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Regulation of the Protease Activity for the Mitochondrial Omi/ HtrA2

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REGULATION OF THE PROTEASE ACTIVITY FOR THE MITOCHONDRIAL OMI/HTRA2

by

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ABSTRACT

Human High Temperature requirement A2 (HtrA2) also known as Omi, is a serine protease located in the mitochondria with an important function in both cell survival and death. My results show the proteolytic activity of Omi/HtrA2 varies under different conditions. I characterized the optimal condition for Omi/HtrA2 protease activity using an *in vitro* assay system. Additionally, I identified a new allosteric regulation of Omi/HtrA2 through interaction with a specific substrate, the MUL1 protein. MUL1 is a multifunctional E3 ubiquitin ligase anchored in the outer mitochondrial membrane with domains both inside mitochondria and in the cytoplasm. The data shown here strongly supports the hypothesis that Omi/HtrA2 activity is modulated by a number of different mechanisms. Some of these conditions, such as pH or substrate denaturation might reflect the state of mitochondria under stress. It has been known that Omi/HtrA2 is a stress activated protease, but the mechanism of its regulation has not been fully elucidated. Furthermore, the allosteric regulation of Omi/HtrA2 by specific substrates, can be another mechanism that provides a feedback loop to increase the activity of the enzyme. The findings from this project contribute new information on the mechanisms of activation of Omi/HtrA2 protease. They support the hypothesis that mitochondrial stress might be involved in the regulation of Omi/HtrA2 protease.

ACKNOWLEDGMENTS

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INTRODUCTION AND LITTERATURE REVIEW

Omi/HtrA2 Isolation and Characterization:

DegP/HtrA was first isolated in *E. coli*, and was considered to be essential to the survival of microbes at high temperatures [2]. Later, it was discovered to be a serine protease in the periplasm of the microbes with a function in the degradation of misfolded proteins [3]. Homologs of the enzyme in *E. coli* include DegS and DegQ [4]. HtrA homologs were then promptly found in many other species and in eukaryotes. Omi was originally isolated in our lab and later by several other groups that named it HtrA2 based on its homology to bacterial HtrA protease [5] [6, 7]. The protease is now referred to as Omi/HtrA2. Humans possess three homologs: HtrA1, Omi/HtrA2, and HtrA3 (Figure 1A). Omi/HtrA2 is normally contained within the mitochondria, HtrA1 and 3 harbor secretion signals that indicate their likely function in the secretory pathway [8].

Omi/HtrA2's precursor form, described in Figure 1B, contains a mitochondrial targeting signal (MTS), transmembrane region, IAP binding motif, serine protease domain, and a C-terminal postsynaptic density of 95 kDa, disk large, and zonula occludens 1 (PZD) domain that functions to regulate chaperone and protease activity [9]. In its precursor form, Omi/HtrA2 is embedded in the inner mitochondrial membrane. Through a process of autocleavage it is released into the mitochondrial intermembrane space (IMS). The serine protease domain of Omi/HtrA2 plays an important role in the formation of a pyramid shaped homotrimer (Figure 1C). In the trimeric form, the PDZ domains usher in denatured or damaged proteins into the catalytic domain [10]. In addition, the Omi/HtrA2 trimer can more easily be regulated by activator peptides that change the oligomer between a closed or an open confirmation. This allows for tight regulation of the cleavage of proteins, preventing any potential uncontrolled degradation of essential proteins [11].

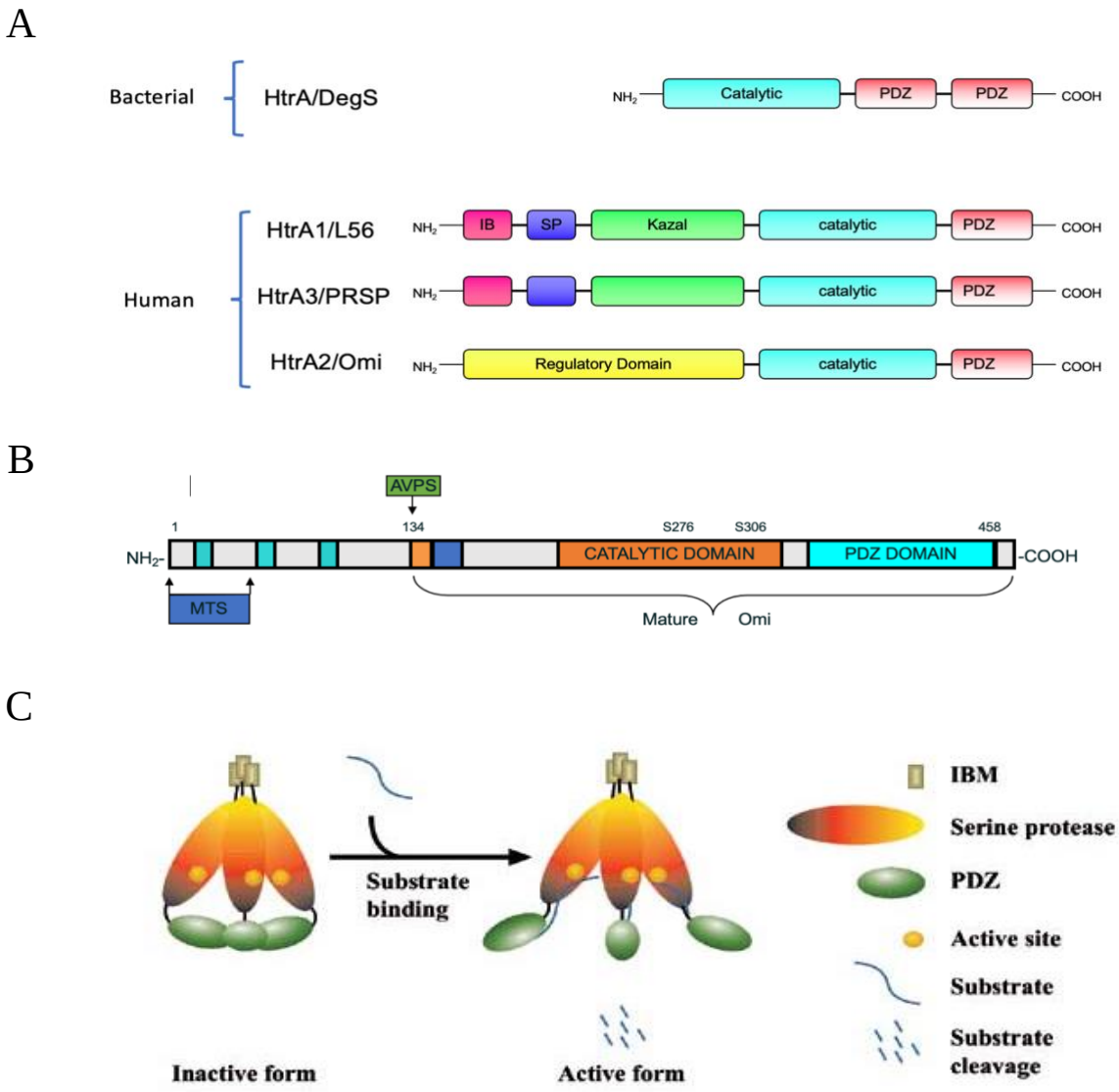


Figure 1: Homology and Structure of HtrA family of proteins

HtrA was first isolated in *E. coli* as DegS. Humans contain 3 homologs of HtrA: HtrA 1, 2, and 3. HtraA1/3 contain secretion domains indicating their involvement in the secretion pathway (Figure A). Omi/HtrA2 is 458 amino acid serine protease, with its catalytic serines located at amino acids 276 and 306. Omi/HtrA2 has a mitochondrial targeting sequence, a transmembrane domain (aa 105 – 125; not labeled), a PDZ domain, an and an IAP targeting AVPS domain (Figure B). Mature Omi/HtrA2 forms a homotrimeric pyramid, which is regulated by the PDZ domains, and can be activated through substrate binding (Figure C).

