HUMAN HTRA2 DELAYS THE AGGREGATION OF THE ALZHEIMER'S DISEASE ASSOCIATED AMYLOID β-(1-42) PEPTIDE

HUMAN HTRA2 DELAYS THE AGGREGATION OF THE ALZHEIMER'S DISEASE ASSOCIATED AMYLOID β-(1-42) PEPTIDE

By

JOEL P. KOOISTRA, B.Sc.

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AUTHOR: Joel P. Kooistra, B.Sc. (McMaster University)

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ABSTRACT

Human HtrA2 is part of the HtrA family of ATP-independent serine proteases that are conserved in both prokaryotes and eukaryotes and localizes to the inter-membrane space of the mitochondria. Several recent reports have suggested that HtrA2 is important for maintaining proper mitochondrial homeostasis and may play a role in several neurodegenerative disorders. One disorder HtrA2 is implicated in is Alzheimer's disease (AD). AD is characterized by the presence of oligomers and fibrils of the amyloid β (Aβ) peptide that is generated from cleavage of the amyloid precursor protein (APP) by β– and γ-secretases. HtrA2 degrades APP at the mitochondria, and binds the neurotoxic Aβ (1-42) peptide. In this report, the ability of HtrA2 to prevent the aggregation of a model substrate CS and the toxic Aβ (1-42) peptide were investigated. Using CS aggregation assays, HtrA2 was seen to have a moderate ability to delay and prevent the aggregation of CS, and this activity was significantly increased following removal of the PDZ domain. Additionally, using EM and 1D-WG NMR analyses HtrA2 was seen to significantly delay the aggregation of the Aβ (1-42) peptide via a dual proteolytic and chaperone-like function. These results show a novel chaperone-like activity for HtrA2 and a model emerges from this work in which HtrA2 monitors the inter-membrane space of the mitochondria using a dual proteolytic and chaperone-like function to turnover stress-damaged proteins. Furthermore HtrA2, along with other quality control factors, may be involved in the metabolism of regular as well as aberrant levels of intra-mitochondrial Aβ (1-42) peptide, which is known to lead to oxidative stress and mitochondrial dysfunction.

INTRODUCTION

The presence of damaged and improperly folded proteins within the cell can be extremely detrimental to normal cellular function. These damaged proteins can interact through surface exposed hydrophobic regions leading to the formation of insoluble and potentially toxic aggregates. Such toxic aggregates are characteristically seen in prion and amyloid diseases, such as Creutzfeldt-Jakob disease, Alzheimer's disease and Parkinson's disease and ultimately lead to neurodegeneration[1;2].

To combat the detrimental effects of improperly folded proteins, due to exposure to environmental stresses, such as heat, chemical and pathogen exposure, both eukaryotes and prokaryotes have developed specific systems to maintain proteins in a fully functional state. These systems lead to the upregulation of genes encoding both chaperones and proteases, often via signal transduction pathways that recognize the cellular stress[3;4].

Proteases act to degrade damaged and misfolded proteins as well as short-lived proteins, such as those involved in gene regulation. Substrate recognition by proteases can occur through exposed disordered regions or through added degradation tags such as Ubiquitin or SsrA. Proteases can function either in an adenosine tri-phosphate (ATP) independent or ATP dependent manner. In the latter case this often involves the help of ATPases associated with various cellular activities (AAA+) unfoldase chaperones that target and fully unfold substrate allowing for degradation. These Chaperones often assume a hexameric structure and use the energy generated through ATP hydrolysis to pull the substrate through a narrow axial pore. This unfolds the substrate and allows access to the catalytic triad within the chamber of the protease. In addition to this

unfolding activity by chaperones, they are also able to refold misfolded proteins or newly formed proteins to their native structure, or bind exposed hydrophobic regions and prevent incorporation into toxic aggregates[5; 6]. Ultimately, both proteases and chaperones work in concert to ensure the maintenance of proteins in a fully functional state.

The HtrA (High temperature requirement A) family of ATP-independent serine proteases are stress response proteins conserved in both prokaryotes and eukaryotes. The most well characterized member of the HtrA family of serine proteases is *Escherichia coli* (*E. coli*) HtrA or DegP. DegP localizes to the periplasmic space and is specifically upregulated in response to extracellular stresses that lead to protein misfolding[1]. DegP maintains proper protein homeostasis within the periplasm by acting predominately as a protease at elevated temperatures and a chaperone at lower temperatures[7]. Recent studies however have suggested that DegP can perform both these functions at lower and elevated temperatures, suggesting that additional mechanisms are necessary for regulating these functions[8; 9].

DegP is composed of three domains, a chymotrypsin-like protease domain, a PDZ1 (PSD-95/Discs-large/ZO-1) domain that is involved in substrate binding[8; 10] and PDZ2 domain that is involved in oligomerization[11]. The basic structural units of DegP are trimers, which arise due to inter-protease domain contacts. DegP trimers associate through PDZ2-protease domain, as well as LA loop (an N-terminal loop which extends from the protease domain) interactions forming a hexameric cage, that encloses all six catalytic triads (Ser-His-Asp)[12]. The active site of the DegP hexamer in the available crystal structure[12] is in an inactive conformation, and represents the resting state for the protease. Upon binding to an unfolded substrate, a condition that often arises due to

cellular stress, DegP is activated and undergoes a structural reorganization to higher oligomeric forms. These 12-meric and 24-meric multimers form macro-cages and represent a fully proteolytically active form of DegP[13]. DegP then encapsulates its substrate and either progressively degrades it to peptide fragments of 12-17 residues[14] or actively assists in the refolding of protein substrates. Whether substrate is degraded or refolded within the DegP cage may depend on initial substrate recognition or the degree of misfolding and the propensity of substrate to refold back to its native structure[10; 13].

Several homologues of DegP exist in humans. One of the most studied of these homologues is human HtrA2, due to its involvement in many diverse cellular processes such as apoptosis and mitochondrial homeostasis. HtrA2 is initially synthesized as a 49 kDa precursor protein containing an N-terminal signal sequence that localizes it predominately to the mitochondria. Once across the outer membrane of the mitochondria HtrA2 undergoes an autocleavage event, which releases the first 133 amino acids and ensures mature HtrA2 (36 kDa) is located within the inter-membrane space (IMS)[15].

In contrast to the structure assumed by DegP, HtrA2 has only a single PDZ domain and a reduced LA loop region and oligomerizes only as a trimer (Figure 1A), similar to the *E. coli* stress sensor protein DegS[16]. Interactions between N-terminal aromatic residues of adjacent protease domains are essential for maintaining the integrity of the HtrA2 trimer.

In the described X-ray structure for HtrA2 the PDZ domain collapses onto the protease domain, inhibiting access of substrate to the catalytic triad (Ser-His-Asp), rendering HtrA2 proteolytically inactive[17]. A study done in 2003 by Martins et al. showed that the proteolytic activity of HtrA2 is activated by three different mechanisms

(Figure 1B). They include increase in temperature, binding of inhibitor of apoptosis proteins (IAP) to an N-terminal reaper like motif (AVPS) exposed following mitochondrial processing, and binding of exposed C-terminal or internal residues of substrate to the PDZ domain. Each one of these factors is then thought to induce a conformational change that releases the inhibitory effect of the PDZ domain on the protease domain[18].

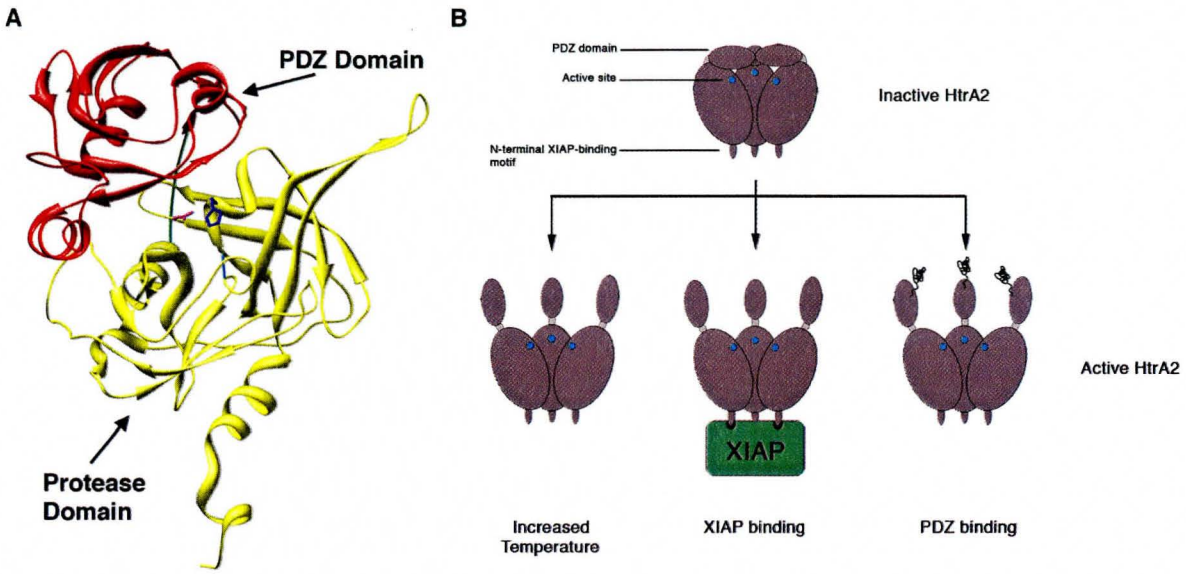

Figure 1. **The Structure and proposed activation mechanism of Human HtrA2.** A) A monomer of HtrA2 (PDB code 1LCY) with the protease domain colored yellow and the PDZ domain colored red. Residues of the catalytic triad are seen as a stick representation. The serine residue of the catalytic triad is replaced with alanine, representing the HtrA2 S306A variant B) Proposed mechanisms of HtrA2 activation, through temperature increase to 37^{O}C, binding of the XIAP, and through interaction of substrate with the PDZ domain. Modified from Martins et al, 2003[18].

Much of the work on HtrA2 has been focused on its role in apoptosis in multiple cell types. Following autocleavage in the mitochondria an N-terminal IAP binding motif (IBM) is exposed. This motif is homologous to those seen in the Drosophila IAP inhibitors Reaper, Grim, Hid and the mammalian protein Smac/Diablo[19]. Following apoptotic stimuli, due to several factors such as DNA damage or death receptor

activation, HtrA2 is released into the cytosol from the mitochondria along with other pro-apoptotic factors. Once in the cytosol HtrA2 promotes apoptosis through both a caspase-dependent and –independent mechanism, both of which are dependent on its proteolytic activity. HtrA2 then binds IAPs in a similar manner to Smac/Diablo and degrades them, releasing their inhibitor influence on caspase-3, -7, and –9[19; 20; 21; 22]. This process allows the activated caspases to continue on the cascade that ultimately leads to cell death.

Despite the evidence suggesting HtrA2 as a major player in apoptosis, recent reports suggest HtrA2 may have an important role in the maintenance of proper mitochondrial protein homeostasis. A study looking specifically at the affinity of different peptides for the PDZ domain of HtrA2, suggest HtrA2 binds C-terminal or internal hydrophobic stretches in proteins. It was seen from this study that no particular residue type was required at any particular position[23]. Therefore, HtrA2 has adapted to be a more promiscuous protease, binding unstructured hydrophobic stretches in a manner similar to DegP in *E. coli*[14]. In addition, it was recently shown that the activity of HtrA2 is increased through phosphorylation at specific serine residues (142 and 400) in the protease and PDZ domain. It was demonstrated that the phosphorylation of HtrA2 by p38 and the PTEN (phosphate and tensin homologue)-induced putative kinase 1 (PINK1) was dependent on activation of the Map kinase/ERK kinase kinase kinase 3 (MEKK3)-p38 stress kinase pathway, which responds to extra-mitochondrial stresses[24; 25]. This suggests that following stress the activity of HtrA2 is upregulated, allowing protection of the mitochondria from potentially detrimental effects.

