SUBSTRATE SELECTION BY THE 20S PROTEASOME AND IMPLICATIONS IN DISEASE PATHOGENESIS

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by

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Alpha-synuclein is a major component of Lewy bodies, large insoluble protein aggregates, found in various regions of the brain in patients with Parkinson's disease, diffuse Lewy body disease, and the Lewy body variant of Alzheimer's disease. Within Lewy bodies, several modifications of alpha-synuclein have been identified, including truncations from either terminus.

Several observations support a role for C-terminally truncated forms of alpha-synuclein in disease pathogenesis. While multiple proteases have been implicated in the metabolism of alpha-synuclein, C-terminally truncated forms of alpha-synuclein produced by the 20S proteasome *in vitro*, through a ubiquitin-independent mechanism, are similar to those found in the cingulate cortex of patients with Lewy body diseases. The work presented here describes an investigation into the mechanism by which alpha-synuclein is recognized and degraded by the 20S proteasome, aimed at the properties of both the substrate and the enzyme required for activity. The results shown reveal a previously unappreciated mechanism by which the 20S proteasome selects its substrates, and this activity is coupled to gate opening in mammalian proteasomes. Additionally, a post-translational modification of the 20S proteasome was identified that correlates with activity of the enzyme against alpha-synuclein.

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11S (also referred to as PA28) - the regulatory subcomplex of the immunoproteasome

19S (also referred to as PA700) - the AAA-ATPase-containing regulatory subcomplex of the 26S proteasome

20S - the core subcomplex of the proteasome that contains the proteolytic sites

26S - the proteasome complex containing a 20S catalytic particle capped on either one or both ends with a 19S (or PA700) regulatory particle

AAA-ATPase (ATPases Associated with diverse cellular Activities) - a conserved family of proteins that serve as molecular motors utilizing the energy of ATP hydrolysis

Aβ - a peptide that is the major component of plaques, a neuropathological feature of Alzheimer's disease

AMC (4-aminomethyl coumarin) - a fluorophore released by proteasome-dependent hydrolysis from fluorogenic peptide substrates

β-ME (beta-mercaptoethanol) - a reducing agent

DLBD - (diffuse Lewy body disease) - a neurological disease characterized by pathological accumulations referred to as Lewy bodies

DMSO (dimethyl sulfoxide) - a solvent for many polar and nonpolar compounds

DTT (dithiothreitol) - a reducing agent

DUB (deubiquitinating enzymes) - proteases that cleave ubiquitin-protein bonds and play an essential role in the ubiquitin-proteasome system

ESI-MS (electrospray ionization mass spectrometry) - a mass-spectrometry technique used to determine the whole mass of proteins

IDP (intrinsically-disordered proteins) - proteins that lack a defined structure in the native state

IPTG (Isopropyl β-D-1-thiogalactopyranoside) - a metabolite mimic that induces transcription of the *lac* operon

LBV (Lewy body variant of Alzheimer's disease) - a type of Lewy body disease that has pathological features overlapping those found in both Alzheimer's disease and Lewy body diseases

LC/MS/MS (liquid chromatography/mass spectrometry/mass spectrometry) - a mass-spectrometry technique by which peptides are first separated by chromatographic techniques, followed by mass determination of the separated peptides

MD (molecular dynamics) simulations - a computational technique used to understand dynamic changes in protein structure, often used when experimental approaches are not feasible

MG132 (N-(benzyloxycarbonyl)leucinylleucinylleucinal Z-Leu-Leu-Leu-al) - a reversible proteasome inhibitor of all three activities of the proteasome

MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) - a precursor to the neurotoxin MPP+ that is commonly used to induce Parkinson's-like symptoms in animal models of PD

NAC (Non-Aβ Component) - a peptide fragment of alpha-synuclein, consisting of amino acids 61-95, that has been found to colocalize with Aβ in Alzheimer's plaques

NMR (nuclear magnetic resonance) - an experimental technique that has been used to determine the structure and dynamics of proteins in solution

OD (optical density) - a measure used for monitoring growth of a bacterial culture corresponding to light scattering equivalent to one absorbance unit across a one centimeter path length

PA28 (see 11S)

PA700 (see 19S)

PAN (proteasome-activating nucleotidase) - a complex of six identical ATPases that binds to and activates the archaeal 20S proteasome

PD (Parkinson's disease) - a neurological disease that is characterized by pathological protein accumulations referred to as Lewy bodies

PDB (Protein Data Bank) - an archive of experimentally-determined structures

pI (isolectric point) - the pH at which a protein lacks a net charge

POPA (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate) - a phospholipid with negative charge

POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) - an uncharged phospholipid

PRE (paramagnetic relaxation enhancement) - a specialized NMR technique by which interactions between a spin label and NMR active nuclei can be observed over long distances (up to 20Å)

RDC (residual dipolar coupling) - a specialized NMR technique by which the orientation of a protein can be determined

SDS (sodium dodecyl sulfate) - an anionic detergent used to denature proteins prior to denaturing electrophoresis

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) - a technique by which proteins are denatured and separated by size through a polyacrylamide matrix

SNARE (soluble NSF attachment protein receptor) - a family of proteins that plays a role in vesicle fusion

UPS (ubiquitin-proteasome system) - the primary mechanism by which cellular proteins are degraded, including the 26S proteasome and all of the ubiquitin activating, conjugating, and ligating machinery

CHAPTER ONE
General Introduction

Living organisms are extraordinarily complex, engaging in a multitude of simultaneous and cooperative processes in order to survive. Not only must cells thrive individually, but they must also be able to communicate with other cells in order to function as a larger physiological unit. These intra- and inter-cellular processes are subject to extensive regulatory mechanisms that are essential in the proper development and survival of species through the ability to reproduce. In addition, regulation of cellular activities must be tightly controlled, yet quick to adjust during times of changing environments and under conditions of stress. Disease is often the result of the inability of an organism to properly regulate functions either within a cell or amongst populations of cells. This improper regulation can cause many downstream effects resulting in pathophysiological consequences, including death.

A universal mechanism by which different cellular processes are regulated is through an alteration of protein levels within the cell. The extent of protein expression in a cell is regulated both by changes in the rate of protein synthesis and the rate of protein degradation. The majority of cellular proteins are degraded by the ubiquitin-proteasome system (UPS). The UPS includes many gene products that make up the catalytic particle, regulatory particle, and all of the

ubiquitin-conjugating enzymes and chaperones that play a role in proteasome assembly, targeting of substrates, substrate recognition, and degradation. The function of the UPS is highly regulated, such that protein levels are maintained at appropriate levels in the cell. An alteration of proteasomal activity or regulation of its activity has been implicated in development, aging, and disease.

The work presented in this document highlights the complexity of proteasomal regulation as it may relate to the incomplete degradation of alpha-synuclein and the pathogenesis of Lewy body diseases. Chapter Two will introduce the proteasome and its role in physiological processes from a genetic and structural perspective. Chapter Three will focus on alpha-synuclein, a known substrate for 20S proteasomal degradation, and the role that alpha-synuclein truncation might play in disease. Both Chapter Two and Chapter Three will include an extensive review of the literature. Chapter Four and Chapter Five will focus on results investigating the role that 20S-mediated degradation of alpha-synuclein might play in disease pathogenesis. More specifically, Chapter Four will focus on identifying the elements of alpha-synuclein that render it a substrate for degradation by the 20S proteasome. Chapter Five will focus on the identification of a post-translational modification that correlates with proteasomal activity against alpha-synuclein. Chapter Six will describe further experimental

ideas that might lead to a greater understanding of 20S-mediated alpha-synuclein degradation and its role in disease.

CHAPTER TWO
The Ubiquitin-Proteasome System: Architecture and Cellular Function

The Ubiquitin-Proteasome System

The ubiquitin-proteasome system (UPS) is composed of many different proteins and subcomplexes, all of which play an important role in the efficient targeting and recognition of substrates for degradation. Homologues of many of these gene products can be found in organisms from all three domains of life (Tanaka et al. 1988; Dahlmann et al. 1989; Benoist et al. 1992), and differences in proteasomes from these sources have been studied both structurally and functionally. The UPS is responsible for the degradation of the majority of cellular proteins (Hochstrasser 1992; Voges et al. 1999). The eukaryotic 26S proteasome, a fundamental component of the UPS, is composed of two major subcomplexes, referred to as the 20S proteasome, or 20S core particle, and the PA700, or 19S, regulatory particle (Driscoll & Goldberg 1990; Yoshimura et al. 1993). While subunits of these two subcomplexes are responsible for many of the activities encompassed by the UPS, the concerted effort of a vast number of other proteins is required for proteasomal degradation to be carried out in the cell. Degradation of substrates by the 26S proteasome is known to be both ubiquitin- and ATP-dependent (Hershko et al. 1984; Hough et al. 1986), but the intricate details of this process are not completely understood.

The first step in generating a functional 26S proteasome is assembly of the 20S core particle (Frentzel et al. 1994; Yang et al. 1995). This assembly is complicated, and requires the assistance of many chaperones (Ramos et al. 1998; Jayarapu & Griffin 2004; Hirano et al. 2005). After 20S formation, the 26S proteasome is assembled through association of the PA700 regulatory particle to one or both ends of the 20S core particle, and this binding process is ATP-dependent (Eytan et al. 1989). PA700 contains polyubiquitin binding sites and ATPase activity, which are coupled to the catalytic core in the assembled 26S proteasome. Thus, the assembled 26S proteasome can recognize and degrade protein substrates that are targeted for degradation by the covalent attachment of specific ubiquitin linkages.

Ubiquitin linkages are added to proteins through the concerted effort of the E1-activating, E2-conjugating, and E3-ligating classes of enzymes. A single E1-activating enzyme attaches a ubiquitin monomer to an E2-conjugating enzyme (Ciechanover et al. 1981; Hershko et al. 1983). The E2-conjugating enzyme then works with a specific E3-ligating enzyme to add ubiquitin moieties to a substrate (Hershko et al. 1983) and, once the chain reaches a certain length, the polyubiquitinated substrate can bind to subunits of the PA700 regulatory particle (Deveraux et al. 1994; Thrower et al. 2000; Lam et al. 2002). Recent studies have indicated that a polyubiquitin tag might not be sufficient to render proteins as

proteasome substrates (Prakash et al. 2004). In this study, the authors showed that an unstructured region of the protein is required, in addition to a polyubiquitin tag, for efficient degradation by the 26S proteasome (Prakash et al. 2004). It was later found that the unstructured initiation site and the polyubiquitin tag can be located on two different proteins of a complex to function as recognition signals *in trans* (Prakash et al. 2009). These observations highlight the complexity of substrate targeting to the proteasome and the many components that must work together for this process to be effective.