The Function of Omi/HtrA2 protease

The main function of Omi /HtrA2 is to act as a chaperone, refolding or degrading denatured proteins in the mitochondrial intermembrane space (IMS) [12]. Through this process, Omi/HtrA2 is able to maintain homeostasis within the mitochondria. In addition, Omi/HtrA2 upon induction of cell death, is released from the mitochondria to the cytoplasm where it participates in apoptosis through caspase-dependent and independent mechanisms (Figure 2) [13-15]. Previous studies have suggested that the proteolytic activity of Omi/HtrA2 could be regulated by mitochondrial stress such as ROS, but the mechanism of this regulation remains unclear [16]. In addition, other studies indicate that specific protein-protein interactions could affect the enzymatic activity of Omi/HtrA2 and target the enzyme to specific substrates [17-19].

My study aims to investigate how various conditions such as temperature, pH, or the denaturation state of the substrate could potentially affect the protease activity of this enzyme in vitro. Furthermore, I will test the hypothesis that MUL1, a recently discovered substrate of Omi/HtrA2, can act as an allosteric activator of the enzyme's protease activity.

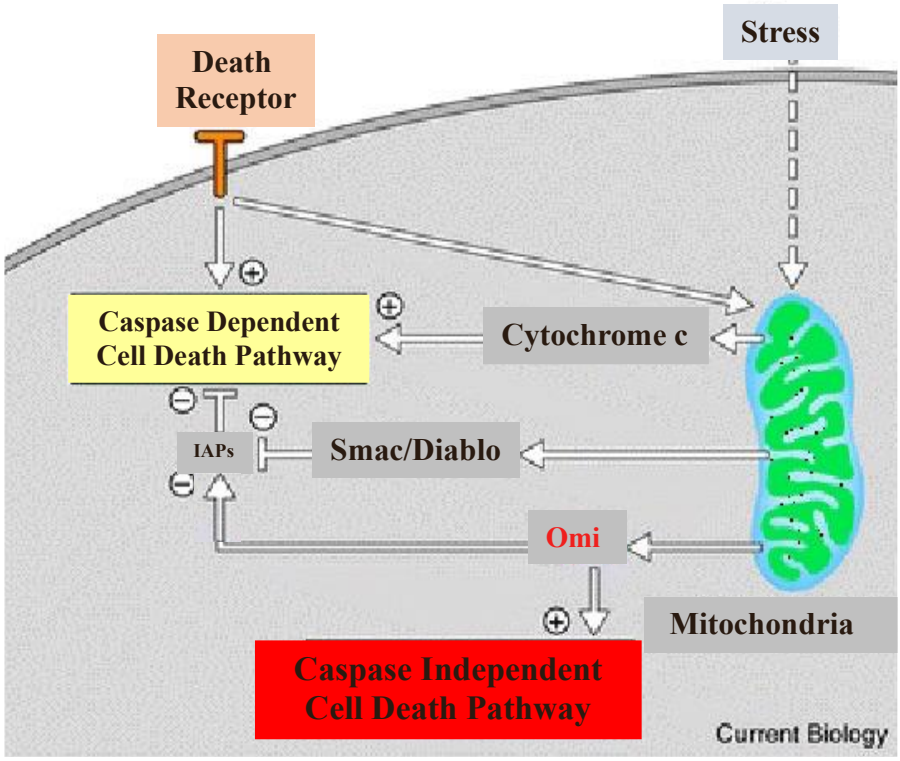


Figure 2: Role of Omi/HtrA2 in Apoptosis

Signaling by pro-apoptotic proteins causes a termination of HtrA2/Omi's normal function of protein regulation inside the mitochondria and releases it into the cytosol together with cytochrome c and Smac/Diablo. There it participates in caspase independent pathway through its serine protease activity, and in caspase dependent pathway through the degradation of the caspase inhibitor IAP proteins.

Implications for Disease

Due to the proapoptotic activity of Omi/HtrA2, it was thought inactivation of the protease in mice would lead to increased proliferation and possibly tumors. However, Omi/HtrA2 KO animals develop normally up until 20 days and then show signs of neurodegeneration with a parkinsonian phenotype, a disorder labeled as motor neuron degenerative 2 (mnd2) in mice [20]. The mnd2 mice have rapidly developed paralysis, muscle loss, shrinking of the body, very small thymus and spleen, as well as the namesake neurodegeneration (Figure 3) [21]. This usually progresses quite rapidly and severely, leading to premature death within 40 days.

In humans, Parkinson's Disease (PD), is classified by loss of dopaminergic neurons in the central nervous system leading to tremors, stiffness, and loss of balance [22]. This disease has been linked to the absence of Omi/HtrA2, as misfolded ∝-synuclein, a normal neuron factor, aggregates and presents strong neurotoxicity causative of the dopaminergic neuronal degradation [23]. Loss of Omi/HtrA2 has been shown to cause oligomeric ∝-syn induced toxicity [24]. This shows the importance functioning Omi/HtrA2, with ideal levels of activity required to keep the body functioning.

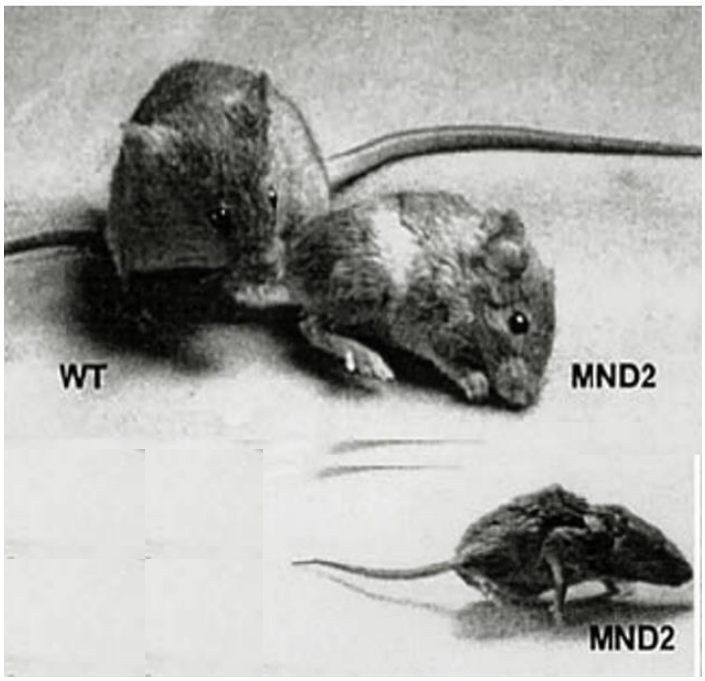


Figure 3: Phenotype of mnd2 mice that carry a mutated Omi/HtrA2.

Mnd2 mice exhibit neurodegeneration, muscle loss, paralysis, involuntary movement, and after a period of 40 days, a premature death. Pictured left is a normal healthy functioning WT mouse. On the right and bottom are mice with the mnd2 phenotype exhibiting shrunken, disheveled bodies and decrease in normal movement.

Known Substrates of Omi/HtrA2 and Mechanism of Action

The mechanism of Omi/HtrA2 degradation is tightly regulated and influenced by a number of factors. The protease forms a trimer that is regulated by the PDZ domains that block access to the catalytic site of the serine protease domain. When activated by stimuli, the trimer progresses into an open conformation allowing substrate access (Figure 4). Numerous important factors have been considered in supporting the activation state.

One such factor is the phosphorylation of the Omi/HtrA2 serine protease. CDK5 dependent phosphorylation of Omi/HtrA2 at residue S400 has been shown to maintain membrane potential in the mitochondria, maintaining function during cellular stress [25]. Additionally, PINK-dependent phosphorylation of Omi/HtrA2 has a similar effect and PINK is often mutated in Parkinson's patients [26].