It is becoming increasingly clearer that HtrA2 may play an important role in several neurodegenerative disorders. Previously, it was shown that mice with inactivated

HtrA2[26], or HtrA2 knocked out[27] showed significant mitochondrial dysfunction and increased susceptibility to cellular stress. Ultimately, the mice experienced degeneration in motor neurons and the striatum, resulting in a disorder with a Parkinsonian phenotype and death by 30 days. In addition to these studies, a recent screen for HtrA2 mutations in German Parkinson's disease (PD) patients identified two mutations alanine 141 to serine (A141S) and glycine 399 to serine (G399S). These two mutations were seen to reduce the ability of HtrA2 to be activated, suggesting their importance in regulating the proteolytic activity of HtrA2. Additionally they lead to a reduction in mitochondrial membrane potential as well as reduced cell viability in response to cellular stress[28]. Furthermore, it was shown that the G399S mutation in HtrA2 lead to a reduction in phosphorylation at serine 400 by p38 and PINK1[24]. These results suggest that the regulation of HtrA2 by p38 and PINK1 in the MEKK3-p38 stress pathway is important and disruption of this regulation can lead to mitochondrial dysfunction and ultimately neuronal cell death.

In addition to a possible role of HtrA2 in the pathology of Parkinson's disease, several studies have been focused on elucidating the involvement of HtrA2 in Alzheimer's disease (AD). AD is characterized by the accumulation of the toxic Aβ peptide, following cleavage of the mature amyloid precursor protein (APP-m) at position 597 and 638 by the β and γ-secretases respectively. This peptide oligomerizes and forms extra-cellular fibers that ultimately accumulate into the characteristic Alzheimer's disease plaques. Such over-expression and aggregation of the Aβpeptide (predominantly Aβ (1–42)) leads to significant neuronal loss in the hippocampus, memory impairment and dementia[29]. In addition to the accumulation of both intra- and extra-cellular

Aβpeptide, it has also been shown that APP accumulation in the mitochondria is increased in the brains of AD patients. Transport of the APP into the mitochondria is arrested through a C-terminal acidic domain, and its subsequent accumulation has been shown to affect proper protein transport and function of the organelle[30; 31]. In 2006 a study showed that APP was a direct cleavage product of HtrA2 and they co-localized at the mitochondria. Cleavage occurred at the C-terminus of the APP releasing a 161 amino acid fragment into the cytosol, and this was seen to occur under normal physiological conditions. This suggests that HtrA2 may play a role in relieving accumulation of APP at the mitochondria, ensuring proper transport and function[32].

In addition to the interaction with APP at the mitochondria, it has also been shown that a small subpopulation of cytosolic endoplasmic reticulum (ER) associated HtrA2 binds immature APP (APP-im) early in the secretory pathway. It was seen that under conditions of proteasome inhibition, a greater amount of APP-im as well as Derlin-1 (a retro-translocation protein involved in the ER associated degradation (ERAD) pathway) was associated with HtrA2. Knockout of HtrA2 lead to a greater association of APP-im with sec23 of COP II vesicles, suggesting increased transport out of the ER to the Golgi for additional processing. Under these conditions a greater association of APP-im with Flotillin-1, a marker for lipid rafts that are known to have high concentrations of β and γ-secretase, was seen. Furthermore, higher levels of Aβ peptide were seen in these cell lines. These results implicate HtrA2 in the regular metabolism of the APP, not only at the mitochondria, but also early in the secretory pathway as part of ERAD. The authors suggested that HtrA2 may function as a shuttling chaperone, ensuring misfolded

proteins at the ER and more specifically APP-im are transported from the ER to the proteasome for degradation[33].

In addition to the role of HtrA2 in the proper metabolism of APP, it has also been shown to bind the Aβ (1–42) peptide, but not degrade it[34]. Both the protease and PDZ domain were seen to be necessary for binding to the Aβ peptide[35]. It is becoming increasingly apparent that intracellular soluble rather than extra-cellular insoluble may be the neurotoxic form of Aβ (1-42)[37; 38]. Interestingly, the Aβ peptide has been seen to accumulate in the mitochondria of AD neurons and the soluble oligomeric form has been implicated in the generation of free radicals and oxidative stress[36]. Due to the mitochondrial localization of the Aβ peptide and the effect of the soluble oligomeric form on its function, it is conceivable that HtrA2 may serve a protective role. This may be through binding and sequestering the Aβ peptide and its soluble oligomers, reducing their effect on regular mitochondrial function.

Much of the work on HtrA2 has suggested it plays an important role in apoptosis, following release from the mitochondria. However, recent studies suggest HtrA2 may play an important role in quality control as well, predominately through maintaining regular homeostasis in the mitochondria. To date this work has shown that the proteolytic activity of HtrA2 may be necessary for its protective role within the mitochondria[24; 25; 26; 27; 32]. In this work we show that in addition to the proteolytic activity of HtrA2, it also possesses a novel chaperone-like function. HtrA2 was seen to have a mild chaperone-like activity through prevention of aggregation of the model substrate citrate synthase (CS) *in vitro*. This chaperone-like activity toward CS was dramatically enhanced following removal of the PDZ domain. In addition, we have shown by electron

microscopy (EM) and nuclear magnetic resonance (NMR) that HtrA2 delays the incorporation of the pathogenic Aβ (1-42) peptide into fibers *in vitro*. These fibers accumulate into the characteristic amyloid plaques seen in the brains of AD patients. HtrA2 delayed Aβ (1-42) fiber formation via a dual proteolytic and chaperone-like function.

We conclude that HtrA2 may have an important role in the regular metabolism of intra-mitochondrial Aβ (1-42) peptide, through a dual proteolytic and chaperone-like function. Additionally, based on the results seen with CS, this dual function may extend to other proteins that misfold in the inter-membrane space of the mitochondria as result of stress conditions (eg. oxidative stress).

MATERIALS AND METHODS

Plasmids and mutagenesis:

The pET21b-HtrA2 ΔPDZ mutant was generated by PCR amplification using the pET21b-HtrA2 plasmid as a template using the following primers that inserted Nde1 and Xho1 restriction sites at the ends:

Forward – CATATGGCCGTCCCTAGCCCGCCGCCCGCTTCTCCC

Reverse – CTCGAGCAGAAACTCTCGAAGACGATCAGAAGG.

The PCR product was purified by agarose gel electrophoresis and ligated with T4 DNA ligase (Invitrogen), into the zero blunt vector (Invitrogen). The vector was digested with Nde1 and Xho1, purified by agarose gel electrophoresis, and ligated with T4 DNA ligase into the pET21b plasmid between an Nde1 and Xho1 restriction site.

The QuikChange site-directed mutagenesis method (Stratagene) was used to obtain proteolytically inactive variants of HtrA2: HtrA2 S306A and HtrA2 ΔPDZ S306A. The following primers were used to generate these mutants:

Forward - GCTATTGATTTTGGAAACGCTGGAGGTCCCCTGGTTAAC

Reverse – GTTAACCAGGGGACCTCCAGCGTTTCCAAAATCAATAGC

The pET21b-DegP S210A construct was generated as previously described[11].

Protein expression and purification:

Expression of all clones in BL21 (DE3) *E. coli* was done in luria broth (LB). Cells were initially grown at 37°C to an optical density at 600 nm (OD_{600}) = 0.7. When the correct OD_{600} was reached 1 mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added and induction of pET21b-HtrA2, pET21b-HtrA2 S306A and pET21b-HtrA2 ΔPDZ S306A was performed at 12°C (overnight), 30°C (5 hrs.) and 37°C (3 hrs.)

respectively. The pET21b-DegP S210A construct was induced with 1 mM IPTG at 37^{0}C for 3 hrs. Purification was performed as previously described[11] for DegP S210A, however for HtrA2 constructs the following buffers were used, buffer A (50 mM HEPES, 500 mM NaCl and 5 % v/v glycerol) and buffer B (50 mM HEPES, 500 mM NaCl, 5 % v/v glycerol, and 0.3 M imidazole). All proteins were dialyzed to 50 mM HEPES and 150 mM NaCl pH 7.3 following purification. Those proteins used in NMR experiments were further dialyzed to 20 mM potassium phosphate buffer pH 7.4.

Protease activity assays:

Proteolytic activity assays were performed as previously described[11] for bovine milk β-casein (Sigma), with some modifications for CS (Sigma) and the Aβ (1-42) peptide (EZBiolab). 20 μg of porcine heart CS (3.4 μM) was incubated with either 180 or 90 μg of Wild type (WT) HtrA2 (14.4 and 7.2 μM respectively) in 120 μL of 50 mM HEPES and 150 mM NaCl pH 7.3 at 43^{0}C. CS degradation assays were resolved by 11% sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie brilliant blue (GE Healthcare Life Sciences); 1.7 μg of CS and 15.1 (4:1 molar ratio) or 7.5 (2:1 molar ratio) μg of WT HtrA2 were loaded at 0 minutes. 283 μg of WT HtrA2 (27 μM) was incubated with 54.2 μg of the Aβ (1-42) peptide (120 μM) in 100 μL of 20 mM potassium phosphate buffer pH 7.4, with 10 % D_2O, 0.02 % NaN_3 at 37^{0}C. The Aβ (1-42) peptide degradation assay was resolved by Tris-Tricine SDS-PAGE (14.5%)[41] and stained with Coomassie brilliant blue (GE Healthcare Life Sciences); 10.8 μg of Aβ (1-42) peptide and 56.7 μg of WT HtrA2 were loaded at 0 minutes.

Aβ (1-42) Peptide Sample Preparation:

The Alzheimer's peptide Aβ (1-42) was purchased from EZBiolab Inc., with a purity greater than 95 %. Soluble Aβ (1-42) sample was prepared by dissolving 1 mg of the Aβ (1-42) peptide in 500 μL of 10 mM NaOH. Sample was sonicated for 1 min and placed on ice for a two minute interval after which was sonicated for another minute. Immediately after sonication, 246 μg of Aβ (1-42) peptide (90 μM) was incubated with 804 or 402 μg of HSA (20 or 10 μM respectively) or 1.26 mg of WT HtrA or HtrA2 S306A (20 μM) or 936 μg of HtrA2 ΔPDZ S306A (20 μM) in 600 μL of 20 mM potassium phosphate buffer at pH 7.4, with 10 % D_2O, 0.02 % NaN_3 at 37^0C. At the indicated time points EM and NMR readings were taken. During the time between the NMR acquisition sessions and EM analysis, the samples were stored in a water bath at 37^0C without mixing.

Electron Microscopy:

For negative staining of all samples, freshly glow-discharged continuous carbon grids (Electron Microscopy Sciences) were floated on 5 μl of the Aβ (1-42) peptide aggregation reaction (seen above) for 2 min. Excess sample was blotted and floated on 5 μl of 3% uranyl actetate (Canemco and Marivac) for 1 min. Excess stain was blotted and air-dried. Samples were imaged at 10,000 and 25,000x magnification respectively using the JEOL 2010F Transmission electron microscope (TEM), operated at 200 kV in low dose mode. EM images were taken on Kodak 50-163 film and digitized using the Nikon Super Coolscan 9000 scanner.

