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ABSTRACT

Mechanisms of Proteasome Inhibition in Neurodegenerative Diseases

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The formation of neuronal inclusions is one of the hallmarks of neurodegenerative diseases. These structures are composed of aggregated proteins, molecular chaperones, and components of the ubiquitin-proteasome system (UPS). Co-localization of aggregated proteins with cell-homeostasis maintaining machinery indicates that the cell may be failing in an attempt to clear these aggregates. Here I explore possible mechanisms of proteasome inhibition in two neurodegenerative disorders, Parkinson's Disease (PD) and Huntington's Disease (HD). These studies were conducted using *in vitro* proteasome degradation assays with radioactive substrates. I have found that the proteasome inhibition that is seen in PD may stem from its inability to recognize ubiquitinated substrates. I show that the degradation of a substrate is inhibited by the addition of aggregated α-synuclein, a protein that is a major component of PD neuronal inclusions. Aggregated α-synuclein has high binding affinity for the subunit of the proteasome that is responsible for recognition of substrates targeted for degradation. Taken together, these results suggest that inhibition of degradation in PD is due to the blockage of substrate recognition by binding of aggregated α-synuclein to the proteasome.

The mechanism of proteasome inhibition in HD may be due to the combination of two effects, processing and inefficient initiation of degradation. A mutation in huntingtin (htt) protein, that causes massive expansion if its polyglutamine tract, is the calling card of HD. This expansion makes the protein highly prone to aggregation. I show that the inability of the proteasome to dispose htt before it reaches aggregate state may be partially mediated inhibition

of proteasome progression. My results show that the proteasome cannot efficiently degrade polyglutamine proteins when in the context of a stable protein, but can proceed with ease when associated with a less stable protein domain. Taken together, this suggests that certain structural components of htt may impede progression of degradation, thereby causing the protein to be processed instead of degraded. I also show that mutant htt provides a poor site for initiation of degradation. Therefore, the proteasome cannot properly engage the protein, thus mediating inhibition.

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Chapter 1

The Ubiquitin Proteasome System

Protein degradation by ATP-dependent proteases is an important part of cellular housekeeping and for maintaining homeostasis in the cell. Its main function is disposal of misfolded, aged, or aberrant proteins. In recent years it has also been shown to be involved in various other processes throughout the cell. In eukaryotes these functions are controlled by the 26S proteasome. Degradation by the proteasome is a highly regulated and tightly controlled system in which specific tagging by ubiquitin determines the fates of cellular proteins. When this regulation or progression of degradation is disrupted, the consequences can be severe. Inhibition of protein degradation can lead to the development of disorders, many of them being neurodegenerative diseases. This thesis seeks to explore inhibition of components of the degradation process and its links to possible consequences in the physiology of neurodegenerative diseases.

Structure of the proteasome

The 26S proteasome is involved in many eukaryotic cell processes. These range from cell cycle regulation (Rape, Reddy et al. 2006) to antigen processing for activation of the immune response (Kloetzel 2001), to activation of gene transcription (Muratani and Tansey 2003), to quality control in the endoplasmic reticulum (Werner, Brodsky et al. 1996). This ~2 MDa protein complex is composed of two basic structures: a 700 kDa 20S core and one or two 900 kDa 19S regulatory complexes (Fig 1.1). The core complex, measuring 15 nm in length and 11 nm in diameter, is a barrel-like structure made up of four seven-membered rings that are stacked on top of each other (Groll, Ditzel et al. 1997). The two distal rings, called the α-rings,

provide structural support for the 19S complex. When not attached to the 19S complex, the α-rings keep the entrance to the 20S core in a closed configuration by blocking the entrance to the degradation chamber with their N-terminal tails. Binding of the 19S complex causes the α-rings to change configuration (Lowe, Stock et al. 1995). The catalytic subunits are housed within the two central β-rings. Proteolytic sites are harbored on each β-ring, making six sites in total (Baumeister, Walz et al. 1998). All of these sites are threonine proteases and have shown three types of hydrolyzing activities: chymotrypsin-like, trypsin-like, and peptidylglutamyl-like, thus allowing for hydrolysis of bonds between all amino acids in a given polypeptide chain (shown in yeast proteasome, Fig 1.2) (Chen and Hochstrasser 1996). Analogous structures in prokaryotes are, ClpP, FtsH and HslV (Pickart and Cohen 2004) (Kihara, Akiyama et al. 1999).

Access to the proteasome's degradation machinery is granted by entrance through one of its axial pores, which is only up to 2 nM in diameter when open (Groll, Ditzel et al. 1997), thus blocking natively folded polypeptides from entering. The 19S regulatory complex structure controls this "gating" process and can be further dissected into two multi-subunit structures, a base and a lid. The base is composed of six homologous ATPases (Rpt1-6) and three non-ATPase subunits (Rpn 1, 2, and 10). Rpn10 serves to connect the distal lid with the proximal base (Fu, Reis et al. 2001). The lid is made up of eight transiently associated non-ATPase subunits (Rpn 3, 5-9, 11 and 12). The overall role of the lid is still unclear, but recent studies have shown that Rpn11 exhibits de-ubiquitination activity, a process that will be touched on later in this chapter. Though this is the only known enzymatic activity of the lid, it may provide clues to the roles of its other subunits (Glickman, Rubin et al. 1998) (Pickart and Cohen 2004). Some complexes that play this gatekeeper role in prokaryotes are ClpA and ClpX, which partner with ClpP, and HslU, which partners with HslV (Fig 1.3). All of these complexes serve a similar role

to that of the 19S complex (Sauer, Bolon et al. 2004).

Figure 1.1: Structure of the 26S proteasome

Electron microscopy detailing the structure of the proteasome. The α and β rings of the 20S core are notated as well as the 19S cap subunits (Larsen and Finley 1997). Protein hydrolysis sites are located within the β-rings, and the ATP-driven ubiquitin recognition sites, and possibly unfolding machinery, are located within the 19S cap.

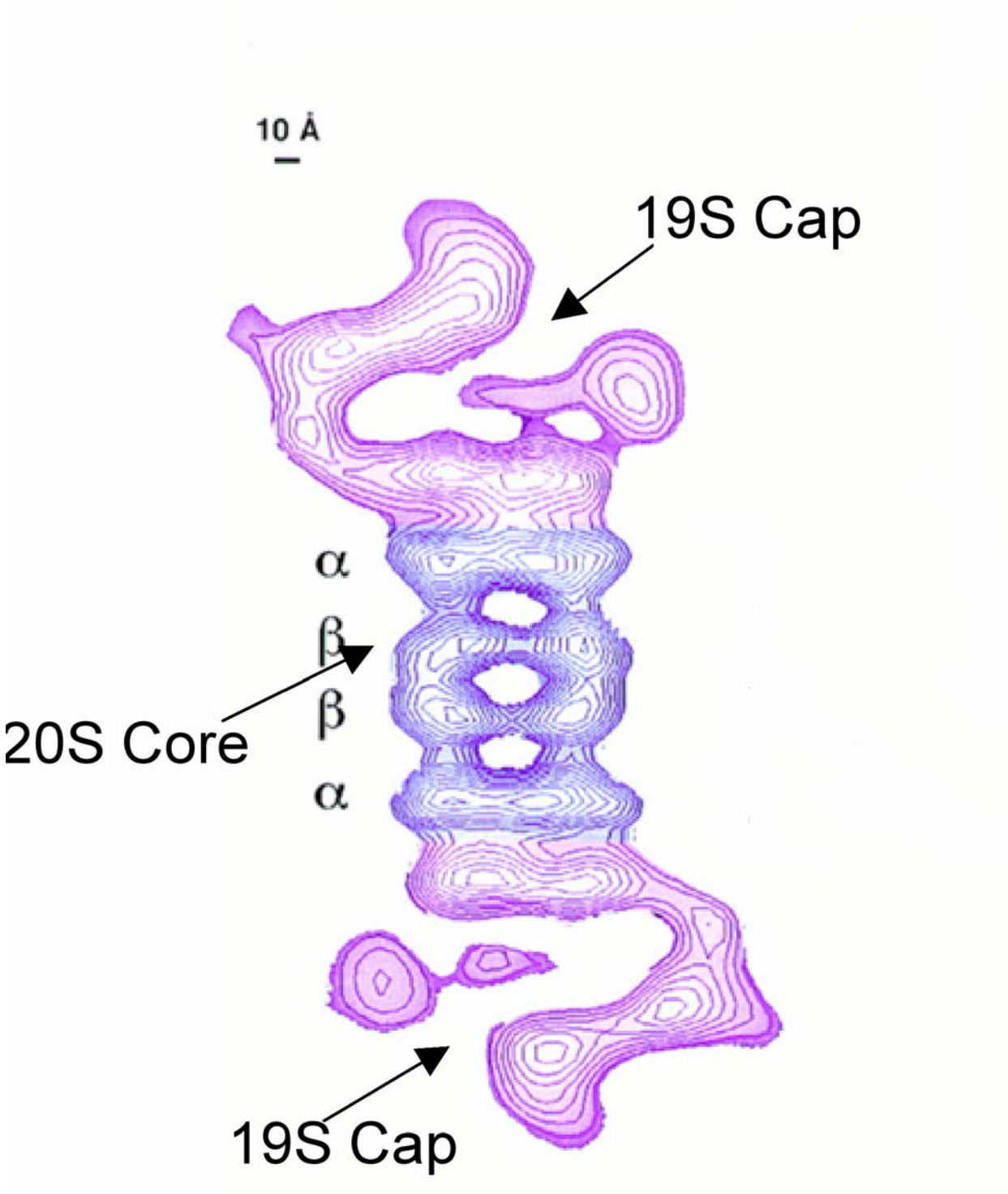

10 Å
19S Cap
α
β
β
α
20S Core
19S Cap

Figure 1.2: Location of hydrolysis sites

A picture adapted from Groll *et al*, (1997) showing the internal structure of the yeast proteasome. The spaces between the red dots indicate the location of the hydrolysis sites on the β-rings of the core subunit (Groll, Ditzel et al. 1997). These sites cleave specifically after acidic, basic, and hydrophobic amino acids in a polypeptide chain.

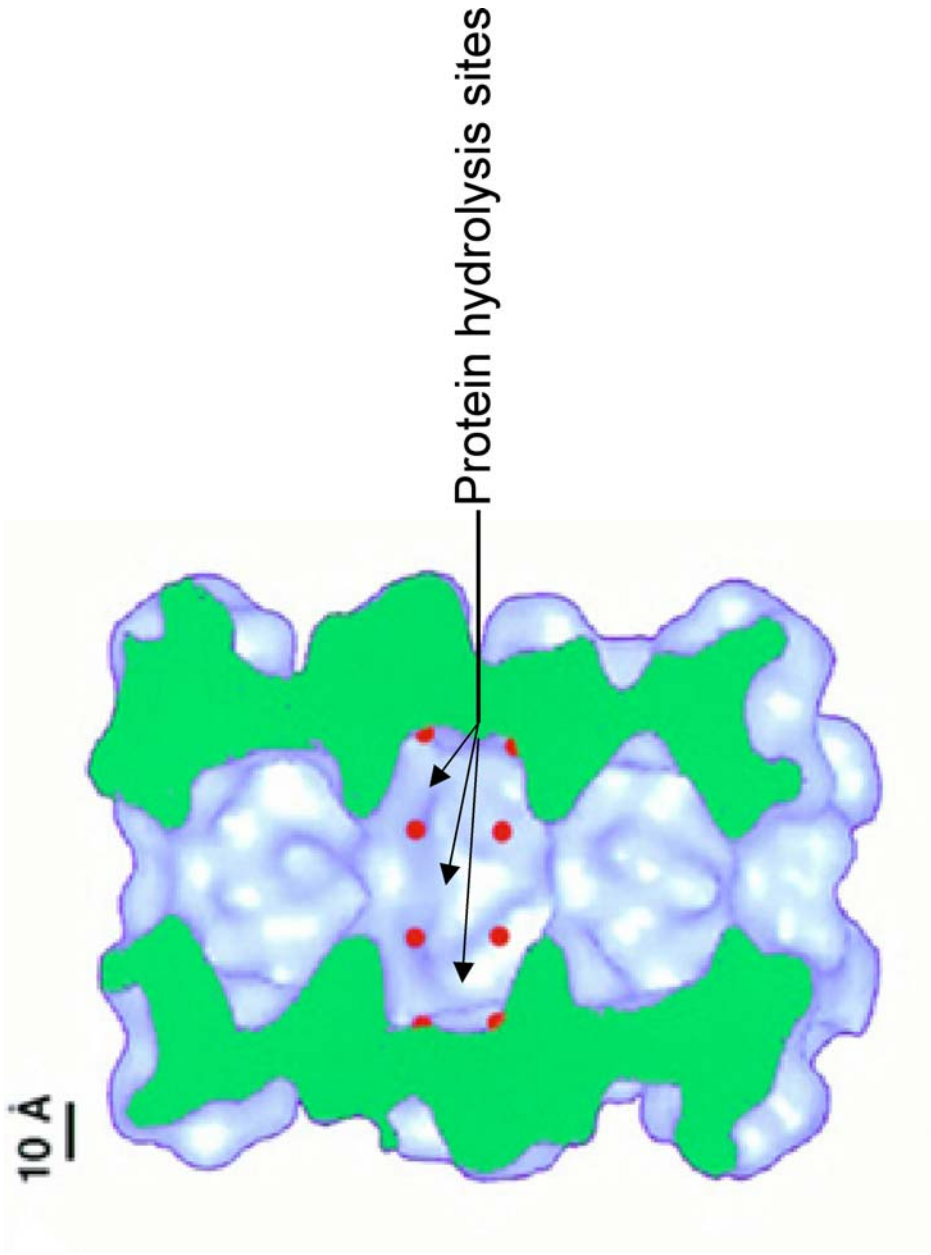
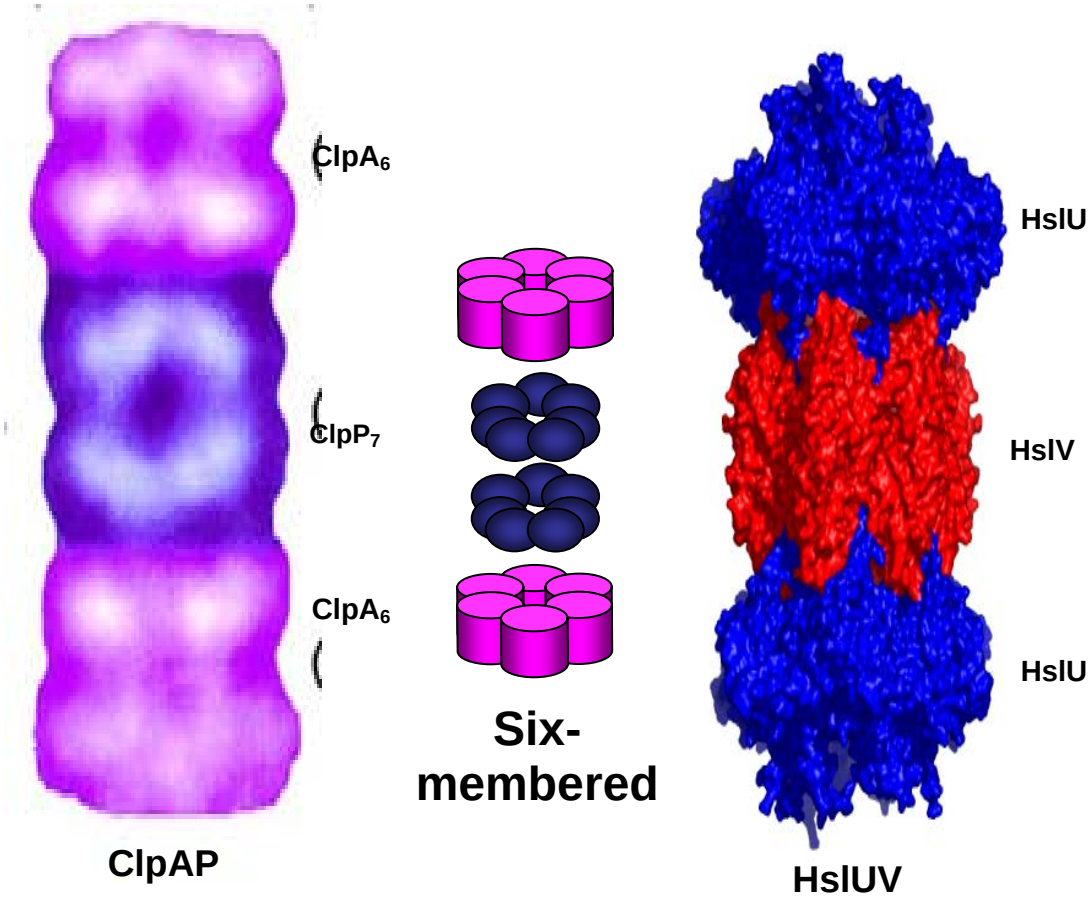

Protein hydrolysis sites
10 Å

Figure 1.3: Structure of ATP-dependant bacterial proteases

The structure of ClpAP and HslUV represented by computer modeling. The multi-subunit ring structure of the ATPase subunits and hydrolysis subunits are depicted in cartoon form (Wang, Hartling et al. 1997; Sauer, Bolon et al. 2004). Each ring is made up of six subunits.

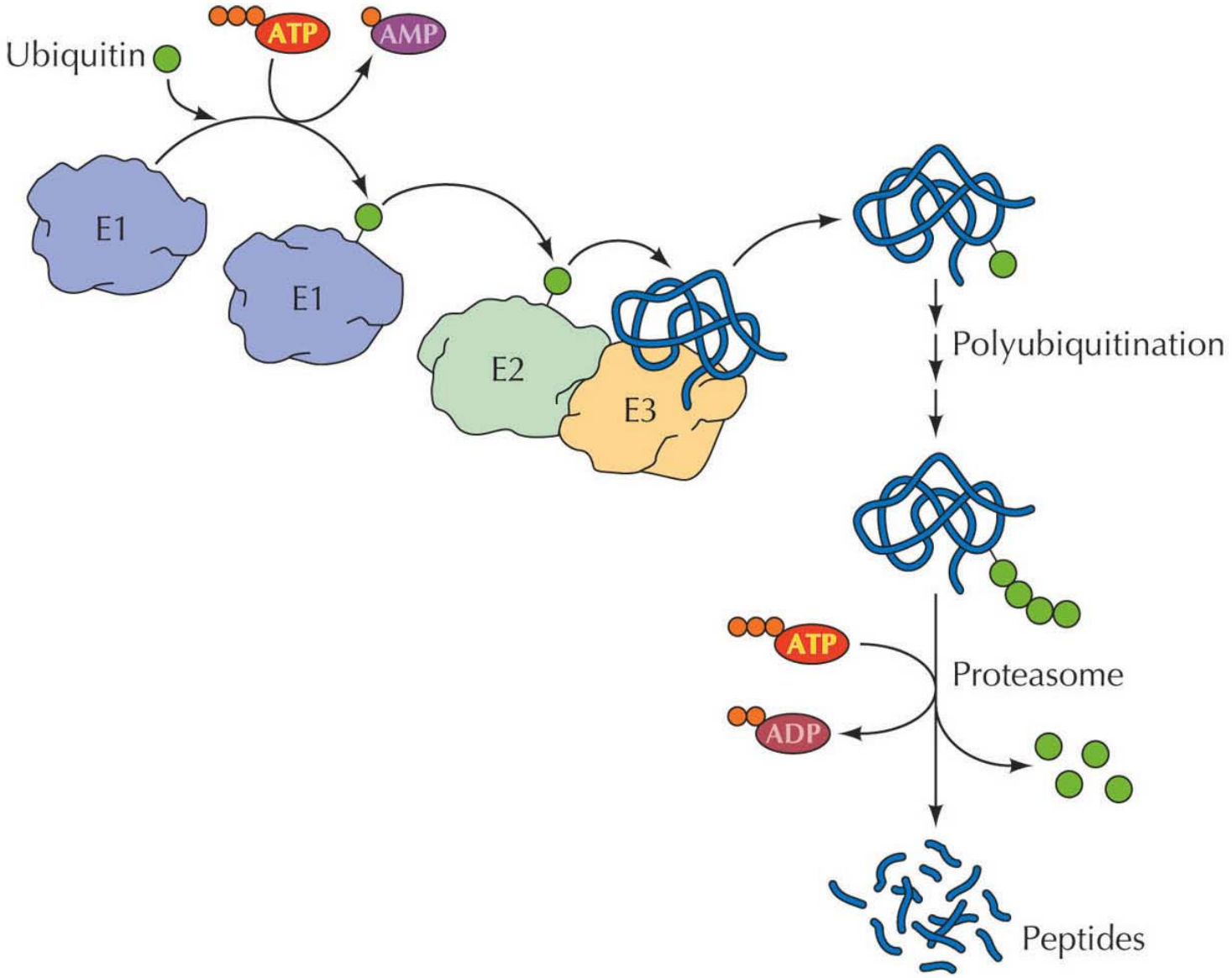

ClpA6
ClpP7
ClpA6
ClpAP
Six-membered
HslU
HslV
HslU
HslUV

Targeting of proteins for degradation by ubiquitin

In order to be degraded by the proteasome, most proteins must be tagged with an 8.5 kDa polypeptide called ubiquitin. The formation of ubiquitin chains is the key step in targeting proteins to the proteasome (Pickart 1997). Ubiquitin attaches to a protein by forming a bond between its C-terminus (G76) and the ε-amino group of a lysine residue in the target protein. The addition of ubiquitin to proteins is mediated by a group of enzymes called E1s, E2s, and E3s (Fig 1.4). It is a hierarchical system in which a single E1 activates ubiquitin for all modifications and can transfer it to several species of E2s. Each E2 then in turn acts with one or several E3s to ubiquitinate the substrate protein (Pickart 2001). E1s, or ubiquitin-activating enzymes, carry two molecules of activated ubiquitin-one as a thiol ester, one as an adenylate. The thiol-linked ubiquitin is transferred to an E2, or ubiquitin-conjugating enzyme (Haas and Siepmann 1997). The E2 then works in concert with one or multiple E3s to catalyze the formation of an isopeptide bond between the ubiquitin and the substrate protein. E3 enzymes are the key players in determining targeting specificity for degradation. There are at least two distinct steps in the E3 reaction: binding to the substrate via the ubiquitin moiety, and covalent ligation of one or more ubiquitins to the substrate. A minimum of four ubiquitins need to form a chain (in most cases) through Lys-48 linkages to guarantee proper targeting to the proteasome (Thrower, Hoffman et al. 2000). Other linkage types that may result in proteasome targeting are Lys-6, and Lys-11 (Baboshina and Haas 1996). Other linkages such as ubiquitin chains linked via Lys-63 play a role in DNA repair (Deng, Wang et al. 2000), but not substrate degradation.

Figure 1.4: The ubiquitination cascade

A cartoon schematic of the ubiquitination cascade in which an E1 mediates transfer of a ubiquitin moiety to an E2, which then works in concert with an E3 to target a protein to the proteasome for degradation (Essential Cell Biology, Garland Science, 2004).


Ubiquitin
ATP
AMP
E1
E1
E2
E3
Polyubiquitination
ATP
ADP
Proteasome
Peptides

As stated above, E3s target specific proteins to the proteasome. There are two major families of E3s: RING (Really New Interesting Gene) finger containing- and HECT-domain E3s (Glickman and Ciechanover 2002). RING fingers are defined by a pattern of conserved Cys and His residues that form a cross brace structure by linking Zn^{++} atoms (Borden 2000). RING E3s may serve as scaffolds that position the substrate and the E2 optimally for ubiquitin transfer (Zheng, Wang et al. 2000). The RING finger domain-containing family is made up of two groups: single and multisubunit proteins. Examples of single subunit proteins are Mdm2 (Boyd, Tsai et al. 2000) and Parkin (Shimura, Schlossmacher et al. 2001). One example of a group of multisubunit complexes is the Anaphase Promoting complex (APC) enzymes. These enzymes are the main coordinators of cell cycle progression in mitosis and G1. Ubiquitin chains are added to substrates with varying degrees of processivity, which then correlates with the temporal order of their destruction. I.e., the more processive substrates are preferentially ubiquitinated, and thus degraded earlier, ensuring proper cell cycle progression (Rape, Reddy et al. 2006). Another extensively studied multisubunit E3 is the Skp-Cullin-F-box ligase. These complexes rely on their F-box protein for their specificity and are involved in the degradation of cell-cycle regulatory proteins and transcription factors (Cardozo and Pagano 2004).

All HECT (homologous to E6-AP carboxy terminus) domain proteins share a 350-amino acid domain that contains a conserved Cys residue, positioned ~35 residues upstream of the C-terminus, to which the activated ubiquitin moiety is transferred from an E2 (Scheffner, Nuber et al. 1995). The unique N-terminus of each HECT E3 interacts with specific substrates, and the HECT domain itself mediates E2 binding and ubiquitination (Pickart 2001). The best-characterized HECT E3 is the yeast enzyme Rsp5. It is known to ubiquitinate various types of substrates, two being transcription factors Spt23 and Mga2 (Hoppe, Matuschewski et al. 2000).

A recently new addition to the HECT family ligases is Smurf1, which selectively interacts with SMADs specific for the bone morphogenetic pathway (BMP) (Zhu, Kavsak et al. 1999).

Another small but well studied subset of E3s is the N-End Rule ubiquitin ligases. These enzymes ubiquitinate substrate proteins based on the identity of the proteins N-end degron. This term refers to the initial or acquired N-terminal residue of the substrate that allows for recognition by an N-end rule E3 and subsequent addition of ubiquitins to internal lysine residues present in the protein. All residues have varying levels of recognition for ubiquitination, with Arg being the best and Met the least effective (Varshavsky 1996). Some of the studies discussed in this thesis utilize the N-end rule for experimentation.