Another important factor looked at is the ideal pH at which Omi/HtrA2 works at. Proteases, like most enzymes, have different activities based on the pH of the surrounding environment. Defining the optimum pH and locating where and when that pH occurs can help define the location and timing that a given enzyme may become more active. The mitochondria boast a wide range of pHs, in part due to the gradient of protons gained through respiration in the IMS [27]. The IMS has a resting pH around 7.4. However, this value has been proven to change based on the conditions of the cell and the state of OXPHOS [28]. The mitochondrial matrix (MM) on the other hand holds a much higher pH range, around 7.9 - 8.0. Changes of the pH of the intermembrane space or the mitochondrial matrix could coincide with defects of the mitochondria or with other cellular process and are thus an important factor to be considered. Omi/HtrA2 functioning at different pH levels could be a method of regulation both within the mitochondria and in the cytoplasm. Unlocking the

ideal preforming pH could play a large role in pinpointing the timing and method of Omi/HtrA2 activity in the cell.

Temperature plays another role in Omi/HtrA2 activity. HtrA, originally isolated as DegP in bacteria, was shown to become more activate when raised from temperatures of 30°C to those of 42°C or above [29]. Additionally, it has been shown that HtrA can alter its activity based on the temperature. At lower temperatures, 37°C, DegP has general molecular chaperone activity and is able to stimulate refolding of chemically denatured substrates. Its proteolytic activity is almost exclusively present at elevated temperatures, 42°C [30]. Although my experiment deals with mammalian Omi/HtrA, the homology between the proteins could mean a similar mechanism could play a role in other members of the HtrA family. Either way, temperature is an important factor to consider is enzyme activity.

Another relevant factor discussed is the denaturation state of substrate prior to interaction with Omi/HtrA2. Although the most common method for denaturing proteins in a laboratory is increased temperature, this is highly unlikely to occur *in vivo*, as the body temperature is maintained. There are many other methods of denaturing of proteins *in vivo*, such as chaperones, changes in pH, or posttranslational modification which can all act independently or in unison to denature a protein. Seeing how this process effects Omi/HtrA2 could help link other proteins to its processes.

Allosteric activation provides another possible regulating mechanism for Omi/HtrA2. As a protease, the function of Omi/HtrA2 is defined by the specific substrates it regulates. There are a number of such substrates present in both the mitochondria and in the cytoplasm that play a role in Omi/HtrA2 activation. HAX-1 is a multifunctional protein that plays a large part as an anti-

apoptotic protein and was shown to interact and be cleaved by Omi/HtrA2 [31]**.** In addition, HAX-1 has been shown to be an allosteric inhibitor of Omi/HtrA2 [18].

Another closely related example, is the GRIM-19 protein (gene associated with retinoic and interferon induced mortality 19). GRIM-19 is a 16 kDa protein and a known substrate of Omi/HtrA2. GRIM-19 was also shown through enzyme kinetics to interact with Omi/HtrA2, and acts as an allosteric activator [32]. Although this case presents a similar set of circumstances to our own, there are still some major differences. The key difference being that the protein interacts with Omi/HtrA2 outside the IMS when Omi/HtrA2 is in the cytoplasm during induction of cell death. Finding an allosteric activator that works inside the IMS to regulate Omi/HtrA2's normal pro-survival role within the mitochondria would be novel and highly significant.

Of particular interest to our laboratory is the protein MUL1 also named MULAN (mitochondrial ubiquitin ligase activator of NFKB 1), GIDE (growth inhibition and death E3 ligase,) MAPL (mitochondria-anchored protein ligase), and RNF218 (RING Finger Protein 218). MUL1 is a E3 mitochondrial ubiquitin protein ligase found imbedded in the outer mitochondrial membrane (OMM)[33]. MUl1 is one of three mitochondrial E3 ubiquitin ligases, with RNF185 and MARCH5 making up the other two [34, 35] (Figure 5). MUL1 has been identified as a specific substrate of Omi/HtrA2. It has a domain located in the IMS that likely interacts with the protease [36]. This newly discovered interaction serves as the basis of our study as the knowledge that Omi/HtrA2 degrades MUL1 in the IMS provides a specific substrate and a possible allosteric activator.

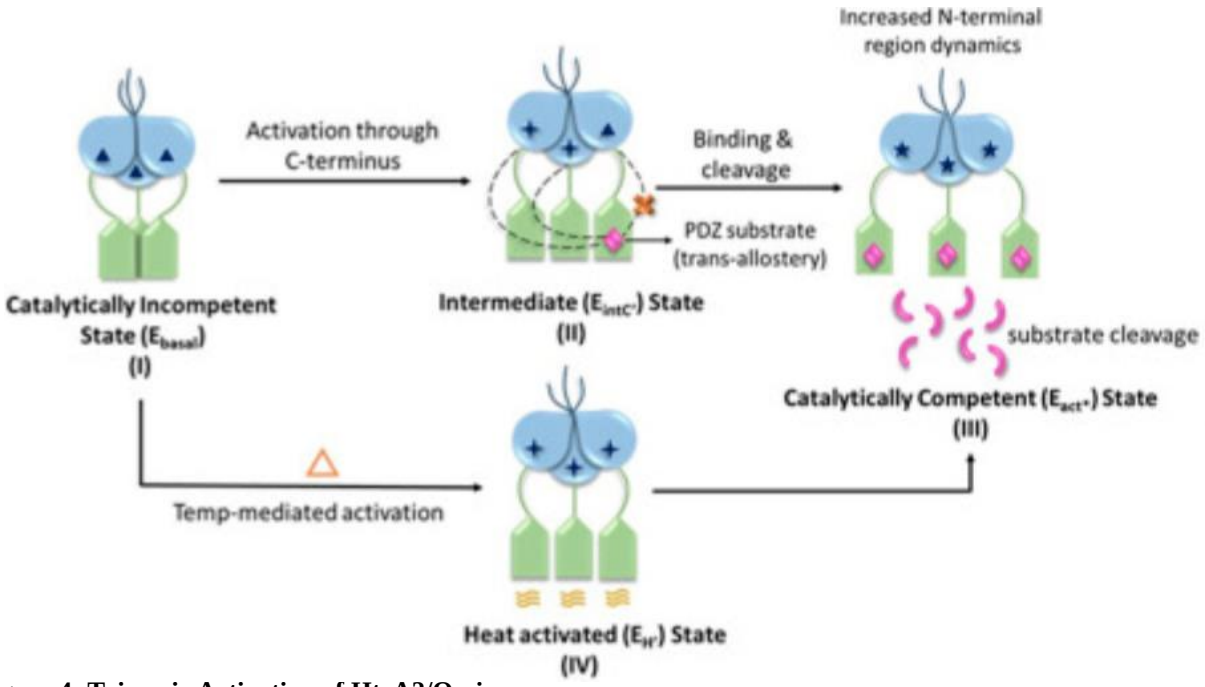


Figure 4: Trimeric Activation of HtrA2/Omi

Omi/HtrA2 activation has been linked to many factors. Circumstances like heat change, pH, and activation by substrate can move the protease from a less active basal state, to an increased activation state. This regulation is tightly managed by the PZD domains that block substrate binding to the serine proteases in the active state. (Image adapted from works of Dr. Ayon Chakraborty)[1]