After a polyubiquitinated substrate is recognized by subunits of the PA700 regulatory particle, the ubiquitin molecules can be removed from the substrate by subunits with deubiquitinating (DUB) activity. Thus, polyubiquitinated proteins that lack an appropriate unstructured region or other attribute(s) required for substrate fitness are deubiquitinated, allowing them to escape proteolysis. Polyubiquitinated proteins with these attributes are deubiquitinated and committed to proteasomal degradation. Several subunits of the PA700 regulatory particle exhibit DUB activity (Lam et al. 1997; Verma et al. 2002), and many other DUBs have been identified that can either associate with the 26S proteasome or function independently from the 26S proteasome complex (Papa et al. 1999; reviewed in Chung & Baek 1999).

Structural studies of the eukaryotic 20S proteasome indicate that substrates must be unfolded in order to access the sequestered catalyic sites (Wenzel & Baumeister 1995; Groll et al. 2000). A subunit with unfoldase activity has been identified in the regulatory particle of the archaeal proteasome (Benaroudj & Goldberg 2000), and this subunit is homologous to subunits of the eukaryotic PA700 regulatory particle (Smith et al. 2007). Substrate unfolding and translocation into the interior of the complex are coupled events that require the energy of ATP hydrolysis (Benaroudj et al. 2003; Liu et al., 2006). Once the substrate has been pulled into the complex, it can access the catalytic sites, where it is degraded by the proteolytic activities of the 20S proteasomse (Arendt & Hochstrasser 1997; Dick et al. 1998). Degradation products that result from both the archaeal proteasome (Kisselev et al. 1998) and the mammalian proteasome (Kisselev et al. 1999) have been found to range from 3 to 30 residues in length. The peptide products are believed to be subsequently degraded by other cytosolic peptidases, and the free amino acids are then recycled for use in *de novo* protein synthesis (Saric et al. 2004).

There are other known activators that bind to the 20S proteasome (Figure 2-1), and these activators afford the complex with alternative cellular functions. One such activator is the 11S (or PA28) regulatory particle that plays an important role in the immune system (Kloetzel 2004). Additionally, proteasomes containing

only one activator complex or with two different types of activator complexes have been found in the cell (Peters et al. 1993; Hendil et al. 1998; Tanahashi, Murakami, Minami, et al. 2000). The function of these singly-capped and hybrid proteasomes are not well understood. The multiple types of activator complexes that can bind to the 20S catalytic core particle under different cellular conditions highlight the complexity of proteasome functionality within the cell.

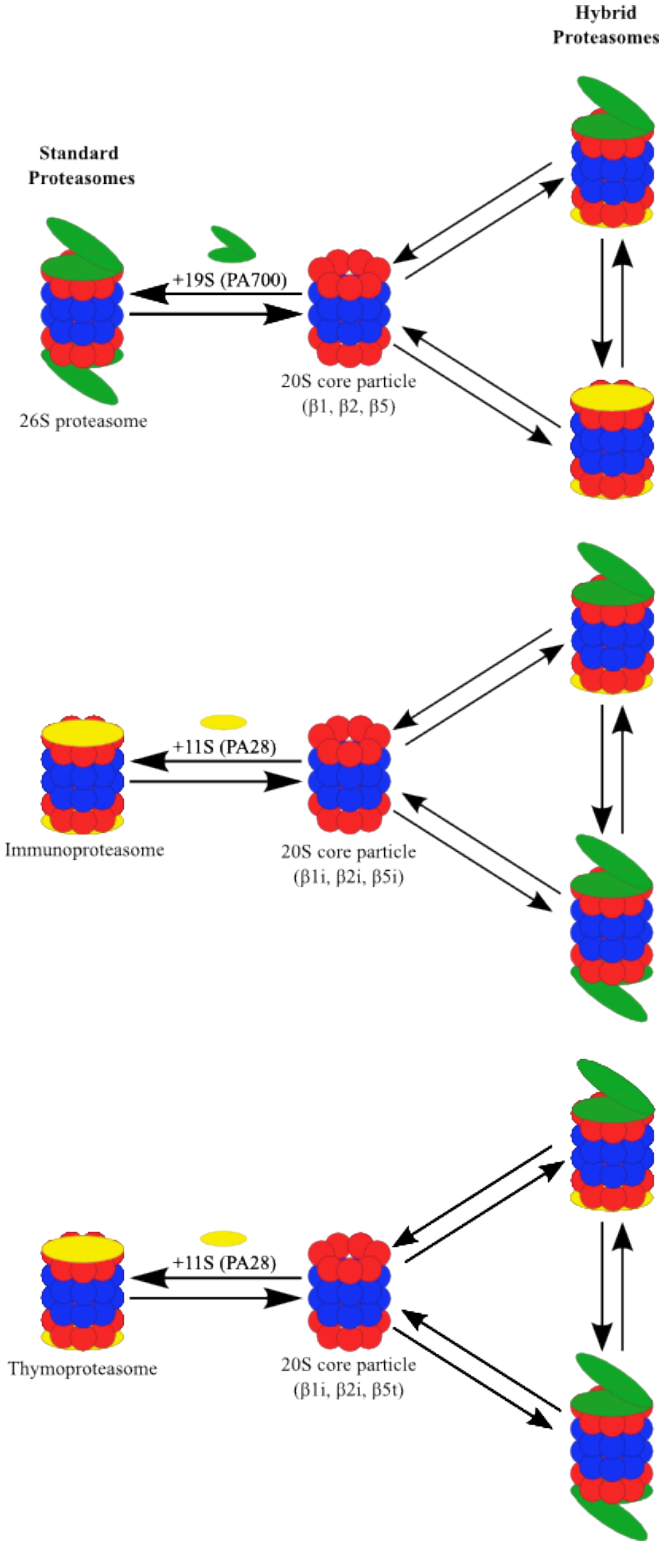
Hybrid
Proteasomes
Standard
Proteasomes
+19S (PA700)
20S core particle
(β1, β2, β5)
26S proteasome
+11S (PA28)
Immunoproteasome
20S core particle
(β1i, β2i, β5i)
+11S (PA28)
Thymoproteasome
20S core particle
(β1i, β2i, β5t)

Figure 2-1. Various forms of cellular proteasomes. The 20S core particle is composed of 28 subunits. Alternate and inducible forms of the three catalytic subunits (β1, β2, and β5) incorporate into the standard immunoproteasome and thymoproteasome. While PA700 (19S) typically binds to 20S core particles with the constitutive active subunits to form the standard 26S proteasome, it can also bind to core particles with the inducible active subunits. Similarly, PA28, normally bound to core particles containing inducible active subunits, can bind to the constitutive 20S core particle. These forms of the proteasome, including core particles with a different activator complex on each end, are referred to as hybrid proteasomes. Each arrow indicates multiple assembly or disassembly steps that have not been completely elucidated.

The number of proteins and cofactors that play a direct and indirect role in the UPS is unknown. These include not only those proteins that directly function in substrate targeting and degradation, but also proteins that bind to recognition motifs on substrates, enzymes that catalyze post-translational modifications of potential substrates to alter their stability, and transcription factors that are involved in ensuring adequate expression levels of each of the above components. In addition, there are many enzymes that catalyze post-translational modifications of proteins involved in the UPS itself, to modulate activity of the proteasome complex and its partners. The interrelationship between each of these processes within a physiological system remains elusive.

The 26S Proteasome

The eukaryotic 26S proteasome, composed of the 20S proteasome with a PA700 (19S) regulatory particle attached to one or both ends (Figure 2-2), has a molecular weight of approximately 2.5 megadaltons and is about 40nm in length (Peters et al. 1991). While the 20S subcomplex contains the activity required for peptide bond cleavage, the PA700 regulatory particle plays an equally important role in the process of substrate recognition and degradation by the UPS.

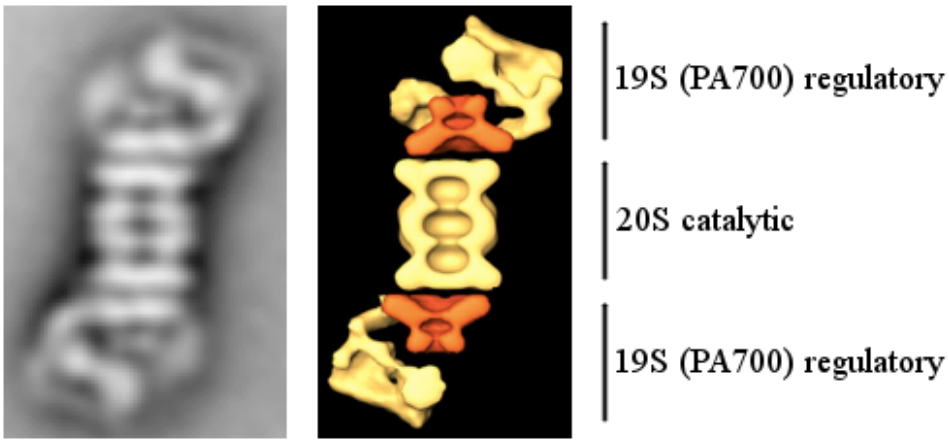

Figure 2-2. Model of the 26S proteasome based on electron microscopy studies. The 26S proteasome is composed of a 20S catalytic particle with a 19S, or PA700, regulatory particle on one or both ends. Electron micrograph was generated by (Adams et al. 1997). Reconstruction from cryo-EM studies was performed by (Nickell et al. 2009).

The PA700 regulatory particle is made up of at least 18 different gene products that form the base and lid components (Glickman, Rubin, Fried, et al.

1998b), and these two components assemble as independent subcomplexes (Isono et al. 2007). The complete assembly of the PA700 regulatory particle utilizes the pre-assembled 20S proteasome as a scaffold (Hendil et al. 2009), and each step in the assembly of the 26S proteasome requires a variety of chaperones (Le Tallec et al. 2009; Funakoshi et al. 2009).

