The active site residue V266 of chlamydial HtrA is critical for substrate binding during both *in vitro* and *in vivo* conditions

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Abstract

HtrA is a complex, multimeric chaperone and serine protease important for the virulence and survival of many bacteria. *Chlamydia trachomatis* is an obligate, intracellular bacterial pathogen that is responsible for severe disease pathology. *C. trachomatis* HtrA (CtHtrA) has been shown to be highly expressed in laboratory models of disease. In this study, molecular modelling of CtHtrA protein active site structure identified putative S1 – 3 subsite residues I242, I265, and V266. These residues were altered by site-directed mutagenesis and these changes were shown to considerably reduce protease activity on known substrates and resulted in a narrower and distinct range of substrates compared to wildtype. Bacterial two hybrid analysis revealed that CtHtrA is able to interact *in vivo* with a broad range of protein sequences with high affinity. Notably, however, the interaction was significantly altered in 35 out of 69 clones when residue V266 was mutated indicating that this residue has an important function during substrate binding.

Introduction

Chlamydia (C.) trachomatis is an obligate intracellular pathogen responsible for the highest number of sexually-transmitted bacterial infections throughout the world [WHO, 2011]. Infections are frequently asymptomatic and without treatment can result in long-term health problems [Ghinsberg and Nitzan, 1994]. One explanation for the severe disease pathology is that the inflammatory response to ascending persistent chlamydial infections results in irreparable tissue damage [Witkin and Linhares, 2002]. Analysis of laboratory models of chlamydial persistence identified high levels of HtrA (high temperature requirement A) [Belland, Nelson et al., 2003]. *C. trachomatis* HtrA (CtHtrA) is an extracytoplasmic (likely periplasmic), serine protease with chaperone and proteolytic activity and demonstrates broad sequence specificity [Huston, Swedberg et al., 2007]. *Chlamydia* is not able to be genetically manipulated and hence *in vitro* strategies to understand the biochemical function of proteins from this important pathogen provide an alternative investigative means. A variety of bacteria require HtrA for their survival under certain stress conditions. HtrA is also an important virulence factor for a number of pathogens, including; *Helicobacter pylori*, *Bordetella pertussis* and *Bacillus anthracis* [Hoy, Lower et al., 2010; Baud, Gutsche et al., 2011; Chitlaru, Zaide et al., 2011]. Biochemically it is known that HtrA degrades misfolded proteins during stress conditions and additionally is a chaperone for outer membrane protein assembly [Poquet, Saint et al., 2000; Hoy, Lower et al., 2010; Baud, Gutsche et al., 2011; Chitlaru, Zaide et al., 2011]. Substrate binding of HtrA occurs initially in the PDZ domain, which leads to conformational changes and subsequent oligomerisation to allow formation of active oligomeric complexes [Krojer, Sawa et al., 2010]. During oligomerisation, the L1 and L2 loops break their interactions with the protruding LA loop from the neighbouring monomer, and arrange into an ordered conformation with resolved

subsite pockets to facilitate proteolysis [Krojer, Sawa et al., 2010]. Studies on the role of the L2 loop in *Escherichia coli* HtrA/DegP (EcHtrA) have shown that the L2 loop, in particular the apical region, is necessary for correct active site conformation [Sobiecka-Szkatula, Gieldon *et al.*, 2010]. Key residues in EcHtrA were identified which are involved in the stabilisation of the L2 loop, in particular residue L229 which when mutated, completely eliminated the proteolytic activity [Sobiecka-Szkatula, Gieldon *et al.*, 2010]. In the present study we conducted a molecular investigation of CtHtrA active site binding specificity, focusing particularly on the role of residues in the L2 loop.