NMR Spectroscopy:

Acquisition of nuclear magnetic resonance (NMR) experiments was preformed at 37^0C. The Aβ (1-42) aggregation was monitored through the loss of signal in the 1D Watergate experiments which occurred over time. 1D-WG experiments incorporated a 30 ms long spin lock pulse with 2.6 kHz strength prior to acquisition to suppress the residual protein signal. All NMR spectra were acquired at 600 MHz using 128 scans and 64 dummy scans and were processed using an exponential multiplication window function prior to zero filling. Spectral region of 0.64-1.07 ppm was integrated and was used as a measure of signal loss due to the aggregation with time. Error on the integral intensities is proportional to the spectral noise and was determined using the signal to noise ratio SINO from the XWINNMR, where the 0.64-1.07 ppm region was chosen for the signal and the 10-10.5 ppm region was chosen for noise estimation (experiments were performed by Julijana Milojevic).

CS aggregation assay:

CS aggregation assays were carried out as previously described[42]. 4.4 μg of CS (0.15 μM) was incubated with 2.6 or 5.2 μg of Lysozyme (0.3 or 0.6 μM respectively); 52 or 104 μg of DegP S210A (0.3 or 0.6 μM respectively); 38 μg of WT HtrA2 or HtrA2 S306A (0.6 μM); 14 or 28 μg of HtrA2 ΔPDZ S306A (0.3 or 0.075 μM respectively) in 600 μL of 40 mM HEPES pH 7.3. Light scattering was measured at 43^0C in a fluorescence spectrophotometer (Cary Eclipse; Varian) with excitation and emission wavelengths set to 500 nm and a slit width of 5 nm in 4 sided quartz cuvettes. Aggregation was monitored for a total of 20 minutes for all reaction conditions.

RESULTS

Purified WT HtrA2 is proteolytically active against a model substrate:

The WT HtrA2 protein was initially purified using metal-chelating Ni-affinity chromatography and peak fractions were resolved using SDS-PAGE (11 %) to asses purity (Figure 2A). No significant contaminating bands were present in the gel, suggesting the purity of the sample was between 90-95 %.

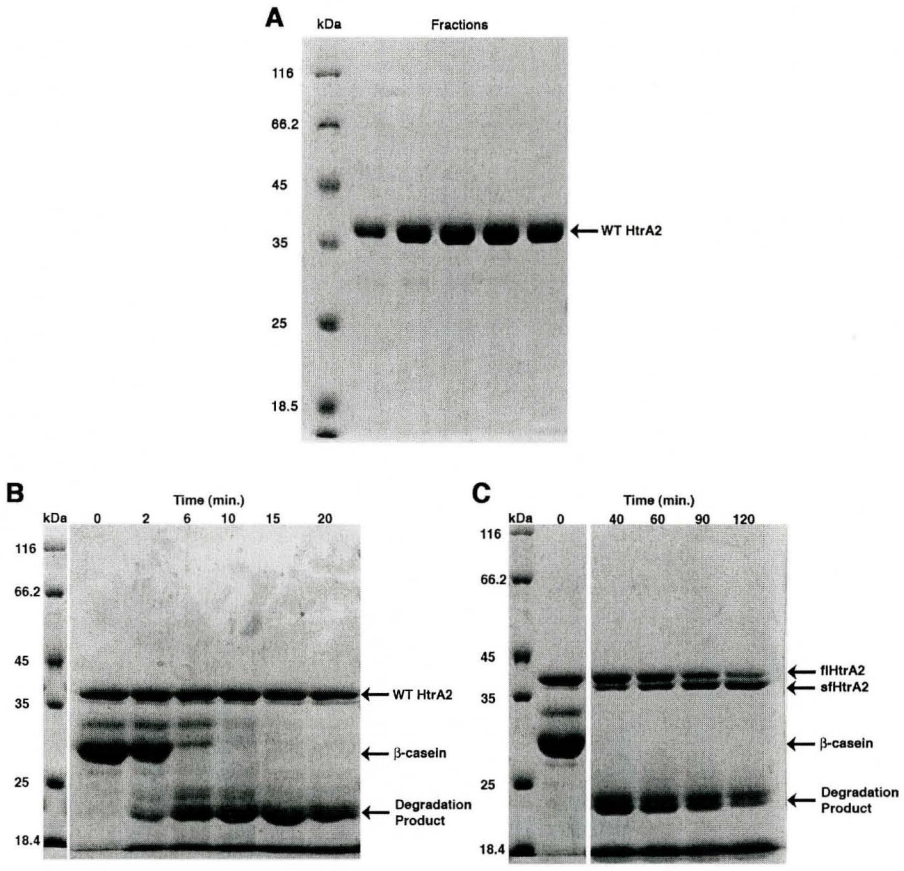

Figure 2. **Purification of WT HtrA2 and characterization of its proteolytic activity toward β-casein.** A) WT HtrA2 was eluted from the Ni-affinity column using 0.24 M Imidazole. Selected peak fractions were resolved by SDS-PAGE (11%) and stained with Coomassie brilliant blue. WT HtrA2 (5 μM) was incubated with β-casein (66 μM) in 50 mM HEPES, 150 mM NaCl pH 7.3 for a total of B) 20 minutes and C) 120 minutes. At selected time points samples were removed from the reaction mixture and placed in 2x SDS loading buffer. Both degradation assays were resolved by SDS-PAGE (11%) and stained with Coomassie brilliant blue. fl – full-length WT HtrA2 and sf – short-form WT HtrA2.

Proteolytic activity of the purified WT HtrA2 was measured against the model substrate β-casein. Initially, WT HtrA2 was incubated with β-casein for a total of 20 minutes, and the rate of degradation was seen to proceed quite rapidly (Figure 2B). After 10 minutes of incubation almost all of the full-length substrate was degraded, and the appearance of an intermediate degradation product was observed at ~ 20 kDa.

The degradation assay was subsequently extended for a total of 2 hrs. and we observed that following the complete degradation of full-length β-casein, an auto-cleavage product of WT HtrA2 began to appear just below the full-length form (Figure 2C). Such an auto-cleavage process has been described as well in *E. coli* DegP following degradation of full-length β-casein, malate dehydrogenase (MDH) and lysozyme[43]. It has been suggested that this represents a self-regulatory mechanism of DegP, to eliminate the excess of DegP after stress conditions. Under such conditions the proteolytic and chaperone-like function of DegP are no longer needed (Jomaa et al, manuscript submitted for publication). It is possible that such a mechanism may exist for HtrA2 as well, however the exact purpose of this auto-cleavage product remains to be elucidated.

Comparison of the HtrA2 and *E. coli* DegP structures:

Based on a large body of evidence suggesting a protective role for HtrA2, it is tempting to speculate that it monitors and controls protein folding in a manner similar to DegP in bacteria. So considering the fact that HtrA2 shows a relatively high degree of structural and sequence homology to DegP, comparing the structures of both these proteins could give insights as to whether HtrA2 could act as a chaperone and where in the structure this function may reside. It had been previously shown that only the protease domain of DegP is necessary for its chaperone activity[11]. Furthermore, a loop

located near the active site with surface exposed hydrophobic residues (L2 loop) was proposed to be responsible for this activity[12]; so the protease domains of HtrA2 and DegP were compared structurally. When the protease domains of HtrA2 (red) and DegP (cyan) were superimposed the L2 loop (seen in yellow for DegP) was also present for HtrA2 (seen in green), however it was somewhat smaller (Figure 3A and B). Additionally, in looking at the intact trimer of HtrA2 it becomes apparent that the PDZ domains block this loop region on the protease domain[17].

When looking at a surface representation of the HtrA2 trimer with the PDZ domains removed, a large number of surface exposed hydrophobic residues (hydrophobic-yellow, aromatic-orange) are seen at the interface between the monomers (Figure 3C). Additionally, the hydrophobic L2 loop (seen in green on the surface representation) is exposed on the surface of the protease domain. Due to the fact that the PDZ domains of the monomers appear to block access to this hydrophobic region, it seems plausible that the chaperone activity of HtrA2 may be activated in a similar manner to its proteolytic activity. Whereby there may be an initial binding to the PDZ domain or elsewhere to stimulate release of the PDZ domain allowing unfolded proteins to access and bind the exposed hydrophobic regions.

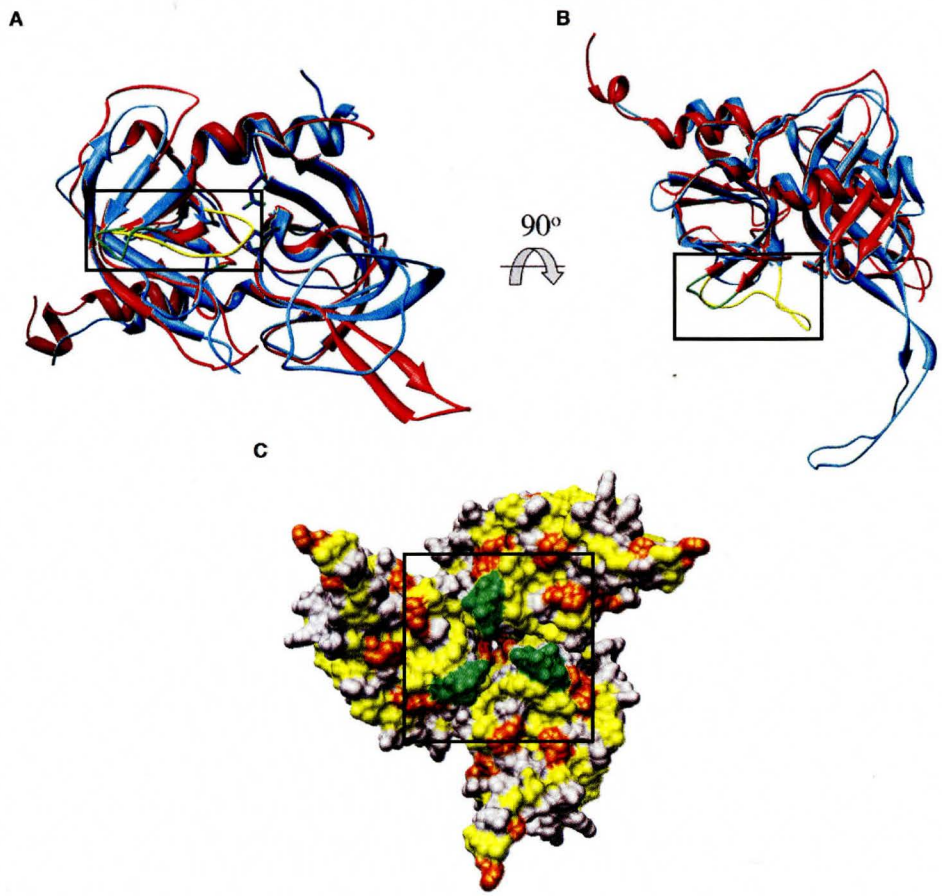

Figure 3. **Proposed Chaperone region for HtrA2.** A) The protease domain of DegP (PDB code 1KY9) (cyan) and HtrA2 (PDB code 1LCY) (red) were superimposed. The loop region (L2 loop) of proposed chaperone activity for DegP is seen in yellow and in green for HtrA2. B) The superimposed regions were rotated 90 degrees to make these loops more visible. C) This is a surface representation of an HtrA2 timer with the PDZ domains removed. The hydrophobic residues are colored in yellow and the aromatic residues in orange. The boxed in area shows the region of concentrated hydrophobic residues at the interface of the HtrA2 monomers. The hydrophobic L2 loop for each monomer is colored in green, similar to 3A and B.

Chaperone activity of HtrA2 against a model substrate *in vitro*:

Based on the previous literature suggesting a protective role for HtrA2 in the mitochondria[26; 27], and the apparent structural similarities to *E. coli* DegP, we decided to test whether HtrA2 had the ability to prevent the aggregation of a model substrate CS. This represented an ideal substrate as the aggregation profile and refolding of CS had

been previously well characterized with a variety of different ATP-dependent and independent chaperones[42].