Recently a new protein has been found to be involved in multiubiquitination. This is a ubiquitin chain elongation factor call E4. In yeast, E4 binds to ubiquitin moieties of short chains and catalyzes elongation in conjunction with E1, E2, and E3 (Koegl, Hoppe et al. 1999). E4s contain a modified version of the RING finger, designated as the U box (Aravind and Koonin 2000). It is thought that these U box proteins constitute a subfamily of E3s that has the ability to elongate short polyubiquitin chains by mediating transfer of ubiquitin to a previously conjugated ubiquitin molecule rather than to the substrate itself (Glickman and Ciechanover 2002). An example of a recently discovered E4 is CHIP, which is involved in the degradation of CFTR. It has been shown to interact with Hsp70, which may indicate that it does not have a substrate binding site, but may ubiquitinate unfolded proteins that are bound to Hsp70, therefore aiding in the heat-shock response (Meacham, Patterson et al. 2001) (Demand, Alberti et al. 2001).

Degradation of ubiquitinated substrates

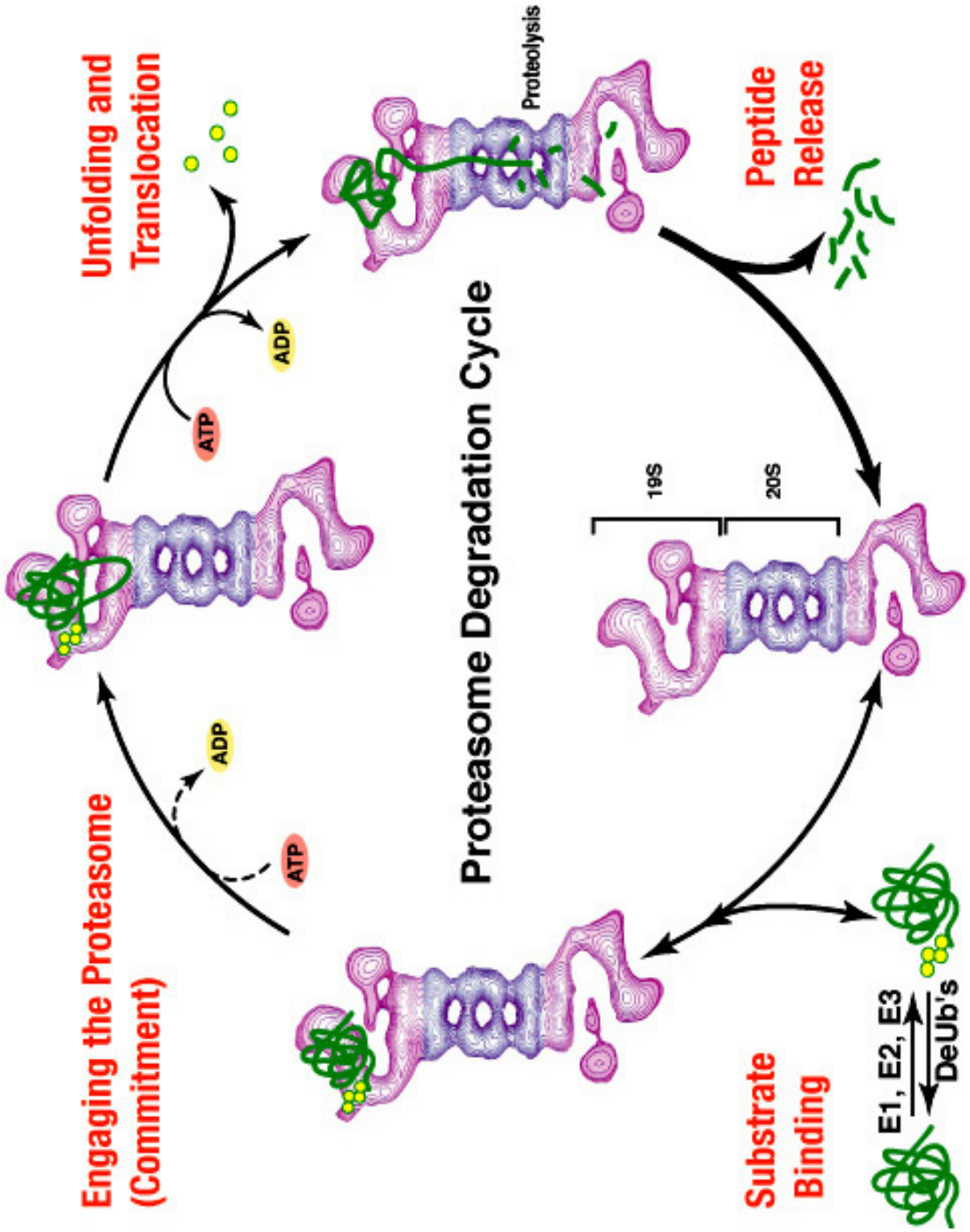
Degradation of proteins by the proteasome is a highly regulated and dynamic process (Fig 1.5). The first step in proteasome degradation is recognition and binding of ubiquitinated substrates. Rpt5, an ATPase subunit in the base of the 19S regulatory complex (Lam, Lawson et al. 2002), is thought to mediate this process. Rpn10 has also been suggested as a recognition site (Elsasser, Chandler-Militello et al. 2004). Recently an alternative recognition mechanism has been suggested. It has been shown that the proteasome can recognize ubiquitinated proteins indirectly through modulator proteins. Two examples are Rad23 and Dsk2. These proteins have N-terminal ubiquitin-like (UBL) domains that facilitate binding to the proteasome, and C-terminal ubiquitin-associated (UBA) domains that bind ubiquitin chains on substrate proteins. These UBA domains show a strong preference for Lys-48-linked ubiquitin chains (Raasi and Pickart 2003). Therefore, these proteins can act like scaffolds that hold substrates within range of the proteasome for unfolding and degradation. The non-ATPase unit Rpn1 mediates the interaction of Rad23 and Dsk2 and may serve as a docking station for several substrates targeted for degradation (Elsasser, Chandler-Militello et al. 2004).

A recent study by Prakash, *et al* has shown the requirement of second signal for degradation, in the form of an unstructured region in the substrate protein (Prakash, Tian et al. 2004). This concept will be revisited later in this thesis.

After recognition and initiation of degradation, the substrate protein must be unfolded and threaded through the 10-15 Å wide degradation channel (Horwich, Weber-Ban et al. 1999).

Figure 1.5: Schematic of the proteasome degradation cycle

Proteins are targeted to the proteasome by polyubiquitination, where they engage with the 19S cap. De-ubiquitinating enzymes then remove ubiquitin moieties from substrates. They are then unfolded and shuttled into the degradation chamber for hydrolysis. The polypeptide chain then emerges after hydrolysis in short pieces which are then further hydrolyzed by cellular proteases (Prakash, Tian et al. 2004).


Proteasome Degradation Cycle
Engaging the Proteasome (Commitment)
Unfolding and Translocation
Proteolysis
Peptide Release
Substrate Binding
ATP
ADP
ATP
ADP
19S
20S
E1, E2, E3
DeUb's

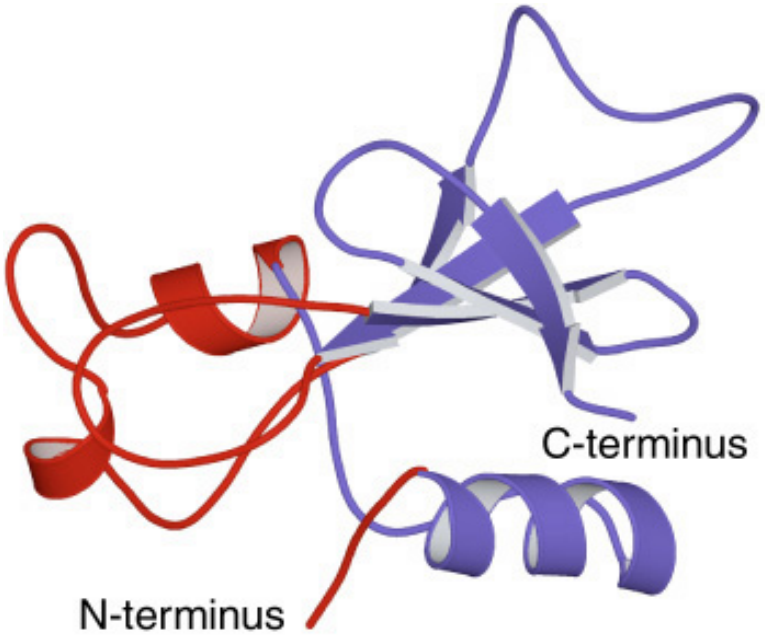
The protein unfolding and ATP turnover that is necessary for degradation are regulated by the AAA+-family ATPases that are present in the base of the 19S complex (Rpt1-6) (Lupas, Koster et al. 1993; Ogura and Wilkinson 2001). The substrate proteins are actively unfolded, as opposed to them unfolding spontaneously. The process of active unfolding was first described through studies of mitochondrial import (Huang, Ratliff et al. 1999). It was shown that the unfolding rate of a substrate protein, barnase, is increased by changing the pathway of unfolding (Fig 1.6). The rate of this mechanism is dependent on the structure of the N-terminal portion of the protein, where import is initiated in this case (Huang, Ratliff et al. 1999).

These concepts were used to study how substrates are unfolded during proteasome degradation (Lee, Schwartz et al. 2001). It was shown that the ability of a substrate to be unfolded and degraded also depends on its structure and its stability (Lee, Schwartz et al. 2001). As was shown with mitochondria, the portion of the substrate where unfolding is initiated plays a key role in this susceptibility to unfolding. Another concept was discovered in this study as well. It was shown that the proteasome degrades proteins sequentially starting from the ubiquitination signal (Lee, Schwartz et al. 2001). The studies and conclusions that will be discussed later in this thesis build on these two aforementioned concepts.

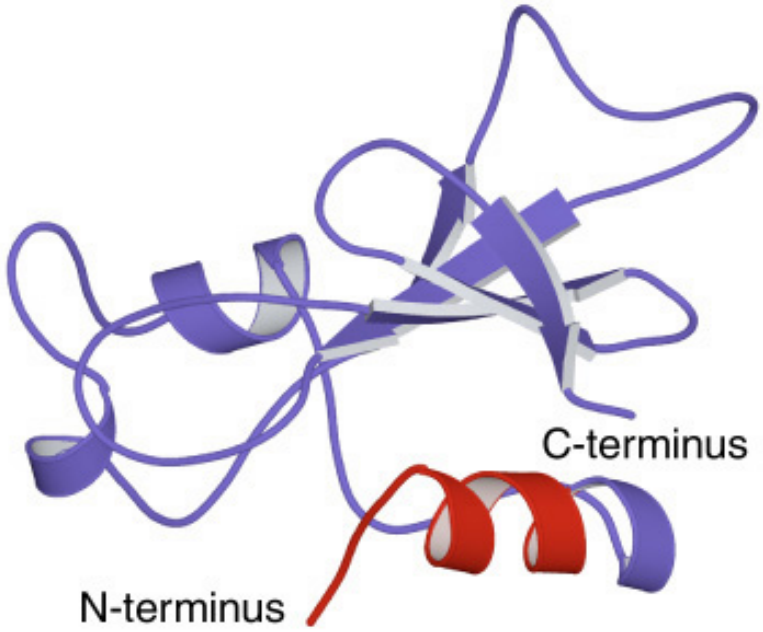
As the substrate protein is fed into the catalytic chamber of the proteasome complex, its polyubiquitin chains are disassembled, resulting in the replenishing of the intracellular pool of ubiquitin (Hough and Rechsteiner 1986). This de-ubiquitination process is mediated by two classes of thiol proteases: ubiquitin C-terminal hydrolases (UCHs) and ubiquitin-specific proteases (UBPs) (Hochstrasser 1996). Finally, small peptide products are produced after degradation, usually 3-23 amino acids in length, where they are hydrolyzed by downstream proteases (Ciechanover 1998).

Figure 1.6: The barnase unfolding pathway

A cartoon depiction of the unfolding pathway of barnase when it is actively unfolded vs when it is allowed to unfold spontaneously in solution. The structures in red unfold early, and the structures in blue unfold late as determined by mitochondrial import studies (Huang, Ratliff et al. 1999).



Unfolding pathway *in vitro*



Unfolding pathway during import

One of the most interesting features of proteasome degradation in terms of this thesis, is that not all substrates are degraded to completion. This phenomenon, called processing, leaves partially truncated proteins that can be used as transcription factors, as in the case of NF-κB (Palombella, Rando et al. 1994). Or it can leave partial proteins that are prone to aggregation as is thought to occur in the case of polyglutamine proteins (Cummings, Reinstein et al. 1999). This mechanism has been shown to be facilitated by the presence of low-complexity sequences- composed largely of one or a few amino acid types- adjacent to folded domains (Tian, Holmgren et al. 2005). This mechanism as it applies to polyglutamine proteins will be explored extensively in chapter three of this thesis.

In summary, degradation of proteins by the proteasome is an ordered and highly regulated process. It requires the cooperation of many components to allow for proper targeting and efficient degradation. If any of these components is disrupted, progression of degradation is therefore disrupted. The studies discussed in this thesis will explore cases in which inhibition of degradation occurs, and draw conclusions as to why these occurrences may contribute to the physiology of common neurodegenerative diseases.

Chapter 2

Inhibition of Proteasome Degradation by Aggregated Alpha-Synuclein

Introduction

Misfolding and aggregation of proteins have been linked to many neurodegenerative disorders. One such family of disorders is called the α-synucleinopathies, which are associated with α-synuclein. The most studied of these is familial Parkinson's disease (PD). Several clues point to α-synuclein aggregation and proteasome inhibition as important factors in PD physiology. In this chapter, I will discuss studies performed in collaboration with Dr. Heather Snyder of the Wolozin lab at Loyola University Medical Center (Maywood, IL). The goal of these studies was to examine the effect of α-synuclein on proteasome function. My results showed that the aggregated form of the protein inhibited degradation by the proteasome. These results, in combination with the results from the studies conducted by my collaborators, propose a mechanism for proteasome inhibition by α-synuclein in PD.

Alpha-synuclein

The proteins of the synuclein family are abundantly expressed in the brain, but their functions are not well understood. The family consists of three members, α-, β-, and γ-synuclein. They are 55-62% identical and range from 127 to 140 amino acids in length (Lavedan 1998). α-synuclein has been implicated in several diseases is. It is a 14.5 kDa protein, which is enriched in pre-synaptic nerve endings where it is distributed between a pool of synaptic proteins and vesicle bound proteins (Ostrerova, Petrucelli et al. 1999). Though the nature of its function is unknown, its localization to synaptic terminals and its structural conformation may provide

clues. α-synuclein is natively unfolded, but it forms a α-helix upon binding negatively charged phospholipid membranes. This may be its mode of interaction with synaptic vesicles (Volles and Lansbury 2002). How the regulation of dopamine release from synaptic vesicles may be controlled by α-synuclein and its possible relationship to α-synucleinopathies will be discussed later in this section.

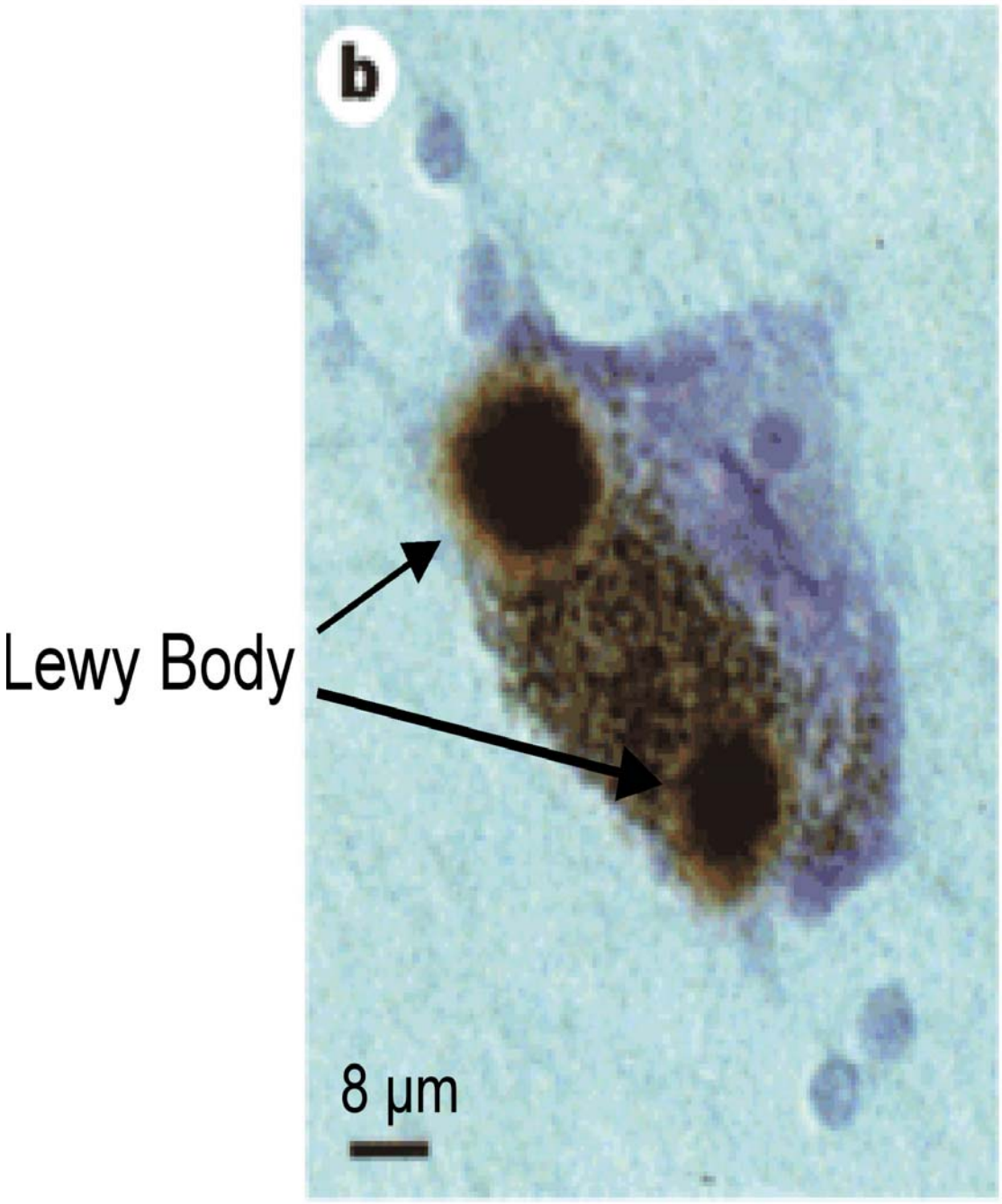
Parkinson's Disease

PD is the second most common neurodegenerative disorder after Alzheimer's disease, occurring in 1-2% of the population over the age of 65. There are three symptoms that are characteristic of PD: muscle rigidity, bradykinesia (slowness of motor activity), and resting tremor. Cognitive impairment is common in the late stages of the disease as well (Goedert 2001). These symptoms have been linked to a deficiency in dopamine release caused by death of dopamine releasing (DA) neurons in the *substantia nigra* region of the brain stem (Masliah, Rockenstein et al. 2000).

There are several diseases that are associated with α-synuclein, two being familial PD and Dementia with Lewy Bodies (Goedert 2001). The formation of Lewy bodies is the most recognizable trait of these diseases (Fig 2.1). Lewy Bodies are composed of α-synuclein, neurophilaments, microtubule associated proteins, and components of the ubiquitin proteasome system (Dawson and Dawson 2002). Their dense cores are granular and their outer rings are formed by 10-16 nm α-synuclein fibrils, which have β-pleated sheet conformations (Spillantini, Crowther et al. 1998). This type of amyloid formation is typical of what is seen in

Figure 2.1: Lewy bodies in PD neurons

Aggregate structures in PD called lewy bodies are always found in the neurons of those with PD. A pigmented nerve cell with two such structures is shown here (Goedert 2001).


b
Lewy Body
8 µm

neurodegenerative diseases. Three rare mutations in α-synuclein are implicated in early-onset forms of PD: A53T, A30P, and E46K (Polymeropoulos, Lavedan et al. 1997; Kruger, Kuhn et al. 1998; Zarranz, Alegre et al. 2004). These mutations accelerate aggregation of α-synuclein, thereby preventing proper function (Conway, Lee et al. 2000).

Various animal models have been used to study synucleopathies. Studies using α-synuclein knockout mice show an increased rate of dopamine release after stimulation with electrical pulse. This suggests a possible role of α-synuclein as a negative regulator of DA release (Abeliovich, Schmitz et al. 2000). Overexpression of human mutant A53T and A30P forms of α-synuclein in Drosophila, showed selective depletion of DA neurons at 30-60 days, formation of Lewy body-like inclusions, as well as loss of motor function; resembling PD (Feany and Bender 2000). Overexpression of α-synuclein in mice, rats, and primates showed similar results (Maries, Dass et al. 2003). The combination of all of these animal studies has provided insight into various elements of PD.

An important question generated from the studies detailed above is how improper α-synuclein function relates to death of DA neurons. In pre-synaptic terminals, dopamine is packaged into phospholipid vesicles and stored for later usage. A decrease in the number of vesicles available for dopamine storage might lead to an accumulation of free dopamine in the cell cytoplasm. This increase in dopamine could then lead to the production of free radicals that are products of dopamine metabolism. These free radicals are prone to cause oxidative damage in the cell, which then in turn may cause cell death (Lotharius and Brundin 2002). In a neurotoxin-induced rat model of PD, injections of chemicals known to induce oxidative stress caused degeneration of DA neurons, mimicking the early stages of PD (Lee, Sauer et al. 1996).

α-synuclein can interact with phospholipid vesicles and is found enriched in pre-synaptic terminals. This enrichment may indicate that it is a regulator of dopamine packaging. Therefore, if α-synuclein is sequestered in Lewy Bodies, it is not able to perform its function and could be a main factor in the death of DA neurons in PD (Fig 2.2).

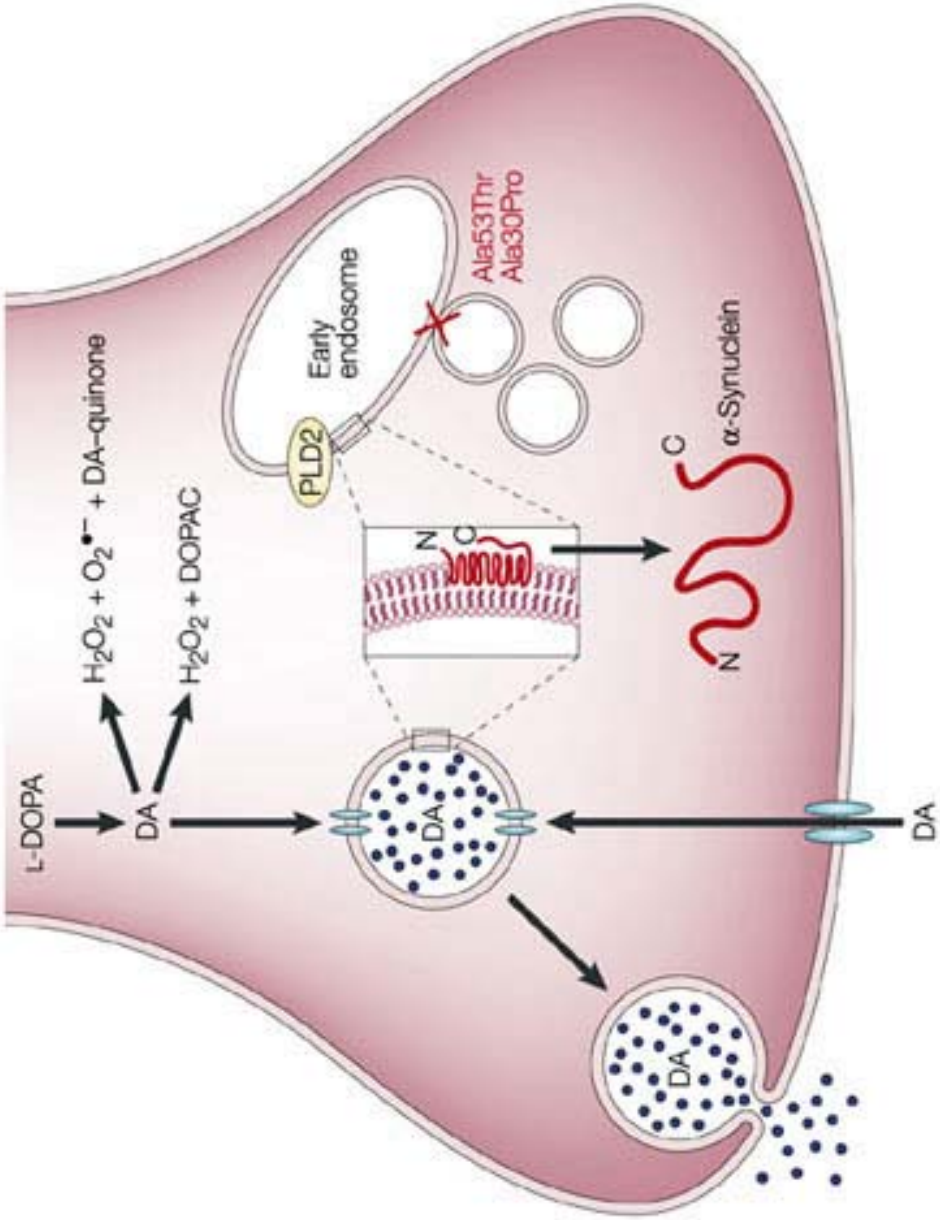
Alpha-synuclein and the ubiquitin proteasome

Impairment of efficient proteasome degradation in PD (McNaught, Olanow et al. 2001), and the existence of ubiquitinated proteins and components of the ubiquitin proteasome system in Lewy Bodies, provides evidence for a link between proteasome inhibition and PD physiology. Two genes that are involved in proteasome degradation are mutated in rare familial forms of PD. One is the parkin gene, which is associated with autosomal recessive, juvenile-onset PD. Parkin is an E3 thought to ubiquitinate α-synuclein (Kitada, Asakawa et al. 1998; Shimura, Schlossmacher et al. 2001). Another is the gene for ubiquitin C-terminal hydrolase L1 (UCHL1), which encodes a de-ubiquitinating enzyme. Malfunction of these two proteins could lead to proteasome inhibition (Tanaka, Engelender et al. 2001).