The base of PA700 is made up of six subunits with AAA-ATPase activity (DeMartino et al. 1994; DeMartino et al. 1996) that form a hexameric ring (Glickman, Rubin, Coux, et al. 1998a). This activity allows the proteasome to utilize the energy of ATP binding and hydrolysis for substrate unfolding, gate opening, and translocation of substrates into the catalytic core of the enzyme (Benaroudj et al. 2003; Smith et al. 2005). The base also contains subunits that lack ATPase activity, including the Rpn10 and Rpn13 subunits. Both the Rpn10 and Rpn13 subunits have been shown to bind polyubiquitin chains and, therefore, likely play a role in substrate recognition (Schreiner et al. 2008; Husnjak et al. 2008). While not all of the PA700 subunits have a known function, several have been shown to play important roles in structural integrity and assembly (Tomko & Hochstrasser 2011; Pathare et al. 2012), while others contain DUB activity and , thus, likely play a role in the deubiquitination of substrates (Verma et al. 2002). Alternative subcomplexes of the PA700 subunits have been observed, and these

subcomplexes can be combined to reconstitute a distinct functional PA700 regulatory particle *in vitro* (Thompson et al. 2009).

Non-homologous proteases with structural and functional similarities to the 26S proteasome, such as ClpP (Kessel et al. 1995; Effantin et al. 2010) and HsIV (Rohrwild et al. 1997), exist in bacterial species. Homologues of the human proteasome are found in all eukaryotes, most archaea, and even a group of bacteria, referred to as antinomycetes (Benoist et al. 1992). The overall architecture of proteasomes from various species is similar; however, there are notable differences between both the core subunits and the identity and function of the associated regulatory complexes.

The 20S Core Particle

The 20S proteasome is composed of 28 subunits and is roughly 700 kilodaltons. Archaeal 20S proteasomes, such as that from *T. acidophilum,* are composed of 14 identical alpha subunits and 14 identical beta subunits. In the eukaryotic 20S proteasome, there are two each of seven different, but structurally similar, alpha subunits and two each of seven different, but structurally similar, beta subunits. Sequence and structural comparisons reveal that the multiple eukaryotic alpha and beta subunits likely diverged from the single archaeal alpha and beta subunits, affording similar functional attributes to these complexes

(Zwickl et al. 1992). Alignments of the seven human alpha subunits (Figure 2-3) and the seven human beta subunits (Figure 2-4) reveal the sequence similarity between each type of subunit.

Figure 2-3. Alignment of the human alpha subunits of the 20S proteasome core particle. Subunits are labeled according to the Baumeister nomenclature (Baumeister et al. 1998). Accession numbers are as follows: alpha-1 (AAH23659.1), alpha-2 (AAH47697.2), alpha-3 (AAH22445.1), alpha-4 (AAH04427.1), alpha-5 (AAI02020.1), alpha-6 (AAH22372.1, and alpha-7 (AAH29402.1). Alignments were generated using the PRALINE multiple sequence alignment tool through the Centre for Integrative Bioinformatics VU. Black background indicates identical residues and gray background indicates similar residues.

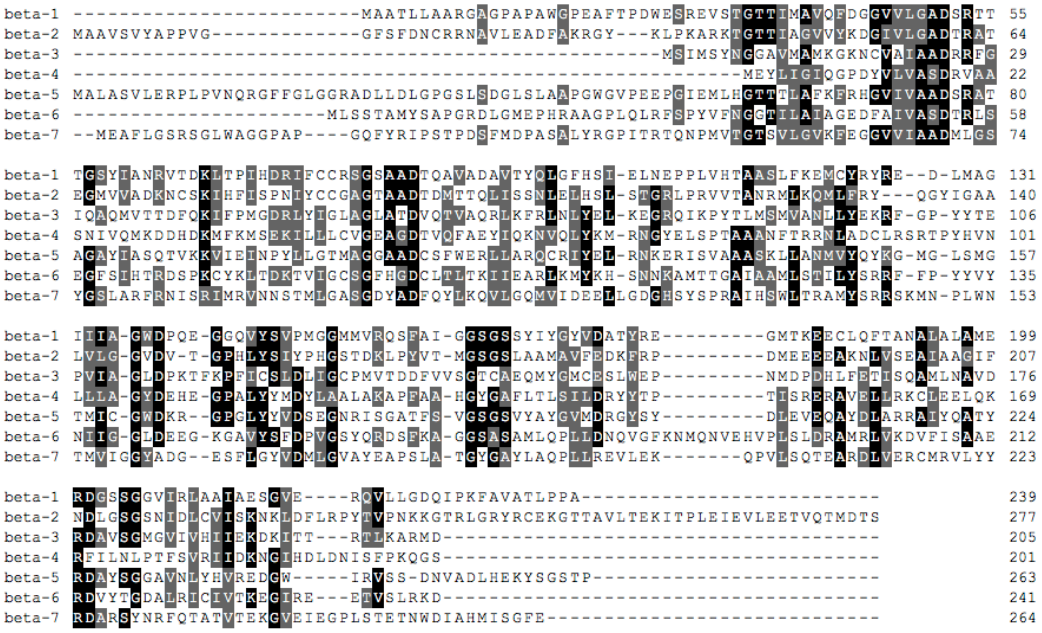

Figure 2-4. Alignment of the human beta subunits of the 20S proteasome core particle. Subunits are labeled according to the Baumeister nomenclature (Baumeister et al. 1998). Accession numbers are as follows: beta-1 (AAH00835.1), beta-2 (AAH00509.1), beta-3 (AAH13008.1), beta-4 (AAI05127.1), beta-5 (AAI07721.1), beta-6 (AAH20807.1), and beta-7 (AAP35563.1). Alignments were performed and represented as in Figure 2.3.

The architecture of the 20S proteasome consists of four stacked heptameric rings, with the two rings composed of beta subunits between the two rings composed of alpha subunits. The beta subunits of the eukaryotic proteasome contain the proteolytic active sites of the enzyme, with the β1, β2, and β5 subunits exhibiting the peptidyl-glutamyl peptide hydrolyzing (PGPH)-like, trypsin-like, and chymotrypsin-like activities, respectively (Arendt & Hochstrasser 1997; Dick et al. 1998). Each beta subunit of the archaeal proteasome contains an active site

threonine and, although the subunits are identical, multiple protease activities have been observed (Akopian et al. 1997; Maupin-Furlow et al. 1998). While the composition of this less complex proteasome is different from that found in higher organisms, the overall structure is very similar. X-ray crystallography studies of the *T. acidophilum*, *S. cerivisiae*, and *B. taurus* 20S proteasomes have revealed a similar architecture and size of each (Figure 2-5), with the dimensions being roughly 150Å in length and 115Å in diameter (Löwe et al. 1995; Groll et al. 1997; Unno et al. 2002).

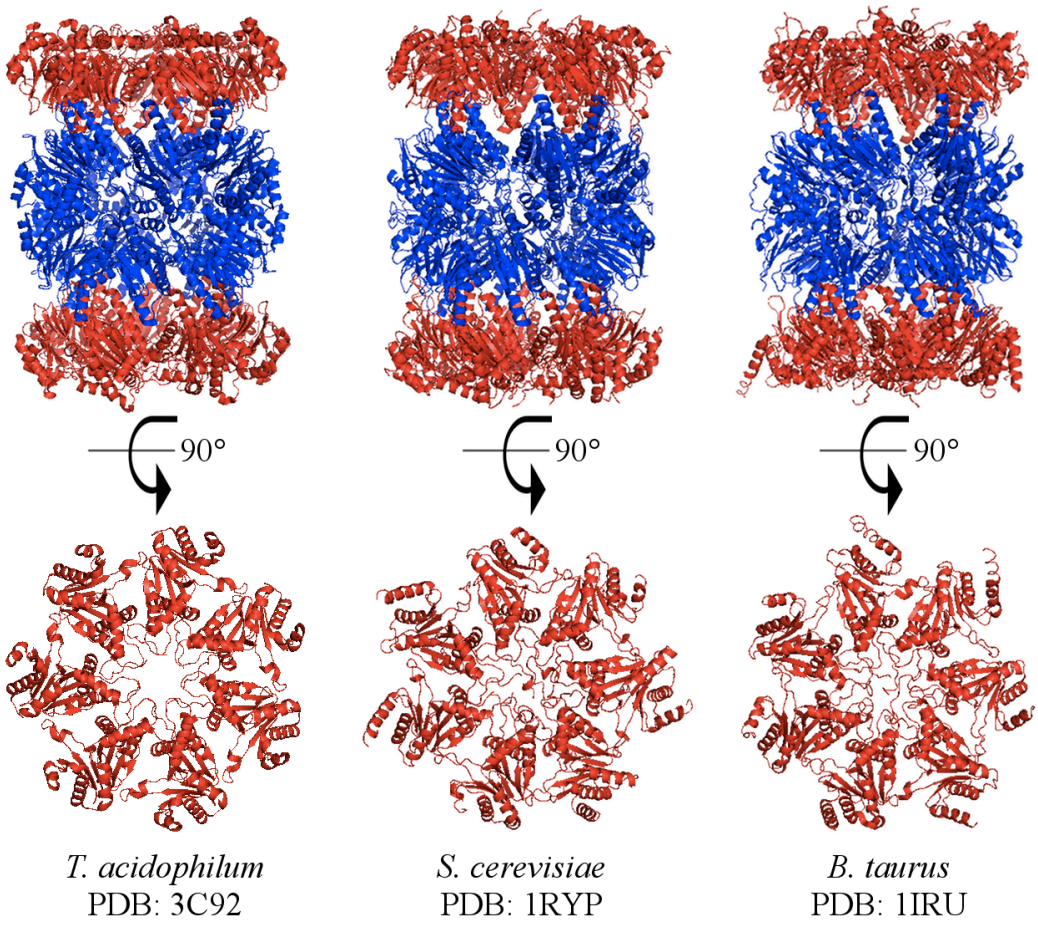

Figure 2-5. Crystal structures of the *T. acidophilum*, *S. cerevisiae*, and *B. taurus* 20S proteasomes. The figures on top show a side-view of the full 20S proteasome, with the alpha subunits shown in red and the beta subunits shown in blue. On the bottom is one ring of alpha subunits, looking into the pore. The N-termini of the alpha subunits of the *S. cerevisiae* and *B. taurus* proteasomes are shown to traverse the pore of the complex. Images were rendered in PyMol.

