The LA Loop as an Important Regulatory Element of the HtrA (DegP) Protease from *Escherichia coli*

**STRUCTURAL AND FUNCTIONAL STUDIES**

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**Background:** An understanding of the HtrA protease activation mechanism is incomplete with respect to its LA regulatory loop.

**Results:** A theoretical model of the LA structure is provided and experimentally verified.

**Conclusion:** LA intersubunit contacts strongly contribute to the stabilization of the inactive HtrA.

**Significance:** This is the first report that simultaneously offers a theoretical three-dimensional structure of LA and its biophysical and functional properties.

Bacterial HtrAs are serine proteases engaged in extracytoplasmic protein quality control and are required for the virulence of several pathogenic species. The proteolytic activity of HtrA (DegP) from *Escherichia coli*, a model prokaryotic HtrA, is stimulated by stressful conditions; the regulation of this process is mediated by the LA, LD, L1, L2, and L3 loops. The precise mechanism of action of the LA loop is not known due to a lack of data concerning its three-dimensional structure as well as its mode of interaction with other regulatory elements. To address these issues we generated a theoretical model of the three-dimensional structure of the LA loop as per the resting state of HtrA and subsequently verified its correctness experimentally. We identified intra- and intersubunit contacts that formed with the LA loops; these played an important role in maintaining HtrA in its inactive conformation. The most significant proved to be the hydrophobic interactions connecting the LA loops of the hexamer and polar contacts between the LA’ (the LA loop on an opposite subunit) and L1 loops on opposite subunits. Disruption of these interactions caused the stimulation of HtrA proteolytic activity. We also demonstrated that LA loops contribute to the preservation of the integrity of the HtrA oligomer and to the stability of the monomer. The model presented in this work explains the regulatory role of the LA loop well; it should also be applicable to numerous *Enterobacteriaceae* pathogenic species as the amino acid sequences of the members of this bacterial family are highly conserved.

HtrA (high temperature requirement A) proteins have been identified in most studied organisms, both prokaryotic and eukaryotic, and are evolutionarily highly conserved. The members of this family are usually involved in protein quality control, degrading abnormal proteins and/or serving as chaperones. The latter activity relies on the binding of improperly folded polypeptides, which prevents them from aggregation and in some cases allows them to achieve their proper conformation and subsequent destination. In bacteria HtrAs are located in the cellular envelope. They protect cells against extracellular stresses and also take part in protein export and folding. Certain HtrAs may also be involved in the regulation of cellular pathways, e.g. DegS from *Escherichia coli* up-regulates the α**+** transcription factor by degradation of the anti-σ factor RseA (for review, see Ref. 1). All such functions may affect the virulence of pathogenic bacteria either directly or indirectly. Consequently, numerous pathogenic bacteria deprived of their functional *htrA* genes are either non-virulent or their virulence is decreased. It has been further demonstrated that *htrA* mutants are deficient in their ability to process certain virulence factors and that they are more susceptible to oxidative and thermal stress; their ability to produce biofilm and to adhere to epithelial cells or survive in macrophages is also compromised (2). Recently it has been shown that certain enteropathogenic bacteria (including *Helicobacter pylori*, *Campylobacter jejuni*, and enteropathogenic *E. coli*) are capable of secreting a portion of their HtrA to the extracellular environment where it can cleave E-cadherin, a component of adherance junctions in polarized epithelial cells (3). HtrA-mediated cleavage of E-cadherin leads to the disruption of the epithelial barrier, and in the case of *H. pylori*, seems to be a crucial step in host invasion (4). The introduction of an extracellular anti-HtrA inhibitor prevents the fragmentation of E-cadherin and consequently strongly prevents *H. pylori* from entering the...
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intercellular space (3). Because HtrA homologs from other Gram-negative enteropathogenic bacteria are suggested to demonstrate similar activity toward E-cadherin, HtrAs appear to be very interesting therapeutic targets, and low molecular anti-HtrA inhibitory molecules seem to be an attractive alternative or supplement to the antibiotic treatment of bacterial infections. Thus, understanding the mechanism of HtrA protein regulation is important not only from a scientific point of view but also because it may provide a basis for the development of therapeutic compounds.

HtrA proteins are serine proteases of the chymotrypsin family; they are composed of the protease and PDZ2 (where PDZ stands for postsynaptic density protein 95 kDa, Disc large and zonula occludens protein 1) domains, which are located at the N- and C-terminal regions of the protein, respectively (5). Serine proteases of the chymotrypsin type share a common structural topology that is characterized by the presence of a β-barrel-shaped proteolytic domain. In a model chymotrypsin protein, this proteolytic domain is formed by two six-stranded β-barrels with the active site triad located at their interface. The β-sheets involved in the formation of the β-barrels are connected by loops, termed LA, LB, LC, LD, LE, L1, L2, and L3 (6). These loops play very important roles in the regulation and maintenance of the enzyme catalytic activity. Four structural aspects are worthy of mention. First, the active site triad is located within the LB, LC, and L1 loops. Second, the amino acid residues of the L1 loop are involved in the formation of an oxyanion hole. Third, the L2 loop is responsible for proper substrate binding (7). Fourth, the L1, L2, and LD loops form the so-called “activation domain,” which plays an important role in chymotrypsin activation (8, 9). The proper organization of these loops is, therefore, crucial to the enzymatic activity of the protease.

HtrA (DegP) from E. coli is a model protease of the HtrA family. The active site triad consists of the His-105, Asp-135, and Ser-210 residues, which are located in a crevice between two β-barrels (10). HtrA exists in two forms: inactive and active. In the inactive state two trimeric HtrA rings form a hexamer. This particular assembly is achieved via interactions of the C-terminal PDZ domains and is additionally stabilized by long LA loops (residues 38–79) that protrude into the opposite trimer, where they are believed to interact with the L1 (residues 203–212) and L2 (228–238) loops (10). In this form the active site is not accessible to substrate as it is not of a proper architecture to do so. The activation process requires the dissociation of the hexamers into trimers, which may subsequently assemble into 12-mers, 24-mers, or other kinds of higher order oligomers. This process is accompanied by significant changes in the interaction network between the loops. First of all, the L1 and L2 loops must be liberated from an interaction with the LA loop from the opposite subunit (LA’). Then the L1 loop must adopt a right conformation so as to correctly orientate Ser-210 toward His-105 and to form the oxyanion hole (11). Transition to the proteolytically active state is believed to occur in two major ways: by (i) temperature-induced or (ii) allosteric activation.

