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Research article

PROTEOLYTIC ACTIVATION OF *Chlamydia trachomatis* HTRA IS MEDIATED BY PDZ1 DOMAIN INTERACTIONS WITH PROTEASE DOMAIN LOOPS L3 AND LC AND BETA STRAND β5

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Abstract: *Chlamydia trachomatis* is a bacterial pathogen responsible for one of the most prevalent sexually transmitted infections worldwide. Its unique development cycle has limited our understanding of its pathogenic mechanisms. However, CtHtrA has recently been identified as a potential *C. trachomatis* virulence factor. CtHtrA is a tightly regulated quality control protein with a monomeric structural unit comprised of a chymotrypsin-like protease domain and two PDZ domains. Activation of proteolytic activity relies on the C-terminus of the substrate allosterically binding to the PDZ1 domain, which triggers subsequent conformational change and oligomerization of the protein into 24-mers enabling proteolysis. This activation is mediated by a cascade of precise structural arrangements, but the specific CtHtrA residues and structural elements required to facilitate activation are unknown. Using *in vitro* analysis guided by homology modeling, we show that the mutation of residues Arg362 and Arg224, predicted to disrupt the interaction between the CtHtrA PDZ1

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Abbreviations used: CD – circular dichroism; CtHtrA – *Chlamydia trachomatis* high temperature requirement A; MCA – 7-methoxycoumarin-4-acetic acid; MOMP – major outer membrane protein; MRW – mean residue weight; OmpA – outer membrane protein A; PDZ – PSD-95/Dics-Large/ZO-1; PmpC – polymorphic membrane protein C; pNA – *para*-nitroalinine; SDM – site-directed mutagenesis; SDS-PAGE – sodium dodecyl sulphate polyacrylamide gel electrophoresis

domain and loop L3, and between loop L3 and loop LD, respectively, are critical for the activation of proteolytic activity. We also demonstrate that mutation to residues Arg299 and Lys160, predicted to disrupt PDZ1 domain interactions with protease loop LC and strand β5, are also able to influence proteolysis, implying their involvement in the CtHtrA mechanism of activation. This is the first investigation of protease loop LC and strand β5 with respect to their potential interactions with the PDZ1 domain. Given their high level of conservation in bacterial HtrA, these structural elements may be equally significant in the activation mechanism of DegP and other HtrA family members.

Key words: *Chlamydia*, HtrA, DegP, Protease, Oligomerization

INTRODUCTION

Chlamydia trachomatis (*C. trachomatis*) is an obligate, intracellular, Gram-negative bacterial pathogen that is responsible for the most prevalent sexually transmitted bacterial infection worldwide [1]. Chronic infection can lead to serious medical conditions, including infertility, ectopic pregnancy, epididymitis, and pelvic inflammatory disease [2], but the pathogenic mechanisms are poorly understood. Recent studies identified the protease/chaperone CtHtrA as a potential virulence factor as it is upregulated during *C. trachomatis* disease models [3] and implicated in the pathogenesis of several other bacteria, including *Legionella pneumophila, Salmonella enterica*, and *Helicobacter pylori* [4-6].

HtrA (high temperature requirement A; also known as DegP) has been extensively studied in *Escherichia coli*, where it has been characterized as both a serine protease and a chaperone that functions in the maintenance of periplasmic integrity through the degrading, refolding, and chaperoning of protein substrates [7-9]. Human homologues are involved in cell growth, the unfolded stress response, and apoptosis [10-12] and are associated with disease conditions such as arthritis, cancer, Parkinson's disease, and Alzheimer's disease [13, 14].

Structurally, HtrA consists of a serine protease domain with a chymotrypsin fold that contains the traditional Ser-His-Asp catalytic triad [15], and two carboxy-terminal PDZ (PSD-95/Dics-Large/ZO-1) domains that are involved in substrate sequestration and oligomer formation [16]. The minimum functional structural unit is a trimer held together by inter-protease domain contacts, while two stacked trimers form an inactive resting hexamer form [17, 18]. The inactive hexamer dissociates into trimers upon allosteric binding of the C-terminus of the substrate to the PDZ1 domain and reassembles into proteolytically active 12-mers or 24-mers mediated by PDZ1:PDZ2* domain contacts ('*' indicates a neighboring monomer) [19].