What could be the cause of proteasome inhibition in cases of PD associated with α-synuclein mutations? The following studies will explore the role of aggregated α-synuclein in the proteasome inhibition seen in PD.

Figure 2.2: Cell death caused by improper dopamine storage my be caused by alpha synuclein aggregation

The function of α-synuclein *in vivo* may be to regulate dopamine (DA) packaging at neuronal synapses through its interaction with phospholipase D2 (PLD2). Depicted here is a mechanism in which α-synuclein aggregation due to A53T or A30P mutations leads to improper DA storage, production of free radicals, and cell death (Lotharius and Brundin 2002). If free, dopamine can auto-oxidize to yield an inert metabolite (3,4-dihydroxyphenylacetic acid, DOPAC) and hydrogen peroxide (H_2O_2). Or alternatively, it may produce H_2O_2, superoxide radical (O_2^-), and a cytotoxic dopamine by-product, DA-quinone. This scenario could lead directly to cell death.


L-DOPA
DA
$H_2O_2 + O_2^{\bullet-}$ + DA–quinone
H_2O_2 + DOPAC
Early endosome
PLD2
Ala53Thr
Ala30Pro
α-Synuclein
N
C
C
N
DA
DA
DA

Materials and Methods

Substrate proteins

Protease substrates were derived from barnase, a ribonuclease from *Bacillus amyloliquefaciens* (Hartley 1975), and *E. coli* dihydrofolate reductase (eDHFR) (Rood, Laird et al. 1980). Genes for both substrates were constructed in pGEM-3Zf (+) vectors (Promega). Barnase was targeted to ClpAP by attaching a 65 amino acid extension to its N-terminus. The extension was derived from the signal sequence of cyotchrome b_2 and contained an Arg to Gly mutation at position 30 and a Leu to Pro mutation at position 62. DHFR was targeted to the proteasome by the N-end rule pathway (Varshavsky 1992). A ubiquitin moiety was attached to the N-terminus of the substrate protein via a 40-amino acid linker derived from the *E. coli* lac repressor (Johnston, Johnson et al. 1995). In reticulocyte lysate, the ubiquitin moiety is rapidly cleaved and the new N-terminus is formed at the amino acid that follows the last residue of the ubiquitin sequence. Depending on the identity of the new N-terminal amino acid, the substrate is then ubiquitinated at two lysine residues in the linker region (Bachmair and Varshavsky 1989). Radioactive proteins supplemented with [^{35}S]methionine were expressed from a T7 promoter by in vitro transcription and translation in *E. coli* S30 extract. The proteins were then partially purified by high-speed centrifugation and ammonium sulfate precipitation (Matouschek, Azem et al. 1997).

Aggregation of alpha-synuclein

Recombinant α-synuclein incubated for two months at 37 ^{o}C in phosphate-buffered saline while shaking at 800 rpm; aggregation was confirmed by performing immunoblot analysis (Snyder, Mensah et al. 2003). Performed by the Wolozin lab.

Proteasome degradation assay

Substrates were generated as described above, then resuspended in 40 μl of buffer (25% (v/v) glycerol, 25 mm $MgCl_2$, 0.25 mM Tris/HCl, pH 7.4) to which 5 μl of the *in vitro* reaction containing the radiolabeled ubiquitinated substrate protein was added with 35 μl of rabbit reticulocyte lysate (Green Hectares, containing 1 mM dithioreitol) that is ATP-depleted (Gonda, Bachmair et al. 1989). The reactions were incubated with the recombinant protein of interest or with no protein as the negative control. Initial cleavage of substrate proteins was enabled by a seven- minute incubation at 37 ^{o}C. Ubiquitination and degradation were initiated by the addition of ATP and an ATP regenerating system (0.5 mM ATP, 10mM creatine phosphate, and 0.1mg/ml creatine phospokinase; final concentrations). Reactions were incubated at 37 ^{o}C and at designated time points (0', 15', 30', 45', 60', 120', 150', and 180'), small aliquots were removed and transferred to ice-cold 5% tri-chloroacetic acid (TCA) to stop proteolysis, and the TCA-insoluble fractions were analyzed by electrophoresis on 10% SDS-polyacrylamide gels and quantified by electronic autoradiography.

ClpAP degradation assay

Purified ClpAP proteolytic digestions were performed in a total of 120 μl degradation buffer (50mM Tris-HCl [pH 8.0], 100 mM KCl, 0.02% Triton X-100, 20 mM $MgCl_2$). *In vitro* translated substrates were resuspended in 20 μl prewarmed degradation buffer, and then added to 100 μl prewarmed degradation buffer containing 4 mM ATP, 1 mM DTT, 20 mM creatine phosphate, 0.1 mg/ml creatine phosphokinase, 3 μg/ml ClpP, and 12 μg/ml ClpA. At designated time points, 10 μl samples were transferred to 100 μl ice-cold TCA to stop proteolysis. After at 10 min on ice, the proteins were pelleted by centrifugation and analyzed by electrophoresis on 10% SDS-PAGE gels and quantified by electronic autoradiography.

Results

Aggregated alpha-synuclein inhibits degradation by the proteasome

Studies conducted by our collaborators (Wolozin lab, Loyola University Medical Center, Maywood, IL) showed selective inhibition by aggregated α-synuclein of 26S proteasome but not 20S proteasome (lacking the 19S regulatory particle) in HEK 293 cells (Snyder, Mensah et al. 2003). We sought to investigate the factors governing this inhibition. We performed *in vitro* proteasome degradation assays in rabbit reticuloctye lysate (Lee, Schwartz et al. 2001) using *E. coli* DHFR (eDHFR) (Fig 2.3) with an N-end rule targeting sequence (Varshavsky 1996)as the substrate. Prior to activating proteasome degradation, the protein was incubated with 4.5 μM monomeric (native) α-synuclein, 4.5 μM aggregated α-synuclein, or no competitor. Substrate protein was then added and its degradation monitored over a three-hour time course. eDHFR lacking competitor was degraded by the proteasome as previously described (Fig 2.4a), as was eDHFR incubated with monomeric α-synuclein (Fig 2.4b). However, incubation of the eDHFR substrate incubated with aggregated α-synuclein inhibited degradation (Fig 2.5).

Figure 2.3: Structure of *E. coli* DHFR

Shown here is a cartoon depiction of wild type *E. coli* DHFR. It is a 154 amino acid protein that forms an α/β structure with an α-helix sandwiched between two β-sheets.

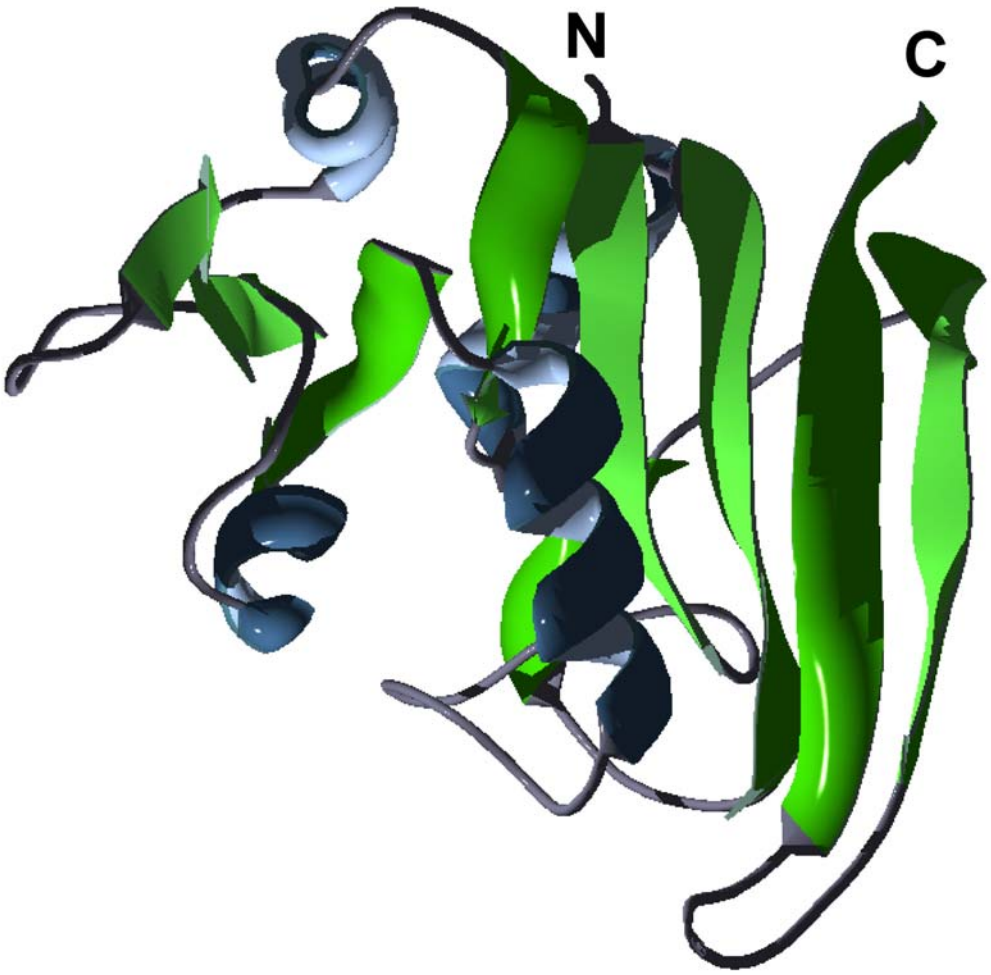
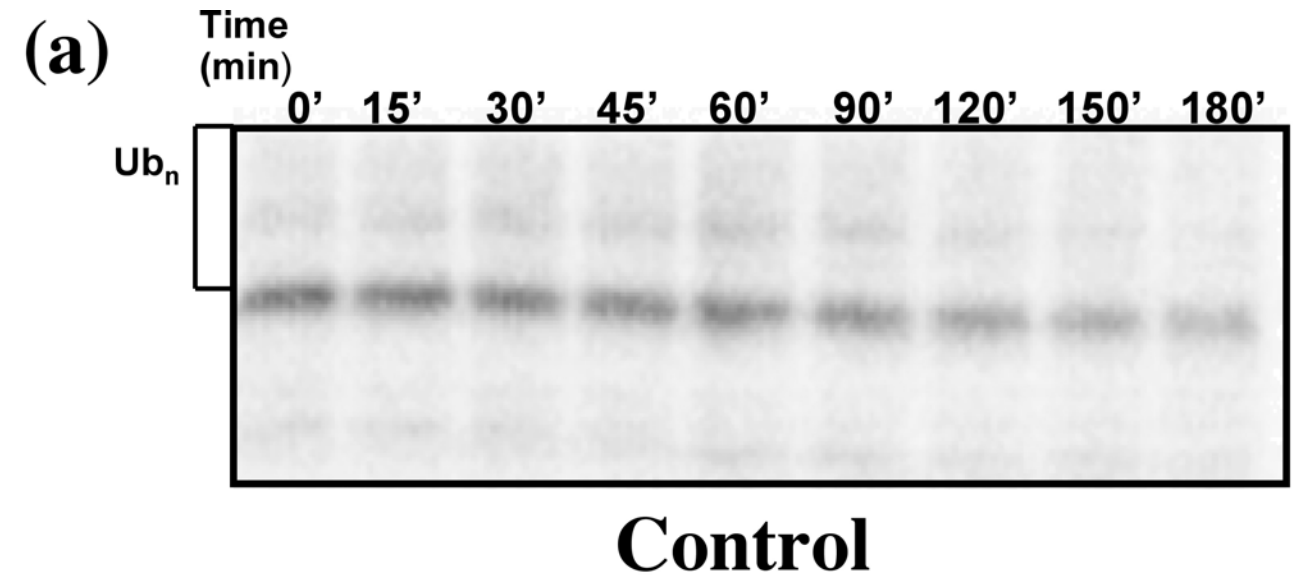
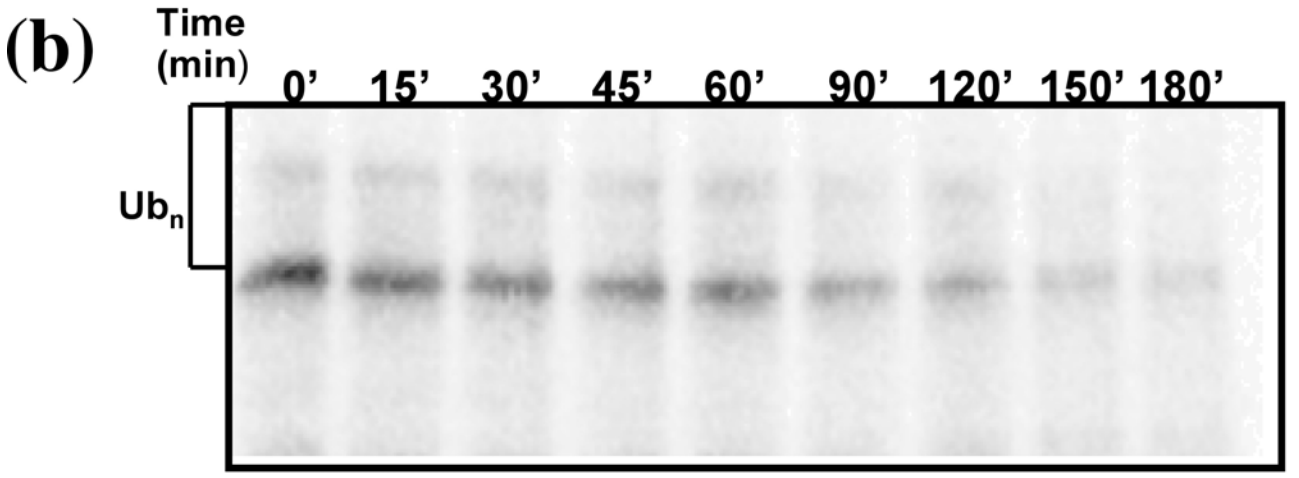

N
C

Figure2.4: Monomeric alpha-synuclein does not inhibit proteasome degradation

Shown in this figure are SDS-PAGE and autoradiography results of degradation of eDHFR when incubated with (a) no competitor or (b) 4.5 μM monomeric α-synuclein. Samples were taken at the indicated time points. In both cases, the substrate protein was degraded efficiently.



Control

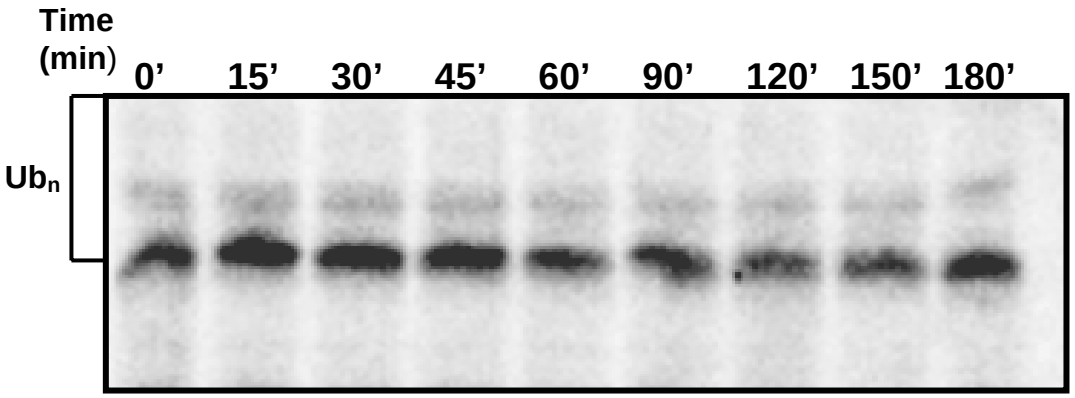


+ 4.5 μM monomeric α-synuclein

Figure 2.5: Aggregated alpha synuclein inhibits proteasome degradation

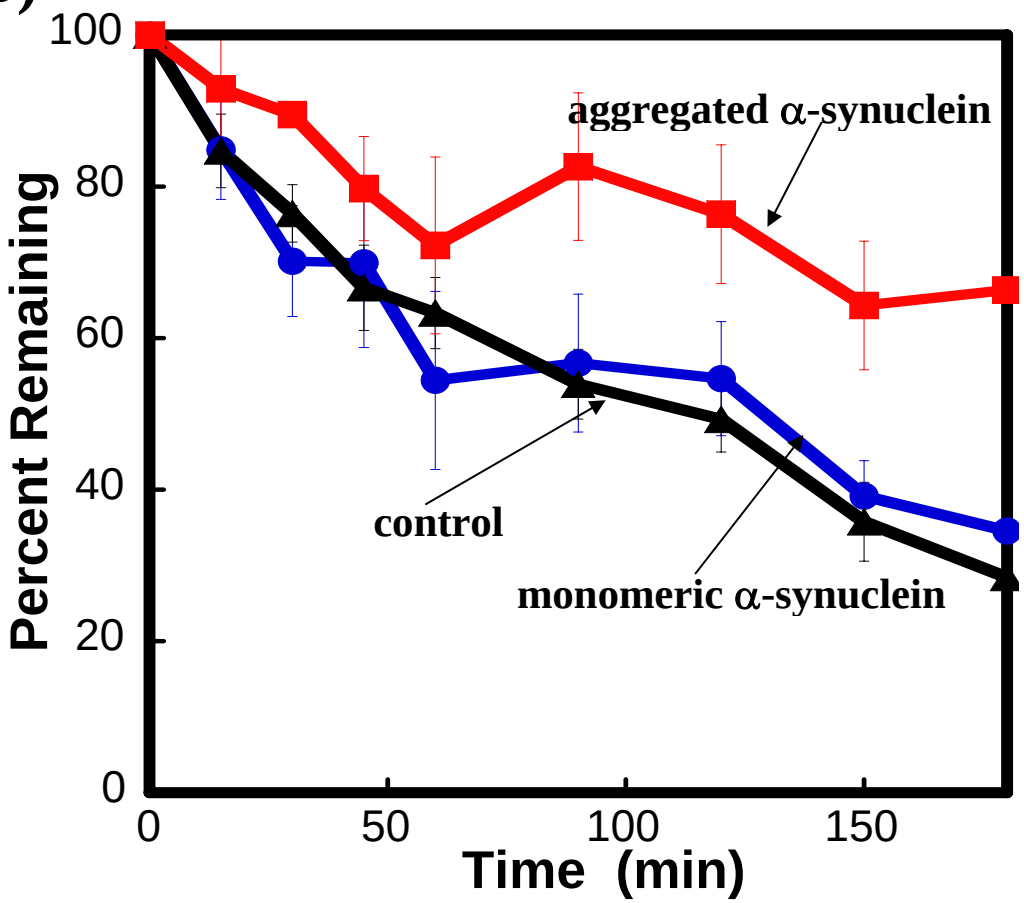
Degradation of eDHFR was inhibited when incubated with 4.5 μM aggregated α-synuclein. This is can be seen from autoradiography on the SDS-PAGE gel, in which radioactive counts from the substrate are not reduced (a). Percent degradation of eDHFR with no competitor, monomeric, or aggregated a-synuclein is represented in graphical form as a function of time (b). Error is represented as standard error.

(a)



+ 4.5 μM aggregated α-synuclein

(b)



These results are consistent with the results shown in HEK 293 cells, and suggest that the resulting inhibition is due to a direct interaction between aggregated α-synuclein and a component of the proteasome degradation machinery or targeted the substrate. This conclusion can be drawn based on the fact the substrate was not inhibited in the presence of monomeric α-synuclein.

Inhibition by aggregated alpha-synuclein is specific to proteasome degradation

The next question that we asked was, is this inhibition specific to the proteasome? To investigate this, I repeated the degradation assays, this time using ClpAP, a bacterial analog of the proteasome (Fig 1.3). The reason this protease was chosen was because of its method of targeting. Targeting to ClpAP is mediated by the presence of an unstructured tail at one terminus of the substrate protein (Wang, Hartling et al. 1997), as opposed to the ubiquitination and recognition process that is necessary for proteasome degradation. The results of this assay would allow me to determine if the inhibition that was seen previously was general to degradation by ATP-dependent proteases, or specific to the proteasome. The substrate chosen for these assays was barnase (Fig 2.6) with a 65-amino acid N-terminal targeting sequence. Degradation of barnase when incubated with 4.5 μM aggregated α-synuclein was similar to what was seen when incubated with monomeric α-synuclein or no competitor (Fig 2.7). These results show that inhibition of degradation by aggregated α-synuclein is specific to the ubiquitin proteasome system.

Monomeric beta- and gamma-synuclein do not inhibit degradation by the proteasome

We next sought to examine the effect of the other members of the synuclein family on proteasome degradation, with the thought that they might behave like α-synuclein. β-synuclein is 78% identical to α-synuclein and their expression levels in the brain appear to be inversely regulated (Rockenstein, Hansen et al. 2001). γ-synuclein is 58% identical to α-synuclein, is up-regulated in late stage breast and ovarian carcinomas, and has been implicated in stimulation of estrogen receptor-α, which can lead to tumorigenesis (Jiang, Liu et al. 2004). Although β- and γ-synuclein do not aggregate as readily, and are not found aggregated in PD inclusion bodies, we used our proteasome degradation system to see if they had an inhibitory effect.

The substrate used was a fusion protein composed of an N-terminal degradation tag, barnase, and *E. coli* DHFR. *In vitro* translated substrates were incubated with 4.5 μM α-, β-, or γ-synuclein, and degradation of the substrate was monitored. The results showed that there was no inhibition of degradation as compared to control (Fig 2.8). Therefore, only aggregated α-synuclein can inhibit degradation by the 26S proteasome. It is unknown whether aggregated β- and γ-synuclein inhibit proteasome function, but since they are not found in this form in PD, we determined this question to be irrelevant in this case.

Figure 2.6: Structure of barnase

A cartoon depiction of barnase, a ribonuclease from *Bacillus amyloliquefaciens.* It is a 110 amino acid protein that forms three α-helices followed by a five antiparallel β-sheets. It will be used with two mutations: histadine 102 to alanine (HA102) to inactivate its ribonuclease activity and glutamine 2 to methionine to allow radioactive labeling.

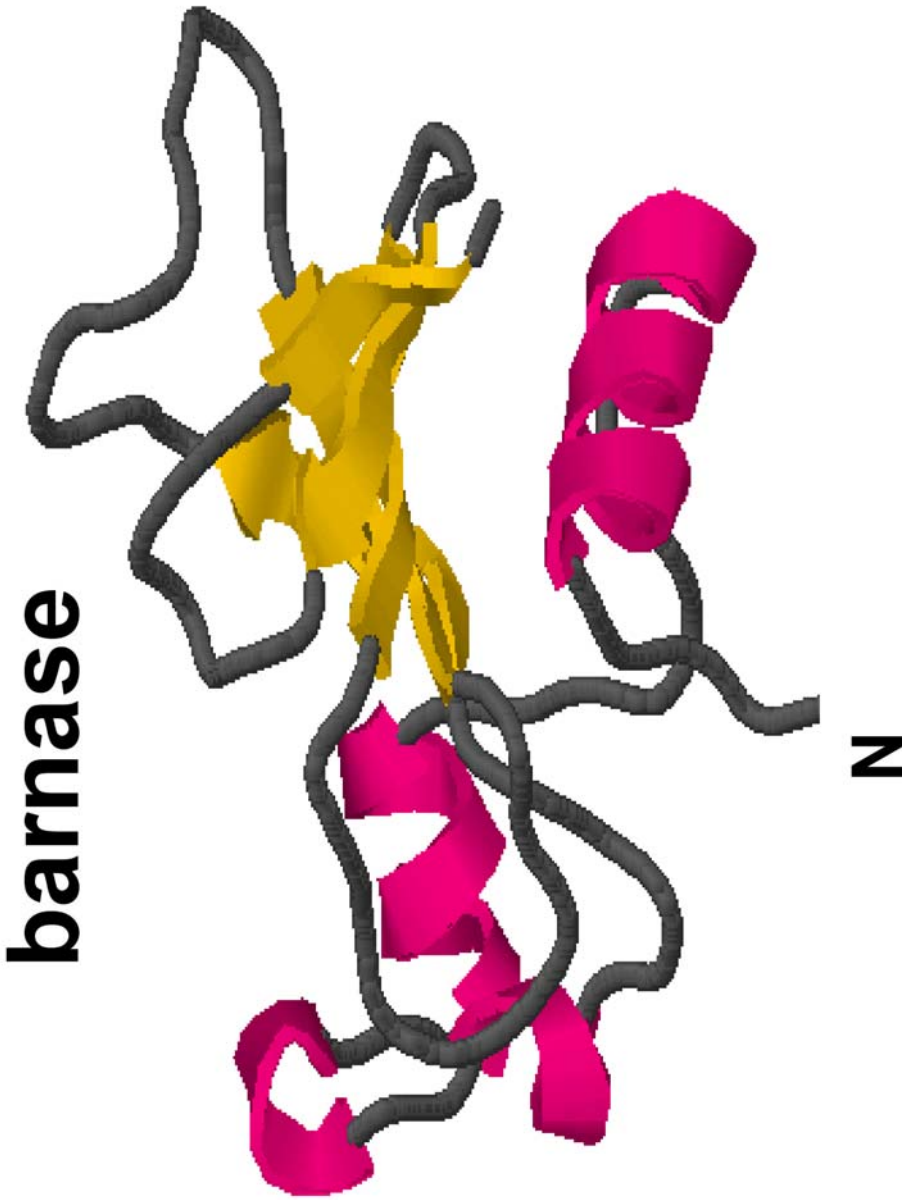

barnase
N

Figure 2.7: ClpAP degradation is not inhibited by aggregated alpha-synuclein

ClpAP degradation of barnase when incubated with no competitor, monomeric, or aggregated α-synuclein is represented as a change in percentage remaining as a function of time. Lack of inhibition is observed in all three cases. Error is represented as standard error.