In our previous work we had shown that upon a shift in temperature, small overall changes within the proteolytic domain as well as structural changes in the loops region could be observed. The LA loop seems to be the first element to react to a change in temperature. It gradually becomes solvent-exposed, and the extent of this conformational change is both proportional and correlated to the increase of temperature effectuated (starting from 25 °C) and to the degree of HtrA proteolytic activity stimulated. The remaining loops (L2 and L1) change their conformation at higher temperatures (above 35 °C or 40 °C, respectively) (12).

The allosteric mode of activation of the HtrA protease is better known. This process is triggered by the binding of an allosteric activator, which consists of a peptide that is long enough to bind to both the active center and to the PDZ1 domain simultaneously. According to the current model the attachment of an appropriate peptide to the PDZ1 domain induces conformational changes in the whole HtrA hexamer. The binding signal is transmitted from the PDZ1 domain of one subunit to the active site of the adjacent subunit. The reorganization of the oligomer enables the PDZ1 domain to interact with the L3 sensory loop, which in turn forwards the signal to the activation domain (the LD, L1, and L2 loops). Additionally, the LA’ loop no longer interacts with the L1 and L2 loops (11).

The LA loop seems to be an important element of this loop-dependent activation cascade: (i) it participates in the stabilization of the inactive hexameric oligomeric state; (ii) it is believed to keep the L1 and L2 loops (elements of the activating domain) in a catalytically incompetent conformation; (iii) it most probably blocks the entrance of the substrate to the active center (for review, see Ref. 13). Deletion of a major part of the LA loop leads to the moderate stimulation of HtrA activity (up to 1.5-fold for the cleavage of β-casein and up to 2-fold for the degradation of lysozyme); in both cases the molecule is found in the trimeric form (14). This observation confirms the inhibitory role of the LA loop. However, deletion of shorter parts of the LA loop may have an adverse or no effect on the activity of HtrA. Simultaneous removal of residues 34–39 and 60–68 leads to a moderate decrease in substrate cleavage rates (20%), whereas deletion of shorter segments (residues 34–37 and 80–83) does not affect HtrA proteolytic activity at all (14). The exact three-dimensional structure of the LA loop is not known, as its pertinent stretch of amino acids (residues 52–78 for the inactive form or residues 36–81 for the active) has evaded tracing by x-ray crystallography, most likely due to its high flexibility. A precise mechanism for the action of the LA loop has, therefore, been only purely hypothetical.

The aim of this work was to further knowledge on the role of the LA loop in the activation of the HtrA protease and to evaluate the contribution of select amino acid residues in this process. The first step was the construction of a model of the LA loop based on in silico structure modeling. The second was the experimental verification of this model. The model predicted certain interactions within the LA loop or between the LA loop and other structures within the proteolytic domain. The most

2 The abbreviations used are: PDZ, postsynaptic density protein 95 kDa, Disc large and zonula occludens protein 1 domain; $K_{SV}$, Stern-Volmer quenching constant; $K_{q}$, bimolecular quenching constant; LA’, the LA loop of the opposite subunit.
important contacts identified were (i) the hydrophobic interactions of the Phe and Pro residues that link the LA loops of all subunits within the hexamer and (ii) a network of hydrogen bonds that connect the LA’ and the L1 loops of the opposite trimer. Using site-directed mutagenesis and disulfide cross-linking techniques, we attempted to experimentally verify our computational model. Our results confirmed the correctness of the proposed LA loop structure in regard to its most important regions. A disruption of the intersubunit hydrophobic or hydrophilic cluster, therefore, appears to be crucial for the HtrA activation process. Furthermore, interactions involving residue 44 appear to be very important to the stability of both the LA loop and the whole HtrA molecule.

EXPERIMENTAL PROCEDURES

Materials—T4 ligase and restriction enzymes were purchased from Fermentas (Vilnius, Lithuania); the polymerase Pfu Ultra Hotstart was from Stratagene (Perlan Technologies, Warsaw, Poland); bis[sulfosuccinimidyl] suberate (BS3) was purchased from Pierce. The primers used in site-directed mutagenesis were purchased from Genomed S. A. (Warsaw, Poland) and were of the highest quality and purity. The substrate peptide NWVSAKFESTDGSTDYGIYQV (22-peptide), whose sequence was based on a peptide described by Kim et al. (15), was synthesized as described in Wysocka et al. (16).

Strains and Plasmids—The E. coli strains and plasmids that were used are listed in the supplemental Table S1.

Construction and Analysis of the LA Loop Model—Because the type of fold formed by the 27-amino acid residues of the LA loop is not resolved in the 1KY9 crystal structure of the inactive HtrA protein, the structure of this sequence was probed using the UNRES Force Field (17). UNRES is a physics-based UNited RESidue force field in which a polypeptide chain is represented by a sequence of Cα carbon atoms having attached united side chains. United peptide groups are located between the Cα knots, and the chain is linked by virtual bonds. For structural calculations, we used Replica Exchange Molecular Dynamics (REMD), which explores the conformational space of the protein much more efficiently than canonical Molecular Dynamics (18).

The calculations were performed at the following conditions: the temperature range was set from 200 to 410 K, a 10 K replica step was employed, and 4 replicas at each temperature were computed. Two million steps were performed per replica, with exchange occurring every 100 000 steps; a 0.1 molecular time step was used. The five lowest free energy structures were selected for the next step. Each of these five LA folds was then attached to the crystal structure of HtrA protein using the SYBYL software package (SYBYL-X 1.2, Tripos International, St. Louis, MO). Next, the hexameric structure of HtrA was generated using the symmetry operator of the original 1KY9 crystal structure (inactive HtrA, determined at 2.8 Å resolution). One of the limitations of protein crystallography is that it only identifies one of several conformational choices that a protein may potentially adopt, and this may be either its native form or a form induced by crystal packing conditions. With low resolution data, the description of side chain-side chain packing becomes unclear (19, 20). Because unconstrained Cartesian simulations are not effective in refining low resolution models (21, 22), we decided to use a statistics-based approach (23, 24) to predict likely side chain-side chain interactions. The architecture of the resolved portions of the six LA loops in the hexamer imply that their missing residues (27 unresolved amino acids per loop) are located inside the protein cage. As such, the space available for the modeling of missing fragments was quite limited. Only one of the modeled loops fitted favorably inside the 1KY9 interior, having only a small number of high energy contacts. These were subsequently removed by repetitive cycles of short, low temperature molecular dynamics simulations that were implemented using the AMBER force-field (25). As a result, we obtained a low energy model of the LA loop that fit well in the published structure of the HtrA protein.