Several HtrA2 mutants were generated in order to fully characterize its chaperone activity and understand what domains may be necessary for this function. A proteolytically inactive variant of HtrA2 was generated, HtrA2 S306A, so only the chaperone function could be tested. Additionally, an HtrA2 mutant lacking the PDZ domain and proteolytic activity was generated, HtrA2 ΔPDZ S306A, because it had been previously shown that only the protease domain of *E. coli* DegP is necessary for its chaperone function. Furthermore, because of the large concentration of hydrophobic residues lining the surface of the protease domain of HtrA2, it seemed that this would be a potential region for its chaperone activity. Initially, both proteins were purified as described above and similarly a purity of 90-95 % was obtained (Figure 4A and B).

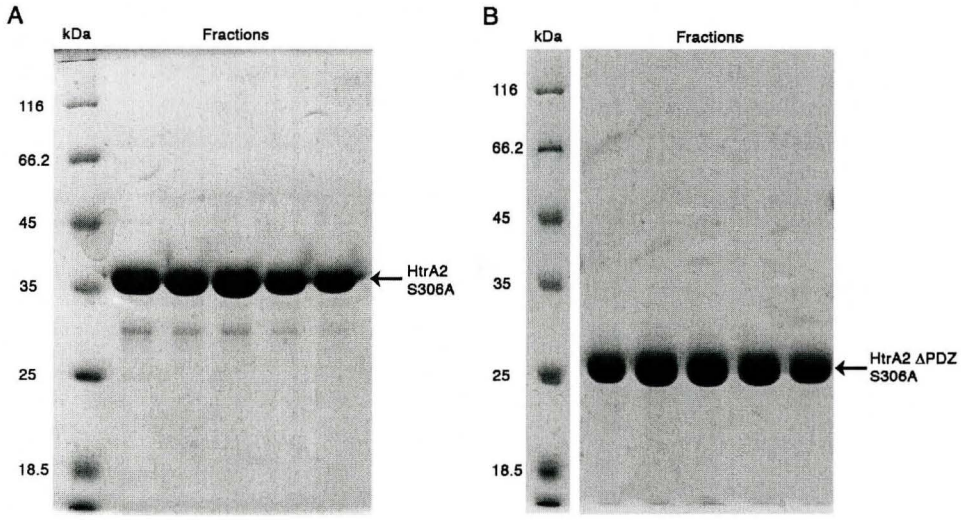

Figure 4. **Purification of HtrA2 S306A and HtrA2 ΔPDZ S306A**. All proteins were eluted from the Ni-affinity column using 0.24 M Imidazole. Selected peak fractions of A) HtrA2 S306A and B) HtrA2 ΔPDZ S306A were then resolved by SDS-PAGE (11%) and stained with Coomassie brilliant blue. The purity of all samples was adequately high, between 90-95 % pure.

To further assess whether CS would be a suitable chaperone substrate for HtrA2, the proteolytic activity of WT HtrA2 toward CS was tested (Figure 5). Initially, the degradation profile of WT HtrA2 to CS at a 2:1 molar ratio respectively was observed (Figure 5A), and it was seen that CS was a weak substrate for WT HtrA2. After 30 minutes incubation very little CS was degraded, and by 2 hours a significant amount of full-length CS still remained in solution.

Subsequently, the degradation profile of WT HtrA2 to CS at a 4:1 molar ratio respectively was observed (Figure 5B); in this case slightly more degradation was seen. However, even after 2 hours some full-length CS still remained in solution. Considering

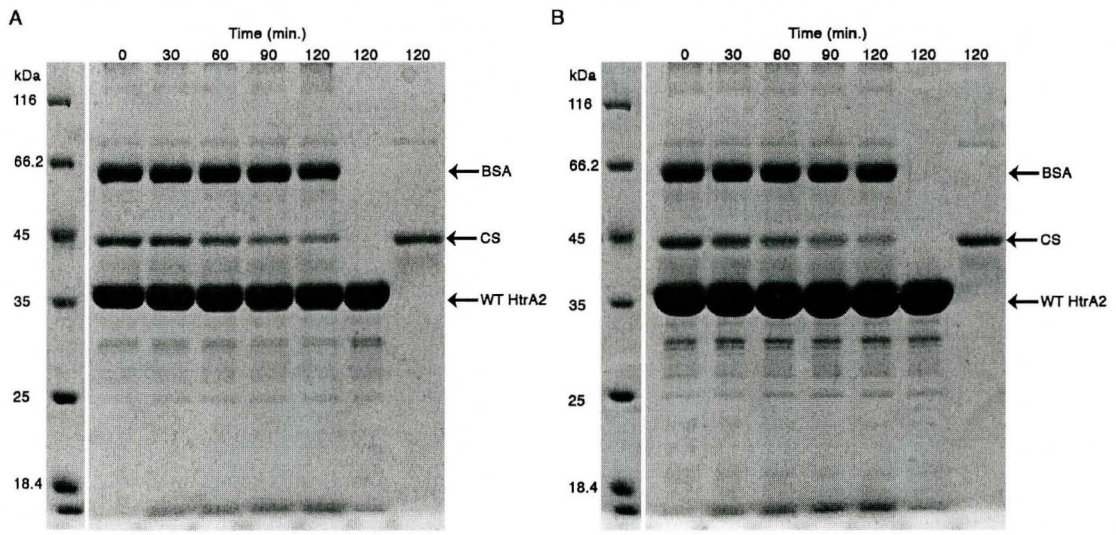

Figure 5. **Proteolytic activity of WT HtrA2 towards CS.** WT HtrA2 was incubated with CS at A) 2:1 and B) 4:1 molar ratios respectively in 50 mM HEPES, 150 mM NaCl pH 7.4 at 43^{0}C. At selected time points sample was removed from the reaction mixture and placed in 2x SDS loading buffer. Both degradation assays were resolved by SDS-PAGE (11%) and stained with Coomassie brilliant blue.

the rate at which WT HtrA2 degraded β-casein, having almost complete degradation of full-length substrate by 10 minutes even with a large molar excess, CS was considered a weak proteolytic substrate and used for further testing.

To test whether HtrA2 acts as a chaperone with the ability to prevent protein aggregation, citrate synthase (CS) aggregation assays were performed. In these assays the extent of CS aggregation is measured at 43^0C (temperature at which CS unfolds and aggregates) in a spectrofluorometer at excitation and emission wavelengths of 500 nm. This is a light scattering technique that measures the turbidity in solution, and the addition of molecular chaperones should reduce this turbidity over time, through aggregation prevention. Initially, the ability of WT HtrA2 (0.6 μM) to prevent the aggregation of CS (0.15 μM) was tested (Figure 6A). WT HtrA2 was seen to delay the aggregation of CS by several minutes, and was able to lead to a slight reduction in its overall aggregation following the 20 minute incubation time. However the degree of prevention was not nearly as pronounced as that seen for DegP S210A (0.6 μM), which is a known chaperone[7]. As a control we tested Lysozyme (0.6 μM) that was completely unable to prevent the aggregation of CS, and it aggregated to the same level as the CS alone condition.

Because it was previously seen that WT HtrA2 does slightly degrade CS over the incubation time of the assay, and at the molar ratio used (Figure 5B), the proteolytically inactive HtrA2 S306A was tested. This was to differentiate how much of the protective effect seen by WT HtrA2 was due to proteolytic activity and how much was due to chaperone-like activity. HtrA2 S306A (0.6 μM) also showed a slight delay and reduction in the aggregation of CS (0.15 μM), however it was somewhat less than what was seen with WT HtrA2 (Figure 6B). So it seems that some of the protective effect seen by WT HtrA2 was probably due to slight degradation of CS over the duration of the assay.

Additionally, DegP S210A (0.6 μM) and Lysozyme (0.6 μM) showed a similar effect on CS aggregation as was seen previously.

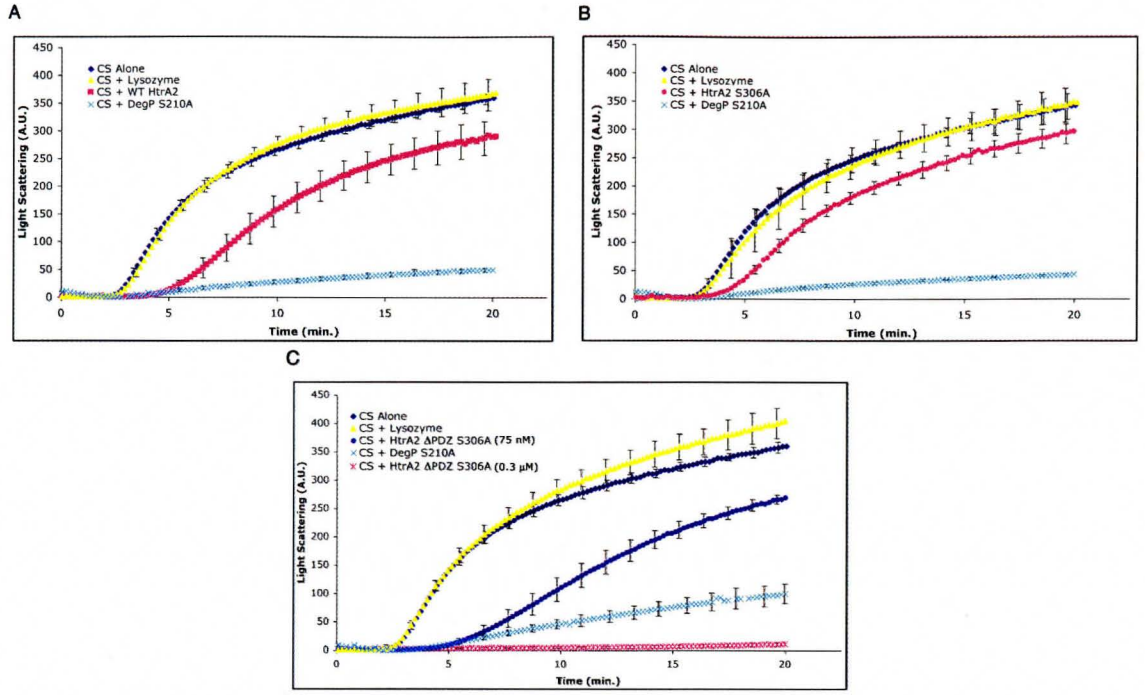

Figure 6. **Prevention of CS aggregation by WT HtrA2, HtrA2 S306A and HtrA2 ΔPDZ S306A.** A) Lysozyme (0.6 μM), DegP S210A (0.6 μM) or WT HtrA2 (0.6 μM) were incubated with CS (0.15 μM) for a total of 20 minutes. B) Lysozyme (0.6 μM), DegP S210A (0.6 μM) or HtrA2 S306A (0.6 μM) were incubated with CS (0.15 μM) for a total of 20 minutes. C) Lysozyme (0.3 μM), DegP S210A (0.3 μM) or HtrA2 ΔPDZ S306A (0.3 μM and 75 nM) were incubated with CS (0.15 μM) for a total of 20 minutes. All reactions were in 40 mM HEPES pH 7.3 and the turbidity in solution was monitored using a fluorescence spectrophotometer with excitation and emission wavelengths set to 500 nm. For all conditions, curves represent the mean of at least three independent runs (± SD).