The structural mechanism of HtrA allosteric activation is characterized by the specific repositioning of loops LD, L1, and L2, which surround the active site catalytic residues [19]. This loop reorientation is initiated by sensor loop L3,

which rearranges upon securing a specific biological signal [20]. The 'L3 – activation domain' cascade is conserved in all chymotrypsin-like and HtrA proteases. However, the origin of the initial signal received by loop L3 appears to vary considerably throughout the HtrA family [21]. For instance, the activation of the *E. coli* HtrA protein, DegP, is initiated by allosteric detection of the substrate C-terminus bound to the 'PDZ1 activation cleft' which facilitates its binding to the 'carboxylate binding loop' and results in an oligomer-induced 'PDZ1 – L3' interaction [22]. The activation mechanism of DegS (another *E. coli* member of the HtrA family) is less defined, as DegS appears to also be activated by a DegP-like 'PDZ – L3' interaction [23], although alternate structural studies suggest that the activation occurs upon direct detection of the substrate C-terminus via a 'C-terminus – L3' interaction [24]. The *Mycobacterium tuberculosis* HtrA2 protein (MtHtrA2) has only been shown to form trimers in solution and remains in a constantly activated state with its PDZ1 domain appearing to bind autoproteolysis products [25]. This mechanistic diversity within the HtrA family is remarkable considering their structural homogeneity [21].

Unlike DegP, DegS and MtHtrA2, the specific structural mechanism of activation for *Chlamydia trachomatis* HtrA (CtHtrA) has not yet been defined. We previously showed that CtHtrA can be allosterically activated by PDZ1-activating peptides (activators) and that this mechanism is potentially mediated by a distinct interaction between the protease and PDZ1 domains [26]. Here, we used a combination of homology modeling and *in vitro* analyses to identify specific residues and structural elements that contribute to this allosteric mechanism, furthering our understanding of the CtHtrA mode of activation. These data will potentially enable us to delineate CtHtrA's potential role in chlamydial virulence pathways and determine its benefit as a possible target for alternate therapeutic strategies for *C. trachomatis*.

MATERIALS AND METHODS

Molecular modeling and refinement

For template identification, we used the sequence profile method of HMMER [27] to search for structural homologues of the CtHtrA sequence. These were chosen based on overall sequence identity and accuracy based on the crystal structure resolution and R-factor (reliability factor). A target-template alignment was generated using Fugue [28] to ensure that the substitution scores remained dependent on the secondary structure. Modeller (Version 9.11) was used for model building [29]. One hundred structures were created and the most reliable model was selected based on its discrete optimized protein energy (DOPE) score, low energy and restraint violations. Loop prediction was achieved using the FALC loop modeling server [30] and the final model was validated using Procheck [31].

Bacterial cultures and plasmid construction

E. coli JM109 cells were used for all cloning and genetic manipulations and *E. coli* BL21 (DE3) cells were used for expression plasmid transformation. Cells were routinely cultured on Luria Bertani broth (LB) or agar plates (when appropriate) with ampicillin at 100 μg/ml. The wild-type *htr*A construct was previously described [32]. It was amplified using PCR and inserted into a pET-22b expression plasmid vector containing a C-terminal 6× histidine tag.

Site-directed mutagenesis

Site-directed mutagenesis was performed with the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene) using *pfu* DNA polymerase (Promega), a *Dpn*I digest of the template for 2 h at 37ºC, and transformation into JM109 cells. The primers used are listed in Table 1. Each mutation was confirmed by DNA sequencing prior to transformation into BL21 (DE3) cells for protein expression.

Table 1. Primers and annealing temperature for the site-directed mutagenesis of each CtHtrA mutant used in this study.

Mutant	Location	Primers	T_A
R362L	PDZ1 activation cleft	FOR: 5'-TTTGAGTGCGTTGTTAAATGCCATTTCCCTAATG-3' REV: 5'-CATTAGGGAAATGGCATTTAACAACGCACTCAAA-3'	63°C
R224A	Loop L3	FOR: 5'-TTAGTGCTAAAGGAGCTAATCAGCTACATATTG-3' REV: 5'-CAATATGTAGCTGATTAGCTCCTTTAGCACTAA-3'	60°C
R299L	Carboxylate binding loop	FOR: 5'-TGATGGGCAGGTAACATTAGGCTTTTTGGGAGTTACC-3' REV: 5'-GGTAACTCCCAAAAAGCCTAATGTTACCTGCCCATCA-3'	70°C
K160V	Beta strand β5	FOR: 5'-TTACTCTCCACGATGGACAAGTTTACACAGCTAAGATCG-3' REV: 5'-CGATCTTAGCTGTGTAAACTTGTCCATCGTGGAGAGTAA-3'	65°C

Protein expression, purification and characterization

Proteins were heterologously expressed in *E. coli* BL21 (DE3) cells, transformed with pET-22b-CtHtrA or SDM plasmids, and purified using affinity chromatography as previously reported [26]. Briefly, cells were grown at 37ºC in LB, and induced at A_{600} of 0.6-0.8 with 0.1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) for 5 h, before being harvested by centrifugation at 4,000 g for 20 min. The cell pellet was resuspended in lysis buffer, sonicated, and centrifuged for 30 min at 30,000 g. The supernatant was then loaded onto a TALON cobalt metal affinity column (Clontech, Australia), washed four times with 50 mM sodium phosphate and 300 mM NaCl (pH 7.0) and six times with increased NaCl (500 mM), and eluted with 200 mM imidazole. The purity of the fractions was confirmed via SDS-PAGE analysis and the protein concentration was determined using the Bicinchoninic acid assay (BCA; Sigma-Aldrich, Australia). These conditions were used for the expression and purification of all site-directed mutant proteins reported in this study.