For the conformational analysis of the Arg-44 side chain, the Penultimate Rotamer Library (based on 500 high resolution structures) was searched, as implemented in the UCSF Chimera software. The probability (% occurrence) for each rotamer both overall and in each secondary structure type (helix, sheet, and other) was, therefore, found (23, 24).

Plasmid Construction—Mutations of the htrA gene were introduced by site-directed mutagenesis according to the protocol of the QuikChange mutagenesis kit (Stratagene) and were verified by sequencing. The plasmids pJS13 or pJS14, pJSFW, or pJS19 were used as templates to generate the pDF plasmids (1–18) or the pMD2, pJSFW, and the pDF 21A, pDF19A, or pDF (20A, 22A) plasmid, respectively. The oligonucleotide primers used for mutagenesis were designed according to the QuikChange mutagenesis kit protocol and are listed in supplemental Table S2. The EcoRI-PstI fragments containing the htrA gene from pJS14 were cloned into the EcoRI-PstI backbone of pTA3 to generate the pJS19 plasmid. The BstEII-HindIII fragments containing the desired mutations were cloned into the BstEII-HindIII backbones of the plasmids pJS13 or pJS14 to generate pMD2wt or the pDF (3A, 5A, 6A, 7A, 10A, and 11A) plasmids, respectively. The BamHI-HindIII fragments containing the desired mutations were cloned into the BamHI-HindIII backbone of the pJS7 plasmid, generating pDF (23, 24, 25) low copy number plasmids. The sequences of the cloned mutated htrA genes were verified by nucleotide sequencing.

Purification of Proteins—The E. coli K38 (pGPI–2) strain transformed with the appropriate plasmids was used to over-produce the mutated HtrA proteins in the T7 promoter polymerase system (26), and the proteins were purified as described previously (27).

The purity of the mutated proteins was estimated to be >95% as judged by SDS-PAGE. The concentration of the HtrA preparations was estimated by staining with Amido Black as described before (28).

Spectroscopic Measurements—Far-UV circular dichroism spectra (185–260 nm) of the HtrA proteins (0.5 mg/ml) were recorded in 10 mM NaHPO4, pH 8.0, at 20 °C in 1-mm path length cells and using a JASCO J-815 (Japan) spectropolarimeter as described in Zurawa-Janicka et al. (29). Steady-state fluorescence, fluorescence quenching, and time-resolved fluorescence decay measurements were carried out as described in Sobiecka-Szkutala et al. (12).
Analysis of Proteolytic Activity—HtrA proteolytic activity was analyzed using β-casein as substrate at temperatures of 20, 35, and 45 °C as described in Sobiecka-Szkatula et al. (30). The proteolytically active variants of HtrA (0.17 μM) were incubated with β-casein (17 μM) in 25 mM Tris-HCl, pH 8.0. The reaction was terminated by the addition of Laemmli lysis buffer and immediately cooled to −20 °C. The samples were then resolved by 12.5% SDS-PAGE, and the gels were stained with Coomassie Brilliant Blue. Electrophoreograms were analyzed densitometrically using a 1DScan EX (Scanalytics Inc.) program. The rates of substrate proteolysis of the mutated HtrA variants were calculated as percentages of the activity measured for the wild type HtrA protein.

Electrophoresis of Proteins and Western Blotting—Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described in Laemmli (31). Gels containing 12.5% (w/v) acrylamide were used. In some cases (as indicated under “Verification of the LA Loop Model by Site-directed Cross-linking”), non-reducing conditions were applied; β-mercaptoethanol was omitted from the system, and iodoacetamide was added to the lysis buffer to a final concentration of 50 mM. Western blotting was performed as described in Oberfelder (32).

Survival Rate Measurements—Bacteria (BL20 strains carrying mutated htra genes on plasmids) were grown in an LB medium at 30 °C to an OD595 of ~0.3. 10-Fold serial dilutions of exponentially growing cultures (4 μl) were spotted on LA agar plates and incubated at 37 and 45 °C for ~18 h.

Protein Cross-linking and Size Exclusion Chromatography—Cross-linking was performed using 10 μM HtrA in 20 mM HEPES, pH 8.0, 100 mM NaCl. Samples (with or without peptide ligand) were preincubated on ice for 20 min then warmed up to 22 °C and incubated in the presence of 0.55 mM bis(sulfo-succinimidyl) suberate (BS3) for 25 min. The reaction was stopped by the addition of Tris-HCl, pH 7.5, to a final concentration of 50 mM. Size exclusion chromatography was carried out on a Superose 12 10/300 GL gel filtration column (GE Healthcare) equilibrated with 150 mM KH2PO4, pH 7.0, 150 mM KCl buffer. Cross-linked HtrA protein samples (100 μl) were analyzed at 25 °C at a flow rate of 0.5 ml/min.

RESULTS

Construction of the LA Loop Model—Using the UNRES Force Field, we obtained a theoretical model of the structure of the LA loop for the inactive form of the HtrA hexamer. The proposed LA structure and the positions of the added-on residues are shown in Figs. 1 and 2 and supplemental file htra_unress.pdb.

In the HtrA crystal structure (PDB code 1KY9) the residues 27–35 and 82–90 form a twisted antiparallel β-sheet that forms part of a pillar connecting two trimeric rings (10). The fragment at residues 36–40 (present in the crystal structure) suggests an extension of this β-sheet. In our model the residues 76–81 revealed an extended conformation (Fig. 1); however, their formation of an antiparallel β-sheet with residues 36–40 is disputable, as the position of Met-85 would cause its side chain to push away the described LA segments.

