The role of HtrA as a chaperone and protease in bacterial pathogenesis

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Abstract

HtrA (High Temperature Requirement A) is a critical stress response protease and chaperone for many bacteria. HtrA is a multitasking protein which can degrade unfolded proteins, conduct specific proteolysis of some substrates for correct assembly, interact with substrates to ensure correct folding, assembly or localisation, and chaperone unfolded proteins. These functions are critical for the virulence of a number of bacterial pathogens, in some cases not simply due to the broad activities of HtrA in protection against the protein stress conditions which occur during virulence. But also due to the role of HtrA in either specific proteolysis or assembly of key protein substrates which function directly in virulence. Remarkably, these activities are all conducted without any requirement for ATP. The biochemical mechanism of HtrA relies both on the chymotryptic serine protease active site as well as the presence of two PDZ (protein binding) domains. The mechanism is a unique combination of activation by substrate motifs to alter the confirmation of the active site, and assembly into a multimeric complex which has enhanced degradation and may also act as a protective cage for proteins which are not degraded. The role of this protease in the pathogenesis of a number of bacteria and the details of its distinctive biochemical activation and assembly mechanisms are discussed in this chapter.

Introduction

HtrA is a critical stress response protease and chaperone for many bacteria and belongs to a large family of related serine proteases. It is found in all domains of life, including humans, and is a key component of protein quality control in the bacterial envelope [1]. Functionally, HtrA contributes to the degradation of unfolded proteins, protein folding, and chaperoning and is essential for the virulence, thermosensitivity, pH tolerance, and H_2O_2 resistance of a number of bacterial pathogens [2]. Human homologs are believed to be involved in arthritis, cell growth, unfolded stress response, programmed cell death (apoptosis), and aging [3]. The proteolysis conducted by HtrA relies on both the serine protease active site and also on the presence of an additional domain consisting of two PDZ (protein binding) motifs. These motifs enable HtrA to assemble into a multimeric protease around the substrate for efficient degradation and may also serve a protective role for proteins which are not degraded. The role of this protease in the pathogenesis of a number of bacteria and the role of its unique biochemical mechanism are discussed in this chapter.

Background

Protein stability is dependent on proper folding and environmental or cellular factors interfering with the folded state [4]. Mis- or unfolding events are monitored in the cell by specialised quality control elements such as chaperones, and proteases [4]. Specifically, proteases function to catalyse the cleavage of damaged proteins, or signal peptides and to inactivate signalling proteins [3], while chaperones allow non-covalent binding and release of polypeptides and contribute to substrate targeting [5]. Heat shock proteins, such as HtrA, are

some of the most prominent chaperones and proteases that are characteristically over-expressed at high temperatures or as a result of other stress conditions [6].

HtrA is a serine protease and a 48 kDa protein induced by heat shock and is indispensible for *E. coli* survival at temperatures above 42°C and in the presence of certain oxidising agents [1, 7-9]. Located in the periplasm of Gram negative bacteria and the outer membrane of Gram positives, HtrA has the unique ability to function as both a protease *and* a chaperone independently of ATP [4, 10] with the physiological role of degrading or reforming aberrant proteins arising in the periplasmic space under stress conditions [1, 11-12]. It is synthesised as an unstable 51-kDa precursor that is processed to a 48-kDa mature form following the removal of the 26-amino acid N-terminal signal peptide that targets the protease to the periplasm [2]. The upregulation and synthesis of HtrA is regulated by both the Cpx and σ^E protein quality control pathways under conditions of protein-folding stress [10, 13]. Biochemical information suggests that HtrA recognises the non-native states of proteins and exhibits a strong preference for cleavage of the polypeptide chain immediately adjacent to small hydrophobic residues such as valine and isoleucine [14].

Structurally, HtrA is intriguing as it is known to convert between several oligomeric forms with each seemingly related to a specific function. The 'resting state' of the enzyme is represented by the hexameric structure formed via the dimerisation of trimers [15]. Following the detection of stress signals within the cell, HtrA oliogomerises into 12-mers or 24-mers that encapsulate the substrate molecule for subsequent degradation or refolding.

While the conversion between oligomeric states provided new insight into the functional capabilities of HtrA, questions remain about the mechanism of the protease/chaperone switch. The conversion between HtrA's chaperone and protease activity was initially reported to be mediated by temperature, where the chaperone activity is predominant at

temperatures around 28°C, while protease activity is dominant at higher temperatures (42°C) [4]. However, recent studies have indicated that proteolytically inactive HtrA mutants are able to prevent the aggregation of unfolded substrates over a wide range of temperatures (30-45°C) [16], highlighting the necessity for further investigation into the structure and function of HtrA. Like other heat shock proteins, HtrA is well-conserved and homologs have been identified in a variety of species, including Gram-negative and Gram-positive bacteria, plants, and mammals [17-18].

History

The HtrA protease was initially discovered in 1983 when it was described as a serine endoprotease and named protease Do [19]. Subsequent research characterised protease Do in *Escherichia coli* according to two phenotypes of corresponding null mutants and were named accordingly [3]. These studies demonstrated that mutants did not grow at elevated temperatures (HtrA for high temperature requirement A) [6] (Figure 1), or failed to digest misfolded protein in the periplasm (DegP for degradation of proteins) [3, 11]. Null mutants also displayed a reduction in growth rate at 37°C and an increased lag phase [20]. Mutations in HtrA have been demonstrated to stabilise unstable envelope-associated proteins but not unstable cytosolic proteins [11], suggesting that the primary physiological function of HtrA is to degrade or chaperone proteins in the periplasm [2]. This was supported by the discovery of abnormal proteins that were found to be stabilised in *htrA* mutants such as a mutant LamB protein [21], a beta-lactamase fusion protein [22], truncated derivatives of diphtheria toxin [23] and a mutant alkaline phosphatase lacking disulphide bonds [24] [2]. The regulation of the HtrA promoter by the alternative sigma factor E was described in 1989 [25] and it was revealed in 1995 that HtrA is controlled by a second signalling pathway, the Cpx two-component regulatory system [26-27]. In 1991, the implication of HtrA in bacterial virulence

was first reported [27-28]. Following the identification of two PDZ domains, that were deemed to mediate protein-protein interaction [29], research began to focus on the structural characteristics of HtrA. In 1996 it was suggested that HtrA might also act as a molecular chaperone that only degrades proteins that it is unable to refold [17]. This hypothesis was supported by the identification of a putative rickettsial homologue of HtrA that lacks all three residues of the catalytic triad [2, 17]. The chaperone function of HtrA was confirmed in 1999 when HtrA was shown to refold periplasmic amylase MalS and the artificial substrate citrate synthase [4]. The initial insights into the architecture of the HtrA structure and oligomeric state were provided by an electron microscopy study reported in 1999 [10, 30]. While the structure of HtrA was resolved in 2002 [15, 31], the functional model was subsequently re-written in 2008, after additional structures showing HtrA in two oligomeric forms were resolved [10, 32-33].

Biochemistry

HtrA substrate proteolysis is conducted in the same way as that described for all serine proteases. For a comprehensive overview of the specific chemical mechanism, see the review by Hedstrom [34-35]. The current review will provide a summary of this process as a necessary prelude to the structural overview that follows.

For effective catalysis to occur, the two important components required are the catalytic triad and the oxyanion hole. The catalytic triad spans the active site cleft, with Ser195 on one side and Asp102 and His57 on the other (amino acid numbers are based on the standard nomenclature for chymotrypsin). This triad is a component of an extensive hydrogen bonding network that assists in maintaining the orientation between Asp102 and His57 [35]

and is suggested to be important for catalytic triad function [36]. The oxyanion hole is formed by the backbone NHs of Gly193 and Ser195. It forms a positive charge to stabilise negatively-charged tetrahedral intermediates and functions to donate backbone hydrogens for hydrogen bonding [35].

As reported by Hedstrom's review, Ser195 attacks the carbonyl of the peptide substrate, supported by His57 which acts as a general base to produce a tetrahedral intermediate (summarised in Figure 2). The resulting His57-H^+ is stabilised by the hydrogen bond to Asp102. The oxyanion of the tetrahedral intermediate is stabilised by interaction with the main chain NHs of the oxyanion hole. The tetrahedral intermediate collapses with expulsion of the leaving group, assited by His57-H^+ acting as a general acid, to yield the acylenzyme intermediate. The deacylation half of the reaction effectively replicates this sequence: water attacks the acylenzyme assisted by His57, yielding a second tetrahedral intermediate. This intermediate then collapses, expelling Ser195 and a carboxylic acid product. As the reaction progresses, changes in bonding and charge at the scissile bond will propagate to more remote enzyme-substrate interactions, and vice versa [35].