Far-UV CD spectroscopy

Far-UV CD data were collected between 190 and 250 nm using 0.2 mg/ml protein solution in 100 mM sodium phosphate buffer (pH 7.0) in a Jasco J-715 spectropolarimeter (Jasco, Easton, MD, USA) with a 0.1 cm pathlength cell. CD data are reported as mean residue ellipticity. Mean residue ellipticity at wavelength λ was calculated according to Eq. 1.

$$[\theta]_{mrw\lambda} = \frac{MRW \times \theta_\lambda}{100 \times d \times c} \quad (1)$$

where θ_λ is the observed ellipticity (degrees) at wavelength λ, d is the pathlength (m), and c is the protein concentration (mg/ml). Mean residue weight (MRW) was calculated using Eq. 2.

$$(MRW) = \frac{M}{(N\text{-}1)} \quad (2)$$

where M is the molar mass (in Da) and N is the number of amino acids in the chain.

Proteolysis assays

The proteolytic activity of each mutant and wild-type CtHtrA was initially confirmed using β-casein cleavage assays by incubating 2 mg of protease with 10 mg of β-casein in 50 mM Tris and 20 mM $MgCl_2$, and examined via SDS-PAGE. Protease assays were conducted with a peptide substrate labeled with 7-methoxycoumarin-4-acetic acid (MCA; fluorophore) and 2,4-dinitrophenyl (DNP; quencher) or peptides labeled with *para*-Nitroaniline (pNA). Fluorophore/quencher assays were conducted in black plates at 37ºC using a POLARstar Optima fluorimeter (BMG Labtech). The assays were excited at 340 nm and emission was detected at 405 nm. MARS data analysis software (BMG Labtech) was used to calculate the maximum rate from a minimum of six time points for each assay. Assays using pNA-labeled peptides were incubated at 37ºC and analyzed using an xMARK microplate spectrophotometer (Bio-Rad) at 405 nm. Readings were taken every 10 sec for 30 min. Proteinaceous and peptidic substrates and allosteric activators are listed in Table 2. All of the peptides were synthesized by Mimotopes (Melbourne, Australia) to 95% purity and were resuspended in 50% isopropanol. Full-length β-casein was commercially obtained (Sigma-Aldrich, Australia). Dr. Charles Armitage supplied the full-length *Chlamydia muridarum* MOMP, which was recombinantly expressed in *E. coli* and purified according to established protocols [33]. The C-terminal sequence of *C. muridarum* MOMP shares 100% conservation with *C. trachomatis* L2. The statistical analysis was conducted using an unpaired *t*-test, calculated with Prism software (GraphPad). Assays were performed twice, in triplicate.

Table 2. Active site substrates and activators used in the protease assays.

I.D.	Sequence	Function	Origin
βcas1	MCA-ENLHLPLPIIF-DNP	Substrate	Fluorophore/quencher peptide based on known β-casein cleavage site*
pNA1	DPMFKLV-pNA	Substrate	Known DegP and CtHtrA substrate[+, *]
pNA2	PMFKLI-pNA	Substrate	P1 substitution*
pNA3	MFKLI-pNA	Substrate	P1 substitution*
pNA4	MFQLI-pNA	Substrate	P1 and P3 substitution*
pNA5	MFRLI-pNA	Substrate	P1 and P3 substitution*
β-casein	[full-length protein]	Both	Full-length β-casein protein*
Act1	NH_2-VLGPVRGPFPIIF-OH	Activator	13 C-terminal residues of β-casein*
Act2	NH_2-CGELGFFYTPKA-OH	Activator	12 C-terminus residues of insulin b-chain*
MOMP	[full-length protein]	Both	Full-length major outer membrane protein*
OmpA	NH_2-DERAAHVNAQFRF-OH	Activator	13 C-terminal residues of MOMP*
PmpC	NH_2-LAHMMNCGARMTF-OH	Activator	13 C-terminal residues of polymorphic membrane protein C*

* Reported in [26]; [+] reported in [34].

Oligomerization assays

Assays were performed as previously described [26]. Briefly, 0.5-1.0 mg/ml CtHtrA samples in 0.1 M sodium phosphate buffer (pH 7.0) were incubated at 37ºC for 30 min. PDZ1 allosteric activators (full-length protein or peptide; Table 2) were added and complexes were fixed after 10 min with 0.5% glutaraldehyde at room temperature. 1 M Tris buffer (pH 7.5) was added to inactivate any excess glutaraldehyde. Samples were incubated for a further 10 min before being examined on 3-8% Tris acetate gradient gels (Novex, Invitrogen) by silver staining. All assays were conducted in duplicate.