A detailed sequence analysis of our LA supplement revealed that the core of the loop (residues 42–75) is rich in proline (positions 43, 55, 62, 67) and glycine (positions 51, 60, 71, 72, 74, 75) (Fig. 1). Such a high percentage (about 30%) of proline and glycine suggests that the core of the loop has a structure-breaking character (flexibility) (33). Indeed, in the existing crystal structure of the LA loop there is a bend at position 43, due to a proline residue at its center. The computer simulations performed implicate two other similar bends at Pro-62 and Pro-67 in the insert, with another bend at Gly-75. In the added-on fragment, the bend is stabilized by two hydrogen bonds: Glu-32–Asn-76 and Asn-30–Gln-73, which are created with the cooperation of facing residues in the opposite trimer. Additionally, we found a type II β-turn formed by Ser-54, Pro-55, Phe-56, and Cys-57. The overall shape of the LA loop consists of two orthogonally oriented loops that are made of residues 50–58 and 59–68 (Fig. 1). Fitting of the LA model into the framework of the HtrA hexamer implicates the presence of two very interesting structures at the interface of the trimers. The first of these structures is composed of Phe and Pro residues (46, 49, 50, 62, 63, 67, and 68) from all subunits, which form a hydrophobic core. This structure separates from the aqueous environment a cluster of polar amino acid side chains comprising Asn-206, Arg-207, Asn-209 (in the L1 loops), and Asn-45, Gln-47, and Gln-70 (in the LA loops of the opposite trimer); these form a hydrogen bond network (Fig. 2A). We assumed that such intersubunit connections involving clustered hydrophobic and hydrophilic interactions should strongly stabilize the integrity of the HtrA hexamer and consequently keep HtrA in its inactive conformation. The LA loop model predicts a set of LA interactions with other parts of the protein, especially with the regulatory loop L2, and possibly with the loop L3. Almost the entire L2 loop is located between two LA’ loops from units belonging to the opposite trimer, and it forms numerous con-
The interactions between (a) Leu-229 (L2) and Ile-236 (L2) with the Cβ atom of Cys-69 (LA') or (b) Ile-236 (L2) with Phe-68 (LA') or (c) Ile-228 (L2) with Phe-56 (LA') appear particularly noteworthy (Fig. 2B). It is hard to describe potential interactions between LA and L3, as a major part of the L3 loop is not resolved in the inactive HtrA crystal structure (PDB code 1KY9). The LA loop may also form hydrogen or electrostatic bonds with the PDZ1 domain, in particular Ser-54 (LA') with Arg-325, Asp-52 (LA') with Asn-273, and Asp-53 (LA') with Glu-271 and Thr-270 (Fig. 2D).

The proposed model predicts the presence of additional contacts between two LA loops belonging to opposite units on two trimers, where an orthogonal structure composed of the residues 62–64 and having a Cα-Cα distance between Gln-64 side chains of about 7 Å (Fig. 2C) is evident.

Construction of HtrA Variants Carrying Mutations within the LA Loop—Using site-directed mutagenesis, we obtained a set of mutated htrA genes, coding for proteins with substitutions of select amino acid residues. For point mutations, we chose residues that, according to the proposed model, are involved in intra- or intersubunit interactions. These mutations comprise the following substitutions: P43G, R44A, R44D, R44L, R44P, F46Y, Q47L, F49Y/F50Y, D52A, D53A, S54A, F56S, F63Y, Q64I, Q64A, F68Y, and Q70A. We have also exchanged certain amino acids that do not seem to form important interactions (E59V, S65Y, S65A/S66A, S66Y); these mutations served as negative controls for the assessment of the model correctness. We avoided the substitution of proline for fear that a lack of proline might disturb the overall structure of the loop, as proline is known as a helix- and β-breaker. The only exception was the P43G substitution. In parallel, we constructed proteolytically inactive variants of the mutants listed above, where the active site serine residue was replaced with alanine (S210A). These variants were used for structural studies, because proteolytically active HtrA proteins undergo autocatalytic degradation and thus are not homogenous.

Analysis of the Proteolytic Activity of Mutated HtrA Variants—To examine the effect of the mutations on the proteolytic activity of HtrA, we performed proteolytic cleavage of β-casein at temperatures of 20, 35, and 45 °C. The replacement of phenylalanine residues at the positions 46, 49, 50, 63, and 68 by tyrosine resulted in an increase in the rate of substrate cleavage with respect to wild type HtrA. Single amino acid substitutions caused a moderate stimulation of proteolytic activity at all the tested temperatures (~1.5-fold) (Fig. 3A). Stimulation was most pronounced when two adjacent phenylalanines were sub-
In this case, β-casein was degraded 6-, 4-, and 3-fold more efficiently at temperatures of 20, 35, and 45 °C, respectively (Fig. 3B).

The substitution of glutamine at position 47 or 70 with a nonpolar residue (Leu or Ala, respectively) also resulted in the stimulation of proteolytic activity (1.5-fold) (Fig. 3C). The substitution of residues that were not expected to be engaged in important intra- or intermolecular contacts did not alter the ability of HtrA to degrade β-casein as predicted (not shown).

To our surprise, the replacement of Asp-52 with Ala, Asp-53 with Ala, Ser-54 with Ala, Phe-56 with Ser, and Gln-64 with Ala or Ile also had no impact on the proteolytic activity of HtrA (not shown). The effect of the P43G mutation was temperature-dependent. At 20 °C the activities of HtrA-P43G and of the wild type HtrA were comparable, whereas at higher temperatures (35 or 45 °C) the mutant HtrA variant showed a higher activity (by 1.5-fold) (Fig. 3C). Interestingly, the substitution of the Arg-44 residue with alanine resulted in a dramatic autocleavage of the protein, occurring both within cells and during their preparation. The resultant degradation products migrated in PAGE similarly to the truncated HtrA forms that were observed in the case of the reduced HtrA molecule. The truncated forms of HtrA-R44A showed an activity that was comparable with that of the full-length wild type HtrA (Fig. 3D). However, the activity of the full-length HtrA-R44A is not known, as it was impossible to purify.

To ensure that the observed differences in activity were not the result of an overall protein secondary structure disturbance by the introduced mutations, we analyzed the far-UV circular dichroism (CD) spectra of the proteolytically inactive HtrA variants at temperatures of 20–85 °C. The shapes of the obtained CD spectra as well as the thermal stabilities of the wild type and the mutated HtrA variants were very similar (not shown). Thus, the analyzed mutations did not affect the HtrA conformation.

Summing up, the predicted hydrophobic interactions involving Phe-46, Phe-49, Phe-50, Phe-63, and Phe-68 and other kinds of interactions formed by Pro-43, Arg-44, Gln-47, and Gln-70 stabilize the inactive conformation of HtrA, as their disturbance leads to increased proteolytic activity or to the destabilization of the HtrA molecule. Thus, these results are consistent with the proposed LA loop model and confirm the

![Figure 3: Effect of point mutations in the LA loop on the proteolytic activity of HtrA.](image-url)
presence of the postulated LA interactions with the other elements of HtrA molecule. Contrary to this, the removal of the polar contact between the LA’ loop (mutations D52A, D53A, S54A) and the PDZ1 domain did not affect the activity of the protease.