Substrates likely access the catalytic sites by entering through an axial channel on either end of the complex (Wenzel & Baumeister 1995). The N-termini of the alpha subunits traverse this channel to occlude entry of substrates

into the catalytic interior of the complex (Groll et al. 1997). These N-termini function as a gate that can be opened upon binding of a regulatory particle. Early crystal structures of the *T. acidophilum* proteasome reveal a lack of electron density traversing the 20S pore, and this observation contributed to the belief that ancestral proteasomes lack a gating mechanism (Löwe et al. 1995). Since then, studies have been performed to suggest that the archaeal proteasome has a gate similar to that found in higher organisms (Benaroudj et al. 2003). In Figure 2-5, the crystal structure of the *T. acidophilum* 20S proteasome appears to lack a gate; however, this lack of density is likely due in part to conformational flexibility of the nine N-terminal residues from each alpha-subunit preventing apparent electron density. Recent studies using TROSY-NMR indicate that the N-termini of the *T. acidophilum* alpha-subunits adopt multiple arrangements that interconvert on a short time scale (Religa et al. 2010). These arrangements, some of which include N-termini that traverse the pore, presumably blocking access to the catalytic interior, exist in equilibrium. In addition, the authors showed that the activity of the *T. acidophilum* 20S proteasome against fluorogenic peptide substrates increases when the equilibrium is shifted to prefer conformations with a more open gate (Religa et al. 2010). It has been presumed that the 20S gate evolved as a mechanism to block the uncontrolled degradation of proteins, and that the 20S proteasome is inactive until binding of a regulatory particle, thereby

preventing degradation of proteins that have not been specifically tagged (Köhler et al. 2001; Groll 2003).

In eukaryotic organisms, the 20S gate is opened by binding of a regulatory particle which, in the constitutive 26S proteasome, is PA700 (Chu-Ping et al. 1994). While archaea lack a regulatory particle as complex as PA700, some archaea express a different activating subunit referred to as proteasome-activating nucleotidase or PAN (Zwickl et al. 1999). PAN was first identified as a homolog of the eukaryotic PA700 AAA-ATPases in *M. jannaschii* (Bult et al. 1996). PAN is similar in sequence and function to the eukaryotic Rpt subunits, in that it encompasses all of the motifs responsible for ATPase activity and exhibits the C-terminal HbYX motif that is required by the Rpt subunits for binding to the alpha subunits and gate-opening (Smith et al. 2007). However, it is different in that its functional form is a homo-hexamer, versus the hetero-hexamer observed in higher organisms (Medalia et al. 2006). When the PAN C-terminal peptide from *M. jannaschii* is added to the *T. acidophilum* 20S proteasome, the pore diameter increases from 9Å to 20Å (Rabl et al. 2008). This observation suggests that the archaeal proteasome has a gating mechanism that allows access of substrates to the active sites only under certain conditions, and that gate opening can be responsive to activator binding. The regulatory particle in *T. acidophilum* that serves to open its 20S gate has very recently been reported as Cdc48 (Barthelme

& Sauer 2012), an AAA-ATPase with structural and functional similarities to both PAN and the Rpt subunits of the eukaryotic PA700 regulatory particle. Binding of Cdc48 to the *T. acidophilum* 20S proteasome led to stimulation of activity against peptide and protein substrates (Barthelme & Sauer 2012).

Several observations have led to the assumption that the 20S proteasome lacks any activity without binding of a regulatory particle. First, structural studies of 20S proteasome from yeast and mammals reveal that the alpha subunits form a gate that occludes entry of proteins into the catalytic interior of the proteasome complex (Groll et al. 1997; Unno et al. 2002). Second, the subunits responsible for substrate recognition are components of the PA700 regulatory particle (Deveraux et al. 1994; Thrower et al. 2000; Lam et al. 2002), and no mode of substrate recognition has been identified for the independent 20S proteasome. Third, the unfoldase activities of the 26S proteasome complex that are responsible for unfolding substrates are part of the PA700 regulatory particle (Benaroudj & Goldberg 2000; Smith et al. 2007). Because of these observations, it has been assumed that the binding of a regulatory complex is required for the recognition, unfolding, and translocation of targeted substrates through the narrow pore, allowing access of substrates to the catalytic sites within the complex.

While the role of the 20S proteasome in the absence of any regulatory particle is not well understood, there is increasing evidence suggesting that the

20S proteasome can function independently of the PA700 regulatory particle. Without the activities encompassed by the PA700 subunits, substrates for degradation by the 20S proteasome are not required to be tagged with polyubiquitin, the energy of ATP binding and hydrolysis is not required, and substrates must be unfolded by alternative mechanisms. Recent reports have suggested that only denatured or intrinsically disordered proteins (IDPs) can be degraded by the 20S proteasome in this manner (Asher et al. 2005; Tsvetkov et al. 2008; Baugh:2009jom Tsvetkov & Shaul 2012). This process has recently been termed "degradation by default", and is believed to be an important cellular event (Asher et al. 2006). It is unknown however, whether these IDPs can access the catalytic interior of the 20S proteasome while the gate is closed or whether there is a more complex mechanism by which these substrates are allowed entry to the catalytic sites.

Functions of the UPS

The widespread role of the ubiquitin-proteasome system in the cell is critical for the survival of organisms. All proteins have a determined half-life under normal conditions, with some proteins exhibiting more intrinsic stability than others. Proteins are constantly and deliberately degraded by the UPS in order to deplete old, possibly damaged, proteins, while making room for newly-

synthesized proteins. Because most functions in the cell are performed and regulated by a change in protein levels, the proteasome and its many components are responsible for ensuring that all of these functions occur simultaneously and efficiently. The balance between protein translation and protein degradation is referred to as proteostasis, and the UPS is essential in maintaining this balance.

In addition to its every day role in proteostasis, UPS-mediated protein degradation has been found to play a role in developmental processes and proper cell cycle progression. Post-translational modifications of certain proteasomal subunits (Haass & Kloetzel 1989) and changes in expression levels of subunits in specific cell types (Klein et al. 1990) have been observed during the embryogenesis of *D. melanogaster*. A change in proteasomal activity during the development of muscle in chick embryos has also been observed, and this change in activity is tissue specific (Ahn et al. 1991). Another mechanism by which the proteasome has been implicated in development is at the transcriptional level, through alternative splicing of the Rpn10 subunit in *Xenopus* oocytes (Kawahara et al. 2000). The alternatively spliced variants were shown to incorporate into the 26S proteasome, affording the proteasome with modified substrate specificity (Kawahara et al. 2000). The UPS also plays an important role in cell cycle progression. One such study found that a post-translational modification of one of the core subunits correlates with different stages of the meiotic cell cycle in

goldfish oocytes (Tokumoto et al. 2000). Additionally, the UPS has been found to play an important role in the initiation of apoptosis, through the rapid degradation of anti-apoptotic factors (Sohn et al. 2006). These are only a few of the many studies that have implicated the UPS in important biological processes.

Several alternate and inducible proteasome subunits have been found in certain proteasomal complexes (Foss et al. 1998), and these alternate subunits afford the proteasome with slightly different activities and roles within the cell. An alternate version of the proteasome, referred to as the immunoproteasome, is upregulated during an immune response and plays a role in major histocompatibility complex class 1 antigen presentation (reviewed in Kloetzel 2004). The immunoproteasome has been found in non-immune cell types (Singh et al. 2002), suggesting that it may also play a role outside of the immune system. The immunoproteasome is structurally and genetically similar to the constitutive 20S proteasome, containing two each of seven alpha subunits and two each of seven beta subunits; however, it encompasses slightly different catalytic subunits, referred to as the β1i, β2i, and β5i subunits. These subunits still exhibit the PGPH-like, trypsin-like, and chymotrypsin-like activities, respectively, but they work together to produce slightly different degradation products (Gaczynska et al. 1996). These alternate subunits have been shown to afford the immunoproteasome with slightly increased chymotrypsin-like and trypsin-like activities, and a slightly

decreased PGPH-like activity (Gaczynska et al. 1996). Immunoproteasomes digest substrates into products that are typically 8 to 10 residues in length (Rammensee et al. 1995), and these products are transported to the endoplasmic reticulum for loading onto MHC class I molecules for use in antigen presentation. The expression level of these subunits in immune cells increases quickly in response to interferon-gamma (Hisamatsu et al. 1996; Nandi & H. Jiang 1996). With the increase in expression and the preferential incorporation of all three immune-specific subunits into the proteasome complex (Griffin et al. 1998), an active and functional immunoproteasome complex can be generated very quickly. The half-life of the immunoproteasome is shorter than that of the constitutive proteasome, allowing the functionality of this complex to decrease quickly when it is no longer needed (Heink et al. 2005). The immunoproteasome core particle is capped with a PA28 regulatory particle that has a stimulatory effect on 20S peptidase activity (Ma et al. 1992), and expression of PA28 is also upregulated during an immune response (Ahn et al. 1995). PA28 is composed of two different gene products (Mott et al. 1994), that are assembled in an alternating pattern to form a hexameric ring (Erlander 1996). Like the Rpt subunits of the 19S regulatory particle, the alpha- and beta-subunits of the PA28 regulatory particle both contain a HbYX motif on their C-terminus that is essential in binding to and activating the proteasomal complex (Ma et al. 1993; Mott 1996).

There is another alternate catalytic subunit that is expressed exclusively in the thymus, referred to a β5t (Murata et al. 2007). The β5t subunit also exhibits chymotrypsin-like activity, but proteasomes containing this alternate subunit have lower chymotrypsin-like activity than proteasomes containing the β5 or β5i subunits (Murata et al. 2007). These β5t-containing proteasomes are referred to as thymoproteasomes. While β5t-containing proteasomes have been found to contain the normal β1 and β2 subunits, β5t-containing proteasomes are more often found to contain the inducible immune-specific subunits, β1i and β2i (Murata et al. 2007). CD8+T cells, precurosors of cytotoxic T-cells, do not mature properly in β5t-knockout mice (Nitta et al. 2010), indicating a specific and important role for this catalytic subunit in the immune response. These results reveal that the immunoproteasome and thymoproteasome work together in the thymus to initiate an immune response. These observations reveal that the proteasome is a highly regulated machine that serves a variety of essential physiological functions.

The UPS in Disease

With the complexity of the UPS, it is easy to imagine that any minor disruption in the balance of this system could wreak havoc on the survival of an organism. Abnormalities in the UPS have been observed in many different disease states. Some of these abnormalities have been shown to be the result of various

mutations in proteasomal subunits that affect proteasomal function. Many other disorders have been shown to correlate with proteasomal abnormalities, however it is unknown whether these abnormalities are causative of disease or merely a downstream result of the disease.