RESULTS AND DISCUSSION

CtHtrA homology models suggest a PDZ1 – protease domain activation signal potentially mediated by protease domain loops LC, β5* and L3

Homology models were generated for both the inactive hexamer (based on DegP, PDB code: 1KY9) and active 24-mer (based on DegP, PDB code: 3OU0) of CtHtrA, based on the alignment in Fig. 1. To investigate the activation mechanism, we identified structural features that were characteristic of the active or inactive models. The rotation of the PDZ1 domain and subsequent reorientation of the protease active site (loops L1, L2, and LD*; '*' refers to a neighboring monomer) was readily apparent, suggesting an oligomer-induced 'PDZ1 – L3 – LD*' activation cascade similar to DegP (Fig. 2).

Fig. 1. Multiple sequence alignment of *Chlamydia trachomatis* HtrA (CtHtrA) and *Escherichia coli* HtrA (EcHtrA). The alignment was generated by the Fugue alignment server [28] and used as the basis for homology modeling of the active form of CtHtrA (24-mer). Residues are colored for amino acid similarity, with green representing 100% conservation and yellow indicating residue likeness (polar, hydrophobic, and charged) according to the Blosum62 matrix. Labeled annotations appear above the sequence, with the signal peptide in yellow, protease domain in pink, PDZ1 domain in blue, and PDZ2 domain in brown. Protein secondary structures appear below the sequences in grey: straight arrows represent β-strands, and undulating lines represent α-helices. Important loops are labeled in yellow, the 'carboxylate binding loop' and 'PDZ1 activation cleft' are shown in maroon. The active site residues (His143, Asp173 and Ser247) are bordered with a red box, while the residues investigated in this study via mutagenesis are bordered with a black box. CtHtrA residue numbers are according to the full-length protein sequence as deposited at the National Centre for Biotechnology Information (NCBI; accession number: YP_001653297.1) [26]. EcHtrA residue numbers are according the standard system used for all published EcHtrA crystal structures [19], which begin at residue position 27 (underlined) in the full-length EcHtrA protein sequence (NCBI accession number: BAL37467.1). The sequence alignment was generated and annotated in Geneious, Version 6.1.4 (Biomatters) and edited using Illustrator CS6 (Adobe).

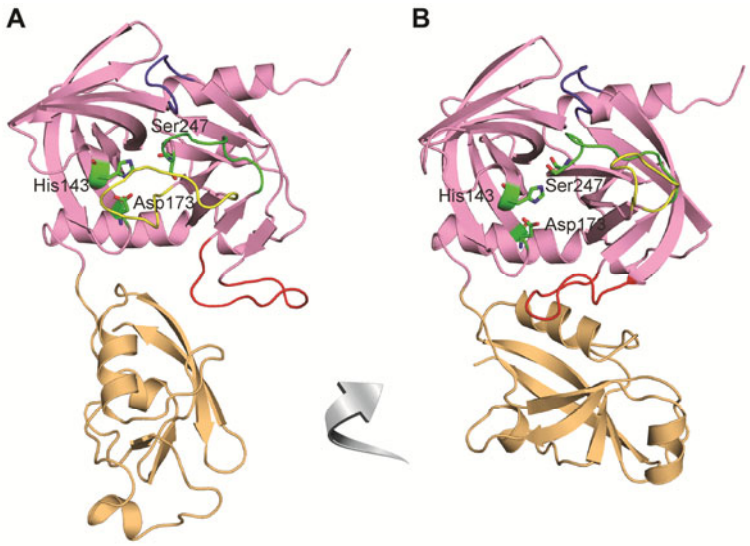

Fig. 2. Conformational change upon the transition from inactive hexamer to active 24-mer. Specific conformational changes occur between the transformation from one monomeric unit within the hexamer (A – inactive; pink protease domain, orange PDZ1 domain) and one monomeric unit within the 24-mer (B – active; pink protease domain, orange PDZ1 domain). The grey arrow indicates the direction and rotation of the PDZ1 domain upon transition to the active state. PDZ2 domains are not shown for clarity. The protease domain loops that characterize the 'activation domain' are shown as loop L1 (green), loop L2 (yellow), and loop LD (blue), in addition to the sensor loop L3 (red). In the hexameric form of CtHtrA, the active site serine (Ser247) is obscured by loop L2 (yellow) while the PDZ1 domain remains separated from loop L3 (red). The 24-mer shows that movement of loop L2 (yellow) allows substrate access to the active site, following its own conformational adjustment. Rotation of the PDZ1 domain allows its interaction with loop L3 of the protease domain.