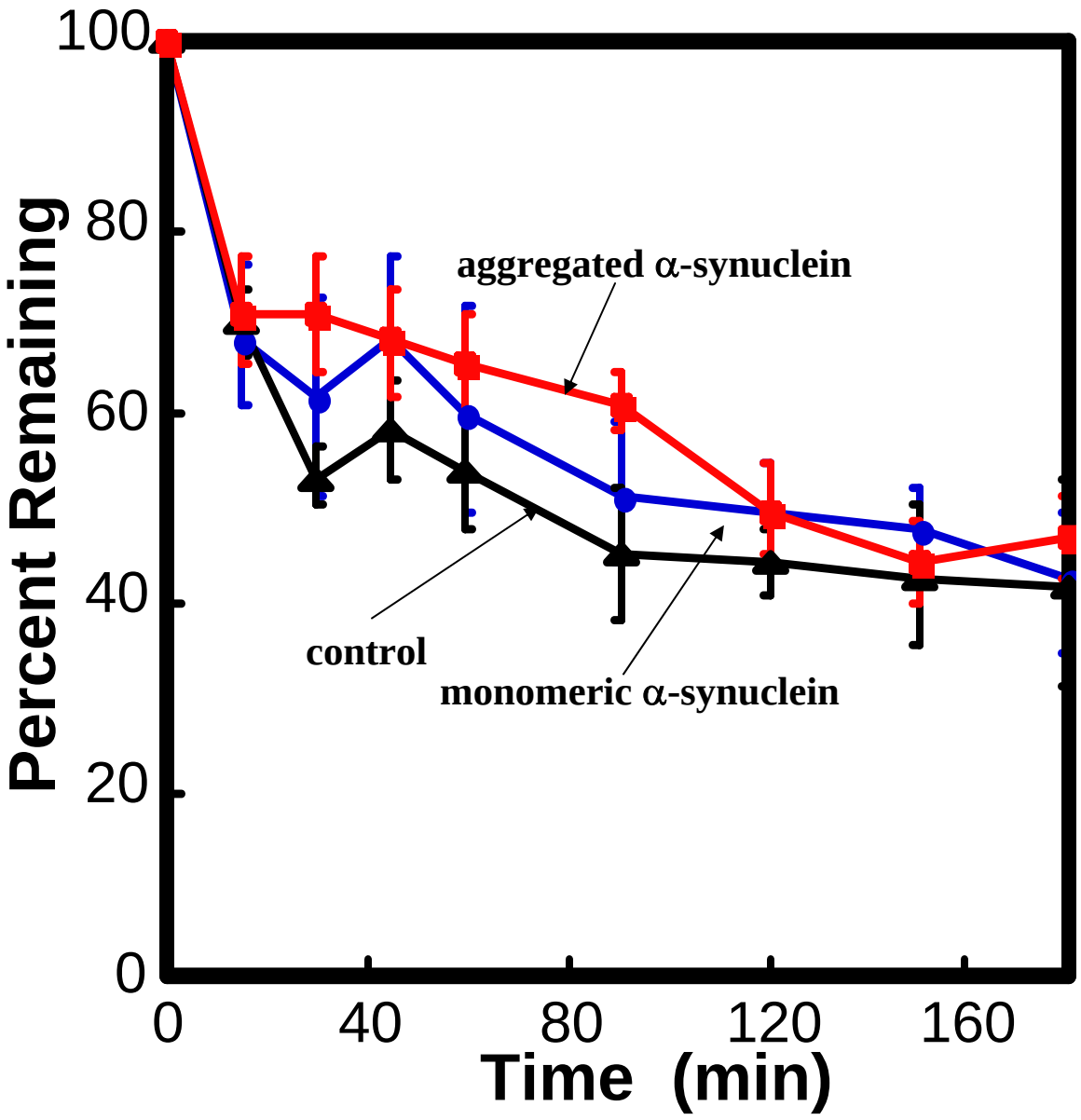
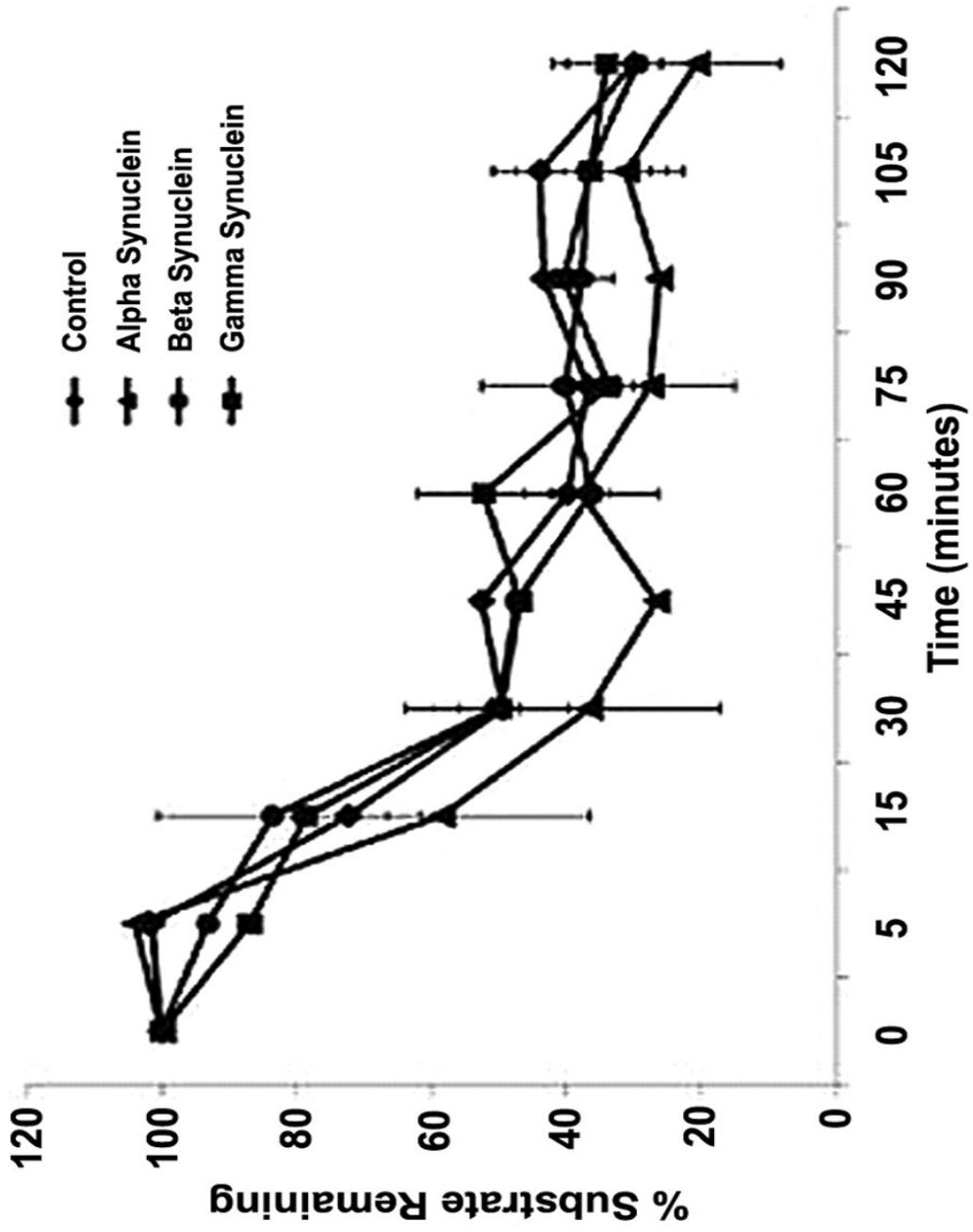

100
80
60
40
20
0
Percent Remaining
0
40
80
120
160
Time (min)
aggregated α-synuclein
control
monomeric α-synuclein

Figure 2.8: Monomeric beta- and gamma-synuclein do not inhibit proteasome degradation

Degradation of a barnase-eDHFR fusion protein incubated with monomeric α–, β-, and γ-synuclein was not inhibited. Results are depicted as a change in percent of protein remaining as a function of time (Snyder, Mensah et al. 2005). Error is represented as standard error.


Control
Alpha Synuclein
Beta Synuclein
Gamma Synuclein
% Substrate Remaining
120
100
80
60
40
20
0
0
5
15
30
45
60
75
90
105
120
Time (minutes)

Discussion

Proteasome inhibition is a hallmark of many neurodegenerative diseases. PD is no exception. Aggregation of α-synuclein, a neuronal protein, has been linked to this inhibition. Here I show that degradation of an artificial proteasome substrate can be blocked by aggregated α-synuclein, and that this inhibition is specific to proteasome degradation. The mechanism could be facilitated by the ability of aggregated α-synuclein to interact strongly the 19S cap. Studies by our collaborators showed that aggregated α-synuclein preferentially binds to the Rpt5 subunit of the base over other subunits Rpn12 and 10b (Snyder, Mensah et al. 2003). It was previously determined that Rpt5 is one of the subunits that mediates recognition of polyubiquitinated substrates (Lam, Lawson et al. 2002). The presence of aggregated α-synuclein may inhibit this activity, thus inhibiting degradation of targeted substrates. Though β- and γ-synuclein are homologous to α-synuclein, they do not aggregate and, just as α-synuclein, their monomeric forms do not inhibit degradation. Interestingly, it was shown β-synuclein can antagonize the actions of α-synuclein by binding to it and preventing its interaction with Rpt5 (Snyder, Mensah et al. 2005).

Based on the studies presented in this chapter, a model for the actions of α-synuclein in PD can be proposed. Mutations, age, or toxins can stimulate aggregation of α-synuclein. In aggregated form, it can act as a negative regulator of proteasome degradation by selectively binding Rpt5. This interaction inhibits the ability of the proteasome to recognize targeted substrates, thus inhibiting degradation (Fig 2.9). The symptoms of PD, including proteasome inhibition, occur

gradually as the disease progresses. This may correspond with the gradual accumulation of aggregated α-synuclein that mediates this process, and the gradual neurodegeneration that accompanies PD. β-synuclein can modulate the activity of aggregated α-synuclein by binding it and preventing its interaction with the 19S cap (Fig 2.10). This taken with the fact that β-synuclein does not itself inhibit proteasome activity, supports the use of β-synuclein as a potential treatment for PD and other α-synucleinopathies.

In summary, I show that α-synuclein in aggregated form is a potent inhibitor of proteasome activity. In the case of α-synucleinopathies, this can lead to severe consequences. The inability of the proteasome to function properly can impede a variety of cellular processes. This can then lead to aggregation of many other proteins, which may inevitably lead to cell death. This effect, when localized to neurons, could be the main cause of the neurodegeneration seen in PD and other α-synucleinopathies.

Figure 2.9: Model for alpha-synuclein interaction with the proteasome

This cartoon depicts α-synuclein interaction with the S6' (Rpt5) subunit of the 19S cap. In monomeric form, α-synuclein does not block Rpt5's ability to recognize targeted proteins. But in aggregated form it binds all the recognition sites to inhibit recognition and thus degradation (Snyder, Mensah et al. 2005).

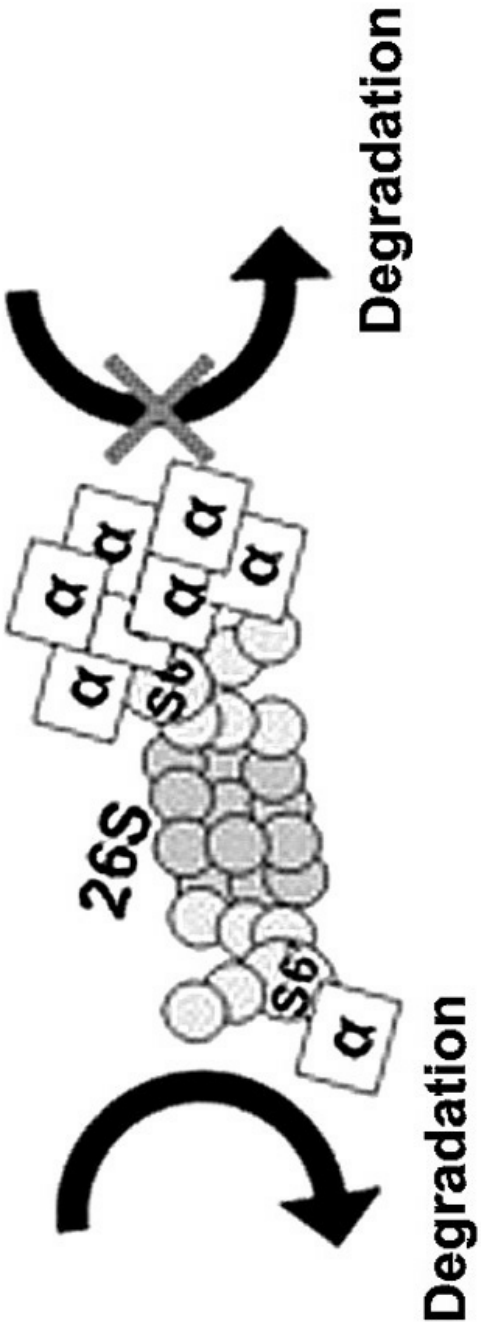
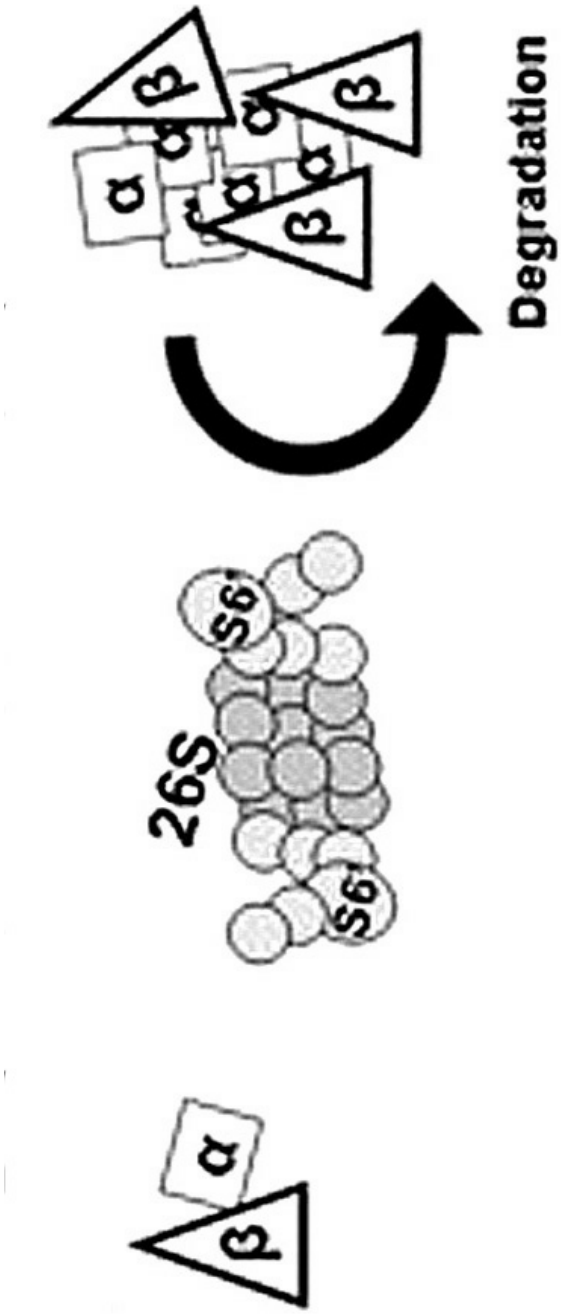

Degradation
α
α
α
α
α
α
α
Sb
26S
S6'
α
Degradation

Figure 2.10: Monomeric beta-synuclein may reduce the effect of aggregated alpha synuclein on proteasome inhibition

A cartoon depiction of β-synuclein's activity *in vivo.* It does not affect proteasome degradation on its own, but may mediate inhibition by binding aggregated α-synuclein (Snyder, Mensah et al. 2005).


α
β
26S
S6'
S6'
Degradation

Chapter 3

Proteasome Inhibition by Polyglutamine Proteins

Introduction

Neuronal protein aggregates are often phenotypic elements associated with neurodegenerative diseases. In many of these cases, components of the ubiquitin proteasome system are found in these aggregates. This implicates the involvement of the proteasome in a possible attempt at clearing them. A well-studied example of this what occurs in Huntington's Disease (HD). A mutation in huntingtin protein (htt), which causes expansion of its polyglutamine region, is the calling card of this disease. The following chapter discusses two studies investigating inhibition of proteasome function by polyglutamine proteins. The first was done in collaboration with Dr. Carina Holmberg of the Morimoto lab at Northwestern University (Evanston, IL). This study shows that expanded polyglutamine repeats can protect a folded protein domain from degradation. The second study explores how this phenomenon is governed by the context of the substrate protein in which it is housed. It shows that overall stability of the protein contributes to its ability to inhibit degradation by the proteasome. It is finally discussed that the hypotheses formulated from these result,s can apply to a possible mechanism of proteasome inhibition by huntingtin protein in HD.

Huntington's Disease

Huntington's Disease is the most common member of a family of diseases caused by expansion of CAG repeats, these repeats code for polyglutamine. There are eight known members of this family including HD, Spinal and bulbar muscular atrophy (SBMA or Kennedy's

Disease), and Spinocerebellar ataxias 1-3, 6 and 7(Gusella and MacDonald 2000). HD itself is a progressive disorder with an autosomal dominant mode of inheritance. It occurs in between three and seven per 100,000 in populations of Western European decent. Its symptoms usually begin between the ages of 35 and 50, but some juvenile cases also occur. HD progresses over 15-20 years in adults and 7-10 years in juveniles, with the symptoms often being fatal (Ho, Carmichael et al. 2001). The most common motor symptom is chorea, which is as alteration in involuntary movement and abnormal voluntary movement. Memory deficits, affective and emotional disturbances, change in personality, and overall slowing of intellectual processes accompany the motor defects (Zoghbi and Orr 2000; Ho, Carmichael et al. 2001).

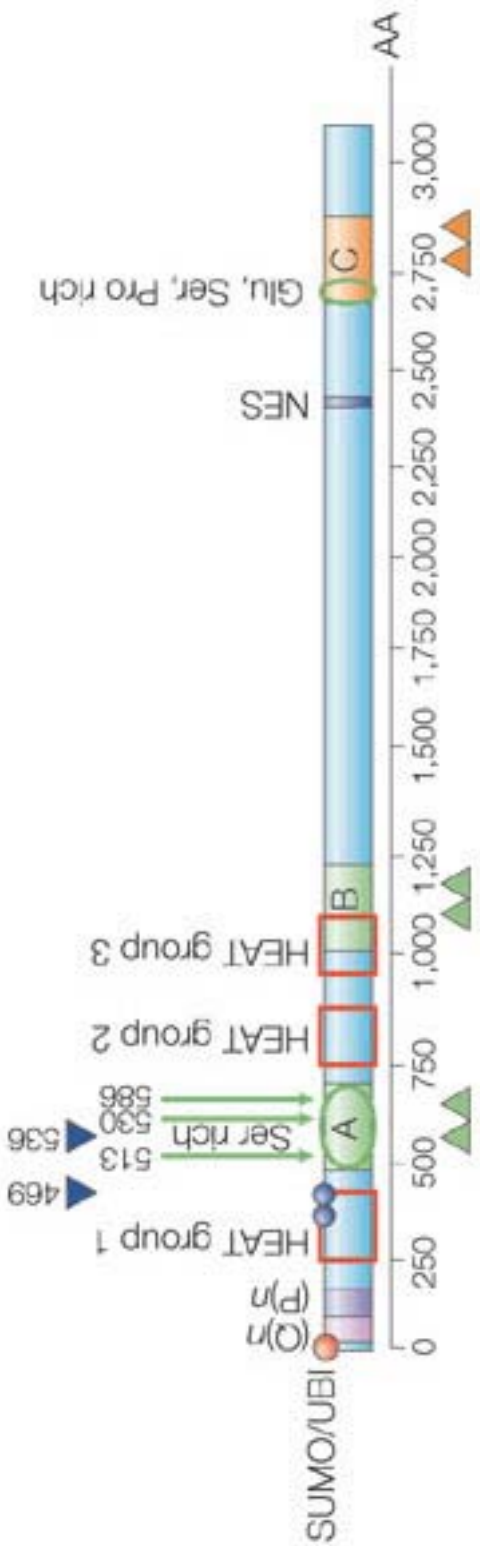
The pathological hallmark of HD is diffuse and severe atrophy of the neostriatum region of the brain. Most vulnerable to this effect are the medium-sized spiny striatal neurons containing γ-amino butyric acid (GABA), making up about 80% of the lost cells. In contrast, early onset cases of HD most often show loss of cerebellar Purkinje cells, along with general atrophy of the brain (Vonsattel, Myers et al. 1985; Vonsattel and DiFiglia 1998).

Huntingtin protein

Each polyglutamine disease has a particular protein associated with it. In the case of HD, that protein is huntingtin protein (htt). Htt is a 348 kDa protein of 3,144 amino acids found ubiquitously expressed in central nervous system neurons (Fig 3.1) (Cattaneo, Zuccato et al. 2005). The polyglutamine stretch in htt begins at the eighteenth amino acid, and wild-type protein contains up to 34 glutamine residues. In higher vertebrates, a polyproline stretch follows,

Figure 3.1: Linear representation of huntingtin protein

This diagram shows all the critical components of huntingtin protein (htt). (Q)n and (P)n indicate the polyglutaime and polyproline tracts respectively, and the red squares indicate the HEAT repeats, which are involved in protein-protein interactions. The green arrows indicate caspase cleavage sites, and the blue arrowheads indicate calpain cleavage sites, with B identifying preferential cleavage sites for the htt in the cerebral cortex, C in mainly the striatum, and A both. A is the region that is most prone to cleavage in mutated htt. The green and orange arrowheads indicate other sites of protease cleavage. NES is the nuclear export signal, the blue circles indicate ubiquitination, and the red circles indicate sumoylation and/or ubiquitination (Cattaneo, Zuccato et al. 2005).


AA
0
250
500
750
1,000
1,250
1,500
1,750
2,000
2,250
2,500
2,750
3,000
SUMO/UBI
(Q)n
(P)n
HEAT group 1
469
513
530
536
586
Ser rich
A
HEAT group 2
HEAT group 3
B
NES
Glu, Ser, Pro rich
C

which is then followed by three HEAT repeats, which are ~40 amino acids in length and are involved in protein-protein interactions (Andrade and Bork 1995; Steffan, Kazantsev et al. 2000). Htt also contains a functionally active C-terminal nuclear export signal and a less active nuclear localization signal (Xia, Lee et al. 2003). Three well-characterized protease cleavage sites (caspase, and two calpain), and also exist in htt (Goldberg, Nicholson et al. 1996). The high susceptibility of mutant htt to proteolysis and its role in protein aggregation will be discussed later in this chapter.

Htt can undergo four types of post-translational modification: phosphorylation (Hackam, Yassa et al. 2000), palmitolyation (DiFiglia, Sapp et al. 1995), ubiquitination, and sumolyation (Steffan, Agrawal et al. 2004). Lysine residues 6, 9, and 15 compete for ubiquitination and sumolyation, and may affect neurodegeneration. Studies have shown that sumolyation exacerbates neurodegeneration, whereas ubiquitination abrogates it (Steffan, Agrawal et al. 2004). Thus sumolyation may inhibit the ubiquitin proteasome system from functioning properly.

Until recently the function of huntingtin protein was virtually unknown. But palmitoylation by its co-partner, huntingtin interacting protein 14, a palmitoyl transferase, provided a clue. Palmitoylated proteins are often involved in the assembly of components that control vesicle trafficking and synaptic vesicle function (DiFiglia, Sapp et al. 1995). Gauthier *et al.* used this information to investigate the function of wild-type htt. They found that it works as a processivity factor that increases the transport efficiency of brain-derived-neurofactor (BNDF)-containing vesicles along microtubules. BNDF acts as a survival factor in the striatal neurons (Baquet, Gorski et al. 2004), and its levels of expression are decreased in the brain of HD patients (Ferrer, Goutan et al. 2000). Gauthier *et al.* proposed that BNDF transport is affected as

mutant htt, via huntingtin-associated-protein-1 (HAP1), disrupts the association of key components of the motor machinery to microtubules. Reduced BNDF transport leads to a decrease in neurotrophic support and to neurotoxicity, which could play a role in HD pathology (Gauthier, Charrin et al. 2004).

Huntingtin mutation and aggregation

Expansion of the polyglutamine region of htt is always associated with HD. Wild-type chromosomes contain 6-34 repeated glutamine codons, and disease-causing alleles have more than 36 (Zoghbi and Orr 2000). Mutation of these genes is caused by slippage. It has been shown that tandem repeats in humans and in mice (CAG-CAG vs. CAG-CAA) are highly prone to slippage during replication (Alba, Santibanez-Koref et al. 2001), thus creating an expanded polyglutamine region. Two features shared by all polyglutamine disorders are the inverse relationship between expansion length and age of disease onset, and a characteristic threshold for polyglutamine tract below which symptoms do not occur (Gusella and MacDonald 2000). Accordingly, juvenile forms of HD are associated with alleles containing more than 70 repeats, and the longest allele currently known contains 121 repeats (Zuhlke, Riess et al. 1993). Almost all patients with HD have a family history of the disease. When passed through the male germline, expansions of CAG repeats tend to occur more frequently than contractions. Thus CAG repeat numbers tend to increase in successive generations leading to a corresponding decrease in age of onset (Ranen, Stine et al. 1995). In mouse models of HD in which transgenic animals expressing mutant htt exon 1 were studied, the animals developed irregular gait, resting tremor, and occasional seizures. All of these symptoms are consistent with HD physiology in

humans (Ordway, Tallaksen-Greene et al. 1997; Zoghbi and Orr 2000). Invertebrate models of both drosophila and *C. elegans* show loss of motility and movement as well as a high instance of protein aggregation (Warrick, Paulson et al. 1998; Morley, Brignull et al. 2002). All these pathogenesis models posit that expanded CAG repeats confer a "toxic gain of function". The effects appear to be caused by abnormal huntingtin protein function. Thus the mutant protein gains a new function rather than the protein losing its function.

