring until spontaneous unfolding makes the polypeptide available to the protease active site (2, 10).

HtrA (also known as DegP) is a second periplasmic protease of *E. coli*, which can also act as a chaperone (11), and was originally identified as a heat shock-induced protein. It is located on the periplasmic side of the inner membrane and has the conserved triad of His, Ser, and Asp residues that is characteristic of the serine proteases. Homologues of HtrA have been found in a wide range of species including Gram-negative and Gram-positive bacteria, cyanobacteria, yeast, and humans. A natural substrate for HtrA is the periplasmic MalS protein, but HtrA is also able to use β-casein as a proteolytic substrate in *vitro* as the latter is largely unordered in solution (11, 12). At low temperatures HtrA acts predominantly as a chaperone and in *vitro* is able to stimulate the refolding of chemically denatured proteins. The proteolytic activity associated with HtrA predominates at high temperatures. Null mutants in the *htrA* gene are thermosensitive and have a decreased ability to degrade abnormal periplasmic proteins. A mutant form of HtrA in which Ser²¹⁰ has been changed to Ala is correctly folded but proteolytically inactive, and has a reduced ability to suppress...
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The thermosensitive phenotype in a strain deleted for the wild-type htrA gene. These observations link the loss of proteolytic activity with the thermosensitive phenotype (13–21). This mutant HtrA retains chaperone activity (21). Transcription of the htrA gene is highly regulated by a complex interaction with inner membrane proteins and a more global system of phosphoprotein phosphatases (22).

The crystal structure of a protease-deficient mutant HtrA has been determined, and is a hexamer formed by staggered association of trimeric rings; the proteolytic sites are located in a central cavity that is only accessible laterally (23). HtrA monomers are composed of three domains, an NH2-terminal domain, a central cavity that is only accessible laterally (23). HtrA has been determined, and is a hexamer formed by staggered inner membrane proteins and a more global system of phosphoprotein phosphatases (22).

Similarly, the gene encoding the GST protein was amplified by the PCR with COOH-terminal extensions of either Ala-Ala-Asn-Ala-Glu-Asp-Ala-Leu-Ala-Ala-Asp-Ala-Ala-Glu. Site-directed mutagenesis, as previously described (28), using a mutagenic oligonucleotide of sequence AACCGTGTTACCGCGGTTGTCGCGT and a non-mutagenic oligonucleotide of sequence ACCCAACAACCGCGCAACGCGTGAATC and plasmid pTR147 as template were used to generate the Ser210→Ala mutant. The amino acids encoded by the various plasmid constructs and the oligonucleotides used in each PCR are shown in Table I. The correct sequences and the absence of PCR-generated artifacts in the cloned sequences were verified by directly sequencing the double-stranded plasmid DNA on an ABI/PerkinElmer Life Sciences 377 automated DNA sequencer.

Protein Overproduction and Purification—Recombinant plasmids designated pTR129 and 130 were used to transform the E. coli strain BL21 (DE3) to screen for E. coli and S. typhimurium HtrA overproduction in the presence of 0.2 mg ml⁻¹ IPTG. Soluble overproduction of both HtrA proteins was achieved, and analysis by SDS-PAGE showed that greater than 90% of HtrA was processed by removal of the signal peptide. For a typical purification of native HtrA, 4 liters of cells grown at 37 °C in 500-ml batches in rich medium were induced by the presence of 0.2 mg ml⁻¹ IPTG when the cells were in mid logarithmic growth and harvested by centrifugation at 2,500 × g. Following disruption by sonication in 450 ml of 50 mM potassium phosphate, pH 7.2, 1 mM EDTA (buffer 1), the cell suspension was clarified by centrifugation at 2,500 × g for 30 min at 4 °C. The clarified supernatant was applied to a DEAE-Sephal column and eluted with 500 ml of the same buffer. The HtrA-containing column flow-through, made 1.0 mM with ammonium sulfate, and applied to a phenyl-Sepharose column. After washing with buffer 1 containing 1.0 mM ammonium sulfate, the phenyl-Sepharose column was eluted with a 1-liter gradient consisting of 500 ml of 1.0 mM ammonium sulfate in buffer 1 connected to 500 ml of 10 mM potassium phosphate buffer, pH 7.2, 1 mM DTT (buffer 2). The HtrA pool was then eluted from the column in a single batch wash of 500 ml of buffer 2 after completion of the ammonium sulfate gradient. The HtrA pool was applied to a hydroxyapatite column and washed with 500 ml of buffer 2. The column was then eluted with an 800-ml gradient consisting of 400 ml of buffer 2 connected to 400 ml of 400 mM potassium phosphate, pH 7.2, 1 mM DTT (buffer 3). HtrA protein was then eluted from the column in a single batch wash of 500 ml of buffer 2 after completion of the ammonium sulfate gradient. The HtrA pool was applied to a hydroxyapatite column and washed with 500 ml of buffer 2. The column was then eluted with an 800-ml gradient consisting of 400 ml of buffer 2 connected to 400 ml of 400 mM potassium phosphate, pH 7.2, 1 mM DTT (buffer 3). At the end of the 800-ml gradient, HtrA was eluted from a batch wash of 100 ml of 500 mM potassium phosphate buffer, pH 6.6, 1 mM DTT (buffer 4). Typical yields from this procedure were 150 mg of HtrA from 4 liters of original culture. The same procedure was used in conjunction with plasmid pTR147 to purify the Ser210→Ala mutant HtrA.