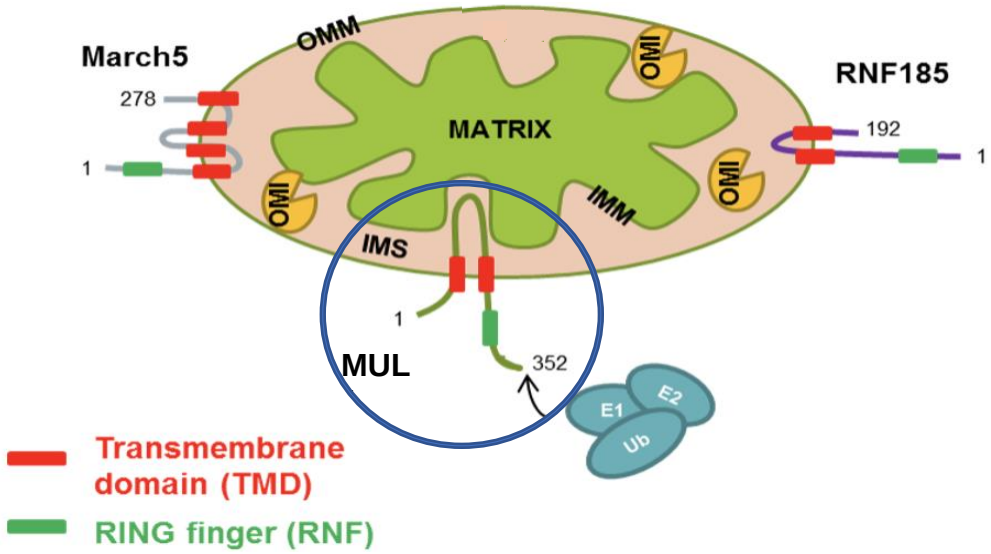


Figure 5: MUL1 and Omi/HtrA2 in the Mitochondria

Omi/HtrA2 is located with the IMS of the mitochondria. MUL1 is one of three E3 Ubiquinone ligase located in the OMM, MARCH5 and RNF185 are the others. All three proteins have a RING finger domain facing the cytoplasm. MUL1, circled in blue, contains a domain located within the IMS that is the likely interactor of Omi/HtrA2

MATERIALS AND METHODS

pGEX-Mul130-352 for Protein Expression Polymerase Chain Reaction

MUL1 (amino acids 30-352) was cloned into pGEX-6P-1 using polymerase chain reaction (PCR). The 5' forward primer, with restriction sites for the enzyme EcoRI: 5'- GCG GAA TTC CGG CAG AAG GCC CGG GTC TC-3' and the 3' reverse primer, with restriction sites for the enzyme SalI: 5'-CTCC GTC GAC TTA GCT GTT GTA CAG GGG TAT CA-3', were used to encode the MUL1 sequences from 30-352. PCR reaction used 20 ng template DNA, 10 ρM of primer 1 and 2 [Eurofins], 5 µl of (10X) PCR buffer + Mg^{2+} [NEW ENGLAND BioLabs], 100 µM dNTPs [Clontech], and 1.5 units of Taq polymerase [ThermoScientific] in dH_2O for a total 20 µl volume. PCR cycles: denatured at 98°C for 30 seconds, annealed at 58°C for 30 seconds, and elongated 72°C for 90 seconds, for 25 total cycles.

Preparation for Loading Gel Electrophoresis

DNA samples were prepared by adding 5 µl DNA with 5 µl of 10X gel loading buffer (30% glycerol and 2% Orange-G in TAE). Prepared DNA samples were loaded along with GeneRuler1kb Plus DNA ladder [ThermoScientific] to estimate the size of the DNA. The gel was visualized with BIO-RAD ChemiDoc MP Imaging System

Precipitation of DNA

PCR-DNA was mixed with precipitation buffer (10% 3M sodium acetate pH 5.5, 2 sample volumes 100% EtOH) Samples were then stored at -20°C for 20 minutes. Samples were centrifuged at 13,000 rpm for 20 minutes at 4°C. Supernatant was removed and 150 µl 70% EtOH was added to the pellet. Samples were again spun 13,000 rpm for 5 minutes this time at room temperature. Supernatant was removed and spun in vacuum till dry. Sample DNA was then resuspended TAE.

Restriction Enzyme Digestion

50 µl DNA of precipitated DNA was combined with 10 µl 10X Restriction Enzyme Buffer [NEW ENGLAND BioLabs], 1 µl BSA (bovine serum albumen) [NEW ENGLAND BioLabs],1.5 µl of given restriction enzyme, EcoRI or XhoI [NEW ENGLAND BioLabs], and 37 µl H_2O. Mixtures were the set to incubate for 1-2 hours when using EcoRI or 4 hours when using XhoI. After one hour, an additional 0.5 µl restriction enzyme was added. DNA was precipitated from solution. Next the process was repeated with a second restriction enzyme.

DNA Ligation

2 µl of DNA was analyzed using gel electrophoresis to determine DNA concentration in 1ul of vector and 1 ul of insert. Then the vector and insert (combined at a 1:2 ration) were combined with water, ligation buffer, ATP, and ligase and incubated at 21°C for 20 minutes, followed by inactivation by raising the temperature to 70°C for 10 minutes. The samples were then stored at 4°C.

Bacterial Transformation

3-4 µl plasmid DNA were added to 50 µl of electro-competent *E. coli* cells. After gentle mixing, samples sat on ice for 5 minutes. Bacteria/DNA mixture was added to 0.2 cm electroporation cuvettes. A pipette was readied with 1 ml SOC. Cuvettes were placed into BIO-RAD Gene Pulser with metal facing cathodes, electroporated with a pulse of 2.5 kV at 400 Ω, then immediately the SOC was added. The mixture was transferred to a 2ml microtubes and placed in the incubator at 37°C on rotator at 200 rpm for 1 hour. Agar plates(+Amp) were dried and warmed in incubator for 20 minutes. 50 μl transformed DNA/Bacteria was plated using glass beads. Plates were labeled and placed in a 37°C incubator overnight.

Isolation of Plasmid DNA from Bacteria

A disposable inoculation loops was used to pick a single colony of bacteria from plate and inoculate a 1.5 ml microtube of LB +AMP at 37°C overnight. The bacteria were then centrifuged at 13,000 rpm for 3 minutes. The supernatant was removed and 250 µl Buffer P1[QIAGEN] was added. Next, 250 µl Buffer P2 [QIAGEN] was added and gently mixed. Then 350 µl Buffer N3[QIAGEN] was added and again gently mixed. The samples were centrifuged at 13,000 rpm for 15 minutes. The supernatant was transferred to a QIAprep spin column and centrifuged for 1 minutes. The flow-through was then removed. This process repeated again with 500 µl Buffer PB[QIAGEN], 750 µl Buffer PE[QIAGEN], and again to dry the column before eluting with 50 µl Buffer EB[QIAGEN]. To confirm that pGEX-MUL1was cloned in frame, the plasmid DNA was sent for DNA sequencing.

Making LB and Ampicillin Plates

LB broth (40 minutes for 500 ml containers) (LB Broth: 2L H_2O, 20g bactotryptone, 10g yeast extract, 20g NaCl, adjust pH to 7.2 with sodium hydroxide pellets) (For plate preparation add agar 15g/L) was sterilized by liquid cycle on autoclave. LB was removed from autoclave and place on low stir until warm to touch. Stirring continued while ampicillin was added at a concentration of 100 μg/ml. Media was poured in plates until the bottom was just covered and sat until set. Plates were stored at 4°C, for up to 1 month.

Isolation of Recombinant Protein

10 ng GST-MUL1 plasmid DNA were added to BL21(DE3) *E. coli* electro-competent cells (bacterial expression strain) and transformation was performed using methods previously described. Inoculation loops were used to pick a single colony of bacteria from plate and inoculate 5 ml tube which was then grown overnight. 500 μL of bacteria was added to 500 ml flask of LB + AMP. Covered flask was placed in shaking incubator at 37°C at 250 rpm for 3 hours until OD = 0.8. 1 mM IPTG was added to the bacteria, and which continued to shake at 25°C at 250 rpm for an additional 3 hours, followed by immediate cooling on ice. Bacteria was then transferred from flasks into 250 ml centrifuge bottle being sure to balance the weights. Then bottles were spun in large centrifuge using JLA rotor at 4°C at 7,000 rpm for 10 minutes. Supernatant was removed and pellet stored at -80°C.