The major effect seen as a result of polyglutamine expansion is the propensity for mutant htt to aggregate. Biochemical analysis of synthetic polyglutamine peptides of varying length have shown that they oligomerize and adopt a β-sheet structure (Chen, Berthelier et al. 2001). Currently, the most supported hypothesis states that the β-strands tend to be linked together by hydrogen bonds between main side-chain amides, which form the links of a polar zipper (Perutz, Johnson et al. 1994). Polyglutamine expansions in htt have also been shown to associate with polyglutamine tracts of other proteins. Two examples are TATA box binding protein (TBP), a central component of the transcription initiation complex, and the transcriptional activator CREB binding protein (CBP). Mutant htt sequesters these proteins in aggregate structures, thus prohibiting proper transcriptional activity, which then contributes to neurotoxicity (Schaffar, Breuer et al. 2004). These structures are located mainly in the cell nuclei of neurons, and are often found to be ubiquitinated and associated with components of the ubiquitin proteasome system (Fig 3.2)(Kaytor and Warren 1999) as well as several molecular chaperones (Cummings, Mancini et al. 1998). This may indicate that the cell is attempting to remove these improperly folded proteins. Some studies suggest that misfolded proteins are sequestered into structures called aggresomes as a means of protection for the cell against neurotoxicity. This mechanism would compartmentalize potentially toxic polypeptides to a place where they can be

resolubilized, refolded, or degraded (Fabunmi, Wigley et al. 2000). Many other studies have suggested however, that nuclear inclusions are a result of incomplete cellular clearing. This hypothesis is what I will focus on in the studies that follow later in this chapter.

Proteasome degradation and huntingtin protein

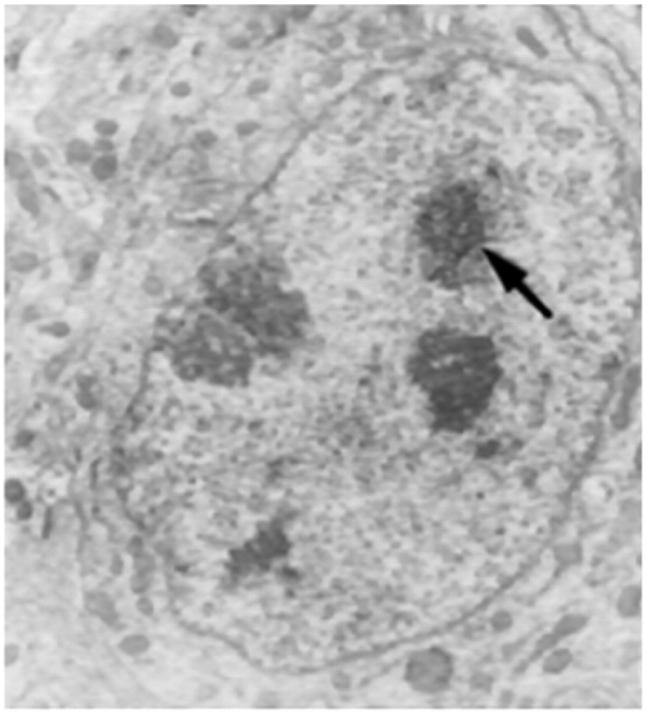
Localization of components of the ubiquitin proteasome system to HD neuronal inclusions indicates that neurons may be attempting to clear mutated htt. The fact that the aggregates persist points to the likelihood that the system is failing in some way. Several studies have shown that neuronal inclusions are largely made up of truncated htt, indicating that some proteolysis is occurring. This is likely mediated by protease cleavage, proteasome degradation, or a combination of both processes. Long polyglutamine sequences make huntingtin more susceptible to caspase-3 cleavage (Goldberg, Nicholson et al. 1996), and several studies suggest that N-terminal cleavage products of mutant htt are more toxic and more prone to aggregation than full-length protein (Lunkes, Lindenberg et al. 2002). In fact, high protein concentration and time are essential parameters for formation of htt aggregates (Scherzinger, Sittler et al. 1999). So as small pieces of fragmented htt build up over time, they reach a critical concentration in which aggregation, proteasome inhibition, and neurodegeneration occur. A breakthrough 2001 study showed direct inhibition of proteasome degradation by aggregated proteins (Bence, Sampat et al. 2001). Using an *in vitro* cell system, Bence *et al*. showed that expression of two unrelated proteins (one with expanded polyglutamine, the other a non-related folding mutant of CFTR), both with the propensity to aggregate, lead to substantial accumulation

of a proteasome-targeted substrate. This was among the first studies to provide direct link between the protein aggregation and proteasome inhibition commonly seen as a symptom of HD.

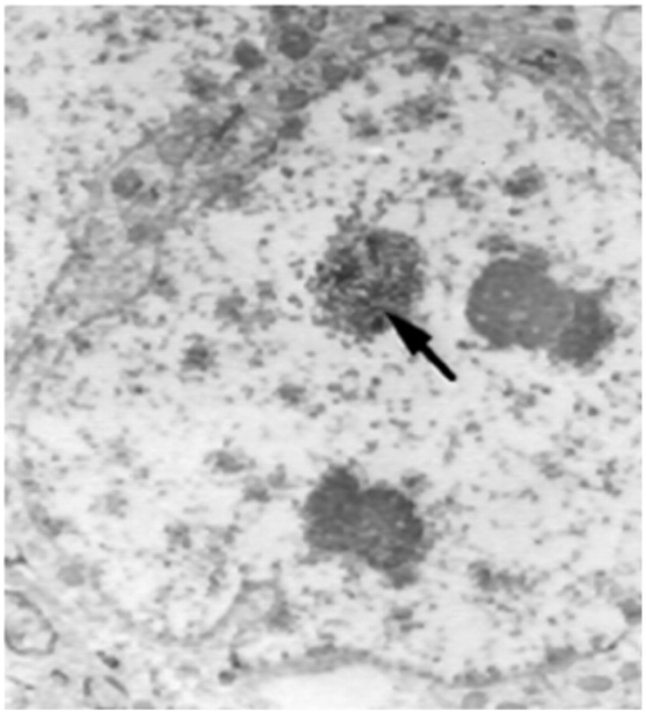
An interesting question is when does the proteasome interact with the mutated proteins? Does it attack ubiquitinated aggregates? Or does inability to degrade the mutants when unaggregated lead to their sequestration into aggregate structures? In a study done in collaboration with the Morimoto lab (Northwestern University, Evanston, IL), we were able to show that the proteasome co-localized with aggregated htt and polyglutamine proteins, and that this aggregation and co-localization only occurred when the polyglutamine tract was above the disease threshold (Fig 3.3) (Holmberg, Staniszewski et al. 2004). These results indicate that the proteasome may attempt to clear mutated htt. The studies discussed in this chapter will explore the mechanism that governs this inhibition.

Figure 3.2: Localization of huntingtin and ubiquitin to neuronal inclusions of HD mice

Electron micrograph localization of (a) htt (stained with anti-htt) and (b) ubiquitin (stained with anti-ubiquitin) in HD neurons of transgenic mice. Both are often found in HD neuronal inclusions (Kaytor and Warren 1999).



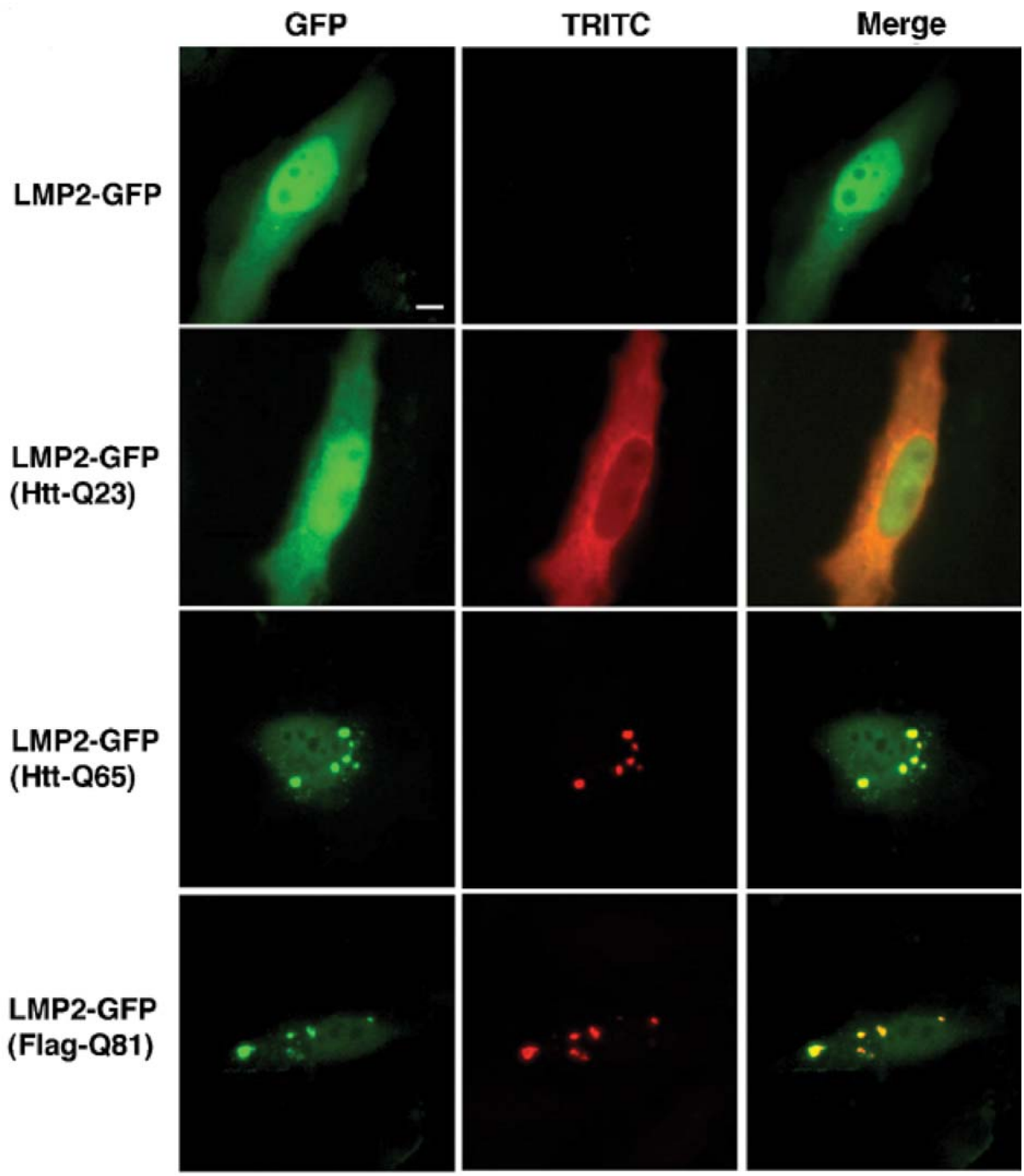
(b)



(a)

Figure 3.3: The proteasome co-localizes with aggregated htt in HEK 293 cells

The proteasome subunit LMP2 was tagged with GFP and overexpressed in HEK 293 cells either by itself, or in combination with httQ23, httQ65, or Q81 peptide. Htt proteins were detected using HP-1 antibody, which recognizes a.a. 80-113 of the protein. Q81 peptide was detected using an antibody against the FLAG epitope. Primary antibodies were detected using TRITC-conjugated anti-rabbit antibody. Results show proteasome co-localization with aggregated htt in this cellular system (Holmberg, Staniszewski et al. 2004).


GFP
TRITC
Merge
LMP2-GFP
LMP2-GFP
(Htt-Q23)
LMP2-GFP
(Htt-Q65)
LMP2-GFP
(Flag-Q81)

Materials and Methods

Substrate Proteins

Protease substrates were derived from barnase, a ribonuclease from *Bacillus amyloliquefaciens* (Hartley 1975), *E. coli* di-hydrofolate reductase (eDHFR) (Rood, Laird et al. 1980) and mouse DHFR (mDHFR). DNA clones for all substrates were constructed in pGEM-3Zf (+) vectors (Promega). Degradation from the N-terminus was mediated by targeting to the proteasome via N-end rule pathway (Varshavsky 1992). A ubiquitin moiety was attached to the N-terminus of the substrate protein via a 40-amino acid linker derived from the *E. coli* lac repressor (Johnston, Johnson et al. 1995). In reticulocyte lysate, the ubiquitin moiety is rapidly cleaved and the new N-terminus is formed at the amino acid that follows the last residue of the ubiquitin sequence. Depending on the identity of the new N-terminal amino acid, the substrate is then ubiquitinated at two lysine residues in the linker region (Bachmair and Varshavsky 1989). The C-terminal ubiquitination signal consisted of amino acids 352-646 of the NfκB precursor protein, p105 (Orian, Gonen et al. 2000). The $(Ub)_4$ degradation signal was constructed by connecting four ubiquitins through their N- and C-termini. The members of the ubiquitin concatamer contain G76V mutations to prevent their deconjugation. The signal was then directly attached to the N-terminus of eDHFR, followed by Q53 or a polyproline region. Polyglutamine constructs for N- and C-terminal degradations were created by inserting Q40, Q73, and Q82, isolated by PCR from pEYFP-N1-Q40 and pEYFP-N1-Q82 (Holmberg, Staniszewski et al. 2004), between barnase and DHFR. Polyproline constructs were created by inserting six repeats of

CCTCCTCCACCTCCGCCA between barnase and DHFR. Huntingtin exon 1 constructs were created by inserting httQ20 or httQ83, isolated by PCR from GST-httQ20 and GST-httQ83 (Scherzinger, Sittler et al. 1999), between barnase and DHFR. Radioactive proteins supplemented with [^{35}S]methionine were expressed from a T7 promoter by *in vitro* transcription and translation in rabbit reticulocyte lysate. The proteins were then partially purified by high-speed centrifugation and ammonium sulfate precipitation (Matouschek, Azem et al. 1997).

Proteasome degradation assay

Substrates were generated as described above, then resuspended in 20 µl of degradation buffer (25% (v/v) glycerol, 25 mm $MgCl_2$, 0.25 mM Tris/HCl, pH 7.4) for N-terminal targeting, and 7 µl processing buffer (240 mM Tris/HCl, pH 7.9, 1.2 M KCl, 100mM $MgCl_2$) for C-terminal targeting. The substrates were then supplemented with 1mM DTT, 20 mg/ml ubiquitin, and 100 µM methionine. 68 µl (N-terminal) or 130 µl (C-terminal) of rabbit reticulocyte lysate (Green Hectares, containing 1 mM dithioreitol) that is ATP-depleted (Gonda, Bachmair et al. 1989) was then added. Initial cleavage of substrate proteins was enabled by three-minute incubation at 30 ^{o}C. Ubiquitination and degradation were initiated by the addition of ATP and an ATP regenerating system (0.5 mM ATP, 10mM creatine phosphate, and 0.1mg/ml creatine phospokinase; final concentrations). Reactions were incubated at 30 ^{o}C and at designated time points, small aliquots were removed and transferred SDS-PAGE sample buffer to stop the reaction. The samples were then analyzed by electrophoresis on 10% SDS-polyacrylamide gels and quantified by electronic autoradiography.

Results

Polyglutamine proteins are partially degraded by the proteasome

To investigate whether proteins containing polyglutamine repeats could be degraded by the proteasome, *in vitro* proteasome degradation assays were conducted using substrates created by our collaborators. These proteins were targeted for degradation by a N-end rule tag, followed by YFP, Q19-YFP, Q40-YFP, or Q82-YFP. The results of these assays provided an interesting result. Disappearance of the full-length substrate was observed, and total degradation of the substrate lacking poly-Q, but degradation of the substrates containing poly-Q yielded a fragment corresponding to the size of a protein slightly larger than YFP and increased over time (Fig 3.4) (Holmberg, Staniszewski et al. 2004). Since degradation occurs sequentially (Lee, Schwartz et al. 2001), these results indicate that polyglutamine can protect an adjacent downstream domain from degradation.

We next investigated whether this effect could be replicated using a different substrate protein. The degradation assays were repeated, placing Q40 or Q73 before eDHFR in the direction of degradation (Fig 3.5a). The results from the N-terminus were similar to what was seen with YFP (Fig 3.6). The protein domain directly following the polyglutamine region was protected from degradation. It has been shown that degradation efficiency from the N- and C-termini can differ (Prakash, Tian et al. 2004), so we investigated whether this protective effect was seen in degradation from the C-terminus (Fig 3.5b). Again, the results were similar to what was previously seen in that eDHFR was protected from degradation (Fig 3.7). Originally these

Figure 3.4: Polyglutamine protects YFP from degradation *in vitro*

SDS-PAGE and autoradiography of the results of *in vitro* degradation shows that polyglutamine causes fragment formation indicative of partial degradation. YFP was efficiently degraded when no polyglutamine was placed upstream of it (Holmberg, Staniszewski et al. 2004). The arrowheads indicate full-length substrate, and the arrows indicate the truncated fragment. The T lane is a sample taken prior to starting the assay.

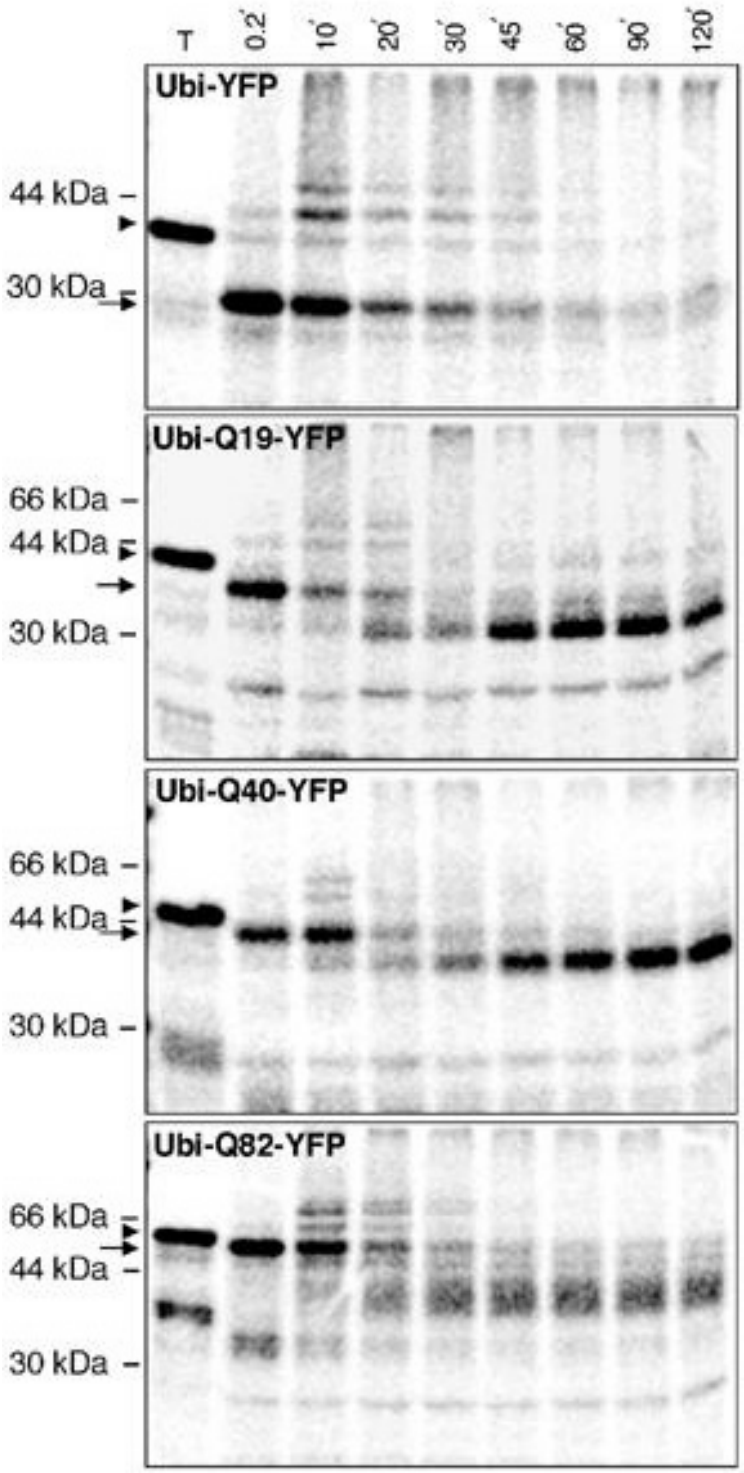

T
0.2'
10'
20'
30'
45'
60'
90'
120'
Ubi-YFP
44 kDa
30 kDa
Ubi-Q19-YFP
66 kDa
44 kDa
30 kDa
Ubi-Q40-YFP
66 kDa
44 kDa
30 kDa
Ubi-Q82-YFP
66 kDa
44 kDa
30 kDa

Figure 3.5: **Linear representation of polyglutamine substrate constructs**

N- and C-terminal tagged substrates that were used in these studies. (a) shows the N-end-rule targeted substrate, and (b) shows targeting by an ~200 a.a. region of p105 The blue folded domain represents DHFR, the main protein domain that will be monitored.

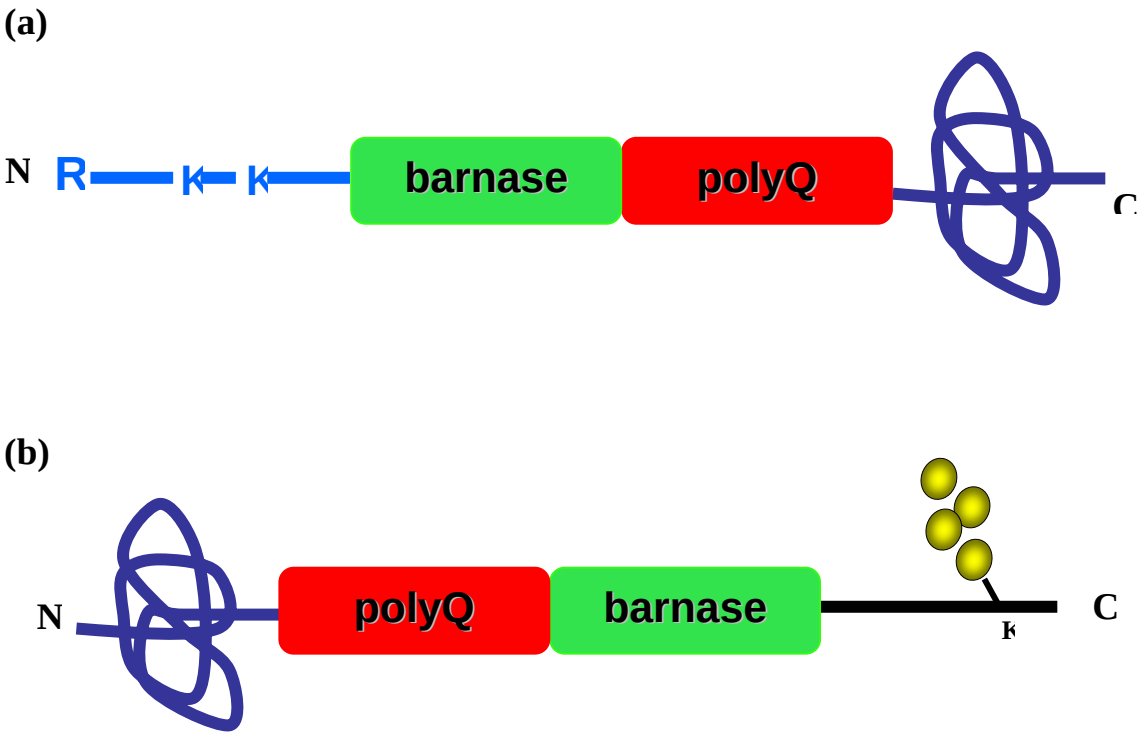
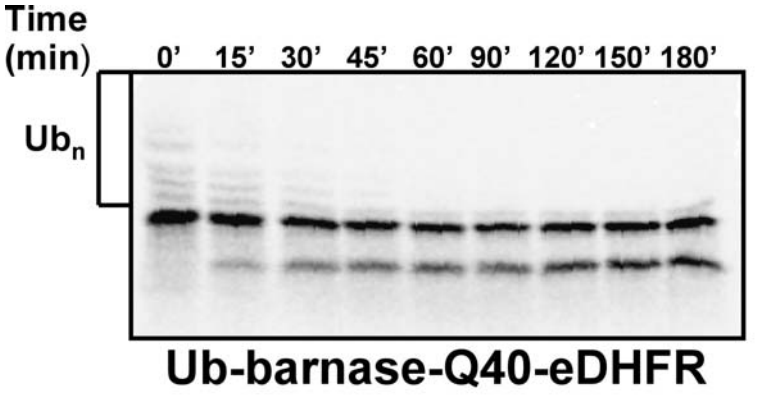
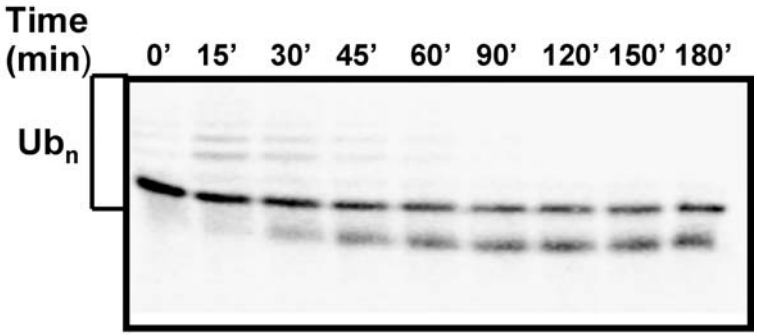

(a)
N
R
barnase
polyQ
C
(b)
N
polyQ
barnase
C

Figure 3.6: Polyglutamine inhibits proteasome degradation from the N-terminus

SDS-PAGE and autodradiography results of degradation of polyglutamine proteins. Samples were taken at the indicated time points. A fragment forms due to Q40 and Q73. Graph represents percent degradation and fragment formation. Error is represented as standard error.