It has been proposed that spontaneous mutations in the constitutive β5 subunit during chemotherapeutic treatment with bortezomib may serve as a mechanism by which these cancers become drug-resistant in some patients (Franke et al. 2012). In cell culture models of leukemia, treatment with increasing amounts of bortezomib leads to mutations around the bortezomib binding pocket of the β5 subunit. These mutations were shown to decrease binding affinity for the drug, and also correlated with a decrease in the chymotrypsin-like activity of the proteasome (Franke et al. 2012). Mutations in the immunoproteasome-specific β5i gene have also been recorded, and these particular mutations play a role in several autoimmune syndromes (Agarwal et al. 2010; Y. Liu et al. 2012).

Many studies throughout the past decade have implicated the proteasome in diabetes. One study established a role for the UPS in causing Type 1 diabetes, by leading to destruction of the insulin-producing beta cells in the pancreas (Pinkse et al. 2005). In this study, the authors identified an antigenic peptide produced through both the constitutive- and immunoproteasome-mediated degradation of insulin. This peptide was found to generate autoreactive CD8+T

cells that could then destroy endogenous beta cells. This mechanism has been proposed in the failure of cell transplants that were once expected to cure Type 1 diabetes, as these transplanted cells are often destroyed in a similar manner. There have also been studies establishing a role for the UPS in insulin signaling (Sun et al. 1999; Haruta et al. 2000). In addition, a change in PA28 expression and different subtypes of 20S populations were found in diabetic rats versus control rats (Merforth et al. 2003). Finally, a mutation in TRIM37, an E3 ubiquitin ligase, causes mulibrey (muscle-liver-brain-eye) nanism, a disorder that has been found to result in insulin resistance (Karlberg et al. 2005).

Alterations in proteasomal activity have also been implicated in many cardiac abnormalities. In one report, oxidative modifications to several proteasomal subunits were observed after coronary reperfusion, and these modifications were found to correlate with a decrease in all three catalytic activities of the proteasome in cell extracts (Bulteau et al. 2001). A proteomic study in human hearts from patients with dilated cardiomyopathy revealed increased expression of several components of the UPS and an increased amount of protein ubiquitination, as compared to control hearts (Weekes et al. 2003). Another study showed that apoptosis of cardiomyocytes can result from proteasomal inhibition by oxidized protein aggregates (Powell et al. 2005).

Finally, many studies have implicated a role for the UPS in neurodegenerative diseases. Neurodegenerative diseases have in common the accumulation of misfolded proteins in the brain. These diseases are pathologically differentiated by which regions of the brain are affected, the identity of proteins that accumulate, and the cellular localization at which this pathology is observed. These pathological differences are associated with various behavioral and movement phenotypes that are used to distinguish between the diseases in a clinical setting. Mutations of UPS components have been shown to cause genetic forms of some neurodegenerative diseases. Mutations in ubiquilin-2, a protein that binds both to ubiquitin and the proteasome, have been shown to cause amyotrophic lateral sclerosis (Deng et al. 2011), while mutations in UCH-L1 (a protein with DUB activity) (Leroy et al. 1998) and parkin (an E3 ubiquitin ligase) (Kitada et al. 1998) have been shown to cause familial forms of Parkinson's disease (PD). Additionally, an intronic variant of the gene coding for Rpn10, a subunit of the PA700 regulatory particle, has been shown to be more prevalent in PD patients than age-similar controls (Wahl et al. 2008).

Within protein aggregates that correlate with neurodegenerative diseases, hyper-ubiquitination has been observed. Large amounts of ubiquitinated proteins have been observed in aggregates associated with fronto-temporal dementia (FTD) (Neumann et al. 2006), amyotrophic lateral sclerosis (ALS) (Deng et al.

2010), Huntington's disease (HD) (Sieradzan et al. 1999), and PD (Kuzuhara et al. 1988). Certain forms of ubiquitin linkages have also been shown to play a role in these diseases. K63-linked polyubiquitin chains, the only type of linkage that cannot target proteins for proteasomal degradation (P. Xu et al. 2009), have been shown to occur more frequently in models and patients with neurodegenerative diseases (Tan et al. 2007; Paine et al. 2009). For example, alternate K63-ubiquitin linkages have been observed on the proteins DJ-1 and alpha-synuclein in mammalian cell culture, and these linkages to DJ-1 promoted aggregation of the protein in mammalian cell culture (Zucchelli et al. 2010).

Additionally, it has been reported that different forms of proteins that aggregate in neurodegenerative diseases impair the 26S proteasome. Aβ oligomers (Tseng et al. 2008) and tau (Keck et al. 2003) have both been shown to inhibit proteasomal function. In addition, alpha-synuclein filaments and oligomers have been shown to impair the proteasome by inhibiting its chymotrypsin-like activity (Lindersson et al. 2004). The 26S proteasome is also impaired by oligomeric forms of the prion protein, PrP, that causes Creutzfeldt-Jacob disease (CJD) (Kristiansen et al. 2007) and a disease-causing form of the protein huntingtin that contains an expanded TAG tract (Bennett et al. 2005). Finally, the expression of immunoproteasome-specific subunits has been shown to be increased in human cases of AD (Nijholt et al. 2011), in ALS mouse models (Cheroni et al. 2009), and

HD mouse models (Díaz-Hernández et al. 2003). While the role of the UPS in these neurodegenerative diseases has not been fully elucidated, these observations reveal that an imbalance between protein synthesis and protein degradation in mantaining proteostasis may play a role in disease pathogenesis.

CHAPTER THREE
Alpha-Synuclein Truncation and Disease

Lewy Body Diseases

Lewy bodies are insoluble, predominantly cytoplasmic, protein aggregates located in the brain that are characteristic of a group of neurological diseases. Lewy body diseases include Parkinson's disease (PD), diffuse Lewy body disease (DLBD), and the Lewy body variant of Alzheimer's disease (LBV). No single event has been shown to cause Lewy body diseases, yet all of these diseases result in similar pathological and physiological characteristics. Lewy body diseases are all pathologically defined by the accumulation of cytoplasmic protein deposits and neuronal cell death (reviewed in Trojanowski et al. 1998). Physiological effects include an increase in cellular oxidative damage (Norris & Giasson 2005) and inflammation (Mackenzie 2000; reviewed in Qian et al. 2010). These diseases are all progressive and correlate with a decline in motor and cognitive functions, and eventual fatality.

The major component of Lewy bodies is alpha-synuclein (Spillantini et al. 1998) (Figure 3-1). Before the identification of alpha-synuclein, Lewy bodies were characterized by the presence of ubiquitin and hyper-ubiquitinated proteins (Kuzuhara et al. 1988). In addition to alpha-synuclein and ubiquitin, Lewy bodies have been found to contain a plethora of other protein components, but not all of

these components are present in every Lewy body in every patient. Other proteins that play a role in the UPS have been identified in Lewy bodies, and these include dorfin (Ito et al. 2003), Nub1 (Tanji et al. 2011), and p62 (Kuusisto et al. 2001; Zatloukal & Stumptner 2002). Other proteins identified in Lewy bodies include microtubule-associated proteins (Gai et al. 1996; Jensen et al. 2000; D'Andrea et al. 2001; Duda et al. 2002) and protein kinases (Arawaka et al. 2006). The mechanism by which these proteins co-aggregate with alpha-synuclein and the significance of their aggregation is unknown.

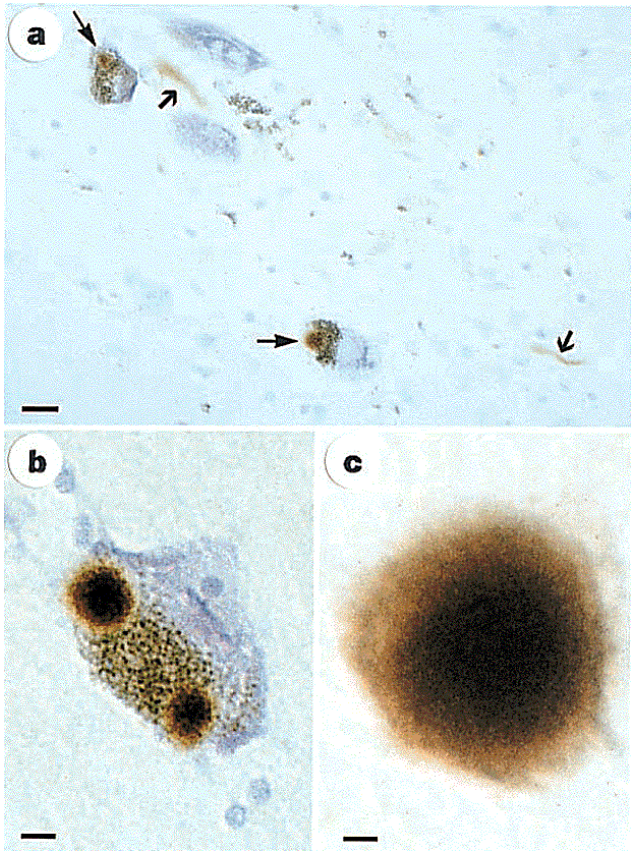

Figure 3-1. Alpha-synuclein-positive Lewy bodies and Lewy neurites. Substantia nigra from PD patients stained for alpha-synuclein (brown) A. Lewy bodies and Lewy neurites. B. A single nerve cell containing two Lewy bodies. C. An extracellular Lewy body. Reprinted by permission from Macmillan Publishers Ltd: Nature (Spillantini et al. 1997).

While a vast amount of information has been generated about Lewy body diseases in the past two decades, many questions remain about the cause and progression of these diseases. One such question is whether the alpha-synuclein-containing protein aggregates are harmful or if they are merely a mechanism by which the cell sequesters individual protein components that might, themselves,

be toxic. Recent results suggest that the alpha-synuclein-containing inclusions may not be toxic themselves, but that the intermediate, oligomeric species along the aggregation pathway might be responsible for proteotoxicity (Volles & Lansbury 2003; Winner et al. 2011). The factors that initiate this aggregation pathway are unknown. Several factors, however, have been shown to contribute to the development and progression of Lewy body diseases, including both genetic and environmental factors. In the case of Parkinson's disease (PD), three independent missense mutations in alpha-synuclein (A30P, E46K, and A53T) have been shown to cause early-onset forms of the disease (Krüger et al. 1998; Zarranz et al. 2004; Polymeropoulos et al. 1997). Duplications (Chartier-Harlin et al. 2004) and triplications (Singleton et al. 2003) of the alpha-synuclein gene also cause early-onset forms of PD. Mutations in other genes, such as LRRK2 (Paisán-Ruíz et al. 2004; Zimprich et al. 2004), PARK2 (Kitada et al. 1998), PINK1 (Valente et al. 2004), DJ-1 (Bonifati et al. 2003), and ATP13A2 (Ramirez et al. 2006) are linked to familial forms of PD; however, these monogenic forms of PD only account for about 5% of all cases. Interestingly, the other 95% of cases have the same pathological hallmarks as the monogenic forms of disease. No mutations in the SCNA gene encoding for alpha-synuclein have been found to cause other Lewy body diseases, yet these diseases exhibit alpha-synuclein pathology similar to that found in PD patients. These observations suggest that a common

mechanism of pathogenesis exists in all Lewy body diseases, but that the initiation of pathogenesis may vary.