Cell Lysis, Purification, and Protein Elution

Pellet was defrosted in 37 °C water bath with 10-15 ml recombinant lysis buffer (25 mM Tris-HCl pH 8, 100 mM NaCl, 0.1% Triton-X, Protease Inhibitors Tablet [ThermoFisher]). An inoculation loop was used to break up pellet and mix. 5 ml pipette was then used tp break up pellet further until resuspended. The solution was then transfer to a 50 ml centrifuge tube, bringing the total volume up to 20ml with lysis buffer as needed. 0.5 ml of lysozyme was added at 1 mg/ml and placed in 30°C water bath for 30 minutes. Lysing bacteria was placed on shaker for 15 minutes until color change and viscosity is clumped. Next solution was sonicated for 1 minutes with 5 second pauses every 20 sec. Process was repeated 3 times per sample followed by centrifugation at 12,000 rpm for 20 minutes at 4°C with JA rotor. GST (Glutathione Sepharose)-resin beads were prepared with wash buffer (at 1/10 volume of protein/lysate) (GST wash buffer: 25 mM Tris-HCl pH 8 = 500 ml). GST charged resin beads were washed with wash buffer (centrifuged and supernatant removed). Protein supernatant of sonicated bacteria was added to column through filter. The column was placed on a rotator at 4°C overnight. The beads + protein mixture was spun for 3 minutes at 2000 rpm, with the supernatant removed and save. GST-wash buffer was added to beads, then beads were slowly added to column ensuring that they lay evenly. GST wash buffer was poured through column (up to 10 times column volume). Protein was eluted with elution buffer (50 mM Tris-HCl pH 8, 20 mM reduced glutathione). Fractions were collected in up to 8 elution tubes, 1.5 ml in 2 ml microtube. 20μL of each elution was loaded and run on SDS-PAGE followed by Coomassie Blue staining to visualize the amount and purity of recombinant protein obtained in each elution.

Processing of Recombinant Protein

When necessary, combined protein elutions of similar purity were placed into dialysis membrane (6000-8000 KDa) about 2-3 inches in length. Air bubbles were removed, and the membrane clamped shut on both ends. The membrane and contents were then placed with PEG (polyethylene glycol compound) to concentrate for 2-4 hours, flipping and moving intermittently. PEG was washed and the membrane was opened to remove any air bubbles from membrane. The membrane was then resealed, and dialysis was performed by submerging the membrane in 2 L wash buffer (200 mM NaCl, 20 mM Na2HPO4) stirring at 200 rpm at 4°C.

pH Variation Experiments

Omi/HtrA2 activity was tested *in vitro* using bacterially expressed mature HIS-Omi, varying pH of sodium phosphate buffer (20mM Na_2PO_4, 200 mM NaCl) between pH 7.5 or 8.0. Activity was monitored first by the degradation of both a generic substrate, β-casein, and then the Omi/HtrA2 specific substrate, MUL1. Samples were incubated at 37 °C for 25 minutes or 90 minutes, respectively. The reaction was halted by the addition of sample buffer (5% SDS, 20% glycerol, 5% 2-mercaptoethanol, 0.004% bromophenol blue, 0.124M Tris HCl pH 6.8) followed by boiling for 5 minutes. 20μl of each sample was then loaded and run on SDS-PAGE (12% acrylamide). Visualization was accomplished using Coomassie Blue staining followed by de-staining with de-staining solution (5% acetic acid, 10% methanol v/v). Pictures were taken on BIORAD ChemiDoc MP Imaging System.

Substrate Denaturing Experiments

The effect of denaturing the substrate before was tested by pre boiling the specific substrate MUL1 prior to incubation with Omi/HtrA2. Sodium phosphate buffer (20 mM Na_2PO_4, 200 mM NaCl, pH 8.0) was added to the enzyme. Samples were incubated at 37 °C for 90 minutes, loaded and run on SDS-PAGE, and visualized via Coomassie Blue staining procedure mentioned previously. Additional visualization of protein degradation was accomplished through Western Blot and analyzed with chemiluminescent and X-ray film.

Temperature Variation Experiments

Enzyme activity was tested at differing temperature by incubating Omi/HtrA2 with substrate, in sodium phosphate buffer (20 mM Na_2PO_4, 200 mM NaCl, pH 8.0), at 37°C or 42°C for equal time periods. The experiment was performed with either β-casein or MUL1. Samples were then incubated for 25 minutes, or 90 minutes respectively, followed by SDS-PAGE and visualization via Coomassie Blue staining.

Allosteric Activation Experiments

The effect of MUL1, a specific substrate of Omi/HtrA2, as an allosteric activator was tested by preboiling the substrate for 5 minutes. Then incubating Omi/HtrA2 in sodium phosphate buffer (20mM Na_2PO_4, 200 mM NaCl, pH 8.0) and MUL1 at 37°C or 90 minutes. The reactions are then removed from incubation, and the generic substrate β-casein is added, and the samples are returned to incubate for an additional 25 minutes. Samples were then separated on SDS-PAGE and visualized via Coomassie Blue staining.

Western Blot Analysis

Samples were run on SDS-PAGE (12% acrylamide) and transferred to PVDF membrane using transfer buffer (5x blot buffer, 20% v/v methanol) and a semi-dry apparat at 20V for 45 minutes. Membrane was then placed in blocking solution of 5% milk in TBST (Tris Buffer Saline) (25 mM Tris-HCl pH 8.0, 125 mM NaCl, 0.1% Tween 20) for one hour. The membrane was then incubated with the primary antibody (rabbit anti-MUL1 1:3000) overnight with shaking at 4°C. The membrane was then bought back to room temperature and washed 3 times for 15 minutes each with TBST. The secondary antibody (anti-rabbit 1:10,000) was added to the membranes and incubated at room temperature for 60 minutes. After this time the membrane was washed with TBST for 3x15 minutes. The membrane was then soaked for 4 minutes with equal parts of Sol-1 and Sol-2 chemiluminescent solution prepared immediately before use, activated by H_2O_2 (Sol 1:10mM Tris-HCl pH8.5, 2.5 mM Luminol, 400 μM ρ-coumaric acid; Sol 2:100 mM Tris-HCl pH 8.5). The membrane was immediate visualized using X-ray films.

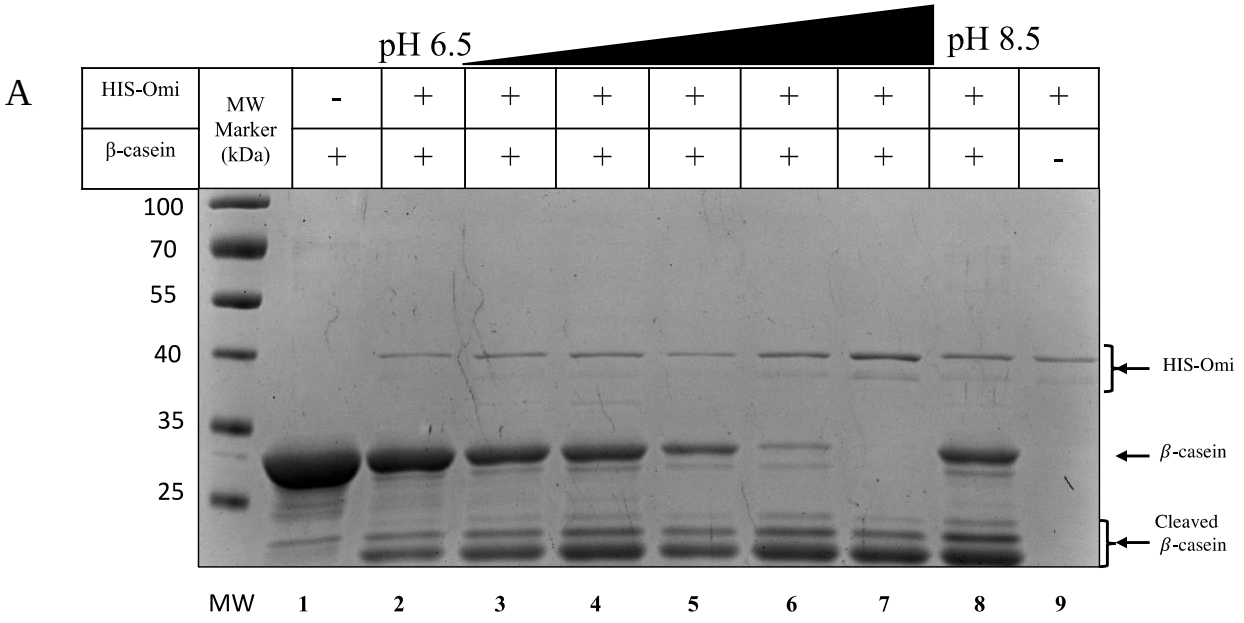
Image Analysis

All gel images were imported from the BIO-RAD ChemiDoc MP Imaging System and uploaded via ImageJ. From here, images were analyzed and fitted. Then Photoshop was used to remove any gel deformity (scratches, bubbles, nicks, blemishes, etc.) and to autotone the image for proper paper presentation.