Plasmids encoding GST fusion proteins (see Table I) were used to transform the E. coli strain BL21 (DE3) leading to the IPTG-inducible soluble overproduction of the appropriate proteins. Typically the cells from two 500-ml cultures, grown at 37 °C and induced in mid-logarithmic growth with 0.2 mg ml⁻¹ IPTG, were harvested by centrifugation at 2,500 × g and sonicated in 200 ml of 0.1 M Tris-HCl, pH 8.0, 1 mM DTT (buffer 5). Following clarification by centrifugation at 2,500 × g, the clarified supernatant was applied to a glutathione-substituted Sepharose 4B column, and the column was washed with 500 ml of buffer 5. Fusion proteins were eluted by a batch wash of 100 ml of buffer 5 containing 10 mM glutathione and dialyzed into 20 mM Tris-HCl, pH 8.0, prior to use in surface plasmon resonance measurements.

Proteins containing a His, COOH-terminal tag were purified by immobilized metal affinity chromatography as described above but with the following modifications; harvested cells were sonicated in 50 mM potassium phosphate, pH 7.2, 0.5 M NaCl, 1 mM DTT, 1 mM benzamidine (buffer 6) and the clarified supernatant applied to a 21-ml chelating Sepharose column charged to 30% capacity with zinc. Following a 100-ml wash with buffer 6 containing 2.0 mM glycine, the column was washed with 100 ml of buffer 6 to remove unbound material, and the column was then eluted with a 100-ml 50 mM potassium phosphate linear pH 6.0 to 4.0 gradient. Following analysis by SDS-PAGE, fractions containing the desired protein were dialyzed into 20 mM Tris-HCl, pH 8.0.
Protease Assays—Protease assays using HtrA and Tsp with β-casein or GST as the substrate were carried out according to the following protocol: 0.1 μg protease was added to 3.7 μg substrate (β-casein or GST) in a final volume of 100 μl of 20 mM Tris-HCl, pH 8.0. The reaction was incubated at 37 °C with Tsp for 2.5 h and at 42 °C with HtrA for 4 h, using a Hybrid thermal reactor. Control experiments (data not shown) demonstrated that HtrA had greater proteolytic activity at 42 °C than at 37 °C. The fragments produced by the proteolysis were analyzed by SDS-PAGE using a 12% separating gel (31). Prior to digestion GST proteins were unfolded by heating at 90 °C for 10 min using a Hybrid Thermal Reactor.

Surface Plasmon Resonance (SPR) Measurements—The interactions among HtrA, Tsp, and their PDZ subfragments with three synthetic peptides were monitored by SPR measurements using the BiAcore™ 2000 from Amersham Biosciences. The synthetic peptides were used as the ligand and the HtrA, Tsp, and PDZ subfragments as the analyte. The concentrations of purified proteins and the synthetic peptides were determined spectrophotometrically from their calculated molar extinction coefficients. For HtrA binding, peptides in the range of 7–30 resonance units (RUs) were immobilized by linkage via a biotin molecule to the streptavidin layer of a SA biosensor chip. Because of the lower affinity of Tsp binding to the peptides, 250 RUs of peptide were bound to facilitate a measurable signal. This immobilization was performed at pH 7.4 in HBS buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% v/v P20 surfactant). GST proteins with and without the wild-type and scrambled SsrA peptide tag were immobilized via amine groups to the surface of CM5 chips at pH 7.4 in HBS buffer in the range 160–180 RUs. The modified biosensor chips were then equilibrated at 25 °C with a running buffer consisting of 20 mM Tris-HCl, pH 8.0. Using the BIAcore KINJECT function, 15 μl of the desired analyte was injected at 5 μl min⁻¹ at varying concentrations. Previous experiments using a range of flow rates showed no evidence of mass transport limitation effects. The regeneration buffer, HBS, was injected at 5 μl min⁻¹ for 60 s. For HtrA, a data set of 30 points was obtained by using concentrations of 5, 10, 20, 30, 40, and 50 nM and repeating this for a total of five times. For Tsp, concentrations of 600, 650, 700, 750, and 800 nM were used six times to generate a data set of 30 data points. The baseline of the sensorsgrams for the experimental and reference flow cells were adjusted to zero immediately prior to injection and the specific changes in the experimental sensorsgram measured by subtracting the values from the reference cell containing peptide 2 (the randomized SsrA sequence). Kinetic analysis was performed using BIAevaluation 3.0 software (BIAcore AB) following the recommendations of the manufacturer for acceptable fits. The 1:1 Langmuir model (29). This analysis showed that the analyte failed to adequately describe the experimental data for peptides 1 and 3.