Structure

The HtrA monomer can be divided into three, functionally distinct domains: a conserved, catalytic protease domain (residues 1-259) and two carboxy-terminal PDZ domains (PDZ1, residues 260-358; PDZ2, residues 359-448) (Figure 3) [15]. As a member of the trypsin family of enzymes the protease domain of HtrA consists of two perpendicular β-barrel lobes with a carboxy-terminal helix with the catalytic triad (His57, Asp102 and Ser195) located at the interface of the two lobes [15]. Eight surface-exposed loops are termed, LA (connecting

strands 1 and 2), LB (strands 2 and 3), LC (strands 4 and 5), those of the C-terminal barrel L1 (strands 9 and 10), L2 (strands 11 and 12) and L3 (strands 8 and 9). The two remaining loops (LD and LE) wrap around from the back of the molecule [37]. The available crystal structures suggest that HtrA proteins differ in their molecular architecture, ranging from trimers with surface-accessible active sites to hexamers, 12 and 24-mers that belong to the class of self-compartmentalising proteases with catalytic sites enclosed within a central cavity [3]. These unique structural characteristics are assumed to result in functionally distinct mechanisms for the chaperone and protease activity which are examined in detail below.

The HtrA Hexamer

The initial X-ray crystallography structure revealed that HtrA oligomerises into a hexamer that encloses its proteolytic sites into a central chamber [10], which is in agreement with the oligomeric size of the wild-type protein as determined by analytical ultracentrifugation [38-40]. The assembled hexameric HtrA structure consists of the staggered association of two trimeric rings [40], separated from each other by a long peptide spacer that is constructed by the enlarged protease loop LA in the N-terminus of the molecule [15, 27]. Thus, a large cavity is formed in the centre of the hexamer that is enclosed at its top and bottom by the six protease domains, while the twelve PDZ domains form mobile sidewalls [15, 27]. Access to the central cavity is only possible via the PDZ domains as the axial pores of the particle are completely blocked [3, 41]. While the core of the protease domain remains highly conserved, there are significant differences in surface loops, which are important for the adjustment of the catalytic triad and the S1 specificity pocket [27]. The enlarged LA loop (Q-linker) protrudes into the active site of one monomer of the opposite trimeric ring where it undergoes numerous polar interactions with other loops [27]. In particular, the long loop LA (residues

38-79) protrudes into the opposing ring monomer where it interacts with the loops L1 (203-212) and L2 (228-238) [37] (Figure 4). This association blocks the access to the active site [37]. Specifically, the L2 loop adopts a conformation that closes the active site, while the L1 loop is kept in such a formation that the active site serine is not properly oriented towards the active site histidine. Furthermore, the NH cradle of the oxyanion hole cannot be formed [3, 15, 37]. Upon activation, HtrA dissociates into trimers which subsequently form 12-mers or 24-mers, leading to dramatic changes to the active site region [37].

The PDZ Domains

PDZ domains are globular domains consisting of approximately 80-100 amino acids and have been found in many intracellular proteins in both prokaryotes and eukaryotes. The name PDZ is derived from the first three proteins in which these domains were identified: PSD-95 (a 95 kDa protein involved in signalling at the post-synaptic density), DLG (the *Drosophila melanogaster* Discs Large protein) and ZO-1 (the zonula occludens 1 protein involved in maintenance of epithelial polarity). Structurally, PDZ domains consist of six β-strands and two α-helices, which fold in an overall six-stranded β-sandwich. The C-terminal ends of a protein substrate usually bind in a groove of the domain formed between one of the α-helices and the adjacent β-strand, functioning as an extra β-strand added to the β-sheet [42-43]. PDZ domains recognise specific C-terminal sequence motifs of approximately five residues in length, however there are infrequent accounts of recognition beyond these five residues [44]. Within the PDZ-binding motif the nomenclature is as follows: the C-terminal residue is referred to as the P_0 residue; subsequent residues towards the N-terminus are termed P_{-1}, P_{-2}, P_{-3}, etc [42].

It was noted that two different conformations of HtrA were present in the crystal structure ('open' and 'closed') which are attributed to two distinct functional states [45]. Although, both molecules consisted of funnel-shaped trimers formed by a central core of protease with both PDZ domains on the periphery [40]. It is suggested that these different conformations are a result of the movement (or flexibility) of the PDZ domains [10]. In the open conformation, the PDZ domains are in an extended position that allows free access between the spacing pillars to the inner chamber, while in the closed position this access is blocked by the PDZ domains [38]. Thus, it is clear that the PDZ domains contain necessary information for the proper assembly of the functional oligomeric states of HtrA [10]. Alternatively, the influence of both PDZ1 and PDZ2 on substrate specificity, binding and the stimulation of proteolytic activity has been the focus of recent studies.

As reported for other PDZ-containing proteins such as Tsp [46-47], HtrA recognises its substrates by binding to exposed C-terminal residues which facilitates the recruitment of the substrate to the enzyme and directs it into the catalytic site which consequently cleaves it at multiple sites [39]. Thus, HtrA uses the C-terminal residues to tether the substrate allowing or directing access to the primary cleavage sites [48]. This data is consistent with the X-ray structure of HtrA, which indicates that the hydrophobic binding pockets for P_0 and P_{-2} in the binding cleft of PDZ1 confer binding specificity [39]. With regard to the involvement of the PDZ domains in HtrA's proteolytic activity, it has been reported that the binding of an allosteric peptide to PDZ1 enhances the peptidase activity of the catalytic domain by converting the distorted active site into an enzymatically competent conformation [49]. This mechanism has also been reported in other PDZ domain-containing enzymes [50-51]. Interestingly, this activating allosteric ligand also functions as a proteolytic substrate and thus enhances its own degradation [49]. Based on these findings, the authors suggest that efficient

protein degradation by HtrA is accomplished by a positive feedback mechanism where the initial cut of a substrate accelerates further degradation of the generated peptide fragments [49]. Protein digestion continues while peptides remain bound to HtrA and upon release of the final cleavage products, HtrA returns to the resting state [49]. While clearly significant in structural conformation and the initiation of proteolysis, studies have also highlighted that both HtrA PDZ domains are dispensable for chaperone activity [39]. As noted by Iwanczyk *et al*., this implies that HtrA is able to recognise substrate molecules targeted for proteolysis and substrate molecules targeted for refolding via distinct mechanisms [39]. It is assumed, therefore, that the two PDZ domains function as proteolytic activity regulators that restrict substrate access and serve as gatekeepers of the proteolytic chamber [15, 40, 45]. Furthermore, it appears that the PDZ2 domain is unrelated to the regulation of activity, but is involved in multimerisation as the isolated PDZ domains retain the ability to form stable complexes [45]. These findings are supported by biochemical studies that demonstrate that PDZ1 is crucial for protease activity [4, 40, 52] as it offers substrates to the catalytic sites, and that PDZ2 is not required for proteolysis but remains essential for assembling the hexameric structure [39-40]. In addition, the sequences of PDZ2 domains are not very well conserved, unlike those of the protease domain or the PDZ1 domain [45]. Therefore, it appears that the PDZ domains of the HtrA family are a prerequisite for proteolytic activity due to their regulation of the rearrangement of the catalytic triad, as well as their substrate-limiting properties [45].

$HtrA_{12/24}$

Recently, two independent groups have reported the structures of large 12-mer ($HtrA_{12}$) and 24-mer ($HtrA_{24}$) oligomeric cages, based on X-ray crystallography and cryo-electron

microscopy (cryo-EM) [15, 32]. Both oligomers are made of identical trimeric units that are triggered by substrate binding [10, 40]. It is suggested that the requirement for the presence of the substrate to trigger the reorganisation of hexameric HtrA into these larger cages explains why the 12-mer and 24-mer oligomeric forms were not described at the time the structure of the hexameric form was first obtained [10] and that the hexameric form represents a resting state of the HtrA trimeric unit [40].