Ub-barnase-Q40-eDHFR



Ub-barnase-Q73-eDHFR

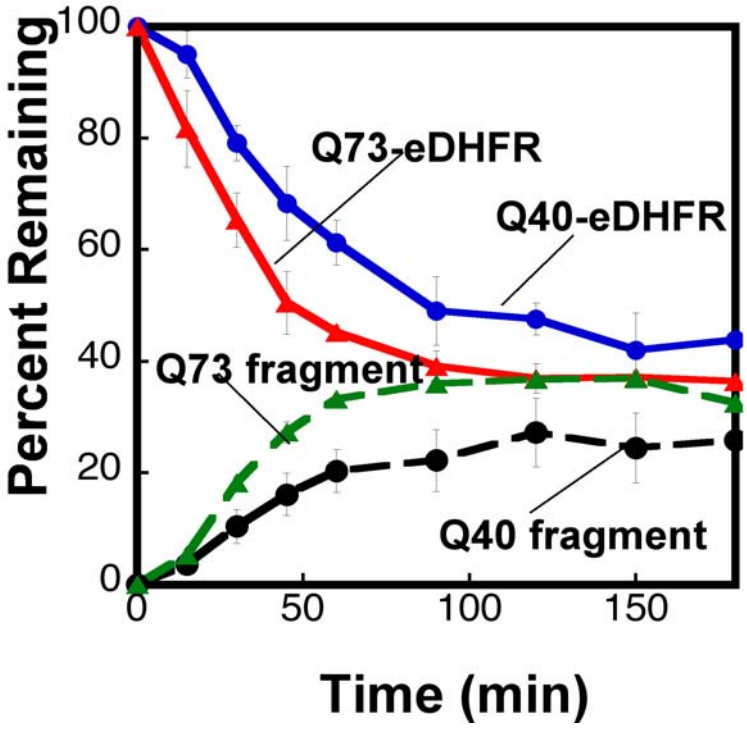
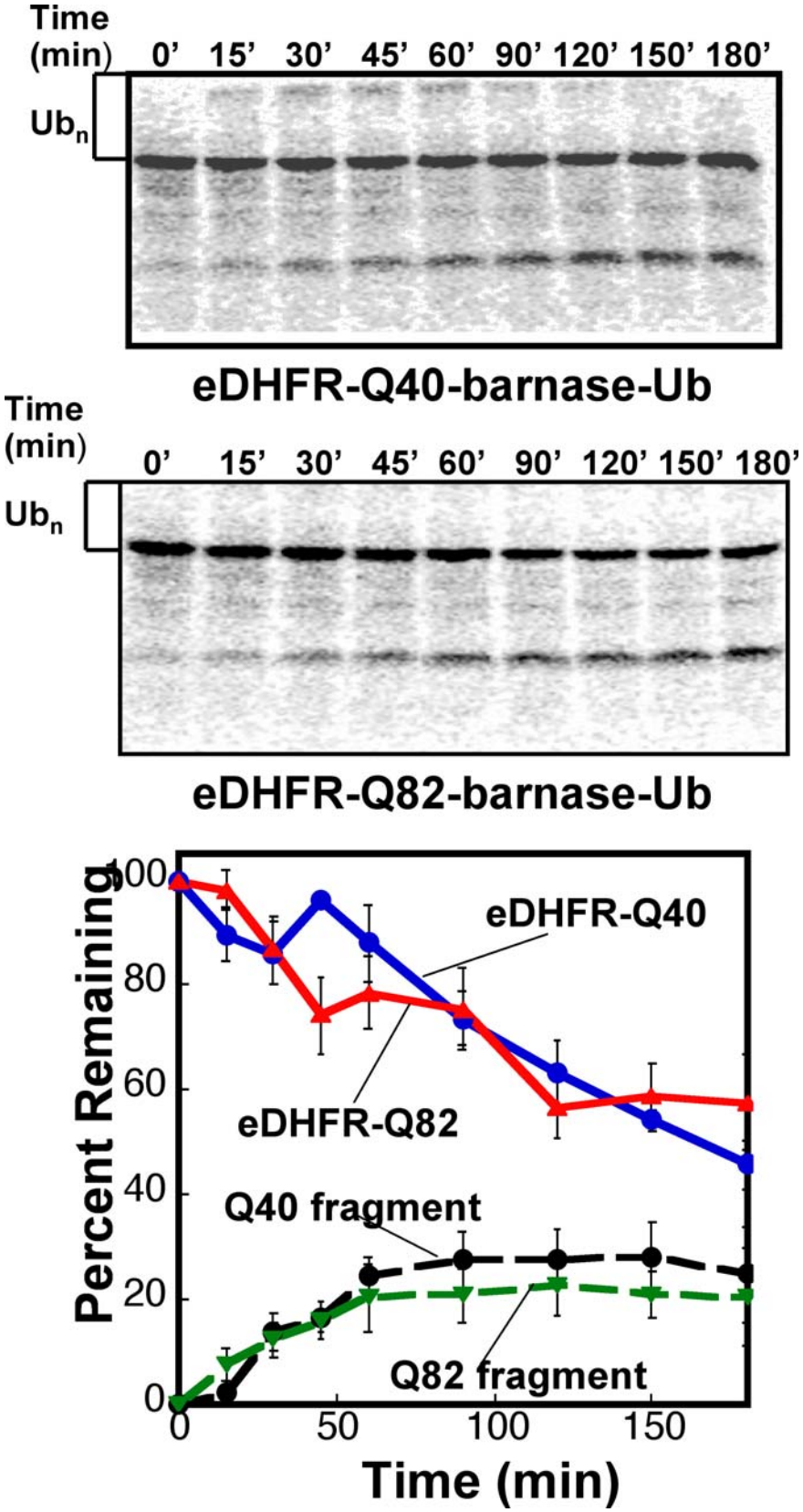


Figure 3.7: Polyglutamine inhibits degradation from the C-terminus

SDS-PAGE and autodradiography results of degradation of polyglutamine proteins. A fragment forms due to Q40 and Q82. Samples were taken at the indicated time points. The effect is identical to what was shown from the N-terminus. Graph represents percent degradation and fragment formation. Error is represented as standard error.


Time (min)
0’ 15’ 30’ 45’ 60’ 90’ 120’ 150’ 180’
Ub$_n$
eDHFR-Q40-barnase-Ub
Time (min)
0’ 15’ 30’ 45’ 60’ 90’ 120’ 150’ 180’
Ub$_n$
eDHFR-Q82-barnase-Ub
Percent Remaining
100
80
60
40
20
0
0
50
100
150
Time (min)
eDHFR-Q40
eDHFR-Q82
Q40 fragment
Q82 fragment

assays were performed using fusion proteins that did not contain a folded domain upstream of the polyglutamine domain. In looking at the results of these assays, it was difficult to determine whether a fragment was forming. This was due to the small difference in size between the full-length proteins and the truncated versions. Therefore, the barnase domain was inserted to better visualize fragment formation. Gel filtration performed of the Q40 fusion protein, and it eluted as would be expected for its size. These results indicated that the protein was not aggregated in the assay system.

Stability of the protein domain that follows polyglutamine affects its protective ability

The initial results of my studies indicated that polyglutamine repeats could inhibit progression of the proteasome. The next question asked was is this mechanism mediated solely by polyglutamine? Does the protein context in which the repeat is housed play a role? To answer these questions, degradation assays were performed in which Q40 or Q82 was placed before a less stable form of DHFR. For the N-terminal degradations a circular permutant of eDHFR named CP P25 was used (Fig 3.8). This was mutant was created by connecting the N- and C-termini of eDHFR with a linker consisting of six glycine residues, and creating new N- and C-termini by clipping the protein at Pro25 (Iwakura, Nakamura et al. 2000). This protein is ~1.7 kcal/mol less stable against unfolding than eDHFR (Lee, Schwartz et al. 2001). Previous studies in our lab showed that the ease of which the proteasome degrades a protein depends on its stability against unfolding (Lee, Schwartz et al. 2001), so any inhibition would indicate a polyglutamine driven effect. For C-terminal degradation, mDHFR was used, which is also ~1.7 kcal/mol less stable against unfolding than eDHFR (Lee, Schwartz et al. 2001) (Fig 3.9).

The results that followed were very intriguing. Replacement of eDHFR with a less stable form of the protein eliminated the protective ability of polyglutamine. This held true for degradation from the N-terminus (Fig 3.10) and from the C-terminus (Fig 3.11). These results indicate that the ability of polyglutamine to inhibit degradation is not mediated merely by its presence alone; it seems that a second component is needed. For full inhibition, a polyglutamine region must be followed by a domain that is stable against degradation. These results mirror those of Tian et al in their studies of proteasome inhibition due to other low complexity domains (Tian, Holmgren et al. 2005).

Polyproline repeat regions inhibit proteasome degradation

To begin investigation of what type of protein domain in htt could serve as the second component necessary to inhibit proteasome progression, first its amino acid sequence was looked at closely (Fig 3.12). Directly C-terminal to the polyglutamine region is a region composed almost entirely of proline residues. Studies observing degradation by immunoproteasomes for MHC Class I presentation showed that cleavage between proline residues is rare. (Nussbaum, Dick et al. 1998; Holzhutter, Frommel et al. 1999). So it was a possibility that polyproline could serve as the second component. Degradation assays were repeated, replacing polyglutamine with polyproline. Degradation from the N-terminus provided the same effect as was seen with polyglutamine, protection of eDHFR and degradation of CP P25 (Fig 3.13). Results of degradation from the C-terminus were somewhat surprising. When polyproline was placed upstream of eDHFR, there was no protection of the folded domain, just as was seen when mDHFR was placed downstream of polyproline (Fig 3.14).

Figure 3.8: Structure of CP P25 DHFR

Cartoon depiction of a circular permutant of *E. coli* DHFR. A polyglycine linker connects the N- and C-termini of wild type DHFR, and a new N- and C-termini is created at Pro 25 (Iwakura and Nakamura 1998).

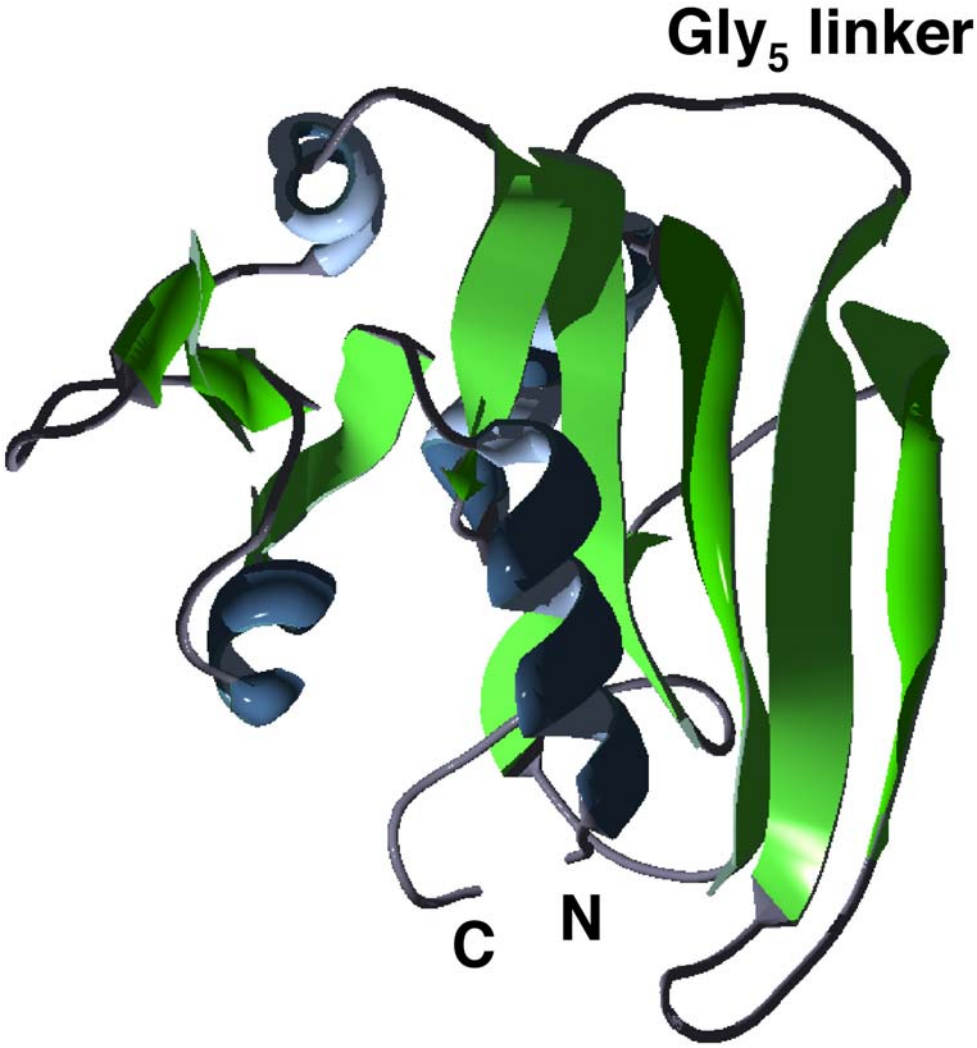
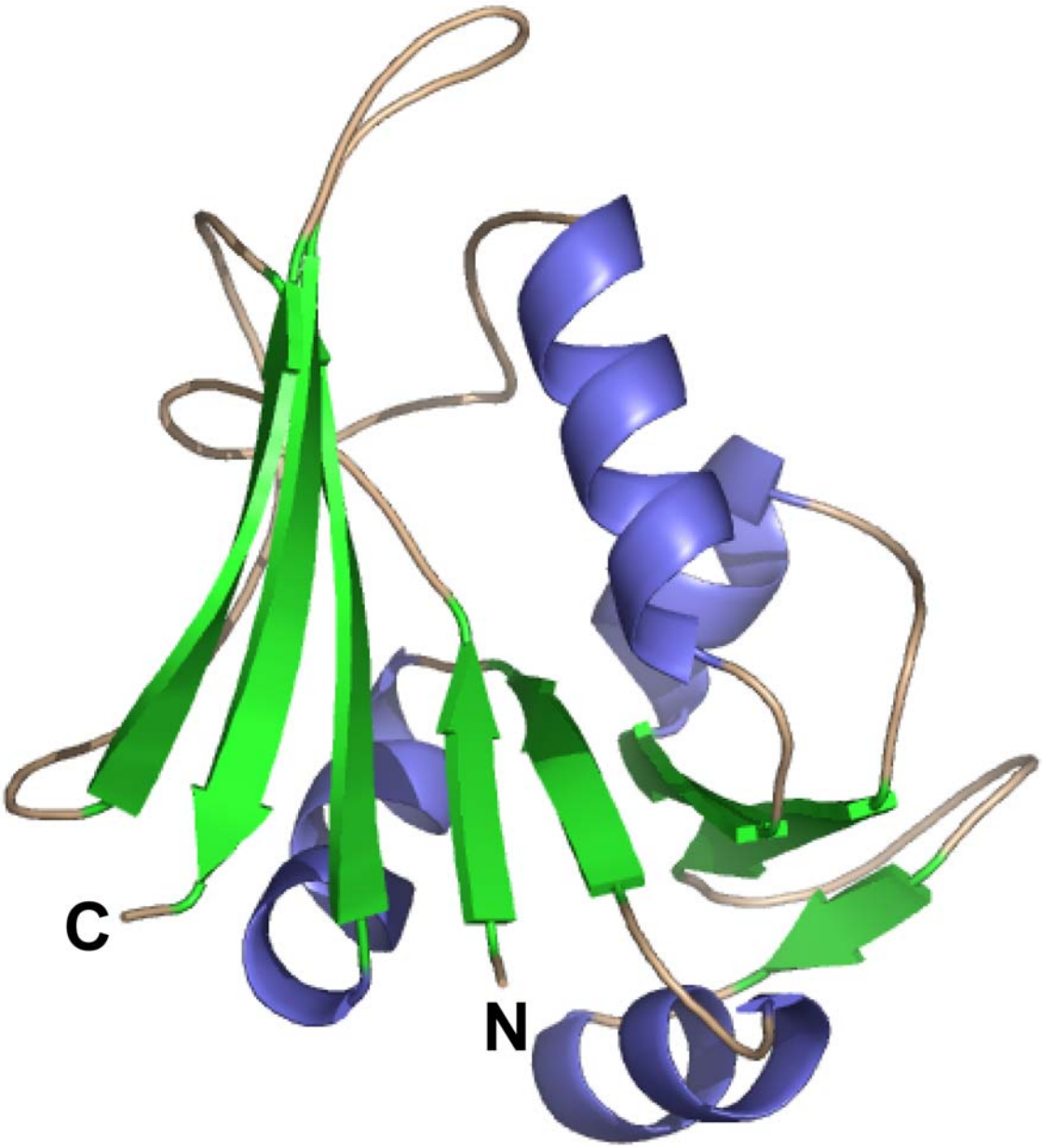

Gly5 linker
C
N

Figure 3.9: Structure of mouse DHFR

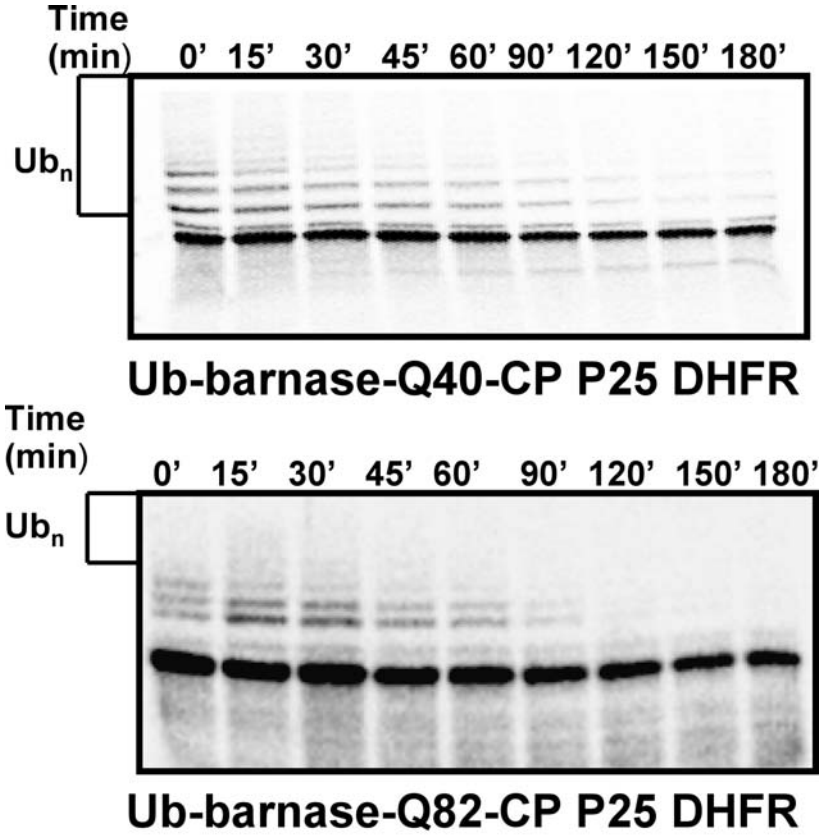
Cartoon depiction of DHFR from mouse. This 173 amino acid version was used as a destabilized protein domain for studies looking at degradation of polyglutamine proteins from the C-terminus.


C
N

.

Figure 3.10: **Polyglutamine does not protect a destabilized protein domain when degraded from the N-terminus**

SDS-PAGE and autodradiography analysis of polyglutamine degradation from the N-terminus in combination with a destabilized protein domain. Samples were taken at the indicated time points. Results show no accumulation of fragment resulting from Q40 and Q82. Graphical representation of results is shown as well. Error is represented as standard error.


Time
(min)
0' 15' 30' 45' 60' 90' 120' 150' 180'
Ub$_n$
Ub-barnase-Q40-CP P25 DHFR
Time
(min)
0' 15' 30' 45' 60' 90' 120' 150' 180'
Ub$_n$
Ub-barnase-Q82-CP P25 DHFR

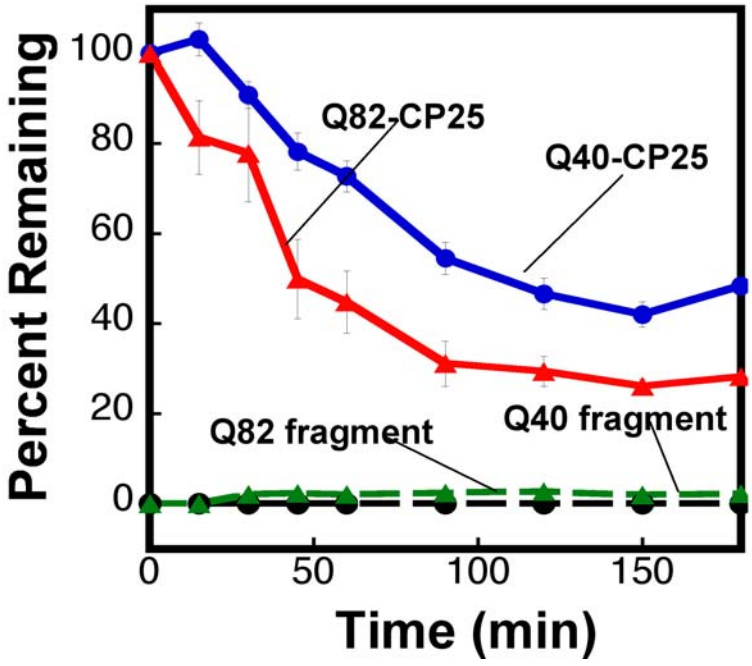
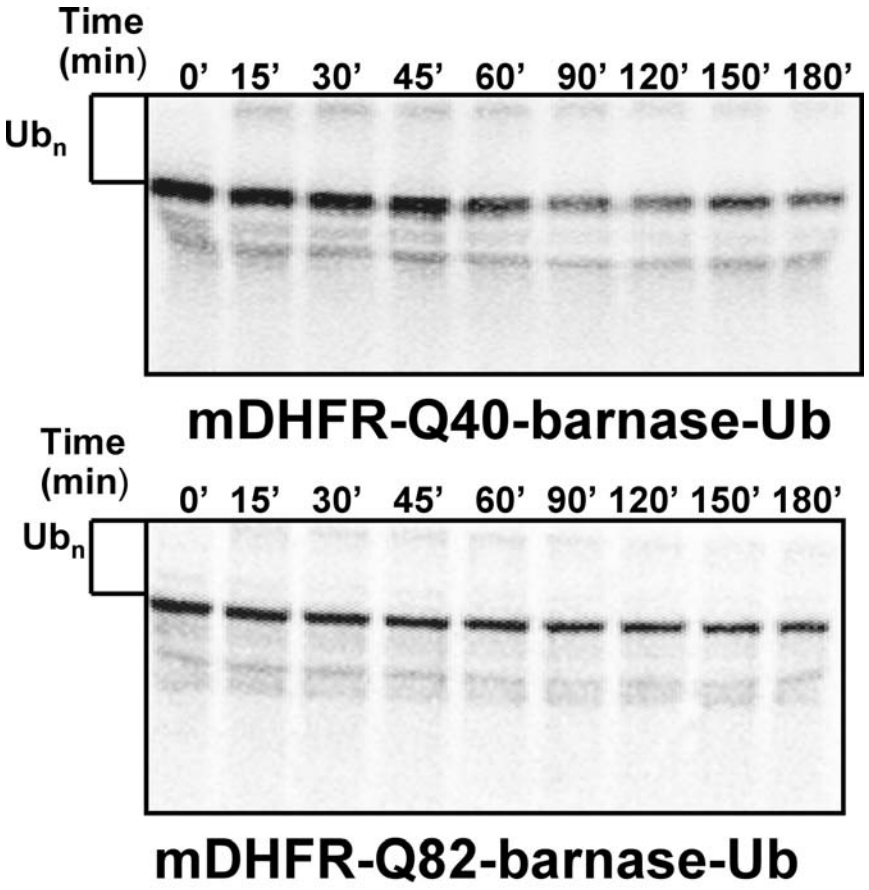
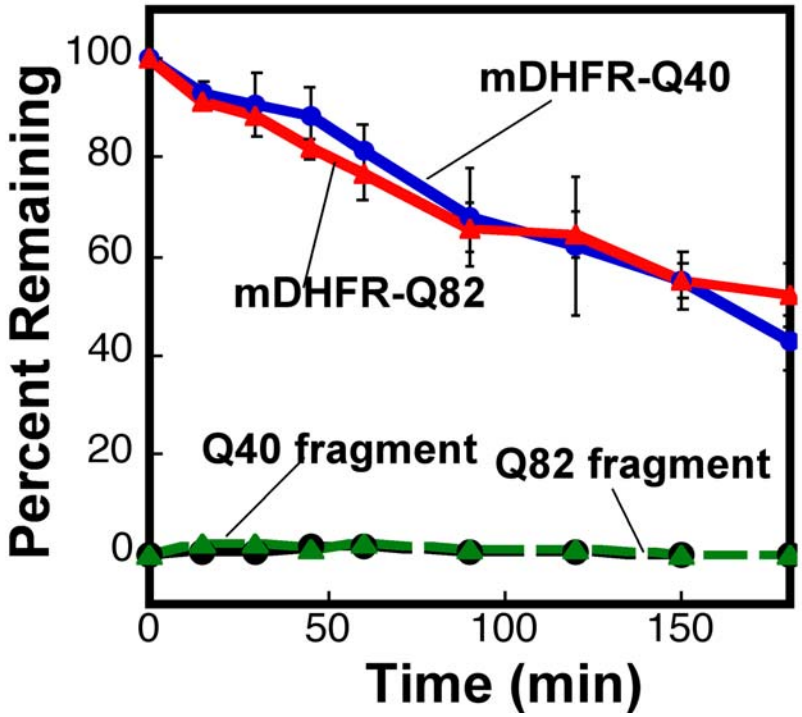

Percent Remaining
100
80
60
40
20
0
Q82-CP25
Q40-CP25
Q82 fragment
Q40 fragment
0
50
100
150
Time (min)

Figure 3.11: Polyglutamine does not protect a destabilized protein domain when degraded from the C-terminus

SDS-PAGE and autodradiography results of degradation of polyglutamine proteins. Samples were taken at the indicated time points. When in combination with a destabilized protein domain, polyglutamine cannot inhibit degradation. Gels show no accumulation of fragment and results are represented graphically. Error is represented as standard error.