However, the models also highlighted specific 'PDZ1 – LC' and 'PDZ1 – β5*' interactions (β5 refers to beta strand 5 of the protease domain), that potentially contribute to an alternative 'PDZ1 – protease domain' activation signal, which may not act via the L3 'sensor' loop mechanism reported for DegP (Fig. 3).
Specifically, these are the residues predicted to mediate these loop interactions. The side-chain carboxylate oxygen of Asp169 of loop LC forms an electrostatic interaction with the guanidinium group of Arg299 of the PDZ1 domain ('PDZ1 – LC'), while a 'PDZ1 – β5*' interaction mediated by the side-chain carboxylate oxygens of Asp310 and Glu312 forms an electrostatic interaction with the terminal side-chain of Lys160 (Fig. 3). In addition, a potential DegP-like 'PDZ1 – L3 – LD*' interaction is mediated by the guanidinium group of Arg362 (PDZ1 domain) forming a H-bond with the carbonyl oxygen of Val230 (loop L3), while the side-chain of Arg224 (loop L3) interacts with the side-chain oxygen of Thr213 (loop LD*; Fig. 3). Each of these interactions occurs in the structural region immediately adjacent to the PDZ1 activation cleft, suggesting their importance in mediating a proteolytic (and/or oligomeric) activation signal

following the binding of the substrate C-terminus to the cleft. Notably, the residues mediating the 'PDZ1 – LC' interaction (Asp169 and Arg299) are conserved between DegP and CtHtrA, suggesting a conserved potential mechanism is mediated by this interaction, while the residues of the 'PDZ1 – β5*' interaction (Asp310/Glu312 and Lys160) are not (Asp310 = Asn273 in DegP). This may result in mechanistic differences between DegP and CtHtrA.

To investigate the contribution of each interaction to the activation of CtHtrA *in vitro*, we focused on the DegP-like 'PDZ1 – L3 – LD*' interactions before examining the potential for an alternative activation mechanism via 'PDZ1 – LC' and 'PDZ1 – β5*'.

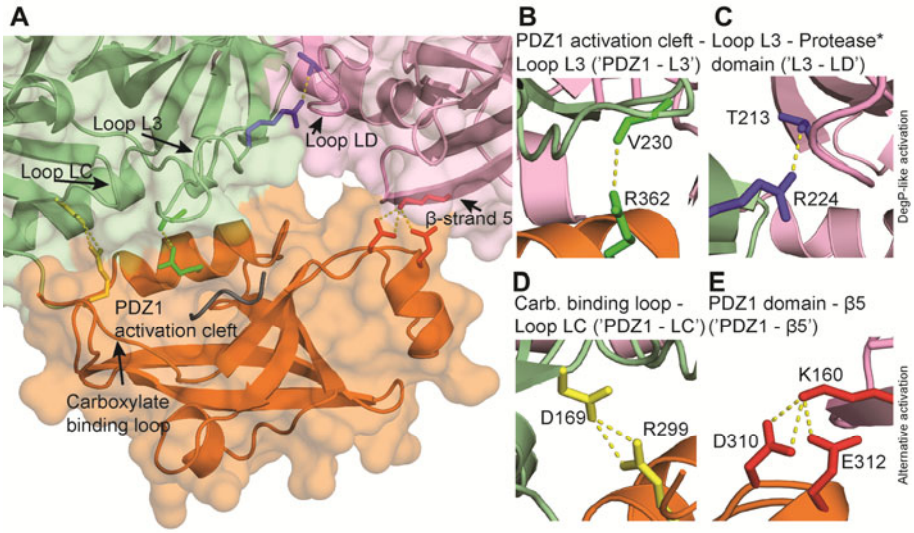

Fig. 3. The CtHtrA substrate C-terminus binds to the PDZ1 activation cleft initiates a series of conformational events to allow the protein to reach the active (24-mer) state. Four key interactions occur between the PDZ1 domain (orange), protease domain (green), and protease domain of an adjacent monomer (pink) upon the activation of CtHtrA. A – Allosteric C-terminal peptide (grey) binds to the PDZ1 activation cleft resulting in the movement of the PDZ1 domain and interaction between the PDZ1 domain and loop LC (yellow), the PDZ1 activation cleft and loop L3 (green), loop L3 and loop LD* (blue), and the PDZ1 domain and β-strand 5* (red). B – The carbonyl oxygen of Val230 forms a H-bond with the side-chain of Arg362; C – The side-chain carboxylate of Thr213 forms a H-bond with Arg224; D – The side-chain carboxylate of Asp169 of loop LC forms an electrostatic interaction with the guanidinium group of Arg299 of the PDZ1 domain (yellow); E – The side-chain carboxylates of Asp310 and Glu312 form an electrostatic interaction with the ε-amino group of Lys160.