Results and Discussion

V266 is predicted to be involved in forming the S1binding pocket of CtHtrA

The structure of CtHtrA was modelled to predict the residues involved in forming the active site binding cleft. Models were generated for both the inactive hexameric form based on EcHtrA as well as an active form based on *E. coli* DegS [Huston, Tyndall et al., 2011]. The model was used to identify putative active site binding residues I242, I265, and V266 which are likely to be involved in forming the S1-3 pockets of the structure (Fig. 1 A and B). The structural loops around the active site of CtHtrA and EcHtrA are well conserved (Fig. 1 C). Therefore there can be some confidence that these residues are important for substrate specificity as the structural loops of EcHtrA, in particular the L2 loop, have been shown to be involved in substrate binding by forming the S1 specificity pocket [Krojer, Garrido-Franco *et al.*, 2002; Krojer, Sawa *et al.*, 2008; Sobiecka-Szkatula, Gieldon *et al.*, 2010]. A microbial protein BLAST and alignment of the results for CtHtrA loop 1 and 2 revealed that from 250 returned matches, I242 and I265 were conserved in 99% of sequences. The conservation of isoleucine among the different bacterial species implies that these residues may play a critical role in the structural integrity of the active site rather than in specificity. In contrast however, only 76% of the aligned BLAST sequences contained a non-polar residue with branched side chains in the CtHtrA V266 position with only 10% having a conserved valine. Different bacterial HtrA potentially have different substrate specificity therefore a reduced conservation of branched non-polar residues, and less still with valine, in the 266 position suggests that this particular residue is potentially involved in binding specificity. To investigate this, each of these residues in CtHtrA was altered and the protein analysed in proteolytic assays.

The identified L2 loop residues are important for protease activity and substrate specificity

In order to assess the role that these CtHtrA L2 loop residues have in substrate specificity, they were altered by site-directed mutagenesis and the proteins purified and examined for proteolytic activity and specificity. The V266G (S1 subsite), I265G (S2 subsite), and V266G-I242G mutant versions of CtHtrA considerably reduced the protease activity of the specific substrates and showed a narrower range of substrates compared to wildtype CtHtrA (wtCtHtrA) (Table 1). CtHtrA activity for these substrates was previously published by Huston *et al.* 2011 [Huston, Tyndall et al., 2011]. The reduced activity observed in the mutated proteins is consistent with a previous study on EcHtrA, where all mutations in the active site affected β-casein hydrolysis activity except I228N [Sobiecka-Szkatula, Gieldon *et al.*, 2010]. All mutated proteins were able to hydrolyse β-casein when assessed by conducting the assay and subsequently monitoring the remaining amount of β-casein using Coomassie stained PAGE. The peptide substrates that were hydrolysed by the mutated CtHrA proteins examined here, did not indicate a marked alteration in the substrate specificity of CtHtrA at P1 or P2. The mutated proteins cleaved very few of the peptides known to be CtHtrA substrates and it is interesting that they retained activity (albeit lower binding and catalytic turnover) on the two shorter peptides (MFKLI-pNA and FKLI-pNA). DPMFKLV-pNA was an efficient substrate for wtCtHtrA and the double mutant, but was not cleaved by the proteins with single mutations in the active site. Interestingly, all three mutated proteins showed a lower Km (increased binding) to the peptide with P at P1, even I265G, despite this residue being predicted to be a site two binding pocket residue. EcHtrA has also been shown to preferentially cleave non-polar, hydrophobic substrates, most often at valine [Jones, Dexter et al., 2002]. This is also the case for CtHtrA, with the data here supporting a key role in L2 loop residues for binding and specificity for protease activity.

The double mutant cleaved two substrates which were not cleaved by wtCtHtrA (GGGAAPL-pNA and GGGEHTV-pNA) further supporting that the L2 loop residues do influence the binding specificity of CtHtrA. These were GGGAAPL-pNA and GGGEHTV-pNA which represent model chymotrypsin and elastase substrates respectively, with additional residues to lengthen the peptide. To determine, whether the loss of cleavage in the mutants, particularly V266G, was due to binding recognition or the structural integrity of the active site an *in vivo* molecular binding assay was employed.