The large 24-meric particles have a diameter of about 195 Å and are built by 8 trimers that are located at the vertices of an octahedron, forming a shell that encloses an internal cavity 110 Å in diameter [10, 27]. These large HtrA particles represent the active state in which loops 1-3 and LD adopt conformations typical of the classical serine proteases [27]. $HtrA_{24}$ also contains wide pores through which outer membrane protein (OMP) substrates could enter the internal cavity [40]. The four trimeric units in the $HtrA_{12}$ oligomer form a tetrahedral shell about 160 Å in diameter surrounding a central cavity 78 Å in diameter [33]. In both forms, trimer interaction occurs via the PDZ domains, resulting in pores in between that are wide enough to allow small folded proteins to diffuse in and out of the inner cavity. As noted by Ortega *et al*., in their HtrA structural review, the $HtrA_{24}$ structure obtained by both X-ray crystallography [33] and cryo-EM [32] suggested that all the PDZ domain interactions are similar and occur between the PDZ1 domain of one trimeric unit and the PDZ2 domain of the contiguous trimeric unit in a pattern that allows each trimer to interact with three other trimers in the oligomer [10]. Both oligomeric states highlight the self-compartmentalised nature of HtrA, as defined by catalytic sites that are enclosed within the inner cavity [10]. However, where the LA loop protrudes into the active site in the hexameric form, the L1 and L2 loops are released from interactions with the LA loop in the $HtrA_{12/24}$ confiramtion [15, 37]. Research has indicated that during thermal activation the structure of

HtrA appears to open gradually, in parallel to the gradual increase of its proteolytic activity [37]. It is reported that the loops react to the temperature shift sequentially: the LA loop first, then the L2 and L1 loops [37]. In the active form the L1 loop adopts a typical turn structure that is essential in forming the oxyanion hole and the active site triad achieves a proper architecture [37]. The loop L2 flips away from the entrance to the active site, resulting in it becoming activated and accessible [33, 37].

Functional Implications of Structure

It is becoming increasingly apparent that the specific structural configurations achievable by HtrA have important implications in terms of both its protease and chaperone function. Specifically, it has been shown that the $HtrA_{12}$ and $HtrA_{24}$ oligomeric forms are induced in the presence of substrates, before reverting back to the hexameric form following degradation of these substrates [53]. This transformation indicates that the hexameric form represents the resting state of HtrA, while the 12/24 oligomers represent the functional state of the enzyme. Furthermore, structural evidence has demonstrated the encapsulation of the substrate into the internal cavity enabling access to the catalytic residues, for degradation or to use the cavity as a shelter for refolding. It is predicted that the association and reassociation of trimeric forms result in the formation of large cages around the substrate. It is also noted however, that it may be possible for substrates to be threaded through the large pores in the shells of the cages, highlighting the need for further investigation into the functional configuration of the HtrA structure. A further question, as posed by Ortega *et al*., [10], remains regarding whether substrate refolding and degradation always occurs *after* internalisation into the inner chamber of one of the large cages. Studies have demonstrated a positive effect on the HtrA-specific activity when adding increasing concentrations of proteolytically inactive $HtrA_{S210A}$, which

was not seen when a non-cage-forming HtrA mutant (HtrA ΔPDZ2) was added [32]. In addition, it was noted that the degradation of a chromogenic peptide substrate that is unable to induce the formation of large cages was significantly accelerated in the presence of lysozyme, a protein substrate that induces the formation of HtrA cage structures [33]. Together, these results suggest that substrate degradation occurs more efficiently in the context of the large cages [10]. Conversely, it has been established that the HtrA ΔPDZ2 mutant which is unable to form the large 12/24 cages remains fully active, demonstrating that substrate enclosure inside the cavity is not the only mechanism for HtrA to perform its degradation or refolding activity [10]. In addition, Ortega *et al*., [10] poses the question that if the dissociation and reassociation of trimers results in the formation of the oligomeric states (and HtrA trimeric mutants are active [32]), how does the enzyme avoid performing any activity as a trimer during the oligomeric state transition? This may be by the activity of the PDZ2 domains to prevent HtrA activity during the oligomeric state transition [32]. Additionally, the PDZ domain has previously been implicated in the inhibition of proteolytic activity in the human homology of HtrA [54]. As noted by Ortega, the inhibitory effect of the PDZ2 domain would then be eliminated following its interaction with the PDZ1 domain of a neighbouring trimer to stabilise the cage structure [10].

The elucidation of the oligomeric forms also added further insight into the HtrA protease/chaperone switch. In the inactive hexameric conformation, loop LA protrudes into the active site of one subunit of the opposite trimeric ring, where it is able to interact with the active-site loops L1 and L2 [33]. The resulting conformation blocks the entrance to the active site and distorts adjustment of the catalytic triad, oxyanion hole and the substrate specificity pocket [51]. However, following oligomerisation into the 12/24 conformation, loop LA is removed from the active site and releases loops L1 and L2 to set up a functional

proteolytic site [33]. Therefore, it is clear from these structures that conversion from hexamers into the 12-mer and 24-mer structures is a critical step for initiating the protease activity [10]. While both the HtrA oligomeric forms result in an active catalytic triad, it is not assured that every internalised substrate is degraded [10]. Krojer *et al*. [33] found that HtrA was able to degrade unfolded OmpA while stabilising the folded protomers, highlighting the chaperone function of HtrA. This finding suggests that the fate of the encapsulated protein is dependent on the folding state of the substrate and its ability to adopt its native conformation fast enough to escape the proteolytic activity of HtrA [10]. As demonstrated by Spiess *et al*., [4] temperature remains an important factor in the regulation of HtrA protease/chaperone activity and these structural findings support this hypothesis, albeit to a degree. As observed by Ortega *et al*., [10] in their review, lower temperatures may allow substrate molecules to readily fold into their native conformations, escaping HtrA proteolytic activity and resulting in chaperone activity only. On the other hand, higher temperatures may induce protein mis-folding (or partial mis-folding) and are subsequently degraded before the native state is reached. Ortega *et al*., [10] also observed that the available structures have not yet been resolved for a temperature range wide enough to rule out any effect of the temperature on the structure of the cages themselves. They importantly suggest that high-resolution structural analyses of the 12-mer and 24-mer cages at 42°C would provide this additional insight into the role of temperature in the conversion of HtrA between protease and chaperone function [10].

Role of Membrane Lipids

The structural model of HtrA provided additional evidence for $HtrA_{24}$ as a membrane-attached protein. Previous studies have highlighted that HtrA is located in the bacterial periplasmic space where it interacts with negatively-charged head groups of phosphatidylglycerol and cardiolipin [55-56]. Krojer and co-workers [33] recent structural work has indicated that clusters of lysine and arginine residues were originating from both PDZ domains resulting in a strongly positive electrostatic potential of the outer rim of the large pores, thereby generating candidate sites for membrane attachment. Further investigation using lipid sedimentation assays indicated that HtrA has exploited the PDZ domains to target cellular membranes, and in some cases may function as a periplasmic macropore allowing the protected diffusion of OMP precursors from the inner to the outer membrane [33]. This data is supported by recent reports of the significance of PDZ domains in membrane localisation [57-60]. A subsequent study provided further insight into HtrA's membrane association by demonstrating its ability to form a range of bowl-shaped structures on lipid membranes, independent of substrate proteins [61]. These structures were seen to display various rotational symmetries (4-, 5-, or 6-fold), where the symmetry axis of each structure is always perpendicular to the membrane plane. Each of the structures consisted of HtrA trimers [61], with a similar configuration to that seen in the $HtrA_{12}$ and $HtrA_{24}$ spherical assemblies, indicating the possibility that the bowl-shaped assemblies recruit new trimers or hexamers form intact cage-like structures after substrate recruitment [61]. While these structures were found to exhibit both proteolytic and chaperone-like activities, dramatically higher proteolytic and lower chaperone-like activities was reported, raising questions about HtrA's previously-suggested role as a periplasmic macropore [61]. It is suggested that the membrane lipids may induce the formation of bowl-shaped structures that function as a reservoir of partially-assembled 12-mer and 24-mer cages that are able to quickly assemble into proteolytically-active (rather than chaperone-active) cages around the substrate [61].

Therefore, as noted by Ortega *et al*., it is tempting to speculate that the presence of the bowl-shaped structures allows faster substrate degradation afforded by the relatively faster assembly of the oligomeric large cages [10]. While these reports imply an appealing model where the bowl-shaped assemblies may represent structural intermediates of the active cage-like assemblies in the periplasm, *in vivo* confirmation is required in future studies [61]. A further question to be addressed is how the influence of all the described factors are detected and integrated by HtrA *in vivo* to ultimately determine whether the substrate will be refolded or degraded [10].