Results

pH Experiments

Previous experiments have shown that Omi/HtrA2 has varying levels of activation under different stress conditions. I have tested the effects of pH by altering the pH of the phosphate buffer used in the reaction of Omi/HtrA2 with its substrate. Figure 6A shows the effect of increasing the pH on the degradation of the generic substrate β-casein. A general trend shows that as the pH increases, the amount of cleavage, and hence the activation of Omi/HtrA2, also increases. There is a sharp drop-off in activation at the last pH value, indicating a bell curve type of activation. Figure 6B shows a more comparative example of the same experiment, choosing to focus on just the pH found in the IMS and MM. There is clearly more degradation of the generic substrate β-casein at the higher pH value, indicating an increase in the activity of Omi/HtrA2. Lastly, in Figure 6C, the comparison is repeated with the specific substrate MUL1. A similar conclusion to the generic β-casein substrate is found, with more degradation occurring at pH 8.0 than at pH 7.5, again showing the increase of Omi/HtrA2 activation at this pH.



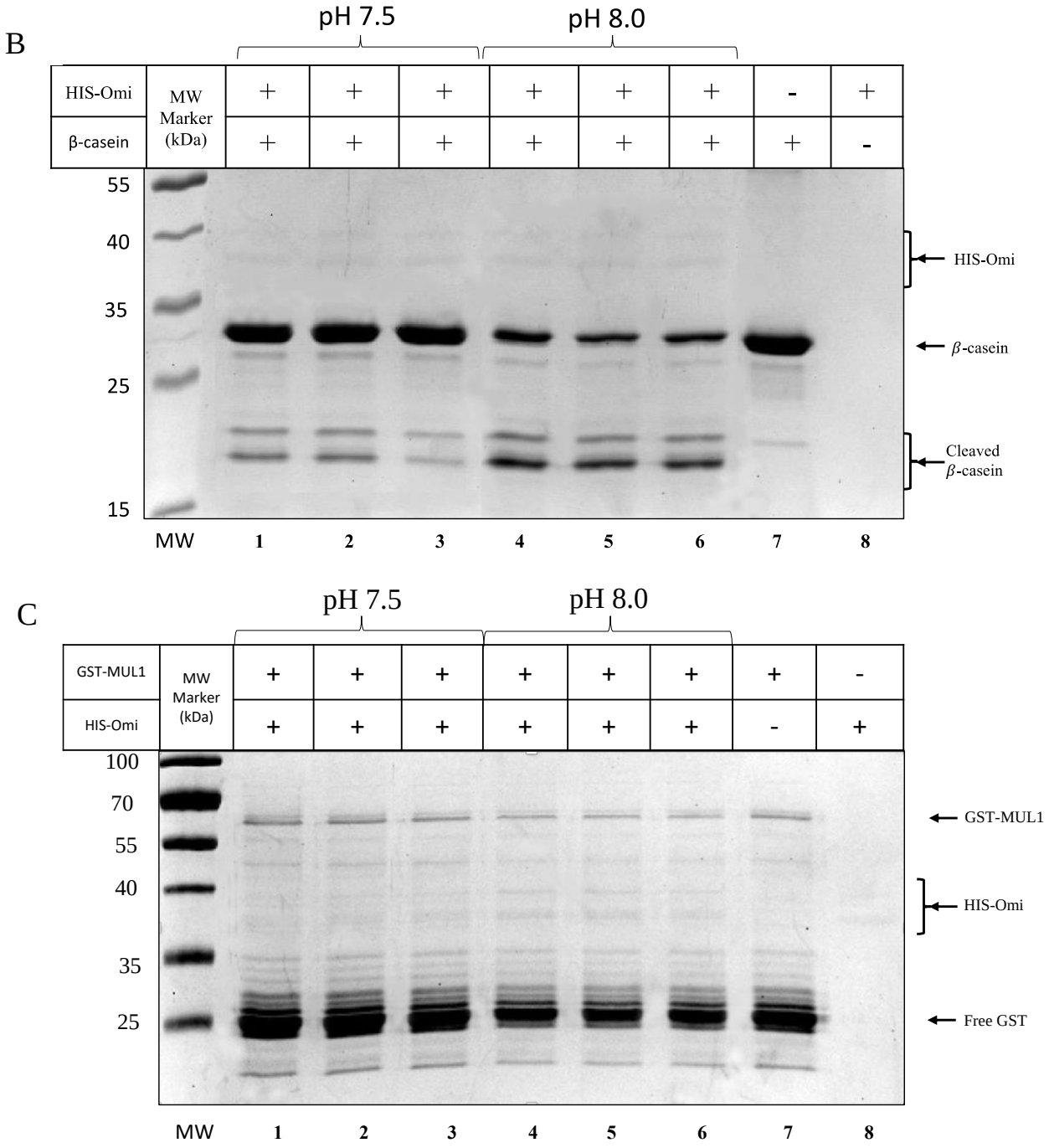


Figure 6: Protease activity of Omi/HtrA2 at different pH

HIS-Omi protein presents at 37 kDa, usually displaying double bands caused by aggregation. β-casein presents at 26 kDa, GST-MUL1 presents at 66 kDa, and free GST at 26 kDa. β-casein, a generic substrate of the protease Omi/HtrA2 undergoes degradation leading to bands of cleaved β-casein at around 18 kDa. In figure 6A, lanes 1 and 9 are controls and show the substrate and enzyme, respectively. Lanes 2-8 show degradation of equal amounts of β-casein by Omi/HtrA2 at increasing. In figure 6B, lanes 1-3 with β-casein are undergoing degradation by Omi/HtrA2 at pH 7.5, while lanes 4-6 show the same process at pH 8.0. In figure 6C, lanes 1-3 with the specific substrate MUL1 undergoing degradation by Omi/HtrA2 at pH 7.5, while lanes 4-6 show the same process at pH 8.0. Proteins were separated using SDS-PAGE and analyzed using Coomassie Blue staining.

Pre-Denaturing Experiments

The effects of denaturing the substrates on Omi/HtrA2 activity was tested in a side-by-side compassion of both versions of the specific substrates (Figure 7A). Only lane 4, containing the denatured MUL1 with Omi/HtrA2 showed any significant change in MUL1 degradation. Because of this, the samples were further analyzed using western blot analysis (Figure 7B). Here it is seen that both the denatured and native MUL1 experience cleavage as a result of adding Omi/HtrA2. Denaturing the MUL1 substrate by boiling has a significant effect, increasing the amount of the degradation MUL1 seen by the smaller kDa protein bands as well as novel bands not found in the non-boiled lane. In addition, there is a notable decrease in the MUL1 bands near the top of the figure.