Time (min)
0' 15' 30' 45' 60' 90' 120' 150' 180'
Ub$_n$
mDHFR-Q40-barnase-Ub
Time (min)
0' 15' 30' 45' 60' 90' 120' 150' 180'
Ub$_n$
mDHFR-Q82-barnase-Ub


Percent Remaining
100
80
60
40
20
0
mDHFR-Q40
mDHFR-Q82
Q40 fragment
Q82 fragment
0
50
100
150
Time (min)

This result was somewhat surprising, so the stability of mDHFR was assessed by binding it to its ligand methotrexate (mtx). If stable, binding to mtx would protect it from degradation. The protein was degraded efficiently, so it was determined that mDHFR was destabilized. To counter this destabilization, a 20-amino acid linker was placed between PRR and mDHFR. Previous studies have shown that placement of low-complexity domains in close proximity to a folded protein domain can cause destabilization (Lin Tian, personal communtcation). However, placing a linker between the two regions did not result in inhibition of degradation. Therefore it was determined that this effect is most likely due to differences in degradation efficiency from the C-terminus vs. the N-terminus, or an effect specific to polyproline degradation from the C-terminus. Though there was some disparity in uniformity of the effect, these results show that polyproline can inhibit proteasome degradation.

The combination of polyglutamine and polyproline protects destabilized DHFR from degradation

So far, results have indicated that polyglutamine and polyproline can both inhibit proteasome degradation when in combination with a stable protein domain. In htt these two domains sit next to each other. In combination, would they protect a destabilized DHFR from degradation? To test this hypothesis, a protein region that mimicked htt exon 1 was created (Fig 3.15). When Q40/PRR or Q68/PRR was placed upstream of CP P25, degradation was inhibited and slight protection of CP P25 was observed, as ~5-7% fragment accumulated (Fig 3.16). These results indicate that the combination of polyglutamine and polyproline can mediate an inhibitory effect on proteasome degradation. It was next investigated whether this effect could

be replicated using htt exon 1. As was mentioned above, polyproline is downstream from polyglutamine in htt coming from its N-terminus. So the polyglutamine/polyproline region used in previous degradation assays was replaced with htt exon 1 containing either 20 or 83 glutamines. Its ability to protect CP P25 was then observed. Human htt exon 1 DNA was graciously donated by the Wanker lab (Max-Planck Institute, Berlin, Germany). No protection was observed with httQ20, which was expected because 20 glutamines is below disease threshold. However, degradation of httQ83 did not cause inhibition, even though this large number of glutamine residues is above the disease threshold for HD (Fig 3.17). Therefore, though inhibition was observed when using an artificial combination of polyglutamine and polyproline, this combination of low complexity regions is most likely does not inhibit progression of the proteasome in vivo. Initiation of degradation is inhibited by polyglutamine and polyproline

Truncated fragments of htt found in HD neuronal inclusions are protected from disposal by proteasome degradation. It was hypothesized that this inhibition is mediated by the inability of the proteasome to progress due to the presence of polyglutamine followed by polyproline. Results showed that though this mechanism was somewhat effective using an artificial combination of the two regions, this effect was not to reproducable using htt exon 1. So what is another possible cause for proteasome inhibition in HD? It may be due to inability to initiate degradation of htt. Previous studies have shown that two signals are necessary for efficient proteasome degradation; the substrate protein must have at least four ubiquitin moieties attached to it, and it also must have an unstructured region that can allow for initiation of degradation (Prakash, Tian et al. 2004).

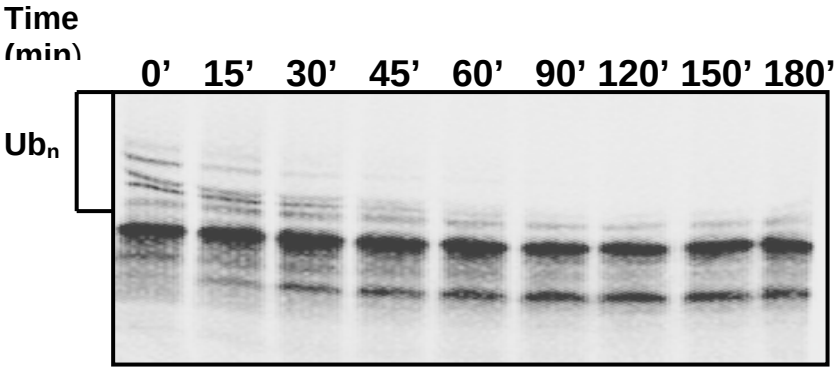
Figure 3.12: Amino acid sequence of huntingtin exon 1

Wild type human huntingtin exon 1. The polyglutamine region is represented in red and the polyproline region in blue. This portion of htt is what is found aggregated in HD neuronal inclusions.

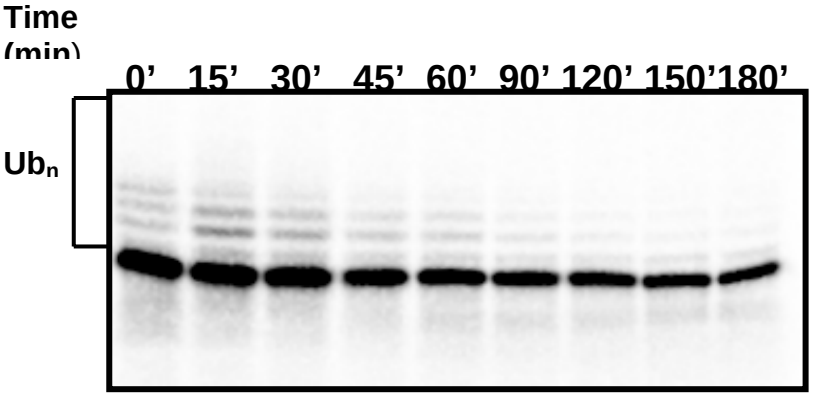
Huntingtin exon 1

MATLEKLMKAFESLKSFQQQ
QQQQQQQQQQQQQQQQQQQQ
QQPPPPPPPPPPPPQLPQPPP
QQPLLPQPQPPPPPPPPPPPG
PAVAEEPLHRPKKELSATKKD

Figure 3.13: Polyproline behaves like polyglutamine when degraded from the N-terminus SDS-PAGE and autodradiography analysis. Samples were taken at the indicated time points Results of proteasome degradation show that polyproline causes inhibition when in combination with a stabilized protein domain, and causes no effect when in combination with a destabilized protein domain. Error is represented as standard error.



Ub-barnase-PRR-eDHFR



Ub-barnase-PRR-CP P25 DHFR

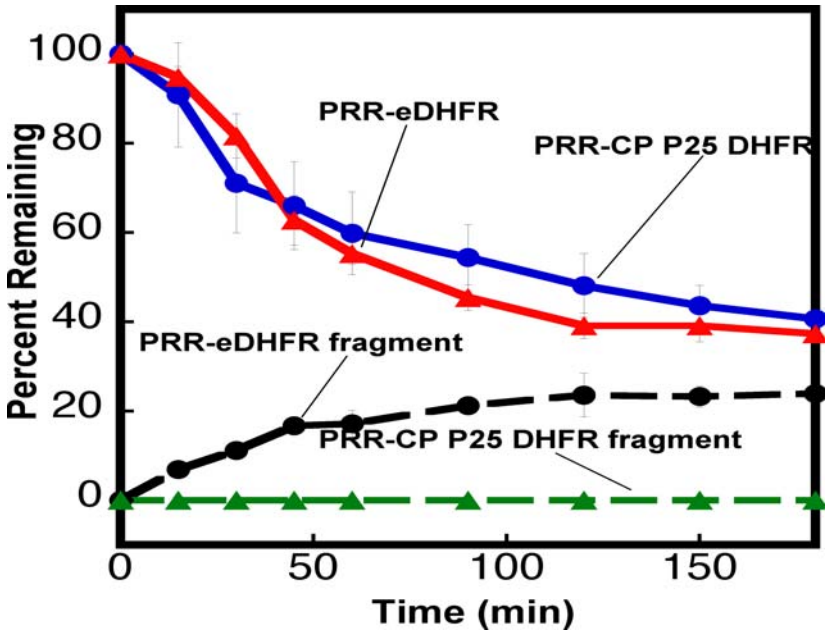


Figure 3.14: Polyproline does not inhibit degradation from the C-terminus

SDS-PAGE and autodradiography results. Samples were taken at the indicated time points Results show no inhibition by polyproline even in combination with a stabilized protein domain. Gels show no accumulation of fragment, and the graph depicts percent degradation. Error is represented as standard error.

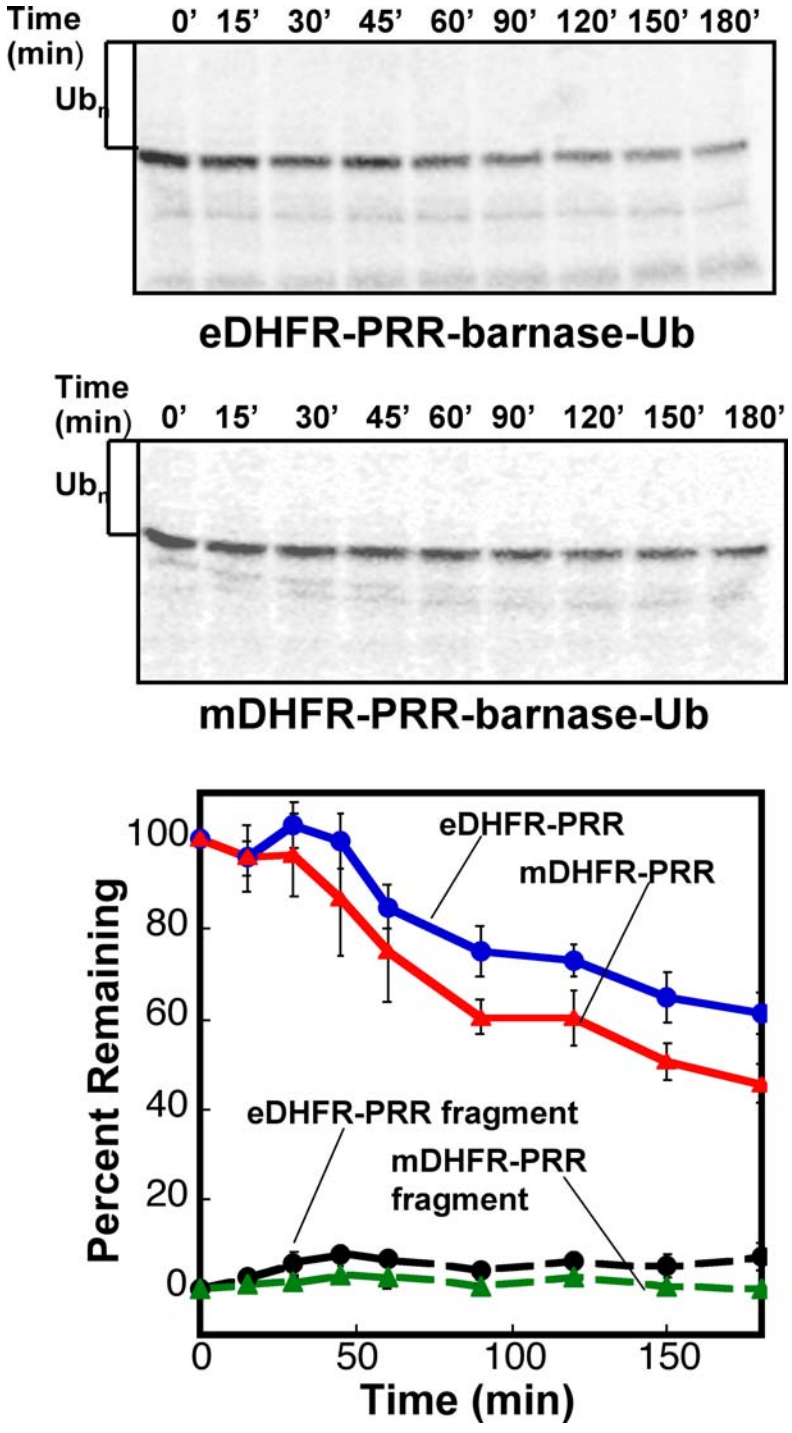

Time (min)
0' 15' 30' 45' 60' 90' 120' 150' 180'
Ub$_n$
eDHFR-PRR-barnase-Ub
Time (min)
0' 15' 30' 45' 60' 90' 120' 150' 180'
Ub$_n$
mDHFR-PRR-barnase-Ub
Percent Remaining
100
80
60
40
20
0
0
50
100
150
Time (min)
eDHFR-PRR
mDHFR-PRR
eDHFR-PRR fragment
mDHFR-PRR fragment

Figure 3.15: Amino acid sequence of experimental polyglutamine/polyproline region

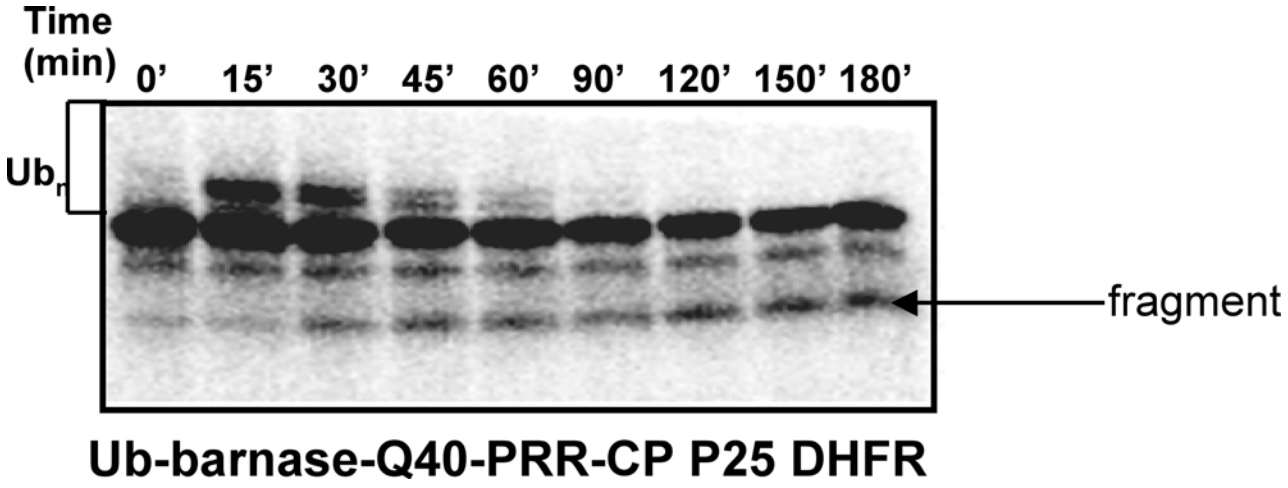
Amino acid sequence of low-complexity combination that was used for degradation assays. Shown here with 40 glutamine residues in red, and 36 proline residues in blue.

Poly-Q/Poly-P

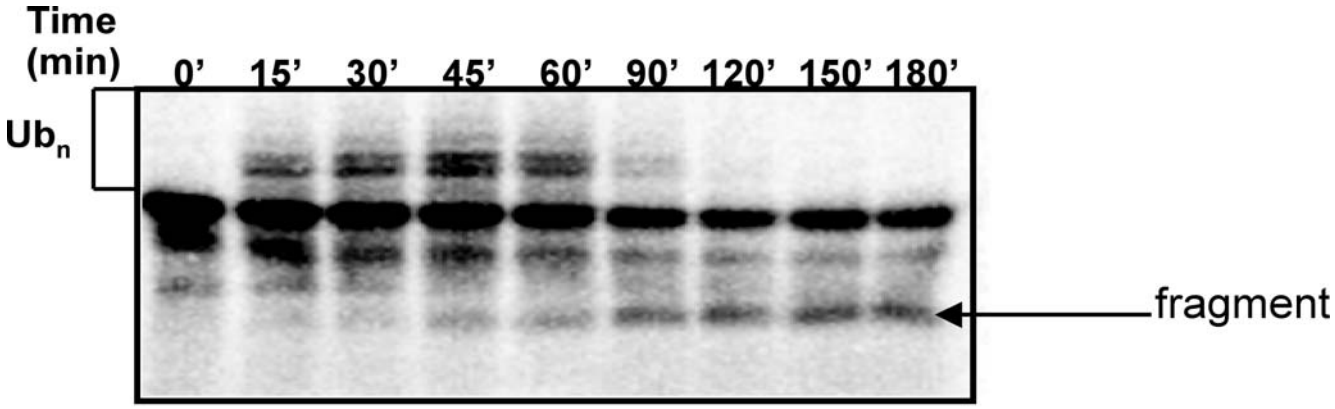
PRHHHHQQQQQQQQQQQQQQQ
QQQQQQQQQQQQQQQQQQQQ
QQQQQHHGTTTDAYQTFTKIRPR
RSPPPPPPGSPPPPPPGSPPPPP
PGSPPPPPPGSPPPPPPGSPPPP
PPGSRS

Figure 3.16: Combination of polyglutamine and polyproline protects a destabilized protein domain from degradation

SDS-PAGE and autodradiography analysis of results of degradation assays with Q40-PRR and Q68-PRR show small accumulation of fragment as indicated by the arrows. Samples were taken at indicated time points.



Ub-barnase-Q40-PRR-CP P25 DHFR



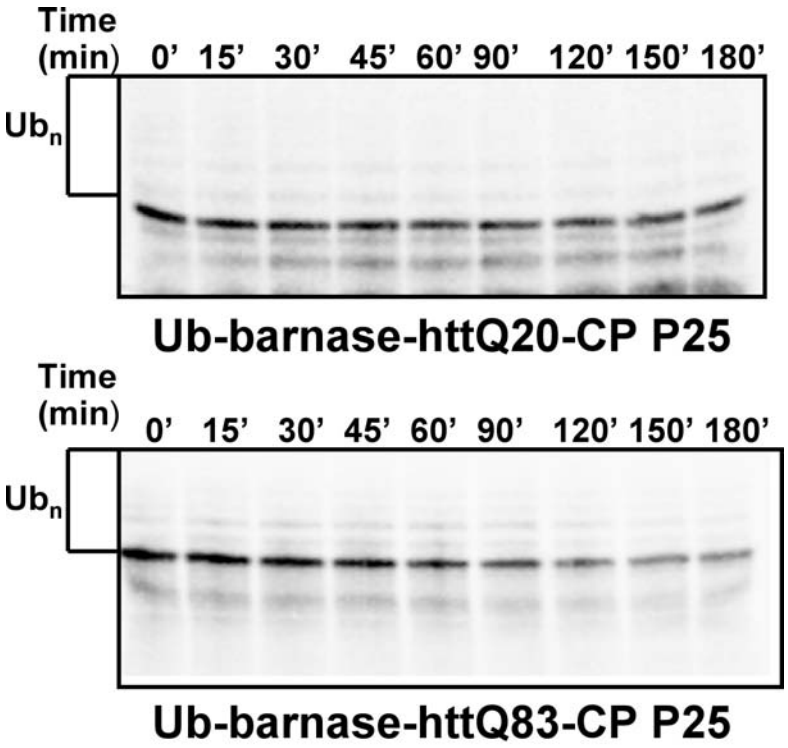
Ub-barnase-Q68-PRR-CP P25 DHFR

In HD truncated versions of htt exist in affected neurons. It would be logical to think that these fragments could be ubiquitinated and targeted for degradation. To address this issue, polyglutamine and polyproline tracts were investigated as initiation sites for degradation. eDHFR was targeted to the proteasome by the fusion of four ubiquitins to its N-terminus, and followed by either Q53 or a 36-amino acid polyproline region. A 35-amino acid region from cytochrome *b2* was used as an initiation control (Fig 3.18). The results showed that neither polyglutamine nor polyproline did not serve as good initiation sites, as degradation was inhibited (Fig 3.19).

This observation could explain why soluble htt fragments are not re-targeted to the proteasome for degradation. I next repeated these experiments using httQ20 and httQ83 as initiation sites. In both cases degradation was significantly inhibited as compared to control (Fig 3.20). These results indicate that inhibition of proteasome degradation may not be due to inability of the proteasome to progress, but rather inability to initiate degradation on truncated htt.

Figure 3.17: Htt exon 1 does not protect a destabilized protein domain from degradation

SDS-PAGE and autodradiography analysis of results of degradation assays of fusion proteins containing htt exon 1 with 20 or 83 glutamine residues. Results show no accumulation of a fragment, as indicated on the gels. Samples were taken at indicated time points. Graphical representation indicates percent degradation. Error is represented as standard error.


Time
(min)
0’ 15’ 30’ 45’ 60’ 90’ 120’ 150’ 180’
Ub$_n$
Ub-barnase-httQ20-CP P25
Time
(min)
0’ 15’ 30’ 45’ 60’ 90’ 120’ 150’ 180’
Ub$_n$
Ub-barnase-httQ83-CP P25

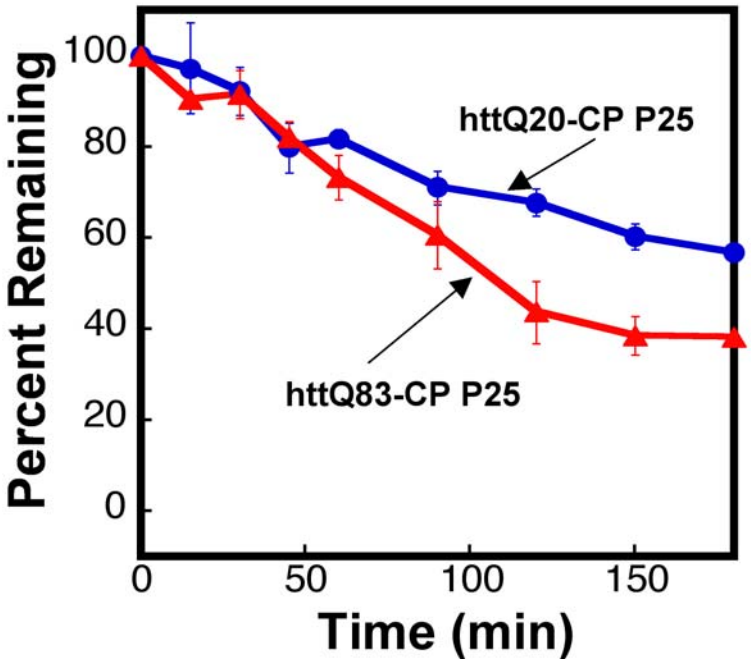

Percent Remaining
100
80
60
40
20
0
0
50
100
150
Time (min)
httQ20-CP P25
httQ83-CP P25

Figure 3.18: Linear representation of proteasome initiation substrate

Gold circles at the N-terminus represent four fused ubiquitin moieties. eDHFR is represented in black, followed by an unstructured region which is noted as either poly-Q or poly-P.

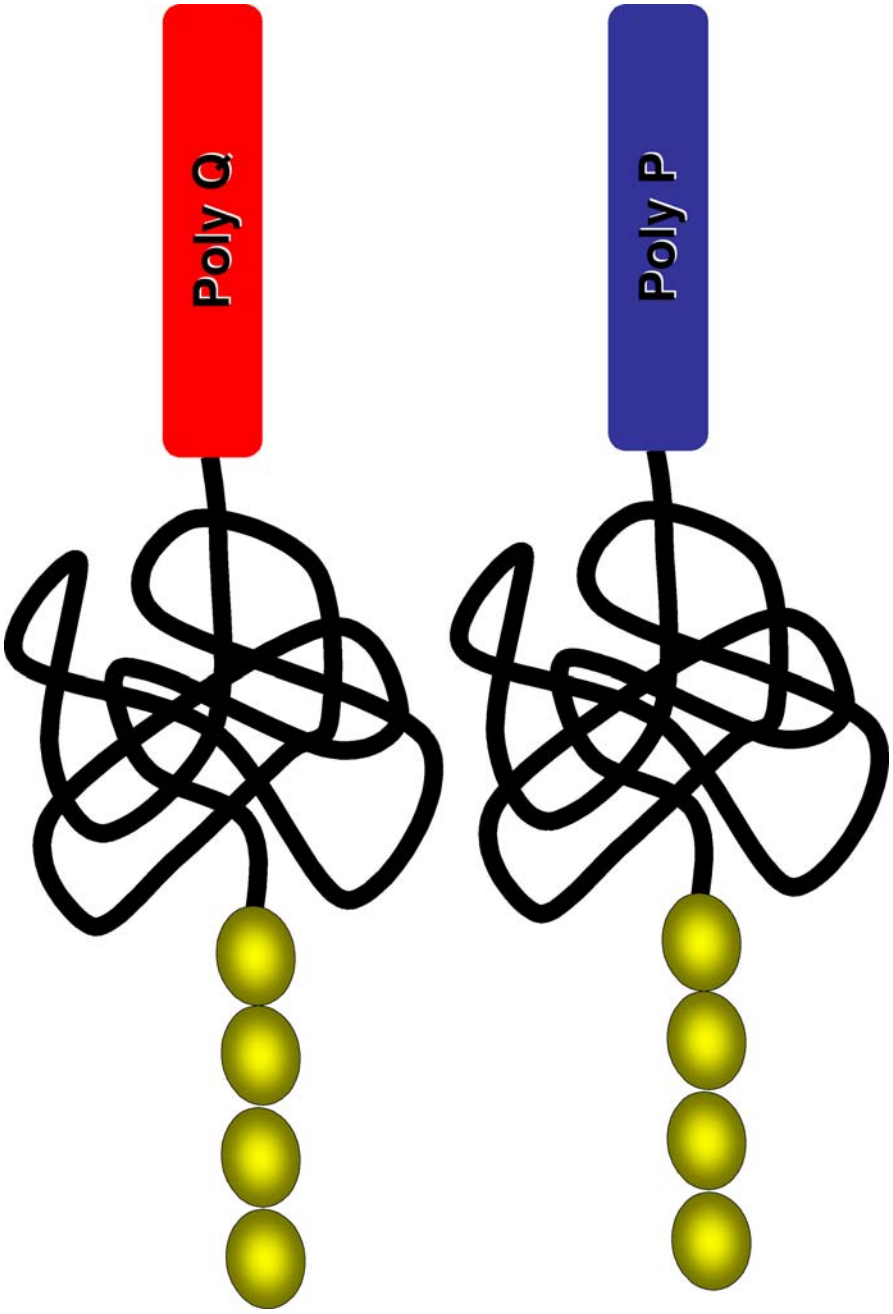

Poly Q
Poly P

Figure 3.19: Polyglutamine and Polyproline do not serve as good initiation sites

SDS-PAGE and autodradiography analysis of results of degradation assays looking at proteasome initiation. Degradation results show no disappearance of substrate protein, which indicates a lack of initiation of proteasome degradation. Error is represented as standard error.