HtrA Self-Regulation

Following the elucidation of the structural configuration of HtrA, further insight was gained into the auto-degradation process of HtrA, a purported mechanism of elimination once cellular stress conditions have subsided. The upregulation of HtrA in conditions of cellular stress that results in the degradation of unfolded and damaged proteins, produces peptide fragments that function to induce the self-cleavage of HtrA [62]. This leads to the reduction in levels of the enzyme, suggesting that the autocleavage process functions to eliminate HtrA once its enzymatic activities are no longer required [62]. It is reported that the hexameric structure of HtrA is necessary for autocleavage to occur and that the cleaved forms appear late in the reaction, once the full-length substrate is degraded [10]. Considering the current structural information, it appears that HtrA remains in the 12-mer and 24-mer oligomeric form in the presence of full-length substrates and undergoes limited amounts of self-degradation. It is not until substrate degradation is complete that HtrA reassembles into the hexameric form resulting in self-cleavage [10]. The structural significance of HtrA self-regulation is also suggested by the X-ray structure of the 24-mer cage [33]. In these large

cages, the LA is displaced from the neighbouring L1 and L2 loops to allow the formation of a functional active site. However, in the hexameric form, the LA loop protrudes into the active site of the subunit in the opposite trimer which forces the L1 and L2 loops into an inactive conformation. Upon its return to the hexameric form following substrate degradation, the peptides resulting from substrate hydrolysis allosterically stimulate HtrA proteolytic activity which in turn may keep the catalytic L1 and L2 loops in an active conformation [10]. It is suggested that this will result in the cleavage of the LA loop, given its proximity to the still-active catalytic site. Furthermore, it is noted that membrane lipids may play a role in preventing the reassembly of HtrA into hexamers by maintaining the enzyme in bowl-shaped oligomers and consequently delaying the autocleavage process. As noted by Ortega *et al*., additional structures and studies are required to provide experimental evidence supporting the proposal of this regulatory mechanism [10].

Molecular Mechanisms of Regulation

Bacterial cells are able to sense surface stresses such as the accumulation of abnormal proteins in the periplasm and the outer membrane, and as a result induce the expression of a set of genes encoding chaperones, foldases and proteases that function to cope with these stresses [63-65]. Two major stress response pathways, σ^E and Cpx, are known [65]: The σ^E pathway utilises this dedicated sigma factor specialised for the extracytoplasmic stress response, while the Cpx pathway utilises the CpxAR two component phosphor-relay mechanism [65-66].

It has been demonstrated that both the σ^E and Cpx regulatory systems respond to envelope stress by up-regulating the production of protein folding and degrading factors, however they

remain distinct at many levels [64]. The σ^E pathway responds to high temperatures and ethanol [25, 67], while the Cpx envelope stress response is activated by elevated pH [68-69] and alteration of the inner membrane lipid composition [70-72]. Furthermore, the σ^E activating signal is mis-folded outer membrane proteins (OMPs), indicated by the elevation of σ^E activity following the over-expression or misfolding of OMPs at a stage after the fate of outer membrane and periplasmic proteins diverges [64, 73-74]. As distinct from the Cpx-inducing signal which consists of aggregated, misfolded proteins associated with the periplasmic face of the inner membrane [64, 71]. This is supported by Cpx pathway activation following alterations in extracellular pH [68, 72], accumulation of enterobacterial common antigen intermediate lipid II [69], overexpression of NlpE [75], overexpression of P pilus subunits in the absence of their periplasmic chaperone PapD [76], and overexpression of the enteropathogenic *Escherichia coli* type IV bundle-forming pilus subunit BfpA [77-78]. Where the Cpx and σ^E pathways overlap however, is in their control of the expression of HtrA [79].

The SigmaE Regulatory System

The alternative sigma-factor E (σ^E) is a transcriptional activator that directs the expression of genes encoding periplasmic chaperones, folding catalysts, and proteases, as well as genes involved in cell wall biogenesis [50, 80]. It is induced by perturbations to outer membrane protein folding [73-74]. Under latent conditions, σ^E is kept inactive by its interaction with RseA, an integral membrane protein with a cytoplasmic N-terminal domain that functions as an anti-sigma factor [67, 81]. This cytoplasmic domain binds to a transmembrane segment and periplasmic segment of σ^E [66] and blocks the major binding determinants for RNA

polymerase [82]. Periplasmic proteins such as RseB and RseC may also participate in the negative regulation of the σ^E activity through its interaction with the periplasmic domain of RseA [67, 81]. Upon folding stress, RseA is degraded by a proteolytic cascade controlled by the membrane-anchored periplasmic protease DegS and the membrane-embedded metalloprotease RseP [50, 83-86]. This degradation pathway is initiated following the binding of the DegS PDZ domain to a stress-dependently exposed outer membrane protein signature sequence [50, 87-88]. This signature sequence is a conserved C-terminal residue found on unassembled OMPs, a phenylalanine, which appears to be a key binding factor for the PDZ domain of DegS, resulting in the transformation of DegS into an enzymatically active protease [51]. When activated, DegS introduces 'site-1' cleavage into the RseA periplasmic region between Val148 and Ser149 [87], which is then subject to RseP-dependent 'site-2' cleavage at a more cytoplasmically proximal site [84, 86, 88]. DegS and RseP are both essential for viability [83-84, 86], but they can be successful deleted if in the presence of increased σ^E by overproduction or by the absence of RseA [65, 84, 86]. Thus, the essential function of DegS and RseP is to provide cells with a sufficient amount of active σ^E [65].

While DegS and RseP cleave RseA in half, the remaining N-terminal part of RseA still binds the σ factor very tightly with pM affinity [66, 89], a reported consequence of multiple interactions between the two proteins [82]. In response, ClpXP and Lon, two ATP-dependent cytoplasmic proteases, are up-regulated to support the rapid degradation of RseA while slower reactions can be carried out by other proteases [66, 89]. Following its release from the anti-σ factor RseA, σ^E associates with the core RNA polymerase and activates transcription from promoters of at least 20 genes [90]. These include genes that encode the periplasmic folding factors, Skp, DsbC, FkpA and SurA, as well as HtrA [6, 25, 90-91]. It is reported that

all these genes have inferred roles in OMP biogenesis [92]. In addition, it has been demonstrated that σ^E null mutations are lethal [67], suggesting that the σ^E pathway is involved in an essential cellular function(s), one of which may be to ensure proper elaboration of OMPs [92].

The Cpx Regulatory System

HtrA transcription is also enhanced by the activation of an additional regulatory pathway, the CpxRA two-component signal transduction system [26]. This pathway is a typical two-component system with a membrane-bound histidine autokinase, CpxA, and a cytoplasmic response regulator, CpxR [78, 93-94]. CpxA is activated by a variety of envelope perturbations including alterations in pH [68-69], membrane composition [69-70], and the over-expression of misfolded envelope proteins [26, 95]. It is suggested that CpxA activation involves relief of an inhibitory interaction between CpxP and the periplasmic sensing domain of CpxA [71]. This is believed to result in an increase in CpxA autophosphorylation which then transfers the phosphate to a conserved aspartate on CpxR [96]. CpxR~P binds to sites upstream of their promoters for multiple genes in the Cpx regulon and activates their transcription [69, 71, 79, 91, 96-98]. These genes include envelope protein folding and degrading factors such as the disulphide oxidase DsbA [79, 91], the peptidyl-prolyl-isomerases PpiA [79, 91] and PpiD [97], and HtrA [26]. Initiation of the Cpx system functions to protect the cell from otherwise lethal envelope protein misfolding [99], indicating that the Cpx response remains significant in maintaining the integrity of envelope proteins in the face of insult. It is also noted that as the Cpx pathway is strongly activated by upregulated assembly of P pili in uropathogenic *E. coli* [95], and *cpx* mutants display P pili

with altered morphology [100]. It is assumed that this envelope stress response is also required for the proper construction of envelope-localised structures like pili [92].

It is also reported that the phosphoprotein phosphatases PrpA and PrpB act as active modulators of CpxA/CpxR-dependent activation of HtrA transcription [63, 81]. It is noted that the Prp phosphatases appear to modulate the activity of other two-component systems besides Cpx and it is suggested that they may have a more general role in protein phosphorylation [81].

Interestingly, both the σ^E and Cpx regulatory pathways appear to be adapted to play a role in pathogenesis [71]. In *Pseudomonas aeruginosa*, the σ^E (AlgU) pathway regulates the expression of *alg* genes required for the expression of the mucoid capsule essential for the establishment of chronic infections [64, 101-102]. In *Shigella* species, Cpx regulates expression of the transcriptional activator VirF, which is required for production of the Ipa proteins involved in cell invasion [64, 68]. Thus, it seems that σ^E and Cpx activating signals are produced during the course of infection [71]. This activation may be the result of insults of a general nature (e.g. elevated temperature) that perturb envelope protein folding or, alternatively, may be due to specific cues that are generated at distinct times during an infection when the products of either regulon are required [71].