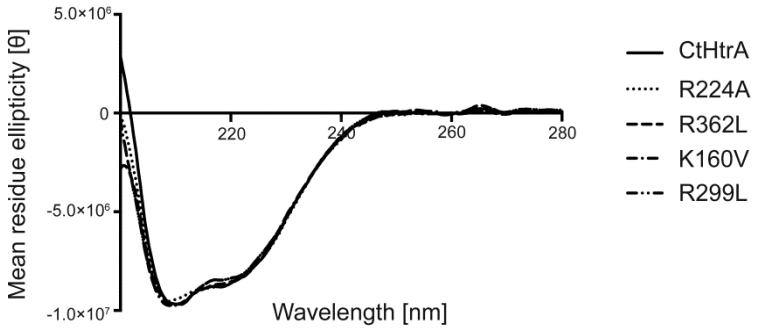

Fig. 4. Far-UV CD spectroscopic analysis of CtHtrA and mutants. Mean residue ellipticity is reported as: degrees m^2 mol^{-1} $residue^{-1}$. CtHtrA: wild-type; R224A: 'L3 – LD'; R362L: 'PDZ1 – L3'; K160V: 'PDZ1 – β5*'; R299L: 'PDZ1 – LC'.

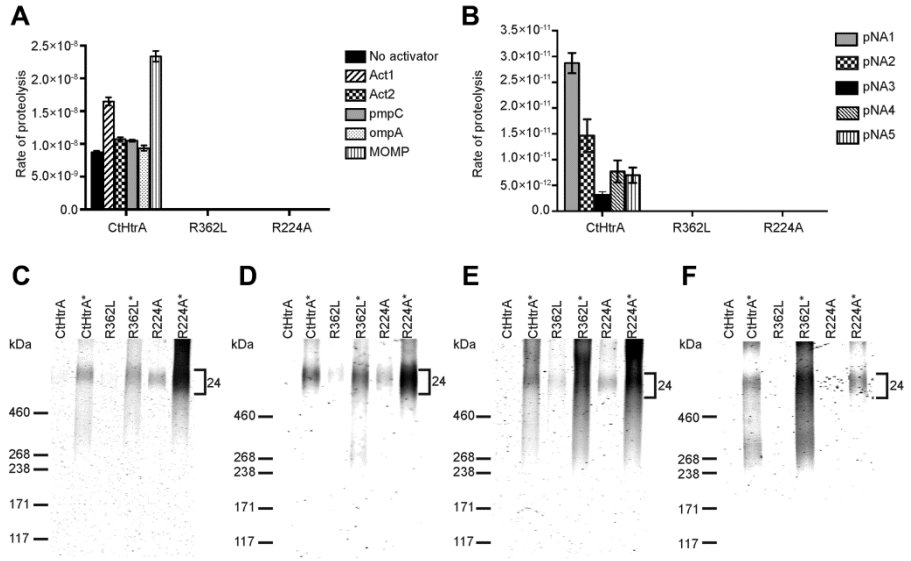

Fig. 5. The rate of proteolysis, impact of allosteric activation, and oligomerization of wild-type CtHtrA and R362L and R224A mutants in the presence of full-length protein or peptide activators. A – Rate of proteolysis of the βcas1 substrate in the presence of activators. Rate of proteolysis is measured as μM MCA min^{-1} μg $CtHtrA^{-1}$; B – Rate of proteolysis of the pNA substrates. Rate of proteolysis is measured as pNA 405 nm min^{-1} μg $CtHtrA^{-1}$; C – Oligomerization to 24-mer in the presence of β-casein; D – Oligomerization to 24-mer in the presence of Act1 activator; E – Oligomerization to 24-mer in the presence of full-length MOMP protein; F – Oligomerization to 24-mer in the presence of OmpA peptide. In the oligomerization assays, * represents the presence of an activator. All substrates and activators are listed in Table 2. Error bars represent standard error of the mean (n = 6). CtHtrA: wild-type; R362L: 'PDZ1 – L3'; R224A: 'L3 – LD'.

CtHtrA 'PDZ1 – L3 – LD*' interaction is required for proteolytic activity but not oligomerization

CtHtrA mutants R362L ('PDZ1 – L3') and R224A ('L3 – LD') were generated and no gross perturbation of overall secondary structure was observed according to CD spectroscopy (Fig. 4). The activity of these mutants was tested against substrates βcas1 and pNA1-5 (Table 2). Proteolytic activity was found to be below the limit of detection for the assay for both mutants, correlating with the results of β-casein degradation assays, where no degradation of β-casein was seen after 40 min (data not shown). To test whether the presence of allosteric activators could promote proteolysis, we assayed these mutants in the presence of full-length MOMP or the allosteric activator peptides, Act1, Act2, OmpA, and PmpC (Table 2). None of the activators could induce proteolytic activity in either the R362L or R224A mutant (Fig. 5A, B), while wild-type CtHtrA proteolysis was activated 2.7-fold in the presence of MOMP, 1.9-fold in the presence of Act1, 1.2-fold in the presence of Act2, 1.1-fold in the presence of OmpA, and 1.2-fold in the presence of PmpC, as previously observed [26]. Given the proteolytic inactivity of these mutants, we then tested whether this correlated with an oligomeric preference for inactive hexamers, but oligomerization assays showed that both mutants readily oligomerized to 24-mer in the presence of each activator (Fig. 5C-F).