Fluorescence-monitored Structural Changes within the LA Loop Due to the R44A Substitution—To explain the decreased stability of the HtrA-R44A variant, we obtained two proteolytically inactive single-tryptophan HtrA versions, HtrA-R44A/F50W/S210A (R44A variant) and HtrA-F50W/S210A (control variant). The presence of Trp at this position should not disturb the LA structure, as the Trp side chain can form hydrophobic interactions with the remaining Phe residues within the hydrophobic cluster and cation–π interactions with the Arg side chain. Thus, we expected Trp-50 to serve as a good indicator of putative structural changes in this region of the LA loop. We analyzed the fluorescence properties of the Trp indole rings in both variants. First of all we monitored the positions of fluorescence emission maxima (λ_{max}), which are indicative of the polarity of the microenvironment of Trp residues (34). We found that the λ_{max} values of the corrected fluorescence emission spectra differed significantly in both HtrA variants (Fig. 4). The mutation R44A caused a significant (8 nm) red shift of λ_{max} with respect of the control protein as measured at 20 °C (349.64 and 341.5 nm, respectively), which indicates that Trp-50 in the R44A variant is in a definitely more polar environment. Moreover, the λ_{max} values of this mutant were almost constant at the temperature range 20–40 °C. Contrary to this, the control protein showed a temperature-dependent 10-nm red shift (341.5 → 351.8 nm). The observed differences in the spectral properties of the Trp residues suggest that the mutation R44A caused a significant alteration of the LA loop structure.

To gain more information about the structural changes caused by the R44A mutation, we performed steady-state fluorescence quenching experiments at temperatures of 20–40 °C. This technique provides information on the accessibility and degree of exposure of Trp side chains within a protein (35). As a quencher we used acrylamide, a molecule that is able to penetrate into a protein and quenches both the exposed and buried tryptophan residues, primarily via a collision mechanism (36). The quenching data were plotted according to the Stern–Volmer equation. The typical Stern–Volmer quenching plots of the single-Trp HtrA mutants at 20 °C are shown in Fig. 5A. The quenching plots were nearly linear at low acrylamide concentrations. At high acrylamide concentrations a small upward curvature was observed, suggesting the occurrence of sphere-of-action quenching (the close proximity of quencher and fluorophore may cause immediate quenching of fluorescence upon excitation). Therefore, the plots were analyzed in terms of both dynamic and sphere-of-action quenching. As expected, in the case of the more exposed Trp residue (in the R44A variant), the upward-curving was observed at lower acrylamide concentrations as compared with the control protein (HtrA-F50W/S210A). The Stern–Volmer quenching constants (K_{sv}) of Trp residues are shown in Table 1.

To directly compare the accessibility of the fluorophores to the quencher the bimolecular quenching constants, k_{q}, were calculated (k_{q} = K_{sv}/r). The mean fluorescence lifetimes (τ) were used for the calculating of the k_{q} values only. The results are shown in Table 1 and in Fig. 5B. At low temperatures the Trp residues of both HtrA variants were moderately exposed, and the k_{q} values were very similar. However, the HtrA variants showed a different relationship between the relative accessibility of their Trp residues and temperature. The k_{q} values of the control HtrA variant increased gradually in almost a linear fashion at a temperature range of 25–40 °C, whereas the k_{q} value of the R44A variant rose rapidly at temperatures of 20–30 °C, then at higher temperatures decreased to reach values similar to those of the control. The Arrhenius plot (Fig. 5C) enables us to draw further conclusions concerning the k_{q} values. A discontinuity in the plot may reflect a conformational change in the protein. The plot of the control protein exhibits a clear change at 25 °C: ln k_{q} versus 1000 /T shows an upward discontinuity. In the case of the R44A variant there are two prominent discontinuities of the Arrhenius plot: a strong downward discontinuity at 30 °C followed by a slightly upward discontinuity at 35 °C. Presumably at these transition temperatures conformational changes occur in the LA loop in the microenvironment of the Trp-50 residue. What should be noted is that at these temperatures the induced structural alterations were different in the studied HtrA variants. The presented data further support our assumption that the R44A mutation causes a significant structural change in the HtrA molecule.

Analysis of Interactions Formed by the Arg-44 Residue—To learn the nature of the role of Arg-44 in the maintenance of LA loop conformation stability, we undertook a detailed analysis of the available HtrA structures. Arg-44 is visible in the crystal structure of the inactive HtrA form only (PDB code 1KY9). Its side chain is directed toward the phenylalanines at positions 49 and 50. Taking into account the geometry of this region, Arg-44 is most probably involved in cation–π interactions with the Phe-49 and -50 phenolic rings. On the other hand, there is an Asp residue (Asp-232 of the L2 loop) in the proximity of Arg-44, suggesting the possibility of a salt bridge formation. To dispel doubts concerning the orientation and interaction partners,
of Arg-44, we did a conformational analysis of its side chain; (see below and Refs. 23 and 24). This indicates that in the database, which represent the intermediate states of Arg-44, showed probabilities of about 19, 33, and 16% (Fig. 6).

Hence, theoretically, Arg-44 is capable of forming both types of contacts: the cation-π with Phe-50 or the electrostatic with Asp-232. To check which type of interaction is more important for maintaining HtrA stability, we performed the following substitutions of the Arg-44 residue: (i) replacement of Arg-44 with Leu, which should break the interactions with Asp-232 but allow hydrophobic contacts with Phe-49 and Phe-50; (ii) replacement of Arg-44 with Asp, which was expected to prevent both types of interactions; however, anion-π interaction with Phe-50 would be possible; (iii) replacement of Arg-44 with Pro, which should provide sufficient rigidity in this region of the LA loop (as opposite to the R44A mutation). Neither of these mutations caused a significant change in the protein activity (Fig. 3D) or stability (no autodegradation) compared with the wt HtrA (not shown). Thus, we conclude that the most essential stabilizing factor is a reduction in the mobility of the LA loop in the vicinity of residue 44 no matter how it is accomplished.