The Bae Regulatory System

Several recent studies have alluded to the existence of a third envelope stress signal transduction pathway, the Bae regulatory system. A number of Cpx- and σ^E-regulated genes of unknown function were recently identified [90, 98] including *spy*, a recognised member of

the Cpx regulon [98]. Spy (spheroplast protein Y) was first identified as a periplasmic protein whose expression is induced by spheroplast formation [103]. It is reported that Spy shares some homology with CpxP, yet its function appears to be distinct [69, 98]. Subsequent research has suggested that Spy may play a role in envelope biogenesis as its removal leads to activation of the σ^E envelope stress response [98]. However, little else is known of its function [92]. In their study, Raffa *et al*., [92] presented evidence that *spy* expression is controlled by an additional regulatory pathway a functionally uncharacterised two-component system consisting of the BaeS sensor kinase and the BaeR response regulator. This system was seen to control *spy* expression in response to a variety of envelope stresses. It is noted that BaeS and BaeR do not affect expression of other Cpx-regulated genes, but *baeR cpxR* double mutants show increased sensitivity to envelope stresses beyond those of individual *baeR* or *cpxR* mutants [92].

The BaeSR two-component system is reported to respond to a set of inducing cues that overlap with those of the Cpx envelope stress response, as both pathways are involved in induction of *spy* expression [92]. Accordingly, it is suggested that the Bae and Cpx envelope stress responses interact synergistically to induce *spy* expression in response to cues [92]. Alternatively, the Bae pathway does not respond to the same type of signal as the Cpx response, as NlpE overexpression, a Cpx activating signal, has no apparent effect on the Bae pathway [92]. It is therefore assumed that the Bae signal transduction apparatus recognises the misfolding of a distinct type of envelope proteins that is induced by some of the same cues that lead to the induction of the Cpx pathway [92]. While additional components of the Bae regulon will continue to be defined and investigated, the Bae system remains an important contributor to the cellular stress response. Nevertheless, the functional relationship

between Bae and the expression of HtrA remains to elucidated, highlighting the necessity for further investigation into this protein quality control system.

Bioinformatics and Genetic Diversity

The MEROPS database represents a classification system based on statistically significant similarities in sequence and structure of all known proteolytic enzymes [104-105]. This system divides proteases into 'clans' which represent groups of 'families' for which there is evidence of common ancestry based on common structural folds [105]. Each clan is identified according to two letters, the first representing the catalytic type of the families included in the clan, followed by an arbitrary second capital letter. The families are grouped by catalytic type, with the first character representing the catalytic type (A = aspartic, C = cysteine, S = serine etc), followed by an arbitrarily assigned number. The current version of MEROPS (release 9.3) includes over 67,000 serine protease sequences, which are further classified into 15 clans and 73 families [105].

The catalytic triad (Asp-His-Ser) that defines the serine proteases has evolved on at least four separate occasions [106], resulting in the characterisation of four distinct clans: chymotrypsin, subtilising, carboxypeptidase Y, and Clp protease [105]. HtrA belongs to the Chymotrypsin-like proteases and is further grouped within the trypsin family based on similarities in substrate specificity [105]. The SA proteases maintain a two-domain structure with each forming a six-stranded β-barrel where the active site cleft is located at the interface of the two perpendicularly arranged barrel domains. Following the initial discovery of HtrA, HtrA homologues were isolated from a variety of species [45]. Several proteins were isolated and characterised as HtrA, united by their inclusion of two conserved core domains, a

chymotrypsin-like protease domain, and at least one C-terminal PDZ domain. It was also indicated that various members of this family included additional domains such as a transmembrane region or an insulin growth factor-binding domain (IGFBP), located at the N-terminal region [45].

In their review of HtrA structure, Kim & Kim [45] note that in *E. coli*, DegP, DegQ, and DegS comprise the HtrA family of proteins. The protease domains of these enzymes are seen to display a high level of sequence homology, DegS contains only one PDZ domain while both DegP and DegQ contain two. Interestingly, many of the HtrA homologues isolated from Gram-positive bacteria, cyanobacteria, and mammals also contain only one PDZ domain [45]. While HtrA cross-species homology is apparent, the Q-linker region in the protease domain is seen to exhibit only minimal sequence homology. From an evolutionary perspective, it has been suggested that the serine peptidases of clan PA are ancestral and were acquired by RNA viruses from their hosts [105]. It is noted that several families of serine peptidases exist in viruses, but on one or more occasions the catalytic serine has been replaced by cysteine with a retention of activity, and the resulting cysteine peptidases have further diversified [105]. Between prokaryotic and eukaryotic HtrA, Koonin & Aravind [107] assert that HtrA-like sequences have entered the metazoan lineages by horizontal acquisition from prokaryotes. Alternatively, the gene flow could have also occurred in the opposite direction given the high degree of sequence conservation between bacterial and human sequences [3].

HtrA Substrates

The *in vivo* substrates of HtrA protease and chaperone activity are likely to be both proteins which have co-evolved with HtrA as specific substrates, and a range of more broad substrates which are simply unfolded proteins with exposed hydrophobic regions which are localised in the same cellular localisation and require chaperoning/degradation. The selection of these substrates seemingly occurs via some of or a combination of the known structural factors, such as oligomerisation and activation sensors of HtrA, binding of the C-terminal of the substrate to the PDZ domain 1, and the presence of the folded state, or exposed/unfolded sequence of the protein substrate. This complexity has largely been elucidated by *in vitro* studies applying model protease substrates. However, there are also been several cases where *in vivo* substrates of bacterial HtrA have been successfully identified or at least implicated indirectly.

HtrA substrate specificity for proteolysis

Serine proteases are generally thought to have broad substrate specificity particularly for their proteolytic activity. However, the self-compartmentalising nature of HtrA and the accessibility of the active site to unfolded polypeptide chains already provide some level of specificity. *In vitro* experiments have demonstrated that HtrA degrades unfolded proteins such as MalS [4] and α-Lactalbumin [30]. The amino acid sequence around the bond which is hydrolysed by proteases and the clefts in the protease structure which co-ordinate these residues are described using a nomenclature originally established by Schecher and Berger [108](Figure 5). A study by Kolmar and co-workers [14] identified that HtrA cleaved most efficiently at Val/Xaa (P1/P1') or Ile/Xaa sites (Xaa indicating any hydrophobic residue) of various model peptides suggesting that HtrA has a preference for small hydrophobic side

chains at the P1 position. A more recent study [109] used LC-MALDI-MS (liquid chromatography-matrix-assisted laser desorption/ionization mass spectrometry) and LC-ESI-MS (liquid chromatography electrospray ionisation mass spectrometry) to determine the specificity of the HtrA2 protease of *Mycobacterium tuberculosis*. Autodigestion products of the dissolved crystals of HtrA2 were monitored using the above methods and the results indicated that there was preference for cleavage of Val residues [109]. Of the 40 potential cleavage sites, 18 were identified at the carboxyl side of the Val residue with the most frequent neighbouring sites being Ala (six sites), Thr (five sites), Ile (five sites), Ser (four sites) and Leu (two sites) [109]. Again, this provides a clear indication that the proteolytic activities of *E. coli* and *M. tuberculosis* HtrAs have a preference for small hydrophobic residues. A study of HtrA in the obligate intracellular bacterial pathogen *Chlamydia trachomatis,* found that the protease had a preference for proline at P1 with the most frequently identified P1/P1' residues being Pro/Pro, Pro/Val or Pro/Leu [110]. Although *C. trachomatis* HtrA specificity has been shown to be different to that of *E. coli* and *M. tuberculosis* HtrA, the cleavage preference still clearly favours hydrophobic residues. In light of this however, not all peptides with these sequences were cleaved. Kim *et al,*. [30] revealed that several other putative Val/Xaa and Ile/Xaa sites were not cleaved and found that the most recognised P1 side chains were buried in the substrates hydrophobic core, indicating that substrate recognition requires unfolding prior to degradation. This activity was also observed by Huston *et al,*. [110] who noted that there were an additional seven sites in β-casein where Pro/Pro, Pro/Leu or Pro/Val bonds were not cleaved.