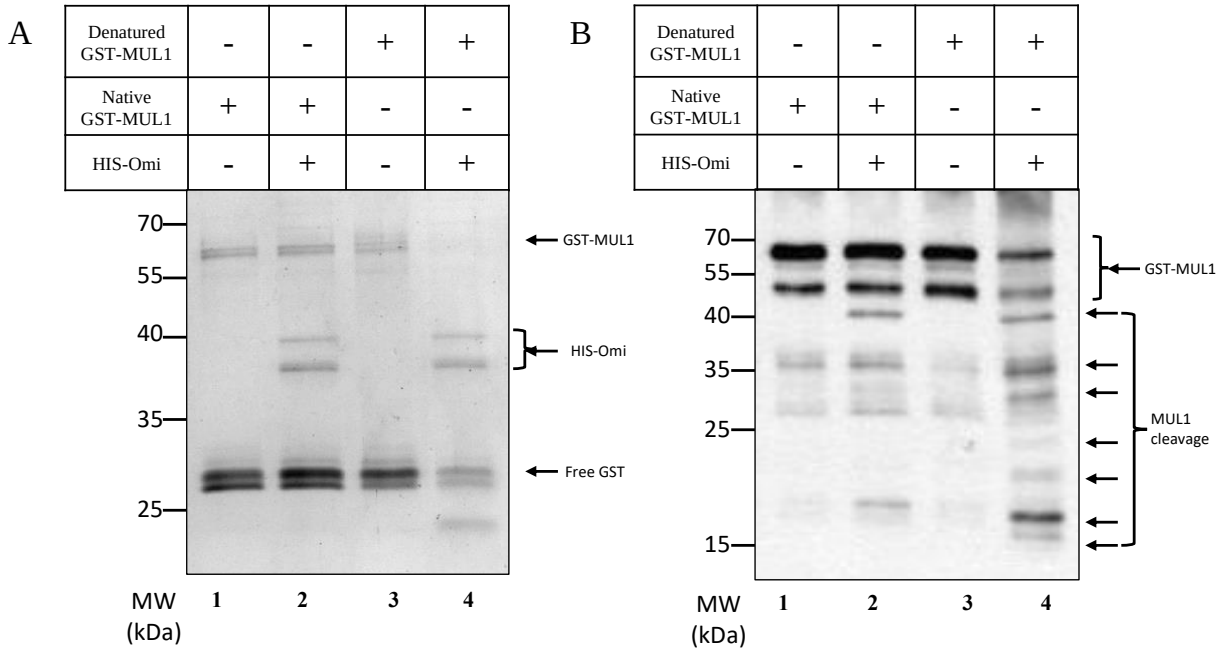


Figure 7: MUL1 denatured vs MUL1 native

GST-MUL1 presents at 66 kDa, HIS-Omi/HtrA2 at 37 kDa, and free GST at 26 kDa. GST presents in two bands due to aggregation, and endures some degradation due to Omi/HtrA2, the cleavage product showing around 20 kDa. This experiment compares denatured MUL1 substrate with and without the enzyme Omi/HtrA2, to its native counterpart. Figure A is separated on SDS-PAGE and analyzed by Coomassie Blue staining, while figure B is analyzed by Western Blotting using mouse anti-MUL1 antibodies.

Temperature Variation Experiments

The effects of temperature on Omi/HtrA2 activity were tested by comparing the degradation of both the generic substrate, β-casein, and the specific substrate, MUL1, at 37 ℃ and 42 ℃. Although repeated numerous times, no indications of change in the activity of Omi/HtrA2 was seen between the two temperatures (Figure 8). Cleavage rates between the generic and specific substrate showed insignificant changes.

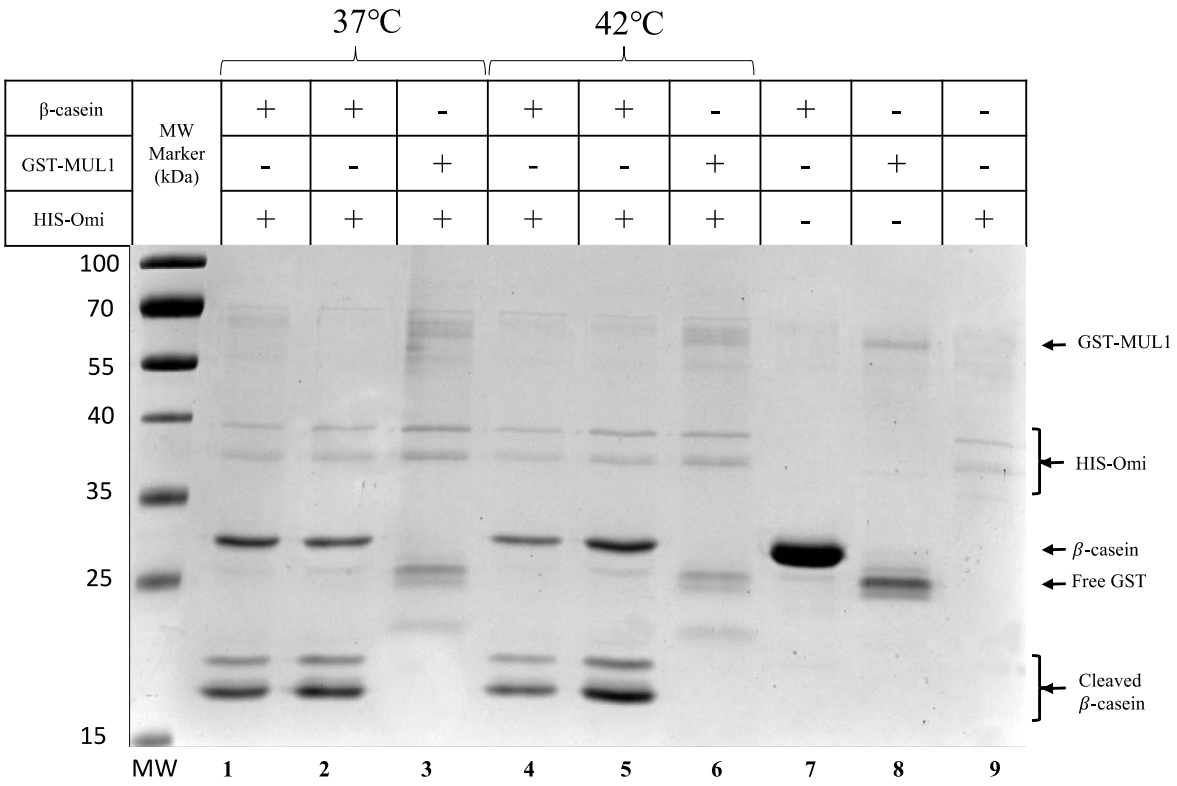


Figure 8: Omi/HtrA2 activity at different temperatures

HIS-Omi protein is shown at 37 kDa, β-casein presents at 26 kDa with cleaved β-casein at around 18 kDa, GST-MUL1 presents at 66 kDa , and free GST at 26 kDa. The experiment tests both the cleavage of the generic substrate β-casein and the specific substrate GST-MUL1 at 37℃ and 42℃. Lanes 7, 8, and 9 serve as the control for β-casein GST-MUL1 and HIS-Omi, respectively. Lanes 1-3 consider degradation of β-casein and MUL1 at 37℃. Lanes 4-6 consider degradation of β-casein and MUL1 at 42℃. Proteins were separated using SDS-PAGE and analyzed using Coomassie Blue staining.

Allosteric Activation Experiments

The effects of MUL1 as an allosteric regulator of Omi/HtrA2 were tested by comparing the cleavage of the generic substrate β-casein, with and without pre-incubation with the specific substrate MUL1. Figure 9 shows the lanes that had a pre-incubation with MUL1 and Omi/HtrA2 (Ln 4-5) had more overall degradation of the generic substrate β-casein, in comparison to lanes that had no MUL1 added before the addition of β-casein to Omi/HtrA2 (Ln 5-6). The increase in β-casein degradation is indicative of increased Omi/HtrA2 activity.