CtHtrA has broad sequence specificity *in vivo*, which appears to favour sequences with an α-helix secondary structure

To further investigate CtHtrA active site binding, an *in vivo* approach was employed. Due to the lack of a suitable genetic manipulation system for *Chlamydia* a bacterial two hybrid approach was employed. To determine if it is possible to utilise this system for CtHtrA, a successful positive CtHtrA control was required. WtCtHtrA and S247A (CtHtrA with the active site serine altered to an alanine to ablate protease activity) were individually cloned into the complementary vectors of the BACTH system and then co-transformed into DHP-1. A positive interaction was expected as HtrA forms homo-oligomers, however this was not observed when wtCtHtrA was present on both plasmids. HtrA is known to undergo self cleavage therefore the inability to detect wtCtHtrA self-interaction may be due to this activity. When S247A was cloned into both vectors however, a positive interaction was observed, providing a positive control and evidence that CtHtrA binding can be assessed using this method.

Previous studies in a variety of bacteria have shown that HtrA is vital for chaperoning and hydrolysing outer membrane proteins to allow for correct assembly [Poquet, Saint et al.,

2000; Baud, Gutsche et al., 2011; Chitlaru, Zaide et al., 2011]. This chaperone activity is believed to be mediated by assembly of HtrA homo-oligomers (up to 24-mer) around the protein substrate [Krojer, Garrido-Franco et al., 2002]. Whilst homo-oligomers and substrate binding cannot be detected in this bacterial two-hybrid, the trigger for this assembly is thought to be by the HtrA PDZ1 binding by a single protein C-terminal sequence [Krojer, Pangerl et al., 2008]. Two membrane proteins which are candidate substrates for triggering HtrA activation by C-terminal binding to the PDZ1 domain were selected from the *Chlamydia* genome to be cloned and tested using the bacterial two-hybrid. In order to control for non-specific interactions both C-terminal and internal fragments of two candidate membrane associated substrates were cloned (both excluding predicted transmembrane domains). As a control a soluble protein was also tested for binding to CtHtrA in the bacterial two hybrid. These candidates were YscC, outer membrane component of the type three secretion system, GspD, outer membrane component of the type two secretion system, and Mip, a secreted protein. These genes were cloned into both T25 vector orientations of the BACTH system and screened with S247A in both T18 orientations, however none of the candidates demonstrated a positive interaction with S247A (data not shown). Thus, either these are not substrates for CtHtrA, or CtHtrA binding to protein substrates is only transient or not possible when it is monomeric based interactions such as in this system. The initial step in triggering the HtrA activation cascade is believed to be direct binding to a single PDZ1 hence if these candidates were substrates which could bind to PDZ1 they would have been detected in this assay.

To further test if there is a *in vivo* sequence specificity for CtHtrA binding a smaller fragment size random clone library was generated in the BACTH vector pKNT25 and screened against pUT18C-S247A. The N-terminus of substrates enters HtrA's active site for processing hence cloning the library into pKNT25 allows the random fragment to be

recognised by the active site. It was calculated that 43, 000 clones were required to cover the genome of *C. trachomatis* L2 once, assuming an average fragment size of 150 bp. This average fragment size was determined by restriction enzyme digest screening of 60 clones randomly from the library prior to screening. A total of approximately 45, 000 clones were generated from the random library of which 500 exhibited a positive interaction when co-transformed with pUT18C-S247A. A selection of the positive clones were randomly isolated, sequenced and analysed for secondary structure. There were 2 vector constructs with no inserts identified in these 80 clones (which had been initially selected based on positive interaction with HtrA) indicating a false positive rate of at least 3.75%, consistent with previous reports using two-hybrid systems [Sontag, Singh *et al.*, 2007]. Furthermore, in a control experiment a small aliquot of the random clone library (not known to interact) was spiked with pKNT25-S247A into the assay (at 10% DNA concentration relative to the library DNA concentration). These were screened and 10 positive interaction colonies were cultured and sequenced, 2 of the 10 positive interaction clones were the S247A construct, supporting that the assay is valid. A large diversity of sequences were identified among this positive population and of these, 53% were predicted to be α-helices, one of the most common secondary structures in proteins. Attempts to develop a consensus or heat map profile of these sequenced interacting clones to construct a more descriptive analysis of the *in vivo* binding determinants of CtHtrA were not successful due to the considerable diversity in length and amino acid sequence of the clones. This suggests that like *E. coli*, CtHtrA binds a broad range of sequences [Jones, Dexter *et al.*, 2002].