The most prevalent risk factor for Lewy body diseases is age. Many physiological processes are altered as an organism ages. Some of the age-related processes that have been correlated with Lewy body diseases include an increase in oxidative damage to cellular components (reviewed in Sohal & Orr 2012), dysfunction of the mitochondria (reviewed in Bratic & Trifunovic 2010), and the long-term exposure to environmental toxins (Masalha et al. 1997). Additionally, many studies have aimed to understand the effects of aging on the cellular protein degradation machinery. Changes in both major pathways of protein degradation in the cell, the UPS and autophagy, have been observed with age. While effects on the trypsin- and chymotrypsin-like activities of the proteasome with age have been inconclusive (Shibatani et al. 1996; Hayashi & Goto 1998; Andersson et al. 1998; Ponnappan et al. 1999), the PGPH-like activity of the proteasome has consistently been shown to decrease with age (Conconi et al. 1996; Hayashi & Goto 1998). Additionally, gene expression studies have indicated a change in proteasomal subunit expression patterns with age in both murine muscle (C. K. Lee et al. 1999) and human fibroblasts (Ly et al. 2000). A decline in autophagy function was observed in rats and human fibroblasts, through a decrease in both substrate binding and transport to lysosomes (Cuervo & Dice 2000).

With these wide-ranging physiological alterations that occur with age, it is reasonable to hypothesize that proteins could be modified over time, leading to enhanced aggregation propensity, and the possible initiation of disease. Lewy body diseases might be the result of the failing protein degradation pathways being unable to compensate for the buildup of damaged proteins. Certain combinations of variables or specific genetic backgrounds may yield an individual more susceptible to these alterations and the lack of compensatory mechanisms, explaining why some individuals succumb to Lewy body diseases while others do not. A further study of each of these processes will allow for a more complete understanding of disease pathogeneis and the generation of targeted therapeutics to slow progression or prevent these diseases altogether.

Alpha-Synuclein Structure

Alpha-synuclein is a 140-amino acid protein that is paralogous to two other nervous system proteins, referred to as beta- and gamma-synuclein. Alpha-, beta-, and gamma-synuclein are similar in sequence, with most of the similarity lying within the N-terminus of the proteins. The N-terminal portion of alpha-synuclein includes seven imperfect repeats of 11 residues containing the KTKEGV consensus sequence, while the C-terminus contains many acidic residues and is, thus, negatively-charged (Figure 3-2). Residues 61-95 encompass

many hydrophobic residues, and a peptide corresponding to this region of the protein (referred to as the NAC region) has been found to colocalize with Aβ plaques in Alzheimer's disease (Uéda et al. 1993).

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  1 MDVFMKGLSK AKEGVVAAAE KTKQGVAEAA GKTKEGVLYV GSKTKEGVVH GVATVAEKTK EQVTNVGGAV
 80 VTGVTAVAQK TVEGAGSIAA ATGFVKKDQL GKNEEGAPQE GILEDMPVDP DNEAYEMPSE EGYQDYEPEA
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Figure 3-2. Human alpha-synuclein sequence. The sequence of the 140-amino acid protein (accession number P37840.1) is shown. Underlined regions are the seven imperfect 11-residue repeats and the sequence in red is the amyloidogenic NAC (Non-Aβ Component) region of the protein.

Alpha-synuclein is among the increasing number of proteins recognized as an intrinsically-disordered protein (IDP), a class of proteins characterized by their lack of a defined structure in the native state (reviewed in Dunker et al. 2001). Upon binding to negatively-charged vesicles, alpha-synuclein adopts a conformation that has a high alpha-helical propensity (Davidson et al. 1998; Eliezer et al. 2001), and a fraction of neuronal alpha-synuclein has been found to associate with membranes *in vivo* (Leng et al. 2001). Structural studies aimed to understand the membrane-bound form of alpha-synuclein have relied on nuclear magnetic resonance (NMR) spectroscopy. A structure of the full-length, membrane-bound form of alpha-synuclein (Figure 3-3) reveals a conformation in

which the N-terminal two-thirds of the protein forms a broken, amphipathic alpha-helix (Chandra et al. 2003; Ulmer et al. 2005). This structured portion of the protein is responsible for membrane binding, and residues at the very N-terminus are essential for this process (Vamvaca et al. 2009). In the NMR structure of alpha-synuclein, the negatively-charged C-terminal tail remains flexible and disordered (Chandra et al. 2003; Ulmer et al. 2005).

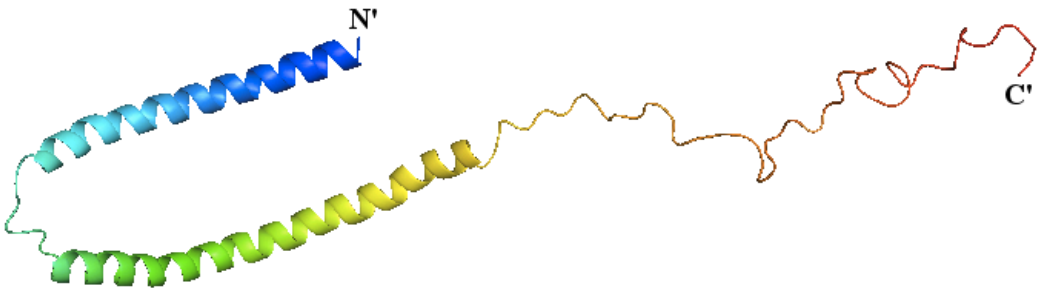

Figure 3-3. Structure of alpha-synuclein bound to lipid vesicles. The structure of the full-length human protein was determined by NMR (PDB ID: 1XQ8) and image was rendered in PyMol. The structure is colored from blue (N-terminus) to red (C-terminus).

Structural studies aimed to understand the unbound state of alpha-synuclein have relied on molecular dynamics (MD) simulations and more complicated NMR techniques, such as residual dipolar coupling (RDC) and

paramagnetic relaxation enhancement (PRE). These techniques have produced results suggesting that alpha-synuclein adopts several thousand structurally distinct conformations, many of which are more compact than expected for a random coil (Dedmon et al. 2005). Many of these conformations include long-range (15Å to 20Å) interactions between the C-terminus and both the N-terminus and central portion of the protein (Bernadó et al. 2005; Bertoncini, Jung, et al. 2005b; Dedmon et al. 2005).

Several observations have suggested that disruption of these long-range interactions facilitates aggregation of the protein. In one study, spermine (a polyamine that has been shown to interact with the acidic C-terminus of alpha-synuclein) was shown to disrupt these long-range interactions while simultaneously promoting *in vitro* aggregation (Bertoncini, Jung, et al. 2005b). A similar result was observed when temperature was increased (Bertoncini, Jung, et al. 2005b). Additionally, studies have shown that the PD-causing A30P and A53T alpha-synuclein mutations both have decreased propensity for these long-range interactions (Bertoncini, Fernandez, et al. 2005a). A disruption of these intramolecular long-range interactions and increased aggregation propensity of the protein may serve as a mechanism by which these two point mutations cause Lewy body formation and disease pathogenesis.

Alpha-Synuclein Physiology and Function

Alpha-synuclein is a protein expressed in all vertebrates. Homology of alpha-synuclein across species is greater at the N-terminus of the protein, with more variability in sequence located toward the C-terminus. Alpha-synuclein is expressed predominantly in the central nervous system and localizes to presynaptic terminals (Iwai et al. 1995). Expression of alpha-synuclein is quite high, consisting of up to 1% of the total protein in certain regions of the brain (Iwai et al. 1995). Within neuronal cells, alpha-synuclein has been detected in both the cytoplasm and nucleus (S. Yu et al. 2007). Studies utilizing fractionated rat brains revealed that about 15% of alpha-synuclein is membrane-bound (H.-J. Lee et al. 2002), and the protein was recently found associated with mitochondrial membranes in normal dopaminergic neurons (W.-W. Li et al. 2007; L. Zhang et al. 2008). The relative subcellular distribution of alpha-synuclein varies among different neuronal cell populations (L. Zhang et al. 2008; G. Liu et al. 2009).

While the function of alpha-synuclein has not been clearly established, observations related to the protein's cellular localization have provided clues to its function. Studies aimed directly at establishing a role for alpha-synuclein have relied on mammalian cell culture and animal models. Alpha-synuclein knockout mice have been generated in several laboratories and these mice are viable, suggesting that other proteins might play a redundant role in the cell. Deletion of

alpha-synuclein in mice causes only mild phenotypes including defects in presynaptic vesicles (Cabin et al. 2002), synaptic transmission (Martín et al. 2004), and the trafficking (Castagnet et al. 2005) and metabolism (Golovko et al. 2007; Barceló-Coblijn et al. 2007) of fatty acids. Additionally, mice lacking alpha-synuclein do not suffer the changes in cellular morphology and cell death in response to MPTP (an inhibitor of mitochondrial complex 1) that are observed in wild-type mice (Dauer et al. 2002). The levels of striatal dopamine were also less affected by MPTP-treatment in alpha-synuclein knockout mice than wild-type mice (Drolet et al. 2004).

Alpha-synuclein has been shown to play a role in neurotransmitter release, as studied by neuronal cell lines expressing alpha-synuclein (Nemani et al. 2010), knockout mice (Abeliovich et al. 2000), and mice overexpressing alpha-synuclein (Nemani et al. 2010). Alpha-synuclein has also been shown to exhibit a non-classical chaperone activity that plays a role in SNARE complex assembly (Burré et al. 2010). Both the N- and C-termini of alpha-synuclein play a role in this process, as the N-terminus of the protein binds to phospholipids while the C-terminus is necessary for synaptobrevin-2 binding (Burré et al. 2010). Alpha-, beta-, and gamma-synuclein triple knockout mice showed an age-dependent decrease in SNARE complex assembly (Burré et al. 2010), indicating that other

members of the synuclein family may serve roles that are similar to or possibly overlap with those of alpha-synuclein.