RESULTS AND DISCUSSION

The HtrA and Tsp Proteases Can Bind to the ssaR-encoded 11-Amino Acid Sequence—HtrA and Tsp proteases were purified according to the protocol under “Experimental Procedures,” and their biological activity confirmed by their ability to digest β-casein in vitro (see Fig. 2). To test directly the hypothesis that HtrA and Tsp could bind to the ssaR-encoded peptide tag, we monitored the interactions between the proteins and various peptides related to the SsrA peptide by SPR measurements. The proteases were used as the analytes and three different peptides as the ligands: the wild-type SsrA peptide (COOH-terminal sequence Tyr-Ala-Leu-Ala-Ala), a variant with the COOH-terminal sequence Trp-Val-Ala-Ala-Ala (the sequence originally used to identify and purify Tsp), and a variant consisting of wild-type amino acid composition but randomly assembled sequence. The randomized sequence was used as a control to check that any protease:peptide interactions were sequence related, and the Trp-Val-Ala-Ala-Ala variant was included as a positive control for the Tsp protease. Fig. 1 shows typical sensorsgrams for the binding of Tsp (panel A) and E. coli HtrA (panel B) to the wild-type SsrA peptide, and Table II summarizes the kinetic data.

None of the proteins (Tsp, Tsp(Lys → Ala), HtrA, and...
Fig. 1. **Sensorgrams showing the binding of Tsp and HtrA to the SsrA peptide.** Panel A shows the binding response of SsrA peptide as the ligand to 600, 650, 700, 750, and 800 μM Tsp; panel B shows the binding response of SsrA peptide as the ligand to 5, 10, 20, 30, 40, and 50 μM HtrA. Plots of the residuals associated with each data set are shown beneath the sensorgrams.
HtrA(Ser → Ala) bound above background values to the randomized peptide, but all four bound to the wild-type and three (Tsp Lys455 → Ala was not tested) to the Trp-Val-Ala-Ala-Ala variant (Table II). The binding of wild-type and mutant E. coli HtrA proteins to the wild-type and Trp-Val-Ala-Ala-Ala variant peptides is similar with an average apparent equilibrium dissociation constant, $K_D$, of the order of 18 nM. The S. typhimurium HtrA bound to the wild-type SsrA peptide with an apparent dissociation constant, $K_D$, of the order of 3 nM. This is the first demonstration that HtrA binds to the SsrA peptide and that an active proteolytic site in HtrA is not required for SsrA-peptide binding. The value for $K_D$ for the binding of wild-type Tsp to the Trp-Val-Ala-Ala-Ala variant peptides is 3.8 μM. The difference of ~100-fold in the values of $K_D$ for E. coli HtrA and Tsp for the same peptide sequence implies that HtrA binds more tightly than Tsp.

The observation that HtrA and Tsp do not discriminate between the wild-type and Trp-Val-Ala-Ala-Ala variant peptides and do not recognize the randomized peptide shows that, although binding is sequence-related, it is not sequence-specific. A comparison of the SrrA-encoded peptide homologues in a range of organisms shows that the prime requirement for recognition is a pentapeptide apolar COOH terminus (10). The randomized peptide we used as a control has glutamate as the COOH-terminal residue, violating the requirement for an apolar COOH terminus, and likely contributes to the inability of HtrA and Tsp to bind to it.

The PDZ Domains of HtrA and Tsp Bind to the Isolated ssrA-encoded Peptide—To test specifically the hypothesis that PDZ domains of HtrA and Tsp are responsible for recognizing and binding to the SsrA peptide, we purified a series of COOH-terminal PDZ-containing fragments (Table I). A deletion analysis of this type has the intrinsic problem that use of the protein fragments may produce atypical kinetic results (in the extreme case, an inability to bind) simply because the protein fragments produced fail to fold correctly or are inherently unstable. To address this potential problem, a series of overlapping HtrA-PDZ-encoding sequences were cloned and expressed from both E. coli and S. typhimurium based on amino acid sequence alignments. Previous work has shown that some PDZ domains are sufficiently stable to allow crystallization and structural determination (11).