A number of other *in vitro* strategies have been applied to probe the protease specificity of HtrA. Hauske [111] used colourimetric labelled peptide substrates (pNA, *para*-nitroanaline) to determine the kinetic cleavage of different peptide substrates for *E. coli* HtrA. These

assays demonstrate that the fastest cleaved substrate was as 7-mer (DPMFKLV-pNA). A peptide library approach was used to determine the binding specificity of human HtrA2 which led to the development of the optimal substrate (MCA-IRRVSYS-DNP; P4-P3-P2-P1(V)-P1'-P2'-P3') for proteolysis [112]. These *in vitro* assays involved another commonly used method for detection of proteolysis a peptide labelled with a fluorophore (MCA) and quencher (DNP) which enables detection of fluorescence as the peptide is cleaved. Another investigation on the substrate specificity for human HtrA2 used a proteomic approach to identify 15 substrates and 50 cleavage sites from the proteome of human Jurkat T lymphocytes. This method identified a slightly different substrate sequence preference from that identified using the library approach described above. In this case valine (V) was the preferred P1 residue in substrates but the other residue sites varied considerably from the ideal substrate described in the previous study, the results of the Vande Walle study have been summarised for the purposes of this Chapter using a WebLogo (Figure 6). These studies demonstrate that there are a number of different strategies that can be used to determine the specificity for HtrA at least the residues surrounding the site of proteolysis. The confounding factors of this complex protease, such as the activation by binding to PDZ1 and oligomersation triggers, likely account for the variability in specificities determined by using different approaches. For example, the proteomic assay involves a mixed pool of proteins and peptides which likely have distinct activation impacts on HtrA when compared to the peptide library assay where no larger proteins or protein fragments were present.

A number of *in vivo* specific protease substrates have been identified for HtrA (detailed in Table 1), supporting that HtrA has an important function as a housekeeping or maintenance protein but has also specific substrates in some bacteria. AcmA, a major autolysin of *Lactococcus lactis*, was found to be proteolytically processed into its two smaller active

forms by HtrA [113]. This study suggests that *L. lactis* HtrA is the sole extracellular protease that degrades abnormal exported proteins as proteolysis was abolished for a number of exported proteins tested in the *htrA* mutant [113]. The same was observed for specific *E. coli* adhesins, intimin and AIDA (adhesin involved in diffuse adherence) which are both involved in host cell attachment [114]. Independent studies found that in an *E. coli htrA* mutant, intimin accumulates in the bacterium and is not inserted into the outer membrane [114] and AIDA was found to not be degraded *in vitro* by a proteolytically inactive HtrA [115]. A relatively recent study by Hoy *et al.* [116] found that HtrA in *Helicobacter pylori* cleaves the ectodomain of E-cadherin, a host cell-adhesion protein. Degradation of host cell E-cadherin disrupts the epithelial barrier and in turn allows *H. pylori* to gain access into the intracellular space [116]. This was confirmed after the study identified a specific *H. pylori* HtrA inhibitor which completely blocked the cleavage of E-cadherin [116]. Complete blockage of E-cadherin cleavage subsequently prevented intracellular entry of *H. pylori* [116].

Chaperone Substrate Specificity

HtrA chaperone activity has been implicated with important functions for cellular protection, bacterial virulence and general house-keeping (summarised in Table 1). Chaperone activity of HtrA was first proposed in 1996 when a *Rickettsia* homolog lacking the catalytic triad was discovered [17]. It was then confirmed and reported by Spiess *et al.*, [4] demonstrating that DegP (HtrA) catalysed the folding of the periplasmic protein MalS as well as the refolding of non-native citrate synthase via DegP's protease deficient form. Overexpression of HtrAS210A (a proteolytically inactive form of the protein where the active site serine has been mutated to an alanine) was able to rescue the lethal high temperature phenotype of the *htrA*$^{-}$ mutant *E. coli*, supporting that the chaperone activity is a significant component of the physiological role of this protein [16]. A recent study in yeast found that an HtrA homologue,

Ynm3, also exhibited ATP-independent general chaperone activity as it was able to successfully bind unfolding intermediates of the non-native substrate, citrate synthase [117]. The eukaryotic study found that Ymn3 protects cells under heat stress by preventing the aggregation of heat-denatured proteins and delivering them in a more soluble state to its protease domain [117]. An interesting study by Sklar *et al*., [118] suggests that DegP is not the major periplasmic chaperone in *E. coli*. The periplasmic protein SurA, has been implicated as responsible for the delivery, biogenesis and assembly of the major outer membrane proteins (OMP) such as the maltose transporter, LamB, a porin-like integral membrane protein OmpA and the multi-component YaeT complex responsible for the assembly of outer membrane β-barrel proteins in *E. coli* [118-119]. Although these studies found that SurA is the major periplasmic chaperone, it was suggested that DegP acts as an OMP 'back-up' chaperone in a separate but parallel pathway to SurA [118]. Sklar *et al.,* (2007) proposed that DegP (as well as another periplasmic chaperone, Skp) rescue OMPs that are not detected by the SurA pathway (or when SurA is depleted) and then either transports them to the outer membrane, re-introduces them to the SurA pathway or degrades them [118]. It is hypothesised that HtrA is one of very few periplasmic chaperones in bacteria and is potentially involved in the successful transport of numerous cellular factors, [reviewed [3]] particularly in the absence of other periplasmic chaperones [118].

The chaperone role of HtrA has largely been described by morphological or other global studies in *htrA* mutants, however, there are some examples where specific *in vivo* chaperone substrates have been identified. One of these includes the member of the OMP autotransporter family, IcsA from *Shigella flexneri* [120]. IcsA is exposed on the bacterial surface and interacts with eukaryotic proteins to aid in the assembly of F-actin tails allowing propulsion of the bacterium through the host cytosol and into adjacent cells [121]. Purdy *et*

al., [120] revealed that a *degP* mutation in *Shigella flexneri* has a defect in the surface expression of IcsA resulting in a small plaque phenotype. This result appears to occur through DegP's chaperone function as the phenotype was not seen when protease deficient DegP was present. However, the study did not have evidence of a direct interaction between DegP and IcsA [120]. EspP, a prototype SPATE (serine protease autotransporters of Enterobacteriaceae) protein in *E.coli* O157:H7 has shown to interact directly with HtrA [122]. The study found that in the *htrA* mutant, the EspP β-barrel domain is still inserted into the outer membrane, however cell lysis results and this is thought to be due to the passenger domain being mis- or prematurely folded [122].

HtrA (DegP) was found to bind to the major adhesin of *Bordetella pertussis,* the causative agent of Whooping Cough, FHA (filamentous Haemagglutinin) with high affinity [123]. *B. pertussis* FHA is important as it forms filamentous structures on the bacterial cell surface that allow it to adhere to ciliated cells [123]. It is therefore proposed that DegP chaperones the extended FHA polypeptide in the periplasm and delivers it to the outer membrane [123].

HtrA substrates in pathogenesis

In addition to the *H. pylori* HtrA virulence function described previously, several other studies have demonstrated that HtrA is essential for virulence. *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae, Listeria monocytogenes, Salmonella typhimurium, Yersinia enterocolitica* and *Yersinia pestis* all had reduced or attenuated virulence in *htrA*$^{-}$ mutant strains compared to the virulence of the original parent strain [124-130]. In many of these cases, the *degP* mutants were used to infect mice and the level of infection, number of deaths and time taken to clear infection were all recorded. In the case of

S. pneumoniae, HtrA-deficient strains were attenuated in both pneumonia and bacteraemia murine models of infection, demonstrating that HtrA is essential for virulence [125]. Histological analysis of the mouse lungs revealed that the *htrA* mutant strain produced fewer lesions indicating that the protease may have a direct action on a substrate that causes host cell damage [125].

Klebsiella pneumoniae htrA mutants were found to have reduced survival and virulence compared to the parent strain [126]. The study also found that capsular polysaccharide (CPS), an essential virulence factor in *K. pneumoniae,* was reduced in *htrA* mutants [126] again indicating that HtrA may have a direct action on a virulence associated substrate and in turn a vital role in virulence. An investigation of HtrA in *Porphyromonas gingivalis*, a causative agent of periodontal disease, found that HtrA protects the bacteria from oxidative stress and suggests this is the reason it has reduced virulence in mice [128]. Microarray data analysis found that a total of 253 genes (ORFs) were differentially regulated in the *htrA* mutant [128]. Of these, 113 were downregulated including a group of stress-related genes including *htpG, groEL/groES, clpB, dnaK, grpE,* a gene encoding the universal stress protein (PG0245) as well as gingipain Kgp/HagD (PG1844) which is a well known *P. gingivalis* virulence factor [128]. The analysis also found that an additional 83 hypothetical proteins, 24 cell envelope genes, 5 regulatory genes and 11 transport and binding protein genes were differentially regulated in the *htrA* mutant [128]. These genes include those involved in various cellular process such as host cell adhesion and degradation of surface proteins suggesting that HtrA interacts with a number of virulence pathways perhaps directly or indirectly by defence against unfolded protein stress [128].