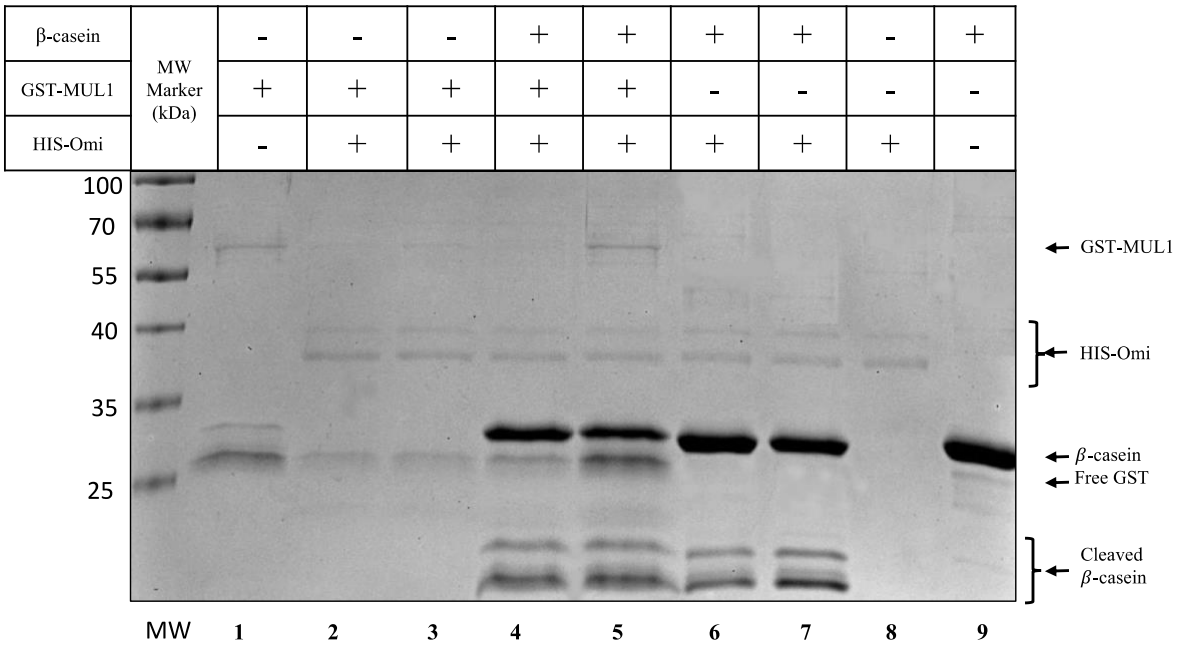


Figure 9: Allosteric Activation of Omi/HtrA2 by MUL1

HIS-Omi protein is shown at 37 kDa, β-casein presents at 26 kDa with cleaved β-casein at around 18 kDa, GST-MUL1 presents at 66 kDa, and free GST at 26 kDa. This experiment tests to see if GST-MUL1 acts as in allosteric activator of Omi/HtrA2. Lanes 1, 8, and 9 serve as the control for GST-MUL1, HIS-Omi, and β-casein, respectively. Lanes 4 and 5 represent the experimental lanes showing the degradation β-casein after incubation with GST-MUL1. Lanes 2-3 and 4-5 represent comparison lanes showing Omi/HtrA2 degradation on just GST-MUL1 and β-casein alone. Indication of MUL1 presence can be verified through the presence of the free GST only located in lanes containing GST-MUL1. Proteins were separated using SDS-PAGE and analyzed using Coomassie Blue staining.

Discussion

Omi/HtrA2 has many functions throughout the cell, including quality control of mitochondrial proteins, autophagy, and apoptosis. Acting as a serine protease, it has many substrates with numerous functions throughout the mitochondria and the rest of the cell. Although much work has been done in investigating the substrates and the functions of Omi/HtrA2, there is still much to be discovered. I looked to establish the environmental changes that affect activity of Omi/HtrA2 to better understand its function, and possibly identify new functions based on the information gathered. In addition, I aimed to identify a novel interaction of allosteric activation between the substrate MUL1, hypothesizing that it would increase the activity of Omi/HtrA2 after being degraded by the protease.

I performed many experiments testing different conditions, observing how the protease activity of Omi/HtrA2 was affected. Figures 6A/B indicate that pH 8.0 is ideal for Omi/HtrA2 activity when presented with the generic substrate β-casein. Figure C is less conclusive, as the cleavage of MUL1 is difficult to ascertain using Coomassie Blue staining, but still showed evidence of the same pattern seen with the generic substrate. Further experimentation using more sensitive methods such as Western Blotting could confirm the ideas presented by the gel. If the optimal pH is 8.0 as suggested in the gels, this is a key finding as the mitochondria is one of the areas of the cell that can alter its pH due to the presence of proton pumps with the ETC. While Omi/HtrA2 most commonly resides and works in the IMS where the pH in nearer 7.5, the matrix is kept at high pH of 7.9-8.0. An increase in the pH in the IMS could be caused if OXPHOS is inhibited and protons are not pumped to the IMS. In that case the activity of Omi/HtrA2 would increase as a response to the mitochondrial condition. In addition, pH change can be an indicator of cellular stress, causing many essential proteins to misfold. Omi/HtrA2's increase in activity

during these times of cellular stress would assist with the removal of misfolded or aggregated proteins due to the change in pH.

The experiment testing the effect of pre-denaturing the substrate MUL1 before incubation with Omi/HtrA2 showed very staggering results. Coomassie Blue staining indicated that denaturing by boiling the substrate drastically effected the protease activity of Omi/HtrA2 during the incubation period at 37 °C. The results of the Western blot analysis (Figure 7B) acted further to confirm this result. There was clear increase in the degradation of MUL1 after denaturing when compared to regular native substrate. This is consistent with the role of Omi/HtrA2 as it is a quality control enzyme for misfolded and denatured proteins, but additionally may indicate that its interaction with MUL1 may be altered *in vivo*. Although our method of denaturation, bringing to 100 °C, is not physiological, there are many other methods for protein denaturation, from pH change as mentioned before, to ROS, to chaperones. Pursuing these ideas could lead to new discoveries in the pathways already presented, or new pathways of regulation entirely. The drastic amount of difference between the amount of protease activity itself warrants further investigation into the relationship between Omi/HtrA2 and the MUL1 substrate.

The temperature variation experiment looked at the changes in protease activity in response to different temperature during the incubation of Omi/HtrA2 with MUL1. Although, as previously stated, the body temperature is highly regulated and unlikely to reach temperatures as high as 42 °C, it is again a placeholder for other cellular mechanisms of increase and enzymes activity. For example, a cellular catalyst could be involved in the process. In addition, increases in temperature and heat shock damage experienced could lead to increased activation of Omi/HtrA2. Although the data found was inconclusive (Figure 8), this pathway could be further ventured into to find a more definite connection or lack thereof.

The final experiment further explored the previously identified interaction between Omi/HtrA2 and MUL1. According to Figure 9, there seems to be a clear increase in activity, against the generic substrate β-casein, when Omi/HtrA2 is pre-incubated with MUL1. This is an important discovery. This suggests that a cleaved pieces of MUL1 bind to Omi/HtrA2 either creating an open conformation or promoting the formation of the active hexamer complex [37]. The MUL1 ∝ sequence contains some promising segments that bare similar patterns to those seen in peptides that promote activation in Omi/HtrA2 [17]. More experiments are necessary to confirm these findings. Although the generic substrate β-casein was used, applying the test to a specific substrate of Omi/HtrA2 would further increase the legitimacy of the claims. In addition, pinpointing the section of MUL1 that is responsible for the allosteric regulation of Omi/HtrA2 could be further explored and well as the cleavage sites in the MUL1 sequence.

In conclusion, the results from my experiments suggest that Omi/HtrA2 activity can be regulated by multiple mechanisms that represent various states of mitochondrial dysfunction or cellular stress. In addition, Omi/HtrA2 is involved in complex protein-protein interactions that invariably affect its protease activity and target its degradation power against specific substrates.

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