The Importance of Stability and Activity of the HtrA Variants for E. coli Survival under Heat Shock Conditions—To check the importance of LA loop stability for the proper functioning of HtrA in a cell, we examined the ability of the HtrA-R44A variant to suppress the temperature-sensitive phenotype of the htrA mutation. We found that at a physiological temperature the growth of E. coli htrA (pDF23) cells, expressing HtrA-R44A from the low copy number plasmid, was similar to that of E. coli htrA (pJS5), producing the wild type HtrA (not shown). However, at 45 °C bacteria carrying the htrAR44A gene showed a significantly lower survival rate (at least 2 orders lower), and their colony size was markedly smaller than in the case of bacteria expressing the wild type htrA gene. Interestingly, the presence of the more active HtrA variants (HtrA-F49Y/F50Y or HtrA-F68Y) provided for a much better survival rate for the E. coli htrA strain at 45 °C (Fig. 7A). The analysis of the HtrA content in the studied E. coli cells revealed that the levels of HtrA-F49Y/F50Y, HtrA-F68Y, and wt HtrA were comparable (Fig. 7B). Thus, the observed effect was most probably a result of the more efficient hydrolysis of substrates performed by the mutated HtrA variants. The HtrA-R44A protein was present in a lower quantity, and it tended to autodegrade (Fig. 7C). Therefore, we concluded that HtrA-R44A was too unstable to fulfill its function under heat shock conditions.

Oligomerization Status of the Selected HtrA Variants—Changes in the activity or stability of HtrA often correlates with alterations in the quaternary structure of this protein (37). As shown before, the R44A and F49Y/F50Y substitutions in the LA loop caused destabilization or increased activity of HtrA, respectively. Therefore, we performed a size exclusion chromatography analysis of the oligomerization states of the proteolytically inactive versions of HtrA-R44A, HtrA-F49Y/F50Y, and HtrA as a control (all carrying additional S210A mutation). As can be seen in Fig. 8, the elution profiles of HtrA-F49Y/F50Y and control HtrA are similar (panels A and C). In the absence of a 22-peptide ligand the majority of protein eluted at the position corresponding to the hexameric form, and only a minor broad peak representing large oligomers was detected additionally. The presence of a substrate peptide is known to
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TABLE 1
Comparison of the fluorescence properties of the HtrA variants

| Temperature (°C) | F50W | R44AF50W |
|-----------------|------|----------|
|                 | Ksv | V | τ0 | kq | λmax | Ksv | V | τ0 | kq | λmax |
| 20              | 2.63 ± 0.13 | 0.81 ± 0.09 | 3.66 ± 0.07 | 0.72 ± 0.05 | 341.46 ± 0.65 | 2.54 ± 0.03 | 1.57 ± 0.02 | 4.06 ± 0.06 | 0.63 ± 0.02 | 349.64 ± 0.564 |
| 25              | 2.46 ± 0.35 | 1.22 ± 0.16 | 3.58 ± 0.06 | 0.69 ± 0.11 | 347.35 ± 1.21 | 2.89 ± 0.18 | 1.37 ± 0.01 | 3.87 ± 0.07 | 0.75 ± 0.06 | 350.73 ± 0.22 |
| 30              | 2.94 ± 0.13 | 1.39 ± 0.08 | 3.47 ± 0.08 | 0.85 ± 0.06 | 350.23 ± 0.43 | 4.09 ± 0.06 | 1.20 ± 0.31 | 3.63 ± 0.07 | 1.13 ± 0.04 | 350.73 ± 1.52 |
| 35              | 2.94 ± 0.15 | 1.62 ± 0.14 | 3.33 ± 0.07 | 0.88 ± 0.06 | 351.24 ± 0.38 | 3.19 ± 0.41 | 1.77 ± 0.20 | 3.37 ± 0.06 | 0.95 ± 0.14 | 351.50 ± 0.50 |
| 40              | 3.00 ± 0.31 | 1.98 ± 0.08 | 3.14 ± 0.06 | 0.96 ± 0.12 | 351.8 ± 0.27 | 3.20 ± 0.05 | 1.87 ± 0.02 | 3.35 ± 0.04 | 0.96 ± 0.03 | 352.11 ± 0.21 |

![Image](66x428 to 282x606)

FIGURE 6. The rotamer analysis of Arg-44. Arg-44 can interact both with Asp-232 and Phe-49 or Phe-30 with almost equal probability of ~6.3 and 8.2%, respectively. The numbers at each Arg-44 conformer represent the probability of occurrence of the most representative conformations for selected highest populated clusters in the database. The numbers in parentheses show the probabilities of all conformations in the cluster (23, 24).

trigger the reorganization of HtrA oligomers, which leads to the formation of higher order complexes consisting of up to 30 subunits (12, 18, 24, 30-mers were detected) (15). In accordance with this, in the presence of the substrate we observed a peptide concentration-dependent decrease of hexamer peak intensity and the appearance of the dodecamer peak. Although the overall patterns of hexamer–dodecamer transition of both HtrA variants were similar, at low peptide concentrations HtrA-F49Y/F50Y formed 12-mers less efficiently than the control HtrA. HtrA-R44A showed a markedly different elution profile (Fig. 8, panel B). In the absence of peptide the protein was present as a mixture of variously sized oligomers: predominantly large oligomers, then hexamers, dodecamers, and a substantial amount of trimers. The addition of the lowest concentration of peptide (30 μM) shifted the hexameric and trimeric forms to dodecamers; the amount of large oligomers remained unchanged.

Verification of the LA Loop Model by Site-directed Cross-linking—Because substitutions of Q64A and S54A had no impact on HtrA proteolytic activity, we decided to further verify the proposed theoretical model of the LA loop by the method of disulfide cross-linking (38). As the HtrA variants are expressed in the periplasmic space, the neighboring sulphydryls are likely to be connected by a natural Dsb system. We introduced cysteine residues at the positions expected to form intersubunit contacts within the HtrA hexamer. This included positions 54 (LA) and 325 (PDZ1) (HtrA-S54C/C575/C69S/S210A/R325C), 64 (LA’-LA) (HtrA-C575/Q64C/C69S/S210A), and 229 (L2) (HtrA-S210A/L229C). In the latter variant according to the proposed model, Leu-229 is placed in the vicinity of Cys-57 (a distance of 7 Å) and of Cys-69 (6.3 Å), which form a natural disulfide bond within the LA loop (39). In the former variants the formation of the natural S-S bond was not possible due to the C575S and C69S substitutions. As a positive control we used the HtrAG233C variant, where the residues Gly-233 of the trimeric unit are known to be located very close to each other in the active form of HtrA. The HtrA-S210C protein, where the 210 residues are separated in both an inactive and active state of HtrA, served as a negative control.