Due to the only slight aggregation prevention activity seen for WT HtrA2 and HtrA2 S306A, we decided to test the chaperone-like function of HtrA2 ΔPDZ S306A. We reasoned that this mutant may give insights as to whether the PDZ domain has an inhibitory role on the chaperone-like function similar to its role on the proteolytic function of HtrA2[17; 18]. In the presence of 0.3 μM of HtrA2 ΔPDZ S306A, the aggregation of CS (0.15 μM) was completely prevented, compared to the CS alone

reaction (Figure 6C). When a significantly lower concentration of HtrA2 ΔPDZ S306A (75 nM) was incubated with CS (0.15 μM), a significant delay and reduction in aggregation was also seen. Consistently with what was previously seen, DegP S210A (0.3 μM) and Lysozyme (0.3 μM) showed both prevention and no effect on CS aggregation respectively. This suggested that the protease domain of HtrA2 is most likely the region for its chaperone-like function, due to the large concentration of exposed hydrophobic residues. Additionally, the PDZ domain may have an inhibitory role, blocking access of substrate to the exposed hydrophobic residues on the protease domain.

WT HtrA2 delays Aβ (1-42) peptide aggregation:

Our initial experiments showed that HtrA2 had the ability to delay aggregation of a model substrate CS *in vitro*. Because HtrA2 has been shown to have a neuroprotective effect[26; 27] and due to the increasing evidence suggesting its involvement in AD[32; 34; 35], we decided to test whether HtrA2 had any effect on the aggregation process of the to Aβ (1-42) peptide. This peptide is generated through sequential cleavage by the β and γ-secretases respectively, and represents the most pathogenic aggregation-prone form [44]. Additionally, HtrA2 had been previously shown to bind the Aβ (1-42) peptide through a combination of it protease and PDZ domain[34; 35].

Therefore, reaction mixtures containing either Aβ (1-42) peptide (90 μM) alone, Aβ (1-42) (90 μM) + WT HtrA2 (20 μM), or Aβ (1-42) (90 μM) + human serum albumin (HSA) (20 μM) were generated. Subsequently, aggregation of the Aβ (1-42) peptide into fibers was monitored by both EM and 1D-WG NMR. Two time points (0 and 4 hours)

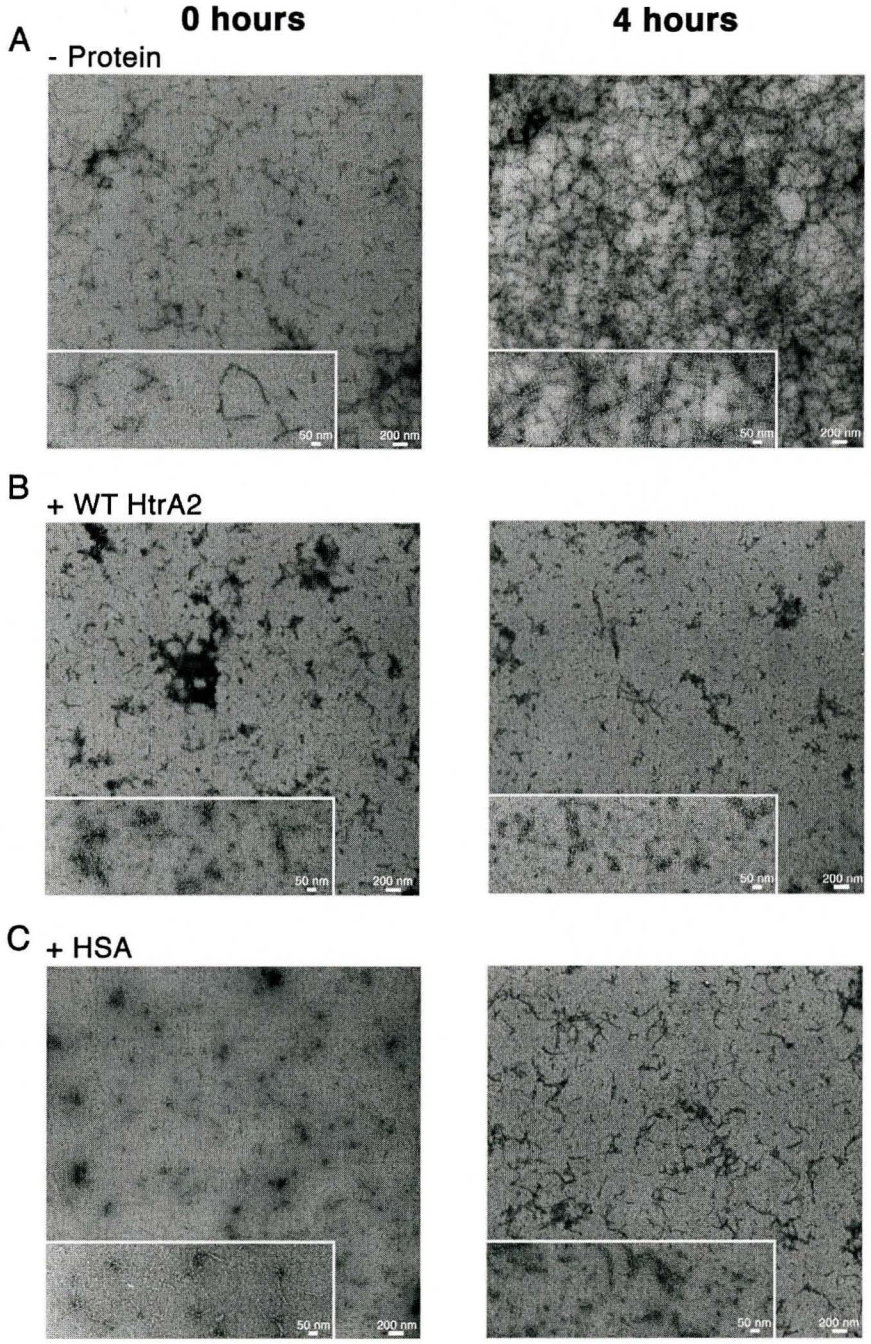

Figure 7. **Visualization of Aβ (1-42) aggregation prevention by WT HtrA2 using EM.** Reaction mixtures containing Aβ (1-42) alone (90 μM) or with HSA (20 μM) or WT HtrA2 (20 μM) were incubated in 20 mM potassium phosphate buffer with 10% D_2O and 0.02% NaN_3 pH 7.4 at 37°C. At selected times aliquots of A) Aβ (1-42) alone at 0 and 4 hours, B) Aβ (1-42) alone + WT HtrA2 at 0 and 4 hours and C) Aβ (1-42) alone + HSA at 0 and 4 hours were added to continuous carbon grids and stained with 3 % uranyl acetate. Samples were imaged at both 10 K (larger area on micrograph) and 25 K (boxed in region on micrograph) magnification under low dose conditions using the JEOL 2010F TEM.

were used for EM analysis (Figure 7). In the absence of WT HtrA2 at 0 hours (Figure 7A) there were only small fiber fragments present in the sample, showing the initial stages of polymerization. Following incubation of this sample at 37^{0}C for 4 hours (Figure 7A), long fibers had polymerized, generating a large tangled network. When Aβ (1-42) was incubated in the presence of WT HtrA2 (Figure 7B) at 0 hours only small fragments of fibers were seen similar to that observed in the sample containing only Aβ (1-42). However, after incubation of the samples at 37^{0}C for 4 hours, WT HtrA2 (Figure 7B) showed a significant delay of Aβ (1-42) fiber formation. As a control Aβ (1-42) was incubated in the presence of HSA (Figure 7C) and at 0 hours again only small fragments of fibers were observed, as was seen before. After incubation of the samples at 37^{0}C for 4 hours, HSA (Figure 7C) showed a significant delay of Aβ (1-42) fiber formation. This was expected for HSA, as it had been shown previously to bind the Aβ (1-42) peptide and be involved in the metabolism of this peptide in the serum[45]. Such activity has not been previously described for WT HtrA2 and suggests a potential role for it in the regular metabolism of intra-mitochondrial Aβ (1-42) peptide.

In addition to the qualitative EM analysis, 1D-WG NMR analysis was used to quantitatively monitor the incorporation of the monomeric Aβ (1-42) peptide into oligomers and larger fibers over extended periods of time (Figure 8). In this technique a reduction in relative intensity relates to a loss of the monomeric Aβ (1-42) peptide signal due to incorporation into oligomers and ultimately fibers. In these experiments the aggregation profile of the samples were monitored for up to a week, and the results were seen to correlate well with the EM analysis. By 4 hours an approximately 40 % reduction in the relative intensity was seen for the Aβ (1-42) peptide alone sample. Such a large

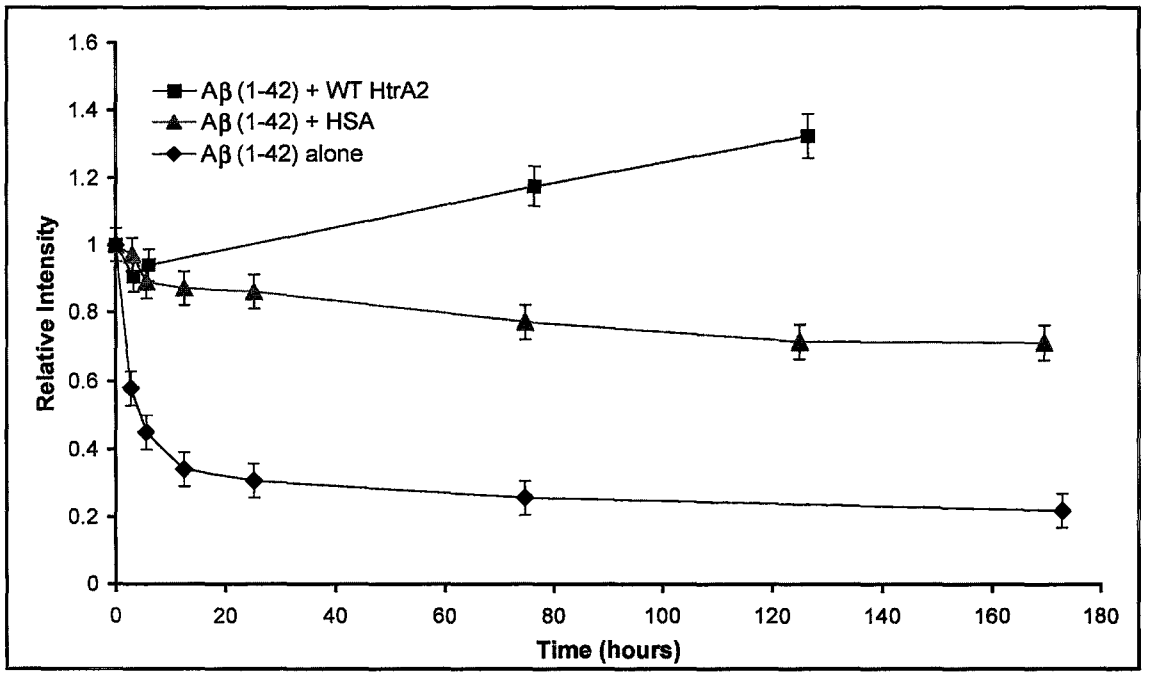

Figure 8. **Prevention of Aβ (1-42) peptide aggregation by WT HtrA2 measured using 1D-WG NMR analysis.** Reaction mixtures containing Aβ (1-42) alone (90 μM) or with HSA (20 μM) or WT HtrA2 (20 μM) were incubated in 20 mM potassium phosphate buffer with 10% D_2O and 0.02% NaN_3 pH 7.4 at 37°C. At selected times each of the samples were subjected to 1D-WG NMR analysis. All NMR spectra were acquired at 600 MHz; the spectral region of 0.64-1.07 ppm was integrated and was used as a measure of signal loss due to the aggregation with time.

reduction in intensity in such a short period of time shows how prone the monomeric Aβ (1-42) peptide is to incorporation into oligomers and ultimately fibers. As a positive control the Aβ (1-42) peptide was incubated in the presence HSA; only a slight reduction in intensity was seen after 4 hours and never went below 20 %. Interestingly, when the Aβ (1-42) peptide was in the presence of WT HtrA2, the relative intensity did not change very much after 4 hours, and increased significantly over an extended period of time. Such an increase in relative intensity can be explained either by a breaking apart of existing oligomers and fibers or degradation of the Aβ (1-42) peptide by WT HtrA2.