Factors that may be involved in the conversion of *Pseudomonas aeruginosa* to its mucoid,

exopolysaccharide alignate-overproducing phenotype associated with chronic respiratory infections in cystic fibrosis patients were found to include HtrA homologues [131]. The study found that the HtrA homologues, *algW* and *mucD*, have partially overlapping but distinct roles in oxidative and heat stress but that mutations in these cause conversion to mucoidy (or lower the induction threshold) suggesting that these factors may suppress alginate synthesis either directly or indirectly by removing physiological signals that may activate this stress response system [131]. Although this study demonstrates that the *htrA* homologues do not directly control virulence through alginate production, it does reveal that they are involved in a stress response pathway important for bacterial survival. Likewise, *Legionella pneumophila* demonstrated an importance of HtrA for survival. Pedersen *et al.,* (2001) revealed that HtrA (at least in part) mediates the stress response that facilitates intracellular replication within mammalian cells both *in vitro* and *in vivo* [132]. Although these studies emphasis survival of the pathogen, it is unmistakable that HtrA, is in turn vital for pathogenic virulence.

Jones *et al.,* (2001) also found that a *degP* knockout strain of *S. pyogenes* had reduced virulence in a mouse model. A similar finding was also observed in the highly virulent foodborne pathogen, *Listeria monocytogenes* and the causative agent of tuberculosis, *M. tuberculosis* which both encode HtrA-like serine proteases that again exhibit reduced and attenuated virulence in mice model respectively [109, 127]. *Salmonella enterica* serovar Typhimurium *htrA* mutants are defective in intra-macrophage survival and therefore their virulence is highly attenuated in mice [28, 133-134].

It is apparent that HtrA has both a general housekeeping and protein maintenance role for many bacteria including pathogens, and that this role is critical for protein stress conditions.

This likely represents why HtrA is an essential virulence factor for many of the bacterial pathogens described here. Within the pathogenic environment these bacteria are likely to encounter a considerable burden of protein stress, innate immune compounds, oxidative burst within phagosomes, temperature changes, osmotic changes, and pH changes are all potential contributors to bacterial protein unfolding during virulence. These factors are all potentially able to impact on protein stability in the periplasmic or extracytoplasmic space where HtrA functions to maintain proteins. However, it is clear that HtrA does not merely function as a stress defence protein. There are several examples where key virulence factors rely on the presence of HtrA for their correct assembly. Several of these are outer membrane or secreted factors, which must be correctly exported and assembled suggesting that the chaperone activity of HtrA for protein assembly is also involved in virulence.

Conclusion

The considerable body of physiological, genetic, and biochemical data reported to date demonstrate that HtrA is a unique and important multitasking protein. HtrA is able to proteolyse unfolded protein substrates, possibly specifically proteolyse some substrates to facilitate their correct assembly, and chaperone numerous other substrates. These activities appear to be self-regulated via a complex process of activation by peptide binding to the PDZ1 domain triggering loop rearrangements in each protein monomer, through to multimeric assembly into larger 12 and 24-mer protein oligos which have enhanced proteolysis activity and are capable of assembling around folded β-barrel outer membrane proteins. Given the complex biochemical and structural mechanisms of activation and the very transient nature of the HtrA proteolysis and chaperone interaction with substrates *in*

vivo, it is remarkable that a considerable number of substrates (as reported here) have been detected to date. The success of these studies has often been due to indirect evidence from genetic and proteomic tools. It seems likely that advances in proteomics will provide new approaches to identify further HtrA studies and may also be applied to further advance the understanding of the biochemical activities. There are a number of tools which are becoming more widely applied for *in vivo* studies such as fluorescence resonance energy transfer (FRET) which may also prove useful to probe both substrate binding and oligomerisation of HtrA. The very elegant biochemical studies on oligomerisation and peptide activation of HtrA have important and unexplored implications for its function *in vivo*. For example, could HtrA detect chaperone substrates directly as they are being transported across the cytoplasmic membrane and the substrates themselves then trigger the formation of the cage (oligomerisation) to facilitate a chaperone mechanism to cross the periplasm? It is clear that more bacterial HtrA will be characterised in the future and that they will have numerous specific substrates as well as unfolded protein response functions which will be critical for both pathogenesis and viability under stress conditions.

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Table 1: HtrA substrates which have been identified in bacteria.

SUBSTRATE	SUBSTRATE FUNCTION	ROLE OF HtrA	ORGANISM	SOURCE
Protease				
MalS	A periplasmic protein that belongs to the α-amylase family and hydrolyzes α-(1,4) glycosidic linkages in long maltodextrins.	Degrades misfolded MalS.	*Escherichia coli*	[4]
AcmA	A major autolysin.	Proteolytically processes AcmA into its two smaller active forms.	*Lactococcus lactis*	[113]
Intimin	An adhesin expressed on the bacterial cell surface and involved in host cell attachment.	Degrades misfolded intimin when not properly inserted into the outer membrane.	*E. coli*	[114]
AIDA (adhesin involved in diffuse adherence)	An autotransporter protein that confers the diffuse adherence phenotype to certain diarrheagenic Escherichia coli strains.	Degrades misfolded AIDA.	Diarrheagenic *E. coli* strains	[115]
Host cell E-cadherin	A host cell-adhesion protein.	Cleaves the ectodomain of E-cadherin which disrupts the epithelial barrier and in turn allows *H. pylori* access into the intracellular space.	*Helicobacter pylori*	[116]
Cal	Colicin A lysis (Cal) protein is responsible for colicin A release. In the outer membrane, Cal is complexed with an outer membrane protein.	Degrades acylated precursor form of Cal after globomycin treatment.	*E. coli*	[135]
SpeB	SpeB is a highly conserved extracellular and cell surface associated protease expressed by most GAS isolates.	Plays an indirect role in the maturation of cysteine protease SpeB (proSpeB).	*Streptococcus pyogenes* (group A Streptococcus; GAS)	[136]
Chaperone				

Citrate synthase	Pace-making enzyme for the first step of the Citric Acid Cycle and is not native in *E. coli*.	Re-folds citrate synthase *in vitro*.	*E. coli* and budding yeast	[4, 117]
Major outer membrane proteins (OMP): - LamB - OmpA - YaeT	LamB – a maltose transporter, OmpA – a porin-like integral membrane protein, and YaeT – a multi-component complex responsible for the assembly of outer membrane β-barrel proteins.	Acts as an OMP 'back-up' chaperone in a separate but parallel pathway to SurA.	*E. coli*	[118]
IcsA	A member of the autotransporter family and is exposed on the bacterial surface and interacts with eukaryotic proteins to aid in the assembly of F-actin tails allowing propulsion of the bacterium through the host cytosol and into adjacent cells.	Appears to occur through DegP's chaperone function as small plaque phenotype still seen in the protease deficient DegP[120]. Noted that, traditionally, autotransporters have no requirement for a periplasmic chaperone therefore suggests that DegP interacts indirectly with IcsA.	*Shigella flexneri*	[120]
FHA	Filamentous Haemagglutinin (FHA) is a major adhesin which forms filamentous structures on the bacterial cell surface that allow it to adhere to ciliated cells.	Binds to FHA with high affinity therefore proposed that DegP chaperones the extended FHA polypeptide in the periplasm to the outer membrane.	*Bordetella pertussis*	[123]
EspP	Prototype SPATE (serine protease autotransporters of Enterobacteriaceae) protein that is inserted into the outer membrane	Correct folding of EspP otherwise in the htrA mutant, the EspP β-barrel domain is still inserted into the outer membrane but cell lysis still results.	*E.coli* O157:H7	[122]
ArgT	*E. coli* has a lysine/arginine/ornithine-binding protein localised in the periplasm and is responsible for binding amino acid substrates during transport into the cytoplasm.	Expression levels varied and both are periplasmic therefore speculated that HtrA processes or modifies ArgT directly or indirectly.	*Shigella flexneri*	[137]

	In *S. flexneri*, the function of ArgT has not yet been elucidated.			
NlpE and YafY	NlpE – an outer membrane lipoprotein involved in Cpx pathway. YafY – inner membrane lipoprotein with unknown function but seems likely that it is involved in the monitoring of certain envelope stresses as speculated for the case of NlpE	Of 90 lipoproteins analysed only NlpE and YafY induced DegP production.	*E. coli* K12	[138]
OutF	Type Two Secretion (T2S) system (a.k.a Out system) secretes extracellular enzymes. OutEFLM proteins suggested to form a platform in the inner membrane that anchors the pseudopilus.	Direct interaction of OutF with DegQ (DegP in abstract).	*Erwinia chrysanthemi* (causes so-called soft-rot disease of dicotyledons)	[139]

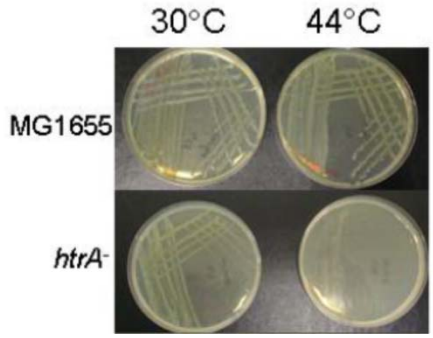

Figure 1. *E. coli htrA*$^{-}$ mutants are unable to grow at temperatures above 42°C. The plate images shown are of *E. coli* MG1655 wild-type strain and *htrA*$^{-}$ mutant cultured over night at 30°C and 44°C [140].