This establishes that interactions mediated by Arg362 and Arg224, potentially mediating 'PDZ1 – L3' and 'L3 – LD*' loop contacts, are critical for the activation of proteolytic activity in CtHtrA, which is consistent with the proteolytic activation mechanism reported for the *E. coli* HtrA (EcHtrA) protein, DegP [20]. Our models predicted that these interactions are achievable upon the formation of 24-mers, which correlates with our *in vitro* data where oligomerization to 24-mer was always observed in the presence of full-length protein or peptide activators. This implies a similar mechanism to that reported for DegP, where oligomerization to 12-/24-mer is shown to be the structural mechanism that promotes the critical 'PDZ1 – L3 – LD*' interactions [18, 22].

The ability of CtHtrA to form 24-mers with a disruption in 'PDZ1 – L3' is notable as it suggests that CtHtrA oligomerization can occur in the absence of the correct formation of the proteolytic site and that oligomerization may result from an alternate structural mechanism. This is in contrast with oligomeric data for DegP, where a 'PDZ1 – L3' disruption mutant remained as a hexamer and could not form any higher oligomers [20]. However, this comparison remains limited as the DegP data is derived from the dynamic nature of size-exclusion chromatography (SEC), while ours relies on the single "snapshot" provided by oligomerization assays, which solely reports the presence/absence of higher oligomers. A true comparison requires validation from SEC data in future studies with a specific focus on the oligomerization mechanism of CtHtrA, but our study provides the first indication that the activation of proteolysis and oligomerization may occur independently for CtHtrA.

CtHtrA proteolytic activity is modulated by interactions between the PDZ1 domain and protease domain loops β5* and LC

We then tested the potential role for a sensor loop L3-independent activation mechanism via alternative interactions predicted to be present in the active conformation of CtHtrA: 'PDZ1 – LC' and 'PDZ1 – β5*' (Fig. 3). R299L and K160V were the mutants generated as they were predicted to result in a disruption of specific 'PDZ1 – LC' and 'PDZ1 – β5*' interactions, with each appearing to retain overall secondary structure composition similar to the wild-type when examined by CD spectroscopy (Fig. 4). The K160V and R299L mutants both displayed a ~2.5-fold reduction in proteolytic rate against the βcas1 substrate compared to wild-type CtHtrA (Fig. 5), while the addition of both full-length MOMP and Act1 activators increased the proteolysis rate by ~2.5-fold and ~1.4-fold respectively, which is consistent with the activation observed for wild-type CtHtrA (Fig. 6). The presence of Act2, OmpA, and PmpC activators were unable to induce an increase in protease activity for K160V and R299L compared to the non-activated control (Fig. 6).

This reduced proteolytic activity for each mutant was also observed during assays with pNA-labeled peptide substrates. The K160V mutant hydrolyzed all pNA-labeled substrates with a similar specificity to wild-type CtHtrA, but at a reduced rate. A 3.5-fold reduction in activity was observed for pNA1, a 2.6-fold reduction for pNA2, a 2.1-fold reduction for pNA3, a 1.5-fold reduction for pNA4, and a 2.4-fold reduction for pNA5 (Fig. 6). Notably, the R299L mutant displayed a 31-fold reduction in activity for pNA1, while a 7.2-fold reduction was observed for pNA2, a 4.1-fold reduction for pNA3, a 1.1-fold reduction for pNA4, and a 5.1-fold reduction for pNA5, and an alternate substrate specificity compared to wild-type CtHtrA. In particular, R299L appeared to prefer an isoleucine at the P0 position and the absence of an aspartic acid at the P6 position, which may be due to disruption of the active site architecture caused by the R299L mutant. This could indicate the involvement of the 'PDZ1 – LC' interaction in promoting the correct orientation of the active site or facilitating the proper binding of substrate to the active site.

These data indicate that CtHtrA is able to maintain proteolytic activity, albeit at a lower rate, and oligomerize to 24-mer in the presence of R299L and K160V mutants, respectively predicted to disrupt 'PDZ1 – LC' and 'PDZ1 – β5*' interactions. While we previously investigated an R299W mutant that was shown to abrogate proteolytic activity, in this case the bulkier tryptophan mutation was intended to alter the flexibility of the loop connecting the protease domain and PDZ1 domain [26]. This study utilizes a more subtle mutation to the relatively small leucine residue to precisely disrupt the PDZ1 domain interaction with loop LC only. As a result, we show that predicted 'PDZ1 – LC' and 'PDZ1 – β5*' interactions via mutations of Arg299 and Lys160 residues are not critical for the activation of proteolysis to occur and further validates the DegP-like 'PDZ1 – L3 – LD*' activation cascade for CtHtrA proteolysis as proposed above.