To explore further whether the later reason is why an increase in intensity was observed, the degradation of Aβ (1-42) peptide by WT HtrA2 was monitored (Figure 9).

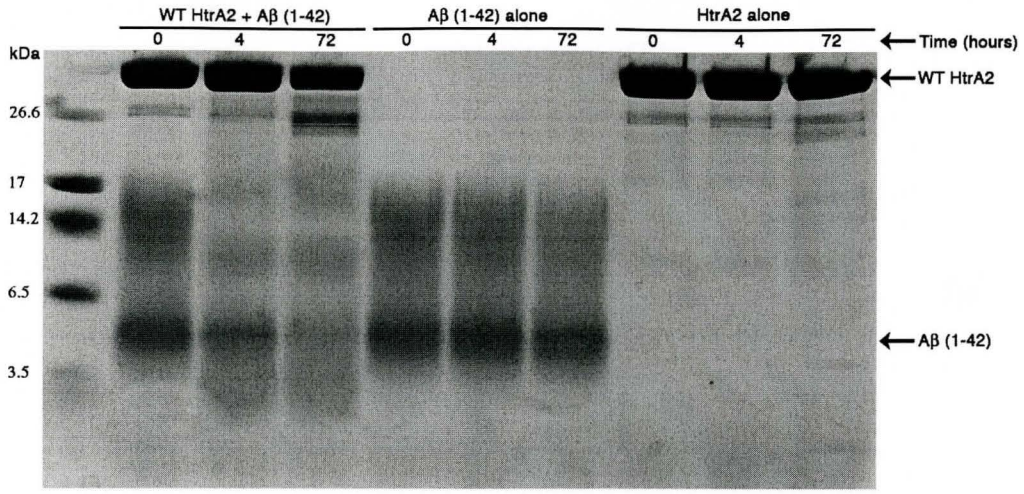

Figure 9. **Proteolytic activity of WT HtrA2 towards the Aβ (1-42) peptide.** WT HtrA2 (27 μM) was incubated with the Aβ (1-42) peptide (120 μM) in 20 mM potassium phosphate buffer with 10% D_2O and 0.02% NaN_3 pH 7.4 at 37°C. At selected time points sample was removed and placed in 2x SDS loading buffer. Samples were resolved by tris-tricine SDS-PAGE (14.5 %) and stained with Coomassie brilliant blue.

The molecular weight (MW) of the Aβ (1-42) peptide is approximately 4.5 kilodaltons (kDa), and a predominant band consistent with this MW was seen on the gel. In addition there was a smearing pattern of larger MW species, which may represent higher oligomeric forms of the peptide that are SDS-insoluble. When WT HtrA2 was added with the Aβ (1-42) peptide there was clear degradation occurring over as little as 4 hours. After an incubation time of 3 days, there was significantly more degradation of the peptide as expected. Both the Aβ (1-42) peptide and HtrA2 alone samples showed little change over the course of the 3 day incubation period, suggesting they were both stable under the conditions of the degradation assay. This new result suggests that WT HtrA2 cleaves the Aβ (1-42) peptide and therefore may ensure the prevention of Aβ (1-42) aggregation through a combination of a proteolytic and chaperone-like function.

Proteolytic activity of HtrA2 is not essential for delaying the Aβ (1-42) peptide aggregation process:

To determine whether the effect of WT HtrA2 on the aggregation process of the Aβ (1-42) peptide was dependent on its proteolytic or chaperone-like activity, the previous experiments were repeated in the presence of the proteolytically inactive HtrA2 S306A (Figure 10). Consistently with our previous results, the sample containing only Aβ (1-42) peptide had only small fibers present after 0 hours (Figure 10A). After 4 hours of incubation at 37^{0}C (Figure 10A) again long fibers had polymerized and aggregated in a pattern that was similar to what was previously seen. The sample containing HtrA2 S306A (Figure 10B) with Aβ (1-42) at 0 hours, had only small fibers present. Interestingly, after 4 hours this sample showed some increase in fiber formation and length, but it was greatly reduced compared to Aβ (1-42) alone (Figure 10B). As a control the effect of HSA on the Aβ (1-42) peptide was observed, and it was shown to have a significant protective effect after 4 hours of incubation at 37^{0}C as expected (Figure 10C).

From the 1D-WG NMR analysis, it was seen that by 4 hours the relative intensity of the Aβ (1-42) peptide alone sample was reduced by approximately 35 % (Figure 11). This reduction in intensity was slightly less than what was seen previously, however the 80 % intensity reduction after 7 days was consistent with what was seen before. In the presence of HSA only a slight reduction in intensity was seen after 4 hours and never went much below 20 %, consistent with what was seen before. In this set of experiments 10 μM instead of 20 μM HSA was used, due to problems getting readings from the 20 μM HSA sample, but in both cases each condition showed the expected protective effect.

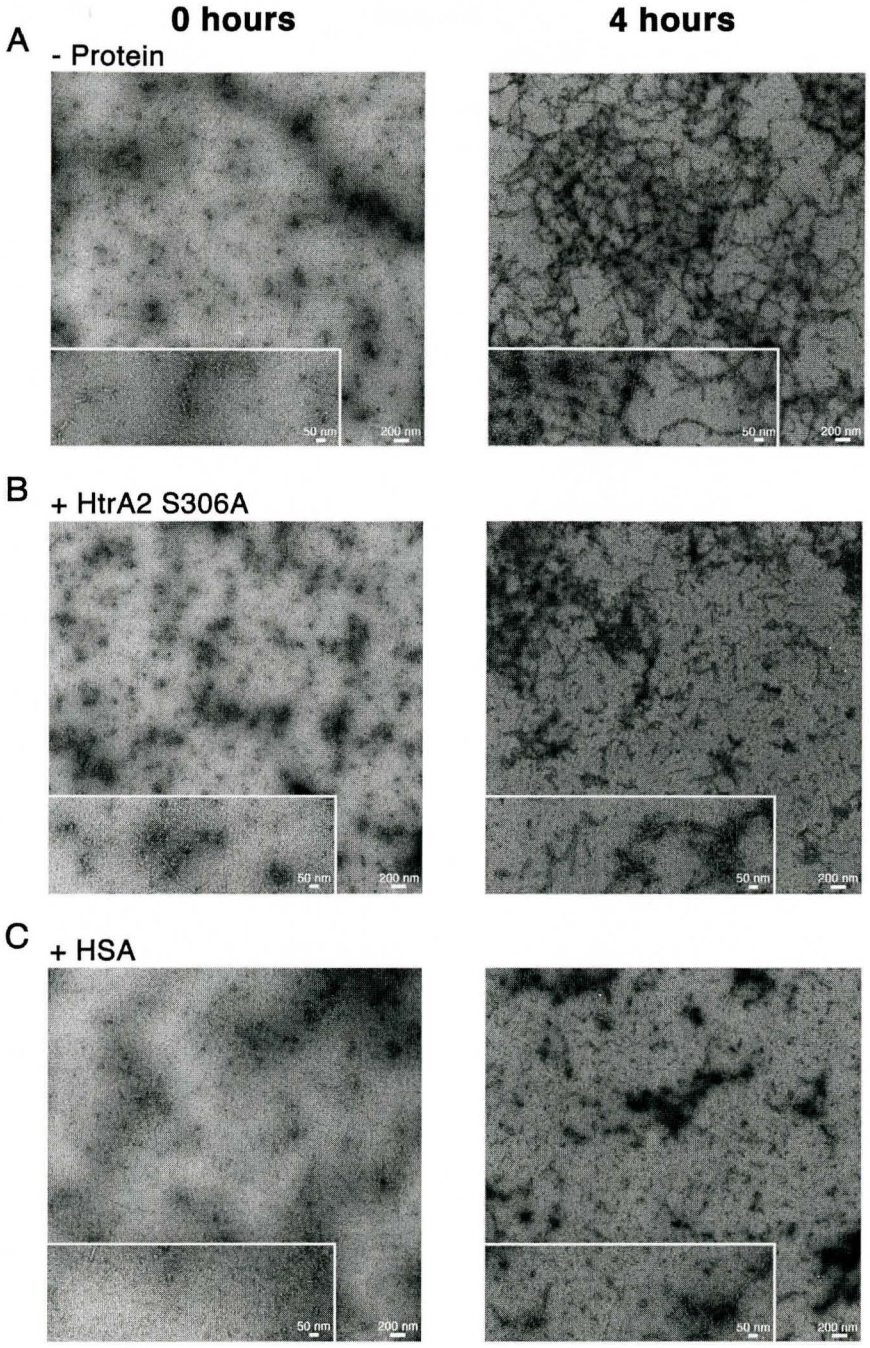

Figure 10. **Visualization of Aβ (1-42) aggregation prevention by HtrA2 S306A using EM.** Reaction mixtures containing Aβ (1-42) alone (90 μM) or with HSA (20 μM) or HtrA2 S306A (20 μM) were incubated in 20 mM potassium phosphate buffer with 10% D_2O and 0.02% NaN_3 pH 7.4 at 37°C. At selected times aliquots of A) Aβ (1-42) alone at 0 and 4 hours, B) Aβ (1-42) alone + HtrA2 S306A at 0 and 4 hours and C) Aβ (1-42) alone + HSA at 0 and 4 hours were added to continuous carbon grids and stained with 3 % uranyl acetate. Samples were imaged at both 10 K (larger area on micrograph) and 25 K (boxed in region on micrograph) magnification under low dose conditions using the JEOL 2010F TEM.

Finally, when the Aβ (1-42) peptide was in the presence of HtrA2 S306A, again only a slight reduction in intensity was seen and never went below 10 %, a protective effect slightly better than seen for HSA.