3.4. Residue V266 is important for CtHtrA in vivo substrate binding

A selection of the clones which interacted with S247A were then examined by β-galactosidase assays to quantify the level of interaction mediated by their co-transformation

with S247A and S247A-V266G. Positive clones were tested in conjunction with either pUT18C-S247A or pUT18C-S247A with an additional site-directed mutation at V266G (pUT18C-S247A-V266G). The level of β-galactosidase activity varied among the clones however all of the positive clones exhibited a level of interaction higher than that of the negative control (pUT18C co-transformed with pKNT25). No sequence type or consensus could be reliably generated using bioinformatic tools, even using only the top 10% of interacting fragments, indicating that CtHtrA is capable of binding a broad range of protein sequences, and potentially the impact of these protein sequences in the context of the Cya fusion may in fact be the determinant of binding to CtHtrA. Interestingly however, when the 69 of the positive clones were co-transformed with pUT18C-S247A-V266G, the complementation was significantly reduced (greater than 1.2 fold, p value <0.05) for 32 of the clones, one clone with a fold change of 1.17 was significantly different (p valuce <0.05), two clones exhibited a statistically significant increase in binding in the V266G mutation (FC <0.8, p value <0.05) (Table 2). A number of the clones which had been randomly selected for sequence were included in this analysis; however there was still no clear consensus of a binding motif based on different β-gal activity observed in the presence of the V266G mutation. These data support that V266 is an important residue for substrate binding in CtHtrA, and this is in a substrate sequence dependent manner. However, it is not possible to tease out what aspects of the clones sequences are affected by the V266G mutation given the size and variability of the sequences of the clones which showed significantly different interactions. This residue could be involved in substrate binding specificity given that the residue in this position differs in other bacterial HtrAs (as indicated by the previous alignment results). Alternatively, and more likely, it could be involved in the structural integrity of the active site and could possibly be involved in the stabilisation of loop L2. Loop L2 blocks the entrance to the active site therefore when HtrA is activated it alters its

conformation to allow activation and access to the active site centre [Krojer, Pangerl *et al.*, 2008]. The residues located at the base of L2 in *E. coli* (L229 and I238) are believed to interact with each other to stabilise the L2 loop which blocks the active site, as substitution of either of these residues with asparagine resulted in EcHtrA losing proteolytic activity [Krojer, Sawa *et al.*, 2010]. In addition these residues have also been shown to contact the LA regulatory loop indicating that they may be involved in the transduction of the allosteric signal from the PDZ [Krojer, Sawa *et al.*, 2010]. Krojer and co-workers (2010) [Krojer, Sawa *et al.*, 2008] also support the existence of protease and PDZ domain collaboration of substrate specificity showing that the EcHtrA protease domain binds with interacting peptide ligands in the same manner as its PDZ1 domain. A similar collaboration may also exist in CtHtrA where V266 functions in the structural activation signal initiated from the substrate binding in the PDZ cleft. Reasonably, substrate specificity of such a complex protein is unlikely to be controlled purely by a single residue. Nevertheless, this study has shown that, V266 seems likely to be a key substrate binding determinant for CtHtrA potentially by either direct formation of a substrate pocket or by a structural role such as stabilisation of loop L2.

Experimental Procedures

Molecular modelling, protein BLAST and secondary structure analysis

The molecular models of CtHtrA structure used were acquired from a previous study by Huston and co-workers (2011) [Huston, Tyndall et al., 2011]. A Protein BLAST was conducted on loops 1 and 2 (residue 240-276) of CtHtrA using *blastp* located at http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins. BLAST results were aligned using ClustalW2 located at http://www.ebi.ac.uk/Tools/msa/clustalw2/. Secondary structures were predicted using the GARNIER software [Garnier, Gibrat et al., 1996] from nucleotide sequences translated by ExPaSy Translate tool ***(http://web.expasy.org/translate/).***

Protease activity and specificity assays

CtHtrA was heterologously expressed and purified as previously described [Huston, Swedberg et al., 2007]. CtHtrA activity was confirmed using β-casein as a substrate [Huston, Swedberg et al., 2007]and confirmed to be purified to homogeneity by checking the preparations on coomassie stained SDS PAGE. Protease assays using recombinant wild type and mutant CtHtrA were performed as previously outlined in [Huston, Tyndall et al., 2011].