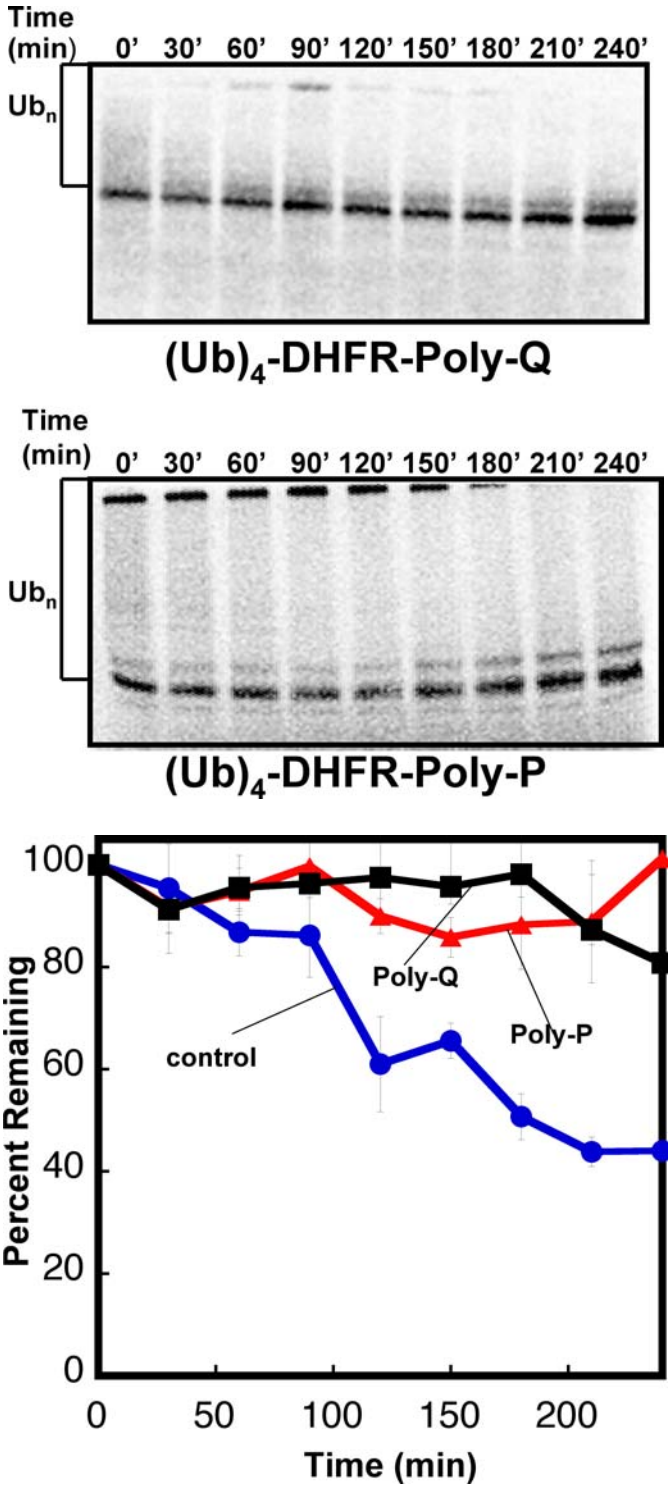
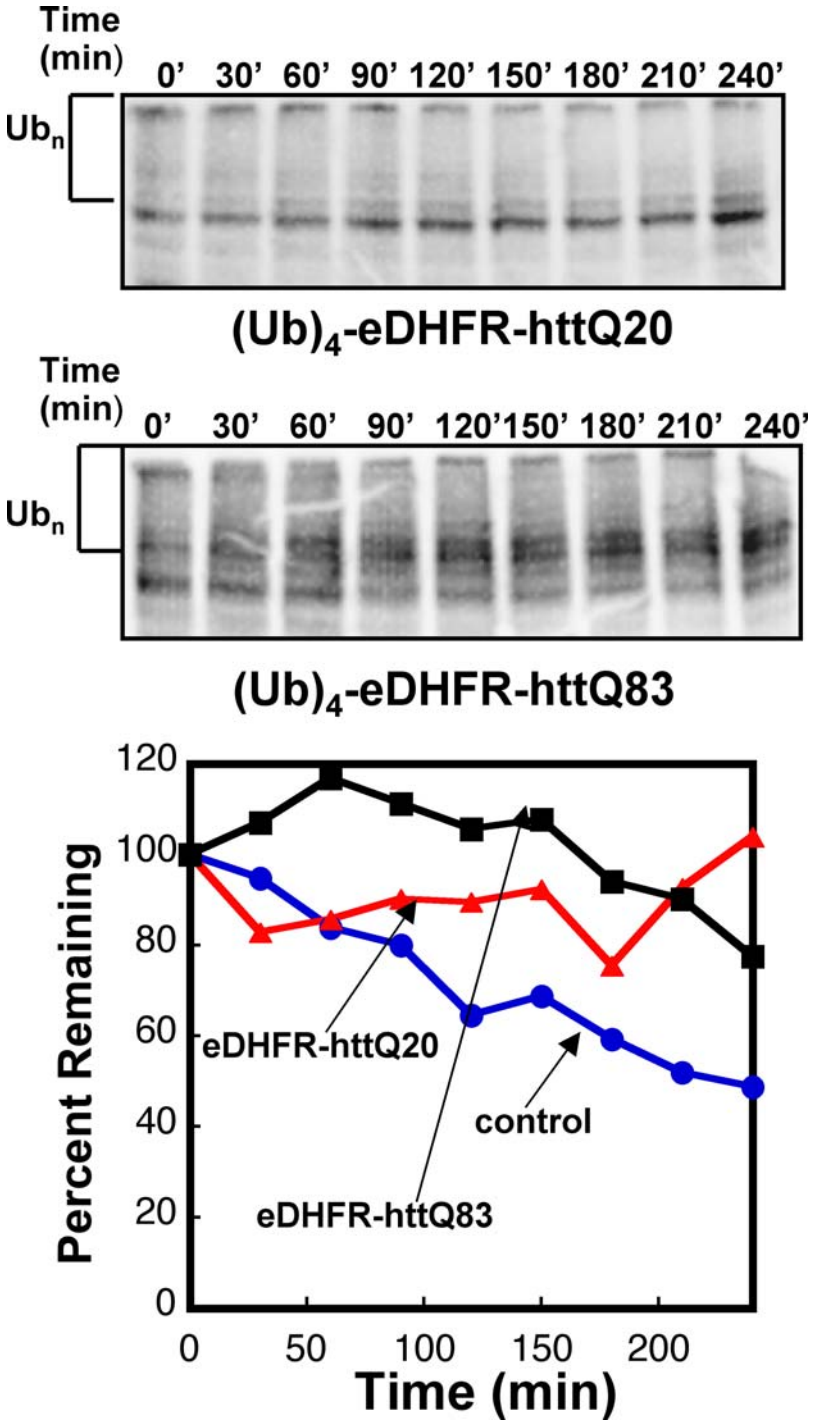

Time (min)
0' 30' 60' 90' 120' 150' 180' 210' 240'
Ub$_n$
(Ub)$_4$-DHFR-Poly-Q
Time (min)
0' 30' 60' 90' 120' 150' 180' 210' 240'
Ub$_n$
(Ub)$_4$-DHFR-Poly-P
Percent Remaining
100
80
60
40
20
0
0
50
100
150
200
Time (min)
Poly-Q
Poly-P
control

Figure 3.20: Huntingtin exon 1 does not serve as a good initiation site for proteasome degradation

SDS-PAGE and autodradiography analysis of results of degradation assays looking at proteasome initiation. Results show that the proteasome cannot efficiently initiate degradation on unstructured region huntingtin exon 1.


Time (min)
0' 30' 60' 90' 120' 150' 180' 210' 240'
Ub$_n$
(Ub)$_4$-eDHFR-httQ20
Time (min)
0' 30' 60' 90' 120' 150' 180' 210' 240'
Ub$_n$
(Ub)$_4$-eDHFR-httQ83
Percent Remaining
120
100
80
60
40
20
0
0
50
100
150
200
Time (min)
eDHFR-httQ20
control
eDHFR-httQ83

Discussion

Neuronal inclusions are the main physiological hallmark of HD. These aggregated structures are composed of a high percentage of mutant htt as well as ubiquitin and other components of the ubiquitin proteasome system. The issue that was investigated was the fate of mutant htt while still soluble. Results from collaborators showed that the proteasome specifically co-localizes with aggregated htt (Holmberg, Staniszewski et al. 2004), so this may be indicative of an attempt to degrade the proteins, possibly before they are in an aggregated state. Results from degradation of a proteasome-targeted substrate that was preceded by an expanded polyglutamine stretch showed that the folded protein domain was spared from degradation. This indicated that polyglutamine could inhibit progression of proteasome degradation.

Many studies have shown that efficiency of proteasome degradation can be affected by the structural components of the substrate protein (Lee, Schwartz et al. 2001; Tian, Holmgren et al. 2005). So the inhibitory effect of polyglutamine when followed by a destabilized protein domain was next observed. These results showed that in order for complete inhibition, a stabilized protein domain must follow polyglutamine. These results were contrary to many previous studies that showed that expanded polyglutamine repeats could not be degraded by the proteasome. The results discussed above showed that polyglutamine can function the same way that other low-complexity domains do in proteasome inhibition (Tian, Holmgren et al. 2005). The next step was to investigate this concept not just in terms of it as a phenomenon in proteasome inhibition, but as something that could cause a specific effect in disease system. In applying this concept to proteasome inhibition by htt, a region that could function in a similar fashion to the stabilized domain in the synthetic test system needed to be determined.

The first hypothesis tested was that the polyproline region in htt could serve as a domain stable against proteasome degradation and mediate inhibition by polyglutamine. Results indicated that polyproline behaved similarly to polyglutamine in its "low-complexity" governed effects, i.e. its ability to protect a stable protein domain from degradation, and in its inability to protect a destabilized domain. When these repeats were used in tandem, protection of a destabilized protein was observed, but only to a slight degree. Initially, it appeared that this small amount of fragment accumulation could correspond with the slow accumulation of htt and formation of aggregates, as well as the late onset of HD that occurs. But when these experiments were repeated using mutant htt, no inhibition was seen. This indicated that although the combination of two low complexity domains could mediate proteasome inhibition, this was most likely not the main factor in determining HD proteasome inhibition *in vivo*.

The inhibitory effect caused by polyglutamine, polyproline, and the combination of the two is most likely due to a phenomenon that is seen with almost all low complexity regions. For reasons that are not yet totally understood, these regions impede the progression of the proteasome. This mechanism of degradation has been termed processing. This may be mediated by allosteric self-regulation of proteasome active sites (Kisselev, Akopian et al. 1999). In most cases in proteins that are processed, their low complexity regions are followed by a stable protein domain. This is the case with p105 processing to p50, and cubitus interruptus processing. A recent study from Tian *et al.* explored these mechanisms. They showed that processing signals may be evolutionarily conserved to mediate partial degradation for activation of cellular processes (Tian, Holmgren et al. 2005). So the effect caused by polyglutamine seems somewhat accidentally driven by the expansion of the polyglutamine tract in htt.

A second hypothesis for inhibition is dependent on initiation of degradation. Initiation on a substrate protein is a critical step in proteasome degradation. This process is mediated by the presence of an unstructured region. Recent studies have shown that this region is essential for efficient degradation, and that low complexity domains serve as poor initiation sites (Prakash, Tian et al. 2004; Tian, Holmgren et al. 2005). The results discussed above (Fig 3.19 and 3.20) are in agreement with what has been previously shown, in that both polyglutamine and polyproline serve as poor proteasome initiation sites. This lack of initiation was also seen when mutant htt exon 1 was used as an initiation site. This implies that the lack of mutant htt disposal in HD is most likely mediated by inability of the proteasome to initiate on the protein.

So in summary, these studies propose a potential mechanism for proteasome inhibition in HD. As discussed earlier in this chapter, htt is highly prone to cleavage by caspase-3 when mutated (Goldberg, Nicholson et al. 1996). These cleavages lead to buildup of fragments that are prone to aggregation and are suggested to be toxic to the cell (Lunkes, Lindenberg et al. 2002). In an attempt to dispose of these proteins, they are tagged with ubiquitin, but the inability of the proteasome to engage them through initiation spares them from disposal. They in fact become “invisible” to the proteasome. If the proteasome were to achieve initiation, it is possible that the combination of polyglutamine and polyproline may slightly inhibit progression of degradation. These ubiquitinated fragments then aggregate and are sequestered into neuronal inclusions. Whether the proteasome stays associated with the proteins before they aggregate, or they attempt to degrade them in aggregated state is unknown. It may be a combination of both phenomena. More studies need to be conducted to isolate the specific causes of the results

observed, but the data presented here provide important clues for what hypotheses to investigate next.

Chapter 4

Future Directions

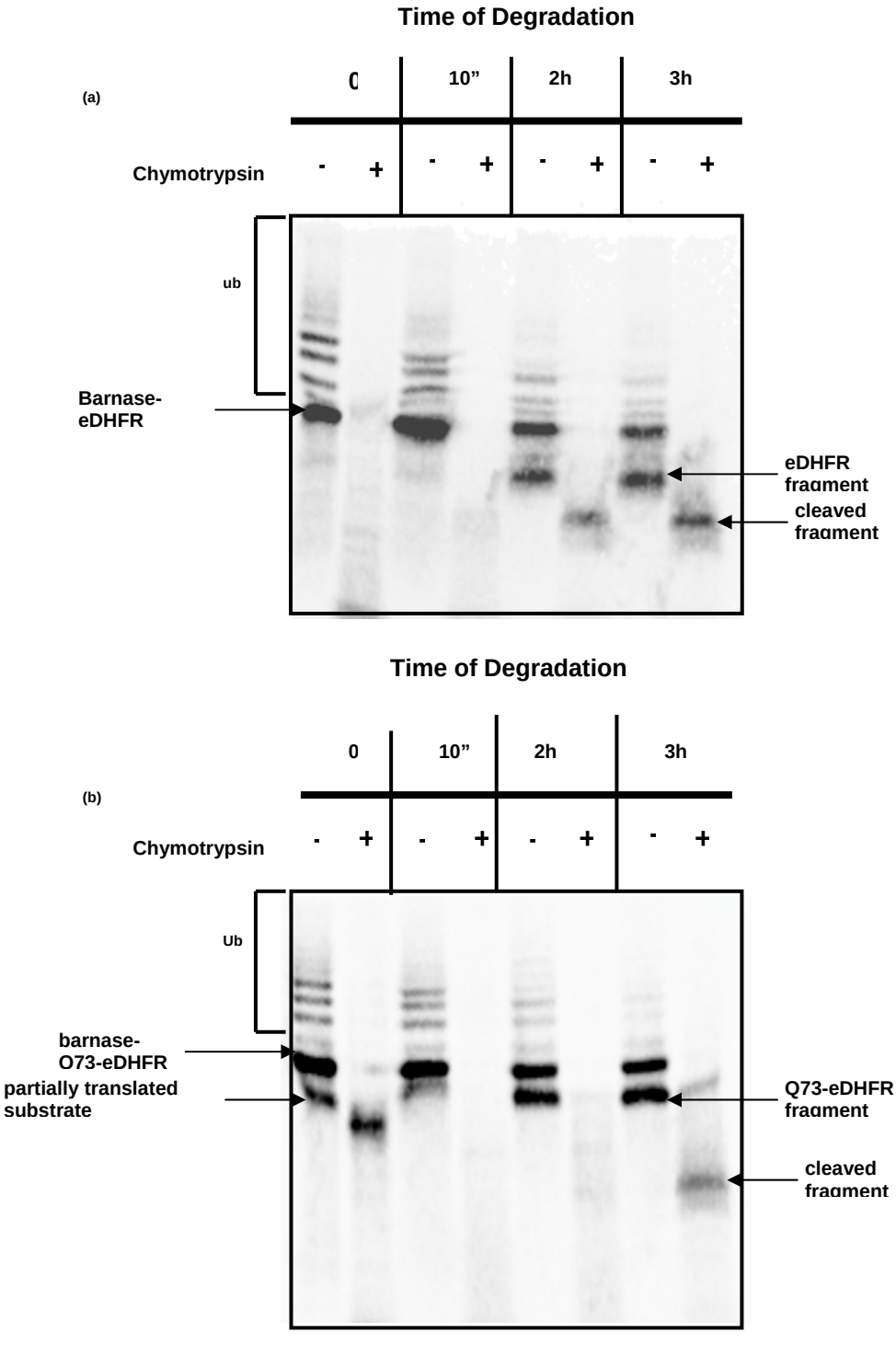
Do truncated proteins stay associated with the proteasome after inhibition?

The studies presented in this thesis, as well as many others, show that protein domains that follow low complexity (LC) regions are protected from proteasome degradation. It is not known, however, if the proteasome disengages with a substrate protein when its progression is inhibited. To address this issue, a degradation assay was performed with the fusion protein barnase-DHFR in which the eDHFR domain was stabilized with methotrexate (Johnston, Johnson et al. 1995). This would insure that the domain would remain folded and protected from degradation. At time points 0, 2 hrs, and 3 hrs, the samples were incubated with chymotrypsin to digest away the ~90 amino acid tail that is present when a protein disengages with the proteasome. The tail would be protected from cleavage if the substrate was still associated with the proteasome, but the shift in size of fragment indicates release from the proteasome (Fig 4.1a). The same assay was performed using barnase-Q73-eDHFR as a substrate, and again, the protein is released (Fig 4.1b). So when low complexity domains inhibit degradation, they most likely disengage with the proteasome. These results agree with what is seen in nature with proteins like p105 and cubitus interruptus (Tian, Holmgren et al. 2005).

In the case of polyglutamine however, one can't be certain that this release occurs. The fact that proteasomes co-localize with truncated htt in aggregates, as well as results that show lack of proteasome migration when HD-like inclusions a subject to FRAP analysis (Holmberg, Staniszewski et al. 2004) suggests that proteasomes and truncated htt may form a more stable type of interaction. The results from the assay system used in the studies discussed here indicate that polyglutamine disengages, but this disengagement is observed after 2-3 hrs, probably a much longer timeframe than in a

Figure 4.1: Polyglutamine proteins may be released from the proteasome after processing

During a three-hour degradation assay, samples of methotrexate-stabilized substrates were removed at 10 sec, 1 hr, 2 hr, and 3 hr time points. The samples were then treated with nothing or 1mM chymotyrypsin for 1 min on ice and analyzed by SDS-PAGE and autoradiography. Shift in fragment size indicates cleavage of an unstructured tail upon release from the proteasome.


Time of Degradation
(a)
0
10”
2h
3h
Chymotrypsin
- + - + - + - +
ub
Barnase-
eDHFR
eDHFR
fragment
cleaved
fragment
Time of Degradation
(b)
0
10”
2h
3h
Chymotrypsin
- + - + - + - +
Ub
barnase-
O73-eDHFR
partially translated
substrate
Q73-eDHFR
fragment
cleaved
fragment

physiological situation. *In vivo*, the interaction may be somewhat transient, but the amount of protein present may expedite aggregation; something that would not be seen in our degradation system. To further analyze this question, studies will have to be conducted in which higher concentrations of proteins are used. The protein-proteasome interaction will also have to be monitored on a shorter time scale, and single molecule studies to determine binding and on/off rates will have to be conducted. These data will help definitively determine whether polyglutamine proteins disengage with the proteasome when its progression is inhibited.

What determines the size of fragments created by low complexity domains?

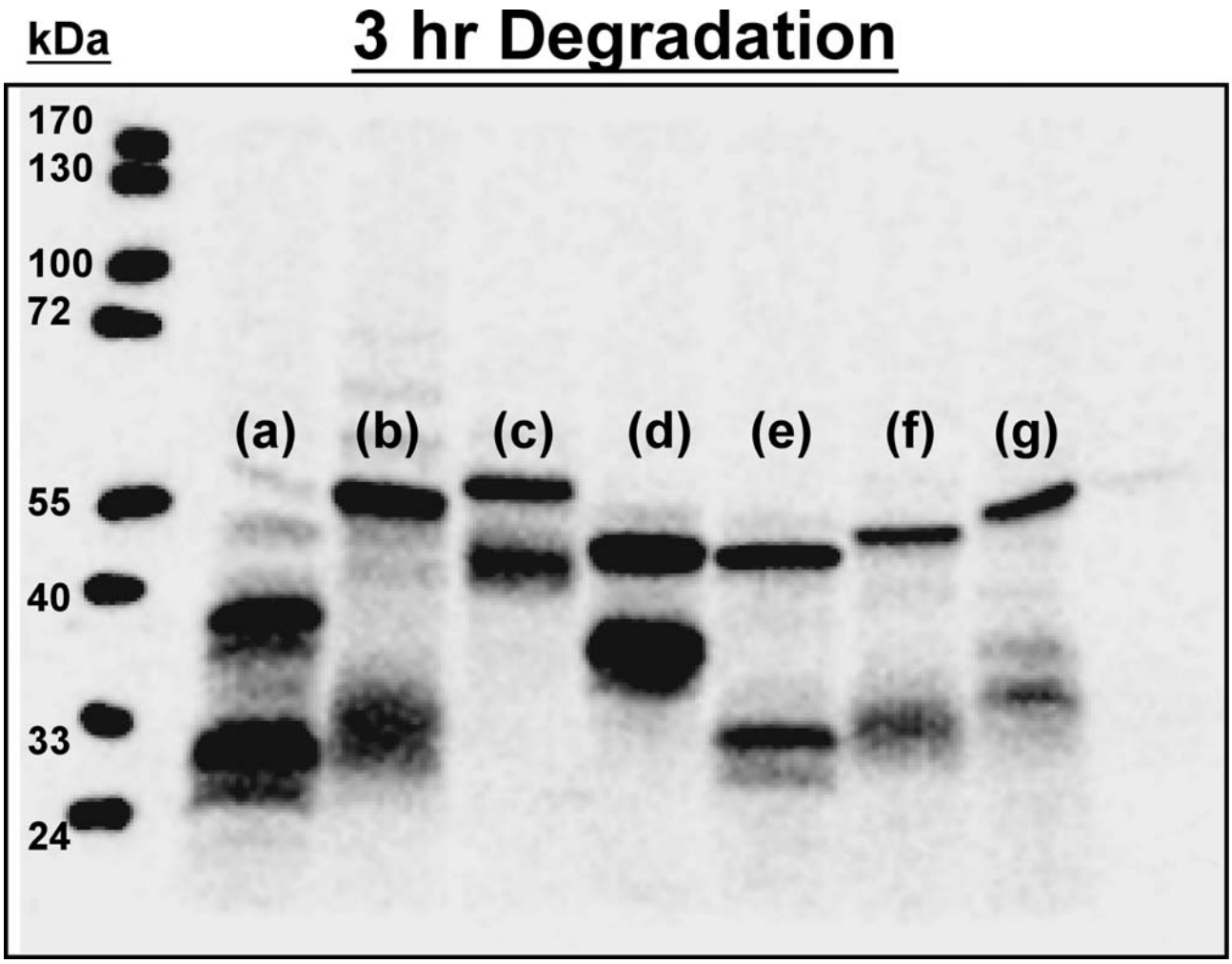
An interesting phenomenon observed in the generation of proteasome degradation fragments by LC regions, is that their fragment size varies in accordance with the length of repeat region (Fig 4.2). The studies discussed previously in this thesis show that inhibition by LC regions is highly mediated by the stability of the protein in which they precede, and that in general, the proteasome has no difficulty degrading these regions. One would think then, that regardless of LC region length, the fragment formed would be the folded domain plus ~90 amino acids. But as figure 4.2 indicates, this is not the case at all. This increase in fragment size was seen in a study by Lee *et al* in which they cross-linked a substrate protein to induce hairpin loops, and stabilized the protein that followed it (Lee, Prakash et al. 2002). Their results suggested that an increase in fragment size was a result of the polypeptide chain entering the 20S particle as a loop. This type of structure could be what is forming due to long LC regions interacting with their cellular environments. In order to determine the structure of these

fragments, degradation assays with purified, chemical amounts of protein will have to be performed. Then, when isolated, the fragments will need to be subjected to analysis by NMR and mass spectroscopy to determine both structure and amino acid content.

In summary, the actual fate of partially degraded polyglutamine proteins after proteasome progression is impeded is unknown. Studies have been initiated that may provide clues as to what this process is. It is hoped that the information and insight provided in this thesis will inspire another scientist to take on the challenge and move forward in attempting to answer these questions.

Figure4.2: Fragment size corresponds with size of low-complexity repeat region

Samples taken after a three-hour degradation assay show differences in fragment size. Fragments observed were (a) eDHRF+mtx, (b) Q40-eDHFR, (c) Q73-eDHFR, (d) 64-a.a. PRR-eDHFR, (e) 50-a.a. glycine-rich region (GRR)-eDHFR, (f) 100-a.a. GRR-eDHFR, and (g) 150-a.a. GRR-eDHFR. The size of stabilized eDHFR is consistent with the folded domain plus ~90 amino acids, and the other fragment sizes correspond to the added length provided by each individual low-complexity domain.


kDa
3 hr Degradation
170
130
100
72
55
40
33
24
(a)
(b)
(c)
(d)
(e)
(f)
(g)

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