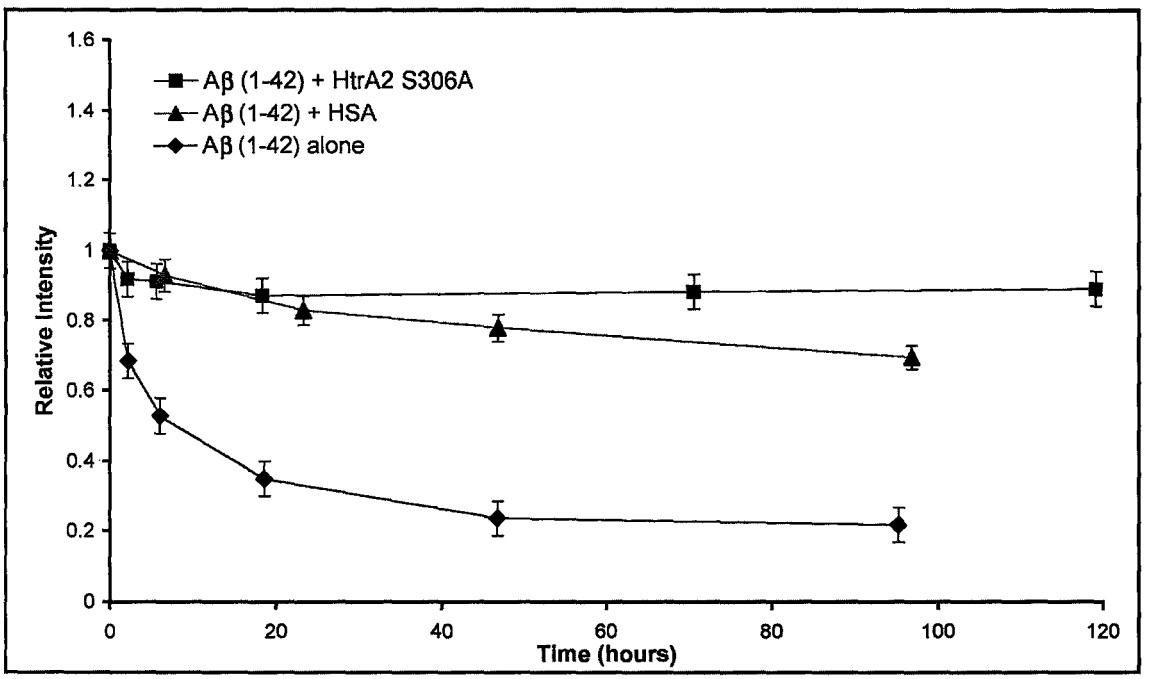

Figure 11. **Prevention of Aβ (1-42) peptide aggregation by HtrA2 S306A measured using 1D-WG NMR analysis.** Reaction mixtures containing Aβ (1-42) alone (90 μM) or with HSA (10 μM) or HtrA2 S306A (20 μM) were incubated in 20 mM potassium phosphate buffer with 10% D_2O and 0.02% NaN_3 pH 7.4 at 37°C. At selected times each of the samples were subjected to 1D-WG NMR analysis. All NMR spectra were acquired at 600 MHz; the spectral region of 0.64-1.07 ppm was integrated and was used as a measure of signal loss due to the aggregation with time.

These results suggest that HtrA2 has the ability to significantly prevent the incorporation of the Aβ (1-42) peptide into fibers, and it is not totally dependent on its proteolytic activity. It seems that WT HtrA2 works via a dual protease, chaperone-like function to prevent Aβ (1-42) peptide fiber formation and aggregation.

The PDZ domain in HtrA2 is not essential for delaying the Aβ (1-42) peptide aggregation process:

Now that it was apparent that HtrA2 has a chaperone-like function against the Aβ (1-42) peptide, we wanted to further test whether the PDZ domain is dispensable for this function, similar to what was seen for CS. This would give further support for a potential inhibitory role of the PDZ domain, through blocking access of unfolded substrate to surface exposed hydrophobic residues on the protease domain. Suggesting a similar mechanism for chaperone-like and protease function, where upon activation of HtrA2 the PDZ domain swings away from the protease domain.

To further explore whether this may be a plausible mechanism, HtrA2 ΔPDZ S306A was tested for the ability to delay the aggregation process of the Aβ (1-42) peptide using EM analysis (Figure 12). In the Aβ (1-42) peptide sample alone, there were almost no fibers present at 0 hours (Figure 12A); only sparsely bound small fiber fragments. After 4 hours (Figure 12A), polymerization occurred and the long Aβ fibers were present. However, the amount of fibers bound seemed less than the previous times the experiments were performed. This could be due to the fact that at 0 hours less fibers were present compared to the previous two experiments, possibly as a result of more efficient sonication and disruption of any initial oligomers that could accelerate the polymerization process. Furthermore, potential variation of the incubation temperature during the course of the experiment may have also contributed to the somewhat inconsistent results. In any event, long fibers were formed and this was expected based on the previous results. In the sample containing HtrA2 ΔPDZ S306A (Figure 12B) at 0 hours, very few fibers were seen and were only small fragments. Interestingly, After 4

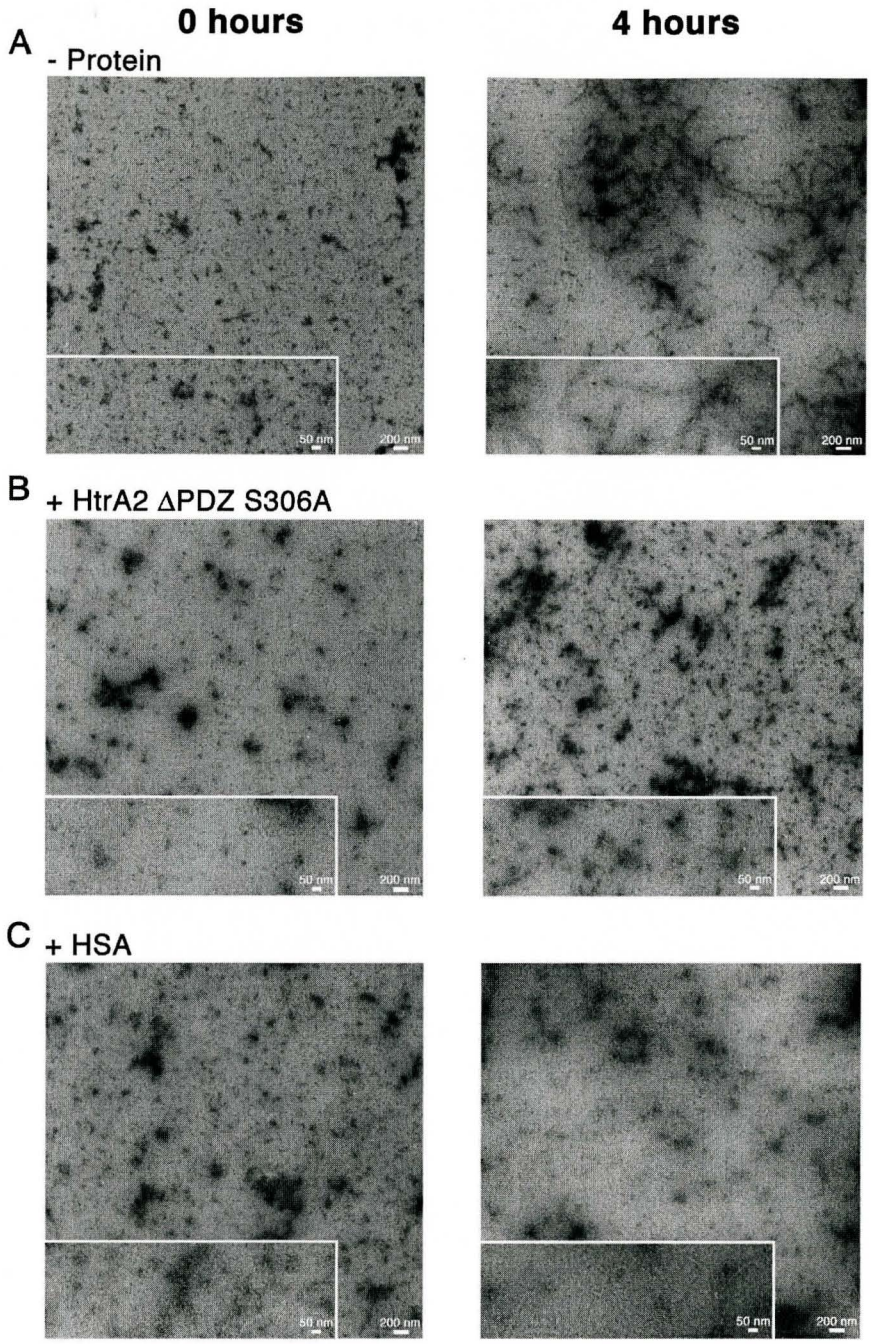

Figure 12. **Visualization of Aβ (1-42) aggregation prevention by HtrA2 ΔPDZ S306A using EM.** Reaction mixtures containing Aβ (1-42) alone (90 μM) or with HSA (20 μM) or HtrA2 ΔPDZ S306A (20 μM) were incubated in 20 mM potassium phosphate buffer with 10% D_2O and 0.02% NaN_3 pH 7.4 at 37°C. At selected times aliquots of A) Aβ (1-42) alone at 0 and 4 hours, B) Aβ (1-42) alone + HtrA2 ΔPDZ S306A at 0 and 4 hours and C) Aβ (1-42) alone + HSA at 0 and 4 hours were added to continuous carbon grids and stained with 3 % uranyl acetate. Samples were imaged at both 10 K (larger area on micrograph) and 25 K (boxed in region on micrograph) magnification under low dose conditions using the JEOL 2010F TEM.

hours (Figure 12B) there was a slight increases in the amount of fibers present, however these levels were significantly lower compared to the Aβ (1-42) alone sample. In the sample containing HSA (12C) only very few fibers were seen at 0 hours and following 4 hours of incubation at 37°C only a slight increase in the amount of fibers was observed, as expected based on the previous two experiments.

The 1D-WG NMR results were again consistent with what was seen through the EM analysis of the different samples (Figure 13). By 4 hours there was an approximately 40 % signal reduction in the Aβ (1-42) alone sample, and this was consistent with the previous two experiments. Furthermore, this significant reduction correlates well with

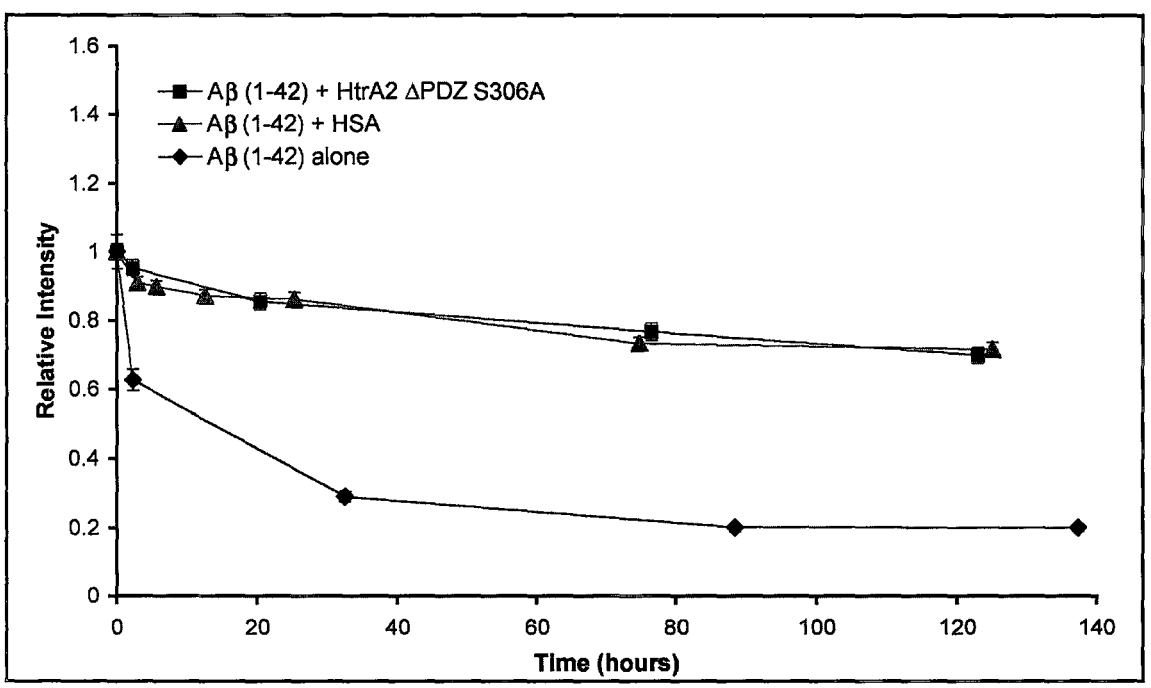

Figure 13. **Prevention of Aβ (1-42) peptide aggregation by HtrA2 ΔPDZ S306A measured using 1D-WG NMR analysis.** Reaction mixtures containing Aβ (1-42) alone (90 μM) or with HSA (10 μM) or HtrA2 ΔPDZ S306A (20 μM) were incubated in 20 mM potassium phosphate buffer with 10% D_2O and 0.02% NaN_3 pH 7.4 at 37°C. At selected times each of the samples were subjected to 1D-WG NMR analysis. All NMR spectra were acquired at 600 MHz; the spectral region of 0.64-1.07 ppm was integrated and was used as a measure of signal loss due to the aggregation with time.

the appearance of long Aβ (1-42) fibrils visualized on the electron micrographs. When the Aβ (1-42) peptide was in the presence of the positive control HSA or HtrA2 ΔPDZ S306A, no significant reduction in the signal was seen after 4 hours. Even after 5 days the signal never went below 20 % for both samples, suggesting that HtrA2 ΔPDZ S306A has a similar protective effect toward Aβ (1-42) than that of HSA. Furthermore, the protective effect seen by HtrA2 ΔPDZ S306A was quite similar to that seen for HtrA2 S306A, suggesting that the protease domain was sufficient for the chaperone-like function of HtrA2 towards Aβ (1-42). This result is consistent with the idea that the PDZ domain has an inhibitory role and is released from its interaction with the protease domain allowing binding to exposed hydrophobic residues. Further supporting the idea of a similar proteolytic and chaperone-like activation for HtrA2.