We expressed the mutated htrA genes in the E. coli htrA BL20 strain, resolved the cellular lysates by a non-reducing PAGE, and performed Western blotting analysis using anti-HtrA antibodies. As can be seen in Fig. 9 the HtrA S54/C/R325C, Q64C, L229C variants (lanes 1, 2, and 3, respectively) formed two major bands in the gel: one double band (A and B) corresponding to a monomer and the other showing a reduced mobility. A similar effect was observed in the case of the positive control: HtrAG233C (lanes 4). In the case of HtrA-S54C/R325C an additional band of reduced mobility could be observed. Most probably two alternative S-S bonds were formed, connecting either two Cys-54 side chains of interacting subunits or the Cys-54–Cys-325 residues. The negative control HtrA variants S210C and S210A (lanes 6 and 7, respectively) migrated as monomers. Hence, the introduced cysteine residues are located close enough in the native HtrA molecule to interact and form covalent bonds.

DISCUSSION

The LA loop is believed to act as an important regulatory element in the activation process of the HtrA protein from E. coli. According to the current model (13), in the inactive protease state the LA’ loop interacts with two other regulatory loops, L1 and L2, and restricts access to the active center. The activation of HtrA involves large conformational changes of the whole HtrA molecule, including a change in the oligomerization state. During this process disruption of the LA’–L1–L2 loop connections occurs. However, little is known about what type of interactions and which elements of the LA loop are involved in the formation of the interloop contacts and in the maintenance of the proper LA structure. As the available HtrA crystal structures do not provide information about the structure and localization of the major parts of the LA loop, we constructed a model of the LA loop by the aid of molecular model-
ing. The obtained model implies the engagement of the LA loop in the formation of the intersubunit interactions, which may be crucial for the HtrA oligomer stability. To prove the correctness of the model and at the same time check the importance of the suggested interactions, we undertook a detailed mutagenic study.

Based on our theoretical model and the available HtrA crystal structures, we appointed regions of the LA loop engaged in the formation of the putatively important inter-subunit contacts. The most significant are interactions of phenylalanines and prolines at positions 46, 49, 50, 62, 63, 67, and 68 of all the LAs within the hexamer that form a hydrophobic cluster placed in the inner cavity of the oligomer. The presence of such moiety has been proposed by (10); however, in the presented crystal structure residues 52–78 were lacking. This intersubunit connection appears to be of special importance in maintaining the inactive conformation of HtrA. Although replacement of a single Phe with a more hydrophilic Tyr led only to a moderate stimulation of substrate cleavage, the double substitution F49Y/F50Y resulted in a strong increase of H9252-casein degradation. Interestingly, bacteria expressing the mutated HtrA variants F49Y/F50Y or F68Y grew at elevated temperatures significantly better than those containing wt HtrA. This result further confirms the importance of the proteolytic activity of

**FIGURE 7.** The effect of point mutations in the LA loop on the ability of HtrA to suppress the temperature-sensitive phenotype of the htrA mutant and on HtrA protein stability. *E. coli* htrA (BL20) cells, deprived of the functional chromosomal htrA gene, were transformed with low copy number pGB2 derivatives carrying selected htrA variants. Bacteria were grown on rich medium (LB or LA (LB medium plus 1.5% Bacto agar) medium). A, growth of *E. coli* expressing selected LA mutated HtrA variants (HtrA-F49Y/F50Y, HtrA-F68Y, or HtrA-R44A) at 45 °C. As controls, bacteria expressing the wt HtrA or proteolytically inactive HtrA-S210A as well as bacteria carrying the empty pGB2 vector (vector) and non-transformed BL20 strain were used. B, HtrA protein content in the tested *E. coli* cells. The *E. coli* bacteria grown at 30 °C in LB medium to OD595 ∼ 0.3 were transferred to 45 °C for 1 h. Extracts of an equal number of cells were resolved by SDS-PAGE electrophoresis (12.5% gel) and subjected to Western blot analysis using anti-HtrA antibodies. C, HtrA-R44A stability in bacterial cells. *E. coli* bacteria expressing the wt HtrA or HtrA-R44A were grown at 30, 37, and 45 °C to OD595 ∼ 0.5. Western blotting of cellular lysates was performed as described above. The arrow indicates the HtrA degradation product.
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HtrA for bacterial survival under stressful conditions. Not all phenylalanine residues within the LA loop participate in formation of the hydrophobic intermolecular core. The residue Phe-56 is not placed in the vicinity of the remaining phenylalanines and consequently its substitution with a hydrophilic residue, serine, had no impact on the HtrA proteolytic activity.

The model also predicted contacts of the LA loop with the remaining regulatory loops and the PDZ1 domain. We expected that point mutations disrupting the proposed interactions would have an impact on protein activity and/or stability. Indeed, weakening of the expected LA’-L1 link (Q47L, Q70A) led to increased substrate cleavage. The substitution of either glutamine with a nonpolar amino acid (leucine or alanine) most probably disturbed the hydrogen network, which in turn could lead to destabilization of the inactive HtrA conformation. Among interactions involving the LA’ and L2 loops, the hydrophobic interaction of Ile-236 (L2) and Phe-68 (LA’) appears to be particularly interesting. As reported previously (30), the substitution I236N resulted in increased proteolytic activity of HtrA at low temperatures (up to 35 °C). This effect was ascribed to the disturbance of the hydrophobic contact with Ile-228, which is expected to stabilize the inactive conformation. However, it cannot be excluded that the interaction of Ile-236 with the LA loop by means of Phe-68 also contributes to fixing the active center in a catalytically inactive form.