While neuronally-expressed alpha-synuclein has been the focus of most studies, many other tissues have been found to express the protein. These tissues include muscle (Askansas et al. 2000), cerebral blood vessels (Tamo et al. 2002), red blood cells (Barbour et al. 2008), plasma (Tinsley et al. 2010), and blood cells of the immune system (S. Kim et al. 2004). The function of alpha-synuclein in these tissues has not been studied. An investigation of alpha-synuclein expression in human fetuses revealed that alpha-synuclein is expressed throughout fetal tissue; however, expression in most of these tissues is reduced in adulthood (Ltic et al. 2004), suggesting that alpha-synuclein might also play a role in development.

Alpha-Synuclein Truncation and Disease

Wild-type and the three PD-causing missense mutations of alpha-synuclein were the primary focus of early studies related to alpha-synuclein aggregation and disease; however, recent studies have acknowledged post-translational modifications of alpha-synuclein and the role that these forms of the protein might play in disease. Within Lewy bodies, alpha-synuclein has been found to exist with several modifications (Table 3-1). These modifications include

phosphorylation (Fujiwara et al. 2002; Anderson et al. 2006; Paleologou et al. 2010), nitration (Giasson et al. 2000), and ubiquitination (Sampathu et al. 2003; Tofaris et al. 2003). In addition to full-length alpha-synuclein, truncated forms of alpha-synuclein have been identified in pathological aggregates (Baba et al. 1998; Campbell & McLean 2001). These truncations have been found to occur from either the C-terminus or both the N-terminus and C-terminus in patient samples (C-W. Liu et al. 2005; Lewis et al. 2010) and transgenic mouse models of PD (C-W. Liu et al. 2005; Li et al. 2005). The C-terminus of alpha-synuclein is negatively-charged, and truncating the protein from this terminus produces species that are more prone to aggregation *in vitro* (I. Murray et al. 2003; Hoyer et al. 2004; C.-W. Liu et al. 2005). Notably, truncated alpha-synuclein can facilitate aggregation of the full-length protein *in vitro* (C.-W. Liu et al. 2005) and *in vivo* (Ulusoy et al. 2010). A truncated form of alpha-synuclein, the NAC region, accumulates in Alzheimer's disease patients (Uéda et al. 1993), suggesting a role for alpha-synuclein truncation in the pathogenesis of multiple diseases.

Protein Modification	Source of Identification	Effect on *in vitro* Fibrillization	Source of *in vitro* Studies
nitration of Tyr residues	(Giasson et al. 2000)	decreased fibrillization	(Yamin et al. 2003; Uversky et al. 2005)
S87 phosphorylation	(Paleologou et al. 2010)	decreased fibrillization	(Paleologou et al. 2010)
S129 phosphorylation	(Fujiwara et al. 2002; Anderson et al. 2006)	inconclusive	(Fujiwara et al. 2002; Paleologou et al. 2008)
truncation (C-terminal)	(C-W. Liu et al. 2005; Li et al. 2005; Lewis et al. 2010)	increased fibrillization	(I. Murray et al. 2003; Hoyer et al. 2004; C-W. Liu et al. 2005)
truncation (N-terminal)	(C-W. Liu et al. 2005)	increased fibrillization	(Kessler et al. 2003)
mono-, di-, tri-ubiquitination	(Sampathu et al. 2003; Tofaris et al. 2003)	unknown	-

Table 3-1. Modifications of alpha-synuclein identified in Lewy bodies and their effects on *in vitro* aggregation. The identification of alpha-synuclein modifications that are present in Lewy bodies might provide insight into disease progression.

Transgenic mice have recently been generated to express truncated forms of alpha-synuclein, and these mice exhibit physiological and pathological similarities to patients with Lewy body diseases (Michell et al. 2007; Wakamatsu et al. 2008; Daher et al. 2009). The three different models generated expressed the human alpha-synuclein (residues 1-120) on a mouse alpha-synuclein null

background (Michell et al. 2007), human A53T alpha-synuclein (residues 1-130) on an endogenous mouse alpha-synuclein background (Wakamatsu et al. 2008), and human alpha-synuclein (residues 1-119) on an endogenous mouse alpha-synuclein background (Daher et al. 2009). The mice expressing human A53T alpha-synuclein (residues 1-130) exhibited loss of dopaminergic neurons in the substantia nigra, lower levels of striatal dopamine, and an alteration in spontaneous locomotor activities (Wakamatsu et al. 2008). Mice expressing human alpha-synuclein (residues 1-119) showed a similar loss of striatal dopamine (Daher et al. 2009). The expression of truncated alpha-synuclein also led to a greater susceptibility to stress (Michell et al. 2007).

The identification of truncated forms of alpha-synuclein that are both prone to aggregation and capable of cross-seeding aggregation of the full-length protein, suggests that alpha-synuclein truncation is a mechanism that contributes to the progression of Lewy body diseases. Some reports have indicated that truncation of alpha-synuclein is a natural process, and that truncated forms of alpha-synuclein are detectable in the brain of healthy individuals (Muntané et al. 2012). However, observations of the truncated alpha-synuclein transgenic mice models described previously (Michell et al. 2007; Wakamatsu et al. 2008; Daher et al. 2009) suggest that truncated alpha-synuclein species might lead to the development of clinical and pathological features if expression exceeds a certain

level. In individuals with disease, the amount of truncated alpha-synuclein species generated might have reached a threshhold that can no longer be tolerated by the cell. The mechanism by which these truncated forms of alpha-synuclein are produced and accumulate in the cell is unknown.

Degradation of Alpha-Synuclein by the 20S Proteasome

Several *in vitro* studies have shown that alpha-synuclein can be degraded by the 20S proteasome in a ubiquitin-independent manner (Tofaris et al. 2001; C-W. Liu et al. 2003; C-W. Liu et al. 2005). While degradation of alpha-synuclein by the 20S proteasome has not been established in an animal model, several *in vivo* observations support a role for the 20S proteasome in alpha-synuclein truncation and disease. C-terminally-truncated forms of alpha-synuclein isolated from A53T alpha-synuclein transgenic mice were identified by mass spectrometry, and some species were identical to those produced by the 20S proteasome *in vitro* (C-W. Liu et al. 2005; Li et al. 2005). Follow-up studies, in which antibodies were generated to specifically recognize the C-terminus of truncated forms of alpha-synuclein, revealed that two C-terminally-truncated alpha-synuclein species, residues 1-110 (syn110) and residues 1-119 (syn119) are present at much higher levels in patients with Lewy body diseases than in age-similar controls (Lewis et al. 2010). Additionally, it was shown that these

truncated species are not always colocalized within the same cell (Lewis et al. 2010), hinting at a mechanism by which their production may be regulated.

Roles of Other Enzymes in Alpha-Synuclein Degradation

While many independent laboratories have shown that alpha-synuclein can be degraded by the 20S proteasome *in vitro* (Tofaris et al. 2001; C-W. Liu et al. 2003; Machiya et al. 2010), other studies have implicated different enzymes in the cleavage and degradation of alpha-synuclein. These enzymes are different in their activities, their cellular localization, and their regulation. It is possible that more than one of these enzymes works in concert to produce truncated forms of alpha-synuclein that promote Lewy body formation and disease progression. Understanding these processes and their cooperativity in normal physiological processes and in disease progression is essential to the understanding and treatment of Lewy body diseases.

Calpain 1 is a calcium-dependent cysteine protease (Ohno et al. 1984; Suzuki et al. 1988). In *in vitro* assays, the monomeric form of alpha-synuclein is predominantly cleaved by calpain 1 after residue 57, while fibrillar forms of alpha-synuclein are degraded at the C-terminus, specifically after residues 114 and 122 (Mishizen-Eberz et al. 2003). Another study by the same group revealed that the cleavage products produced by calpain 1-mediated degradation of soluble

alpha-synuclein inhibited aggregation of the full-length protein, while cleavage products produced by calpain 1-mediated degradation of fibrillar forms of alpha-synuclein were aggregation-prone and capable of cross-seeding aggregation of full-length, monomeric alpha-synuclein (Mishizen-Eberz et al. 2005). It has also been reported that the activities of calpain I and the 20S proteasome may act concertedly in the production of aggregation-prone C-terminally truncated forms of alpha-synuclein (H. Kim et al. 2006). In this study, a product of calpain 1-mediated degradation that was resistant to further degradation by the enzyme, was able to enhance the degradation of full-length alpha-synuclein by the 20S proteasome and this enhancement was specific for alpha-synuclein, as no enhancement was observed for the degradation of azocasein or peptide substrates (H. Kim et al. 2006).

Another protein that has been suggested to play a role in the accumulation of truncated forms of alpha-synuclein is cathepsin D. Cathepsin D is a lysosomal protease that has been shown to cleave alpha-synuclein both *in vitro* and in the lysosomal fraction of cells expressing alpha-synuclein (Sevlever & P. Jiang 2008). In one study, reduced proteasomal activity was observed in cathepsin D-deficient mice (Qiao et al. 2008), implicating a connection between these two mechanisms of proteolysis. Additionally, RNAi knockdown of cathepsin D in a mammlian cell culture model (Sevlever & P. Jiang 2008) and cathepsin D knockout mice (Qiao et

al. 2008) exhibit alpha-synuclein accumulations. Another study, utilizing a *Drosophila* model of human synucleinopathies, revealed enhanced toxicity when cathepsin D was depleted (Cullen et al. 2009), suggesting that this enzyme might play a role in the normal clearance of alpha-synuclein.

In light of recent reports suggested that alpha-synuclein is found extracellularly (Borghi et al. 2000; El-Agnaf et al. 2003; Sung et al. 2005; H.-J. Lee et al. 2005), proteases that might act on these extracellular forms of alpha-synuclein might also play a role in this process. One such enzyme is neurosin, a serine protease that is highly expressed in the nervous system (Yamashiro et al. 1997). Neurosin has been shown to co-aggregate with alpha-synuclein in Lewy bodies (Iwata et al. 2003) and, in *in vitro* assays, specific cleavage products of alpha-synuclein were produced in which the protein was cleaved in the NAC region and at several sites within the C-terminus (Kasai et al. 2008). A recent study has revealed that neurosin-mediated cleavage of alpha-synuclein can only occur extracellularly, once neurosin is activated upon secretion (Tatebe et al. 2010).