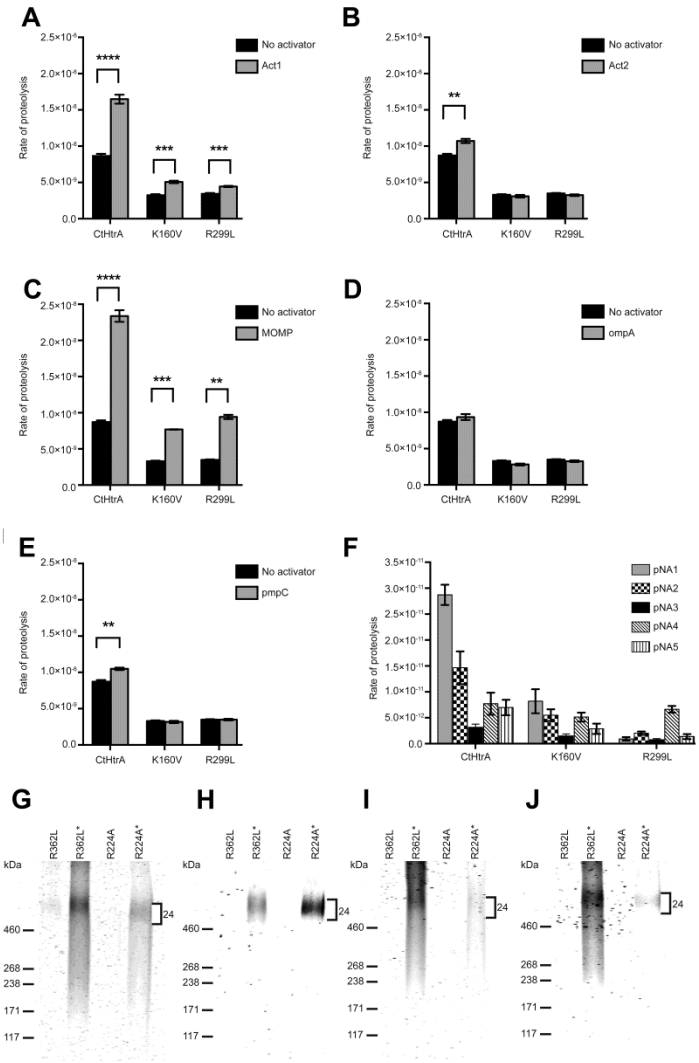

Fig. 6. The rate of proteolysis, impact of allosteric activation, and oligomerization of wild-type CtHtrA and K160V and R299L mutants in the presence of full-length protein or peptide activators. Rate of proteolysis of the βcas1 substrate, measured as μM MCA min^{-1} μg $CtHtrA^{-1}$, in the presence of: A – Act1 activator; B – Act2 activator; C – full-length MOMP; D – OmpA activator; and E – PmpC activator. F – Rate of proteolysis in the presence of pNA1-5 substrates. Rate of proteolysis is measured as pNA 405 nm min^{-1} μg $CtHtrA^{-1}$. G – Oligomerization to 24-mer in the presence of β-casein. H – Oligomerization to 24-mer in the presence of Act1 activator. I – Oligomerization to 24-mer in the presence of full-length MOMP protein. J – Oligomerization to 24-mer in the presence of OmpA peptide. In the oligomerization assays, * represents the presence of an activator. All substrates and activators are listed in Table 2. ** indicates $p < 0.01$, *** indicates $p < 0.001$, **** indicates $p < 0.0001$. Error bars represent standard error of the mean ($n = 6$). CtHtrA: wild-type; K160V: 'PDZ1 – β5*'; R299L: 'PDZ1 – LC'.

It should be noted that disruptions of 'PDZ1 – LC' and 'PDZ1 – β5*', as predicted with the R299L and K160V mutants, remain able to influence the proteolysis rate to some degree and may also contribute to the CtHtrA activation mechanism. Notably, these data demonstrate that the reported 'PDZ1 – L3 – LD*' interaction is not the only requirement for the activation of proteolytic activity in CtHtrA and suggest a complex structural interplay that potentially includes PDZ1 interactions with loop LC and protease domain strand β5*. These interactions may facilitate PDZ1 domain stability in the CtHtrA oligomer, but given the differential proteolytic response to different activators, it is more likely that they influence the correct binding of the substrate C-terminus to the PDZ1 carboxylate binding loop and subsequent proteolytic response. This may be the means by which the proteolysis and chaperone activities can be distinctly mediated. This is the first investigation of loop LC and strand β5 with respect to their potential interactions with the PDZ1 domain. Given their high level of conservation in bacterial HtrA, these structural elements may be equally significant in the activation mechanism of DegP and other HtrA family members.

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