Bacterial Two Hybrid system and random library generation

The *cthtra* gene was cloned in frame with the pUT18C (ampicillin) and pKNT25 (kanamycin) vectors provided with the Bacterial Adenylate Cyclase Two Hybrid (BACTH) kit (Euromedex) by PCR amplification using the primers BACTH-CtHtrA-F 5'-3': CGGGTACCGATGCTAGGCTATAGTGCGTCAAAG, BACTH-CtHtrA-R 5'-3':

CGGGTACCGCCTCGTCTGATTTCAAGACG and cloned with restriction enzyme sites (underlined: KpnI). The *mip* gene and segments of the *gspd* and *yscc* genes were cloned in frame into the pKT25 and pKNT25 vectors using the primers outlined in the supplementary data (Supplementary Data Table 1). Positive interactions were observed when complementary plasmids (*i.e.* one plasmid containing *cya* fragment T18 and one containing fragment T25) were co-transformed into a *cya* deficient *E. coli* strain, DHP-1, and a blue colony obtained on supplemented Luria Bertani media (final concentration of 40 μg/ml X-gal, 0.5 mM IPTG and 100 μg/ml ampicillin or 50 μg/ml kanamycin) as per manufacturer's protocol outlined in the BACTH kit (Euromedex, France). A random clone library was generated by digesting purified *C. trachomatis* L2 with *Sau3A* and cloning into the *BamHI* site of pKNT25. The number (N) of colonies required to obtain a random plasmid library representing 90-99% of one times coverage of the entire *C. trachomatis* L2 genome was determined using the calculation described by Clarke and Carbon (1976), $P=1-(1-f)^N$, where f is 0.000082857 (based on 150 bp divided by 1.4 Mb of the genome) and P is 99% (confirmed by sequencing) [Clarke and Carbon, 1976].

Site-directed mutagenesis

Site directed mutagenesis was used to alter the active site residues S247 (tca) to alanine (gca), using primers published by Huston *et al.* (2007) [Huston, Swedberg et al., 2007]; V266 (gtc) to glycine (ggt); I265 (att) to glycine (ggt) and I242 (att) to glycine (ggt) using primers previously used in Huston *et al.* (2011) [Huston, Tyndall et al., 2011].

β-galactosidase assays

Quantification of the functional complementation of the interacting proteins was measured using β-galactosidase assays of permeabilised cells from overnight culture using o-nitrophenol-β-galactosidase (ONPG) as a substrate as per the method provided by the

manufacturers (Euromedex, France). Empty vectors were used as a negative control and the positive controls included one provided by the BACTH kit (pUT18C-zip and pKT25-zip; Euromedex) as well as the CtHtrA-S247A positive control (pUT18C-CtHtrA-S247A and pKNT25-CtHtrA-S247A; this study). Statistical difference between pUT18C-S247A and pUT18C-S247A-V266G β-galactosidase units per microgram protein were assessed using an equal variance t-test corrected for multiple testing[Benjamini and Hochberg, 1995] in the R statistical environment (version 2.13.1).

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Figure legends

Figure 1

Molecular model of the structure of CtHtrA protease domain. **(a)** Structural model of CtHtrA protease domain showing catalytic triad (S247, H143, D173; yellow) and flexible loop 1 (I242; S247) and loop 2 (I265; V266) encompassing the substrate binding sites S1-S3. The residues predicted to be important for specificity I242, V266 and I265 shown in brown. **(b)** Schematic showing relative orientation of specificity determining residues with respect to a substrate (P1-P3). **(c)** Alignment of CtHtrA and EcHtrA loops 1 and 2; asterisks represent mutated residues I242, S247, I265 and V266 respectively.