Preliminary SPR experiments showed that the individual E. coli PDZ domains did not bind to the SsrA peptide; however, the S. typhimurium PDZ domains did bind, and were used for the collection of a full data set (see Table II). In each case (for both E. coli and S. typhimurium), PDZ domain 1 was truncated by 4–9 amino acids at the carboxyl terminus as defined by the recently published x-ray structure (23). Interestingly, the E. coli PDZ domain 2 construct incorporated the full PDZ domain and showed no SsrA binding, whereas the equivalent S. typhimurium sequence was truncated at the carboxyl terminus by 4 amino acids from the full domain and was able to bind the SsrA sequence. The inability of the E. coli PDZ domains to bind to the SsrA peptide may be because the small differences in amino acid sequence compared with S. typhimurium cause the isolated PDZ domains to be unstable. The isolated Tsp PDZ sequence was also stable and bound to the wild-type SsrA peptide. However, caution must be exercised in interpreting the apparent $K_D$ values shown in Table II, as the PDZ domains were fused to GST, and it has been argued that the use of GST fusion proteins can overestimate the affinity of the measured interaction (31). This has been proposed to be the result of GST fusion dimers binding simultaneously to two ligand molecules at the sensor chip surface; this divalent attachment is described as an avidity effect (31). However, despite this caveat,
ase concentrations up to 0.5 M exposing susceptible peptide bonds. However, all three GST result of nonspecific loosening of the GST tertiary structure of Tsp, and this may be the insensitive. These digestions were carried out multiple times, repeated the experiments with heat-denatured substrates. In this case, the GST variant modified with the wild-type SsrA proteolytic activity toward the SsrA-modified GST but not HtrA. Tsp has previously been shown to bind to a peptide of sequence Ala-Ala-Arg-Ala-Ala-(fluorescent marker)-Glu-Asn-Tyr-Ala-Leu-Ala-Ala. This sequence is a substantially modified derivative of the SsrA peptide (Ala-Ala-Asp-Glu-Asn-Tyr-Ala-Leu-Ala-Ala), incorporating an artificial substrate cleavage site (between alanine and arginine) and a fluorescence marker (N-[4-(4-dimethylamino)phenylazo][benzoyl]lysyl-(6-aminocaprooyl)2) (32). In the same report, the Tsp PDZ domain was shown to bind to the peptide Gly-Arg-Gly-Tyr-Ala-Leu-Ala-Ala, leading to the conclusion the Tsp PDZ domain is responsible for substrate recognition. However, the wild-type SsrA peptide is not itself a substrate for proteolysis; its function is to tag a larger protein or peptide for proteolysis, and this larger protein or peptide is the true substrate. The simplest interpretation of the data we present here is that Tsp binds initially to the modified GST via the PDZ domain interacting with the SsrA peptide and subsequently increases its affinity of binding by a second interaction with the GST protein. To test this interpretation experimentally, we purified the proteolytically inactive Tsp Lys455 → Ala mutant protein and used it as the analyte in SPR experiments with the wild-type SsrA peptide and the GST protein modified with the wild-type SsrA peptide. The results summarized in Table II show that the mutant Tsp Lys455 → Ala protein bound to the modified GST protein with a $K_D$ over 2 orders of magnitude greater than the wild-type Tsp. This observation is consistent with the interpretation that an active protease domain is required to increase the affinity of wild-type Tsp binding through an interaction with the GST protein.

Clues to the reason that HtrA does not show an increased proteolytic activity toward the SsrA-modified GST are provided by its structure. The protease domain forms a chamber of which contains possible binding sites for misfolded proteins. Access to the proteolytic active site is controlled by the action of three mobile loops that interact and must undergo large conformational changes to adopt the classical catalytic model of serine proteases (23). HtrA has apparently recruited PDZ domains to a gating function, and this may allow the coupling of substrate to translocation within the HtrA multimer (23). Consequently it has been speculated that PDZ domains may act as tentacular arms that bind substrates and transfer them to the inner cavity (23). Once in the chamber, the fate of the substrate (proteolysis versus refolding) will depend on the interplay of the three mobile loops that interact to form the classical serine protease conformation. It is possible that the nature of the secondary structure elements translocated into the inner chamber can influence the interaction between these loops in the HtrA protease domain and hence the balance between protease and chaperonin activity for different protein substrates.

The data presented here show that the HtrA PDZ domains can bind to the isolated SsrA peptide and that addition of this peptide to a heterologous protein facilitates binding of the full-length HtrA.
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