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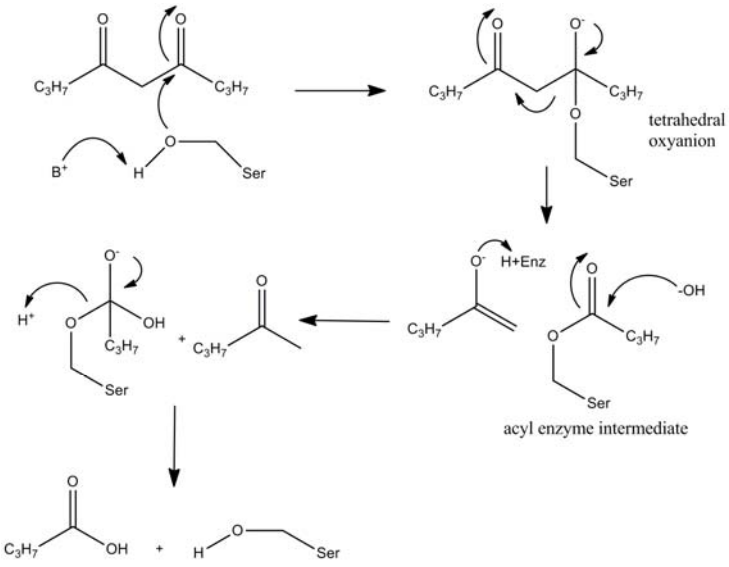

Figure 2. Chemistry of serine protease hydrolysis. The figure shows the hydrogen bonding around the catalytic triad in the active site cleft. The oxyanion hole formed by Gly and Ser enable a positive charge which stabilises the tetrahedral intermediate formed by the nucleophilic attack of the serine residue. The serine attacks the carbonyl of the peptide substrate forming the tetrahedral intermediate which is stabilised by the His. The leaving group is forced to expel by the collabpse of the tetrahedral intermediate forming the acylenzyme intermediate. Water attacks this intermediate leading to the release of the serine and a carbocylic acid product. The figure has been developed based on the review by Hedstrom and figures presented wherein [34].

Figure 3. A schematic representation of *E. coli* HtrA sequence. The figure shows the components of each HtrA monomer. This includes a signal sequence which directs export to the periplasm or extracytoplasmic compartment, the chymotryptic protease domain, and the two C-terminal protease domains.

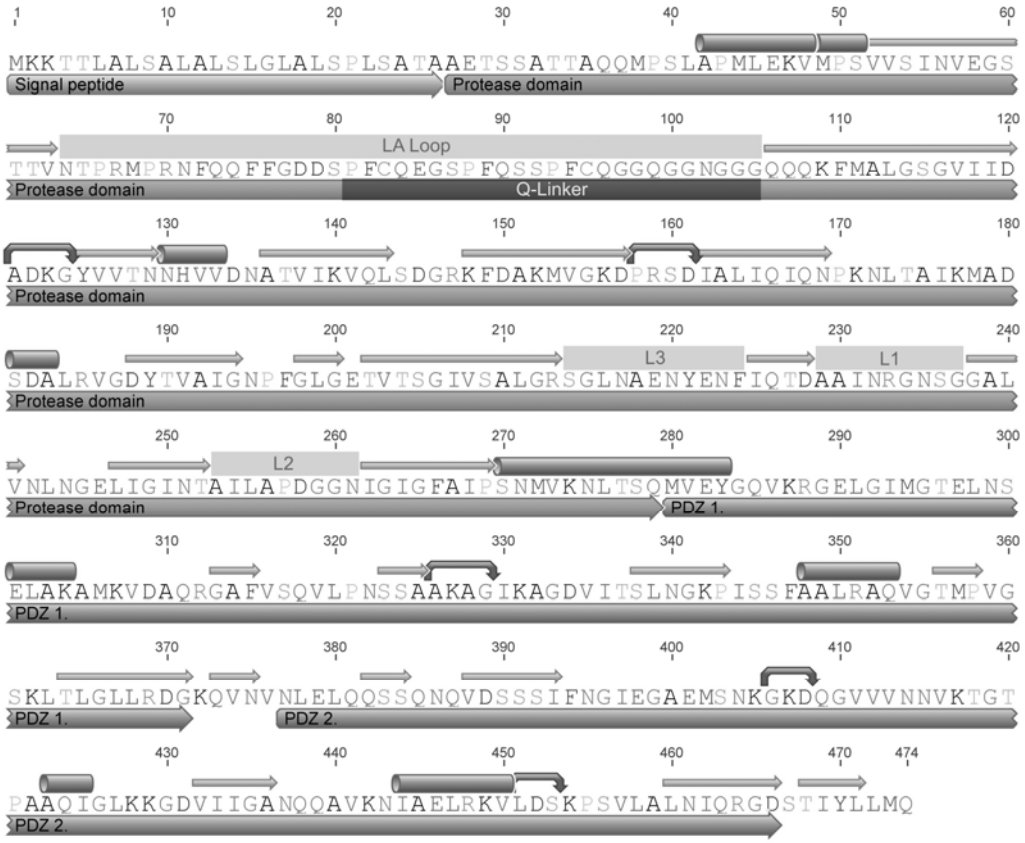

Figure 4. Sequence map of HtrA identifying all of the key structural motifs. The figure shows the HtrA sequence, all of the key loops and structural regions which have been identified by analysis of the structure of protein crystals are annotated on the figure. The signal peptide, protease domain and PDZ domains are annotated below the sequence. The Q linker which is unique to HtrA is also shown. The substrate coordination loops L1 to L3 are shown above the sequence. The flexible loop (LA) which occludes the active site of an opposite trimer subunit in the resting hexameric form is shown. Above the sequence the structural motifs are shown, with α-helix represented by the cylinder, β-sheets represented by the arrows and turns represented by the hooked arrows. The sequence shown is the *E. coli* DegP sequence and this figure has been constructed using Geneious Pro 5.1.4 (Biomatters) with modifications for the purposes of this chapter.

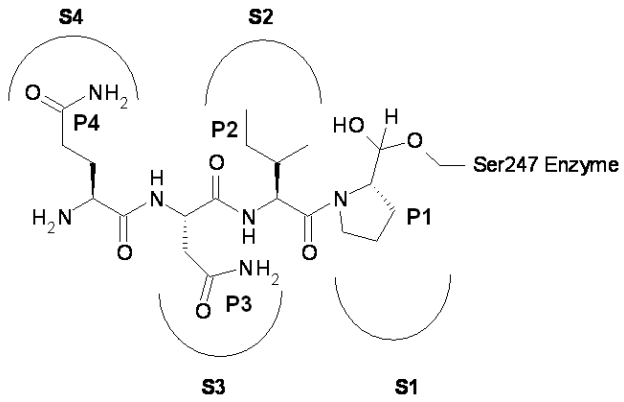

Figure 5. The figure shows a model of a substrate and protease binding interaction. The figure shows the nomenclature of the substrate sequence (P1-P4) and the nomenclature used to describe the pockets in the protease active site cleft (S1-4) [108].

Figure 6. A WebLogo representing the protease specificity of Human HtrA2. The specificity for protease activity of HtrA can be determined using a number of different approaches, one study used a proteomic wide analysis of human Jurkat T lymphocytes to identify sequences and proteins cleaved by HtrA [141]. The figure has been constructed from the data published in this paper using the WebLogo program [142] for the purposes of this Chapter.