The P43G substitution resulted in a moderate temperature-dependent stimulation of the proteolytic activity of HtrA. Proline 43 forms a kink in the LA loop; however, the presence of a glycine at this position favors loop bending as well. Nevertheless, the presence of a proline is known to stiffen loop structure and to reduce its mobility. Therefore, the lack of Pro-43 may increase the flexibility of the LA loop locally and thus facilitate the thermal activation of the protease. Interestingly, in the vicinity of Pro-43 one of the active site residues, His-105, is located (at a distance of ~3 Å). Hence, it is tempting to speculate that the interaction of Pro-43 with His-105 contributes to the inhibitory effect of the LA loop. The replacement of the succeeding residue, Arg-44, with Ala caused in turn a dramatic increase in the autodegradation rate of the HtrA molecule. The central portion of the LA loop became more exposed to the environment, as judged by the fluorescence properties of a Trp residue placed at position 50. According to the crystal structure, the Arg-44 side chain is directed toward two phenylalanine residues, Phe-49 and Phe-50, and most probably forms the cation-π type of interaction with Phe-50. However, Asp-232 of the L2 loop resides in close vicinity to Arg-44. The protein structure is not tightly packed in this region, so it might be possible for Arg-44 to turn toward Asp-232 and form a salt bridge. According to torsional angle analysis, the probabilities of both Arg-44 side chain arrangements are almost equal. In aqueous solution the energies of electrostatic contacts and the cation-π type of interaction may be comparable (40). Therefore, it is difficult to assess which kind of interaction is more likely to occur in nature. The analysis of further substitutions of Arg-44 revealed that the presence of arginine is not necessary to maintain protein stability and activity. Different types of amino acids are suitable at position 44 as long as their side chains are able to restrict the loop mobility. The most striking example is the R44P substitution. According to the crystal structure and our model, proline placed at position 44 is not likely to form any significant interactions. Thus, its role relies on providing local rigidity of the LA loop. This assumption is further confirmed by the fact that in HtrA from several bacterial species belonging to the Enterobacteriaceae family, the positions corresponding to Arg-44 are occupied by prolines (not shown). In these cases there are two neighboring prolines to ensure the proper rigidity of LA. The increased flexibility of the LA loop seems to have a strong impact on the protein functionality, especially under stressful conditions. The R44A substitution resulted in a decreased HtrA stability in the cell, generally at all the tested temperatures. Although at physiological temperatures the destabilization of HtrA did not seem to affect E. coli growth, the mutated HtrA-R44A variant was less protective of the cells against the consequences of the heat shock conditions. As the activity of HtrA-R44A was not lower than that of wt HtrA, the decreased survival rates must have been caused by deficiency of HtrA-R44A in cells due to its instability. Interestingly, the disulfide bridge connecting Cys-57 and Cys-69 also has a prominent role in providing HtrA molecule stability. The lack of this S-S...
bond results in HtrA autodegradation as well (39); however, the extent of auto-cleavage is lower, and no harmful effect of C57AC69A (Δ Cys) mutation on bacterial survival under heat shock conditions has been observed.3

Our model predicted contacts between LA (residues 52–54) and the PDZ1 domain (residues 270, 271, 273, and 325) and between two LA loops of facing subunits (involving Gln-64) as well as the position of the disulfide bridge. We were not able to verify the presence of these interactions by the activity assays; however, the disulfide cross-linking analysis in vivo indicates that the HtrA segments described above were in sufficient proximity to form a covalent S-S link. The lack of effect of mutations breaking the proposed LA-PDZ1 contacts implies that these interactions may not participate in the inhibitory role of LA. Indeed, careful analysis of the HtrA crystal structure and the published reports (15, 37, 41) indicate that the residues 270, 271, and 273 (PDZ1) are not engaged in signal transmission to the proteolytic domain upon the binding of the activating peptide; thus, this region does not take part in the allosteric activation of HtrA. To the contrary, Arg-235 forms a salt bridge with Glu-193 (L3), which is indispensable for the proteolytic activity of HtrA. For this reason, a much weaker contact with Ser-54 does not play any significant role in protease regulation. While analyzing the mechanism of the inhibitory role of the LA loop, one should bear in mind its highly dynamic nature. For this reason it has not been traced by crystallography. Moreover, the crystal of the HtrA hexamer has been obtained at low temperature (4 °C). The LA loop responds to shifts of temperature by gradual exposition to solvent (as judged by the fluorescence properties of Trp placed at position 50 or 63) (Ref. 12 and this work). The overall structural changes within the proteolytic domain occur in parallel (12). Furthermore, the dynamic light scattering analysis indicates that the diameter of the HtrA hexamer increases with an increase in temperature, suggesting loosening of the oligomer structure (not shown). These data imply that the LA loop may in fact have more space available in the hexamer even at relatively low temperatures (30 °C). Thus, its position within the HtrA molecule is most probably not strictly fixed but may be represented by a population of conformations oscillating around the structure described by our model. As a consequence, the effect of single point mutations may not be dramatic. Moreover, certain predicted interactions may be very weak and their role in structure stabilization negligible.

The LA loops were shown previously to be engaged in the maintenance of the HtrA oligomeric structure. The mutants lacking the majority of the LA loop are found as trimers (14). Also point mutations may affect the oligomerization of HtrA. It has been demonstrated that N45F substitution leads to destabilization of HtrA hexamer and facilitates formation of higher order complexes (12- and 24-mers) in the presence of substrates (41). In our work we identified additional residues of the LA loops whose absence affected the size of the HtrA oligomer. The mutation introduced next to Asn-45, R44A, also caused destabilization of HtrA hexamer and strongly promoted the assembly of dodecamers and larger oligomers at low peptide concentrations. The mutation also resulted in instability of the

3 D. Figaj, A. Sobiecka-Skatula, T. Koper, M. Denkiewicz, B. Lipinska, and J. Skorko-Glonek, unpublished data.
HtrA monomer manifested in its autodegradation. The self-cleavage product retained its proteolytic activity though its oligomers were rather unstable. The other studied mutant F497Y/F505Y had a decreased ability to form higher order assemblies. Interestingly, the proteolytic activity of this mutant was markedly higher compared with wt HtrA. This observation is in line with previously published results showing that protease activation does not depend on higher order oligomers (42).

The results presented in this work let us draw the following conclusions. (i) The proposed theoretical model of the LA loop fits into the HtrA hexamer crystal structure well and explains effectively the observed effects of the point mutations analyzed in this work. (ii) According to our model the LA loops are engaged in important intersubunit interactions of two types. These include (a) strong hydrophobic contacts of Phe and Pro residues and (b) a hydrogen bond network with the L1 loop. Both kinds of interactions stabilize inactive HtrA conformation, as their disturbance (even by a single point mutation) leads to an increased proteolytic activity of HtrA. (iii) The maintenance of the proper rigidity of the LA loop is crucial for the stability of the HtrA polypeptide. Increased flexibility of the LA loop may lead to autodegradation of HtrA. (iv) The LA loop plays an important role in the assembly of trimeric HtrA units into larger complexes. It is important not only in maintaining the inactive hexameric structure, but it also participates in substrate-dependent formation of higher order oligomers. However, activity level does not correlate with the ability of HtrA to switch its oligomerization states.

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