DISCUSSION

Human HtrA2 is an ATP-independent serine protease that shows significant sequence and structural homology to the *E. coli* periplasmic protein quality control factors DegP and DegS[17]. HtrA2 has been shown to play a role in apoptosis following release from the mitochondria through degradation of the XIAP in the cytoplasm[20; 46]. Recent reports suggest a broader role for HtrA2 in maintaining proper homeostasis within the mitochondrial through its proteolytic activity, and suggest a more protective role [26; 27]. In addition several studies have shown an association of HtrA2 with the APP and toxic Aβ peptide, suggesting a potential role in the pathology of Alzheimer's disease [32; 34; 35].

Due to mounting evidence suggesting a significant role for HtrA2 within the protein quality control system, as well as recent work proposing a chaperone-like function[33], we decided to further explore this possibility. Initially, we looked at the ability of HtrA2 to prevent the aggregation of the model substrate CS *in vitro*. HtrA2 showed a mild ability to delay and reduce the aggregation of CS, and this activity was significantly enhanced when the PDZ domain was removed. Additionally, we monitored the ability of HtrA2 to delay the aggregation of the toxic Alzheimer's disease associated Aβ (1-42) peptide by EM and 1D-WG NMR. HtrA2 was seen to delay the aggregation of the Aβ (1-42) peptide *in vitro* via a dual proteolytic and chaperone-like function. The PDZ domain was dispensable for the chaperone-like activity against the Aβ (1-42) peptide similar to what was seen for CS. This consistency between the two *in vitro* assays gives support to the idea that the PDZ domain may have an inhibitory role on the chaperone-like function of HtrA2. Ultimately these results suggest a potential role for HtrA2 in the regular metabolism of intra-mitochondrial Aβ. Furthermore, HtrA2 may

protect the inter-membrane space of the mitochondria in a manner analogous to DegP in the bacterial periplasm, via a dual proteolytic and chaperone-like function against protein substrates effected by differing stress conditions.

There is increasing support for the idea that the intracellular soluble oligomers and proto-fibrils rather than the traditional extracellular plaques are the neurotoxic forms of Aβ[38]. One Aβ-mediated mechanism for the development of AD that has gained increasing support is mitochondrial dysfunction. Several studies have shown that soluble oligomers of Aβ localized in the mitochondria inhibit the activity of cytochrome c oxidase, an important component of the electron transport chain[36; 40]. Furthermore, Aβ has been shown to bind and inhibit the activity of alcohol dehydrogenase in the matrix of the mitochondria, leading to the increased generation of free radicals and oxidative stress[39]. Ultimately, this accumulation of Aβ within the mitochondria can lead to its dysfunction and eventually neurodegeneration.

Many studies have suggested that quality control proteins are important for the prevention of intracellular Aβ aggregation[47]. Heat shock protein (Hsp)70 and 90 have been shown to prevent the aggregation of Aβ (1-42) *in vitro*[48] and Hsp70 has been shown to have a neuroprotective effect in response to intracellular Aβ[49]. Additionally, the presequence peptidase (PreP) localized within the mitochondrial matrix has been shown to significantly degrade Aβ (1-42) *in vitro*[50]. Based on our results it seems that HtrA2 may play a role in the prevention of Aβ (1-42) aggregation in the inter-membrane space through a dual chaperone-like and proteolytic function. In combination with other protein quality control factors HtrA2 may be involved in the clearance of regular or aberrant levels of Aβ within the mitochondria.

Recently, the ubiquitin-proteasome system has been implicated in proper quality control of proteins localized to the inter-membrane space of the mitochondria. Upon inactivation of the proteasome, aggregation and clustering of cytochrome c and endonuclease G (endoG) in the inter-membrane space was observed using immunofluorescence and EM. Furthermore, under these conditions HtrA2 was shown by immunofluorescence to cluster in a similar manner as cytochrome c and endoG and was seen to degrade endoG[51]. This gives further support for a protective role for HtrA2 in the mitochondria. Additionally, Observing whether HtrA2 has a chaperone-like function towards substrates like endoG, may give further insight as to whether this function is important for HtrA2 in maintaining proper mitochondrial integrity.

Based on the ability of HtrA2 to prevent the aggregation of CS and the Aβ (1-42) peptide, and the fact that the protease domain is sufficient for this activity, a model for the chaperone-like function of HtrA2 is proposed (Figure 14). In this model HtrA2 recognizes potentially detrimental misfolded substrates, leading to the release of the inhibitory PDZ domain away from the protease domain, similar to what had been previously proposed[18]. This allows substrate access to the catalytic triad as well as exposed hydrophobic residues on the surface of the protease domain. Presumably, HtrA2 can then use a dual chaperone-like and proteolytic activity to sequester and ultimately degrade misfolded protein in the inter-membrane space of the mitochondria. This would relieve the potential toxic effects of protein aggregation that could ultimately lead to mitochondrial dysfunction and cell death.

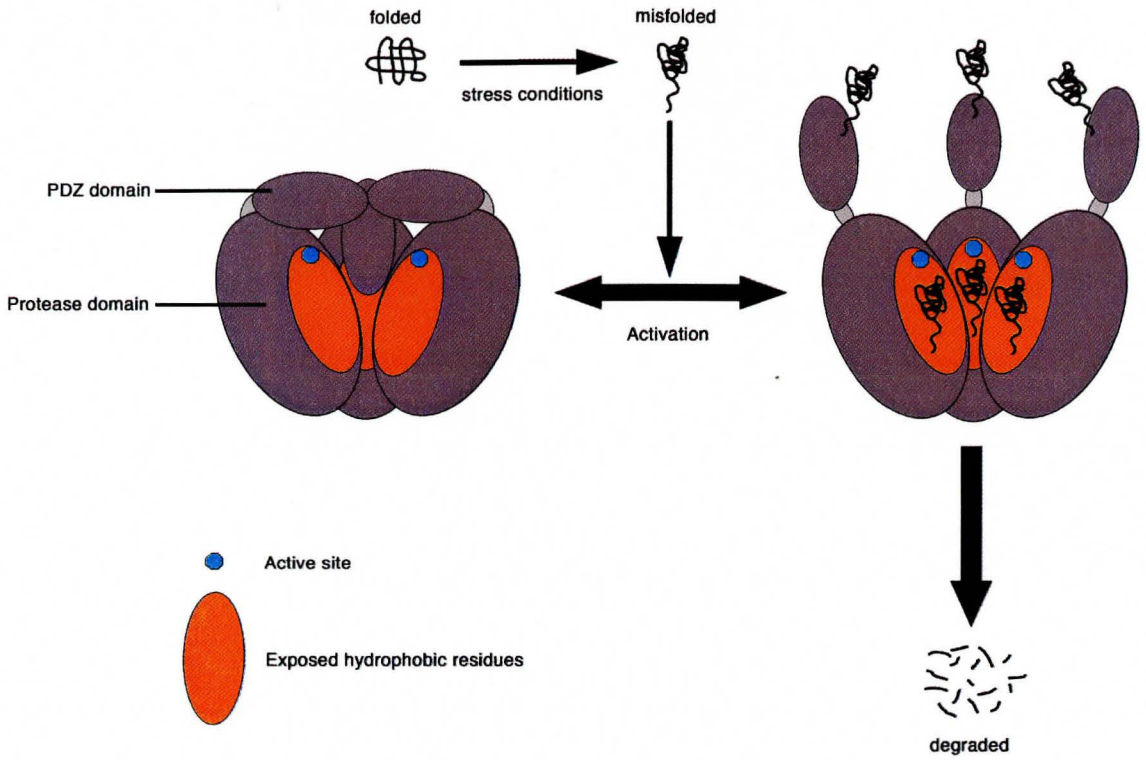

Figure 14. **Proposed mechanism for the chaperone-like function of HtrA2.** Initially HtrA2 is in an inactivate conformation where the PDZ domains collapse on the protease domain. This blocks access of substrate to the catalytic triad and the surface expose hydrophobic residues on the protease domain. Upon activation by binding misfolded substrate, the PDZ domains may swing away from the protease domain, allowing binding to the hydrophobic patches. Both sequestration and degradation of misfolded substrate can then be performed by HtrA2.

In order to address the weak chaperone-like effect of full-length HtrA2 towards CS, we attempted to activate HtrA2 using C-terminal peptides from presenilin-1 (PS1). Previously, these peptides were shown to activate the proteolytic activity of HtrA2 toward the model substrate β-casein in a dose dependent manner[52]. Using a 5x molar excess of peptide to HtrA2 had no effect on the ability of full-length HtrA2 to prevent the aggregation of CS (data not shown). Further experiments with larger amounts of stimulating peptide will need to be performed in order to fully understand whether there may be a similar activation mechanism for the proteolytic and chaperone-like function of HtrA2. It is plausible however that HtrA2 is more specific and because the Aβ (1-42)

peptide represents a more physiologically relevant substrate than CS, it may more efficiently activate the chaperone-like activity of HtrA2. Furthermore, significant size differences between CS (46 kDa) and the Aβ (1-42) peptide (4.5 kDa) may also be relevant, as many mitochondrial intermembrane proteins are no larger than 25 kDa[53]. Further studies looking at the ability of HtrA2 to prevent the aggregation of more physiologically relevant substrates may give further insights into its chaperone-like mechanism.

Finally, HtrA2 was seen to have significant proteolytic activity towards the Aβ (1-42) peptide under our experimental conditions. This result is in contradiction to what had been previously shown by Liu et al, who showed that WT HtrA2 only interacted with Aβ (1-42) but did not directly lead to its degradation[34]. In this paper however, a 40x molar excess of Aβ (1-42) peptide to WT HtrA2 was used and incubated for a total of only 2 hours at 37^0C. In contrast we used an approximately 4.5 molar excess of Aβ (1-42) peptide to WT HtrA2 and incubated for as long as 3 days at 37^0C. Although the conditions used suggest the Aβ (1-42) peptide is a somewhat weak proteolytic substrate for WT HtrA2, this may still represent an important activity for WT HtrA2 within the mitochondria. Furthermore, human HtrA1 has also been shown to degrade Aβ (1-42) peptide *in vitro* and also the C99 precursor of Aβ. Additionally, HtrA1 secreted from astrocytes was seen to colocalize with β-amyloid deposits in human brain samples[54]. These studies suggest members of the human HtrA family may play important roles in the metabolism of Aβin both the extracellular and intracellular space through their proteolytic and chaperone-like activities.

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