Matrix metalloproteases (MMPs) are a class of enzymes that are secreted, and they are known to play a role in the degradation of extracellular and membrane-bound proteins (reviewed in Blobel 2000). Several studies have implicated MMPs in the cleavage and aggregation of alpha-synuclein. In one

study, a dopaminergic neuronal cell line was transfected with alpha-synuclein, and the over-expression of alpha-synuclein led to its secretion (Sung et al. 2005). Additionally, when these transfected cells were subjected to oxidative stress, the expression of matrix metalloprotease-3 (MMP-3) was increased and alpha-synuclein fragments were observed in the media. Generation of alpha-synuclein fragments was blocked by pre-incubation with a matrix metalloprotease inhibitor. In addition, results from this study showed that alpha-synuclein can be cleaved at several positions, and the products generated facilitate aggregation and cell toxicity. Other studies have also shown that matrix metalloproteases can cleave alpha-synuclein, and cleavage by both MMP-1 and MMP-3 were shown to increase aggregation propensity (Levin et al. 2009). Another study showed C-terminal cleavage by MMP-3, and found that MMP-3 cleavage of the disease-causing A53T mutation of alpha-synuclein resulted in an increased number of degradation products (Choi et al. 2011). By analyzing postmortem brain tissue from PD patients, the authors revealed that over 50% of Lewy bodies contain MMP-3 (Choi et al. 2011). Recently, plasmin, a serine protease in the blood, was also implicated in alpha-synuclein degradation and disease pathogenesis (K. S. Kim et al. 2012).

These *in vitro* and *in vivo* results reveal that the formation of truncated alpha-synuclein species is a complex process that likely plays a role in disease.

The major goal of studying these diseases is to develop therapeutics to halt or slow down the progression of the disease. Elucidating the mechanism by which these enzymes produce partially-truncated and aggregation-prone alpha-synuclein cleavage products is an initial step in identifying therapeutic targets. With the number of enzymes that have been shown to produce truncated alpha-synuclein species *in vivo*, it is likely that alpha-synuclein degradation is the result of a combination of enzymes that either work independently or together to produce specific aggregation-prone species.

CHAPTER FOUR
Substrate Selection by the 20S Proteasome

INTRODUCTION

It has only recently been shown that some proteins can be degraded by the 20S proteasome in a ubiquitin-independent manner. Neither the mechanism by which these substrates are selected for degradation, the means by which they access the catalytic sites, nor the physiological consequences of this process are well understood. The current hypothesis in the field is that proteins that are unfolded or contain disordered regions can be degraded by the 20S proteasome in the absence of a polyubiquitin tag (Baugh et al. 2009), and this process has been referred to as "degradation by default" (Asher et al. 2006). While it is still uncertain whether the ubiquitin-independent degradation of proteins by the 20S proteasome is a widespread and regulated phenomenon, there are several examples suggesting that this process may play important and specific roles within the cell.

One example in which the 20S proteasomal-mediated degradation of a protein produces a specific and functional degradation product is processing of the p105 protein, a precursor of the p50 component of NFκB, an important player in the immune system. The inactive p105 precursor is cleaved at a single site by the 20S proteasome in a ubiquitin-independent manner to produce the active p50

subunit (Moorthy et al. 2006). Processing of the p105 precursor occurs from the C-terminus, and a glycine-rich region within the protein serves as a signal to prevent further degradation (Moorthy et al. 2006). Additionally, it was shown that truncated forms of the p105 precursor are still processed into p50, but processing of the full-length p105 occurs more efficiently (Moorthy et al. 2006). It was shown previously that the p105 precursor is located only in the cytoplasm, while the processed p50 is also found in the nucleus (Blank et al. 1991). Processing of the p105 precursor protein leads to removal of the C-terminus that is responsible for blocking nuclear entry (Blank et al. 1991) and, once the C-terminus is removed, the active p50 protein can enter the nucleus and function as a transcription factor. These studies together suggest a mechanism by which the 20S proteasome is responsible for activating a protein and altering its cellular localization by a specific cleavage event.

LC3, a protein that has been shown to play an important but not well understood role in autophagy (Tsukada & Ohsumi 1993), can also be processed by the 20S proteasome (Gao et al. 2010). LC3 was found to be cleaved toward the C-terminus of the protein (Gao et al. 2010). This cleavage event produces a species that is no longer able to bind to the autophagosome, thereby, preventing autophagy. This process was shown to be specific for LC3, as other proteins that play a role in autophagy were not substrates for 20S proteasomal degradation. If

this cleavage event of LC3 is relevant *in vivo*, this may serve as a mechanism by which the 20S proteasome can alter autophagy, indicating interplay between the two major cellular processes that play a role in protein degradation.

The number of proteins shown to be degraded by the 20S proteasome in a ubiquitin-independent manner has been growing steadily for the past decade. Some of the early proteins that were identified to be degraded in this manner include ornithine decarboxylase (Glass & Gerner 1987), $p21^{WAF1/CIP1}$ (Sheaff et al. 2000), NS2 (Miller & Pintel 2001), alpha-synuclein (Tofaris et al. 2001), and p53 (Asher et al. 2005). The mechanism by which these proteins are targeted for degradation and recognized by the 20S proteasome are unknown. It is unlikely that IDPs are rapidly degraded upon their translation, and alpha-synuclein has been shown to have a relatively long half-life in both HEK293 cells (Paxinou et al. 2001) and dopaminergic neurons (W. Li et al. 2004). Therefore, some mechanism must exist to affect propensity of the substrate to be recognized and degraded by the 20S proteasome. Several proteins that can be degraded by the 20S proteasome have been shown to bind directly to proteasomal subunits (Touitou et al. 2001; Yuksek et al. 2009). $p21^{WAF1/CIP1}$ has been shown to interact with the alpha-7 (Baumeister nomenclature) subunit of the 20S proteasome through its C-terminus, and removal of the C-terminus of $p21^{WAF1/CIP1}$ prevented its degradation *in vitro* (Touitou et al. 2001). An additional study showed that the

oncogene MDM2 can interact with $p21^{WAF1/CIP1}$, inducing a conformational change that strengthens its interaction with alpha-7 (H. Xu et al. 2010). The increase in interaction between the substrate and enzyme correlated with more efficient degradation of the substrate (H. Xu et al. 2010). This result strongly suggests that the binding of $p21^{WAF1/CIP1}$ to the alpha-7 subunit of the 20S proteasome plays an important role in its recognition and degradation. This mechanism highlights the complexity of substrate recognition and degradation by the 20S proteasome.

Previous studies have shown that the presence of disordered regions within a protein are necessary for its recognition and degradation by the 20S proteasome; however, there is no evidence that the presence of this feature is sufficient for degradation. The studies described above with the p105 precursor protein and $p21^{WAF1/CIP1}$ reveal that recognition and degradation of proteins by the 20S proteasome might be more complex than previously appreciated. As shown for $p21^{WAF1/CIP1}$, other proteins might play a role in altering degradation propensity of particular substrates, and these alterations could occur through a variety of mechanisms.

While some degradation products of ubiquitin-independent degradation have an established role in the cell, the effects of other degradation products are unknown. These products might play particular roles in the cell, but they could

also merely be by-products of an inefficient degradation pathway. For some proteins, the generation of partially-cleaved by-products have been shown to play a role in disease by either hindering (Nunan et al. 2003) or enhancing (C-W. Liu et al. 2005) disease-related pathology. Because the 20S proteasome produces partially-truncated alpha-synuclein species that accumulate in patients with Lewy body diseases, understanding the mechanism by which these truncated species are not further degraded will provide further insight into disease pathogenesis. The following studies aim to further uncover the mechanism by which proteins are recognized and degraded by the 20S proteasome, with an emphasis on understanding degradation propensity of specific pathogenic alpha-synuclein species.

RESULTS

Unfolded proteins are preferentially degraded by the 20S proteasome.

Studies have suggested proteins that are unfolded or disordered are substrates for 20S proteasomal degradation (Asher et al. 2005; Tsvetkov et al. 2008; Baugh et al. 2009; Tsvetkov & Shaul 2012). To further test this hypothesis, wild-type RNase Sa and various mutations of the protein were analyzed for their propensity toward degradation by the 20S proteasome (Figure 4-1). RNase Sa is a small, folded, and thermodynamically stable protein based on its receptiveness to high resolution structural studies (Sevcik et al. 1996; Laurents et al. 2001) and calculated melting temperature (Pace et al. 1998). Point mutations and deletions within RNase Sa have been identified that alter the thermodynamic stability of the protein as determined by thermal denaturation (Pace et al. 2001).

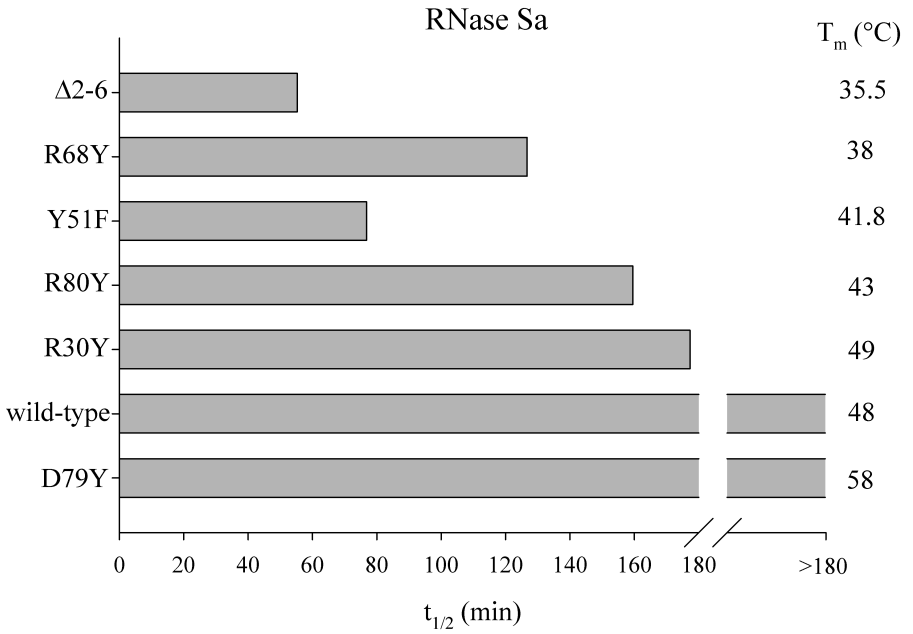

Figure 4-1. Degradation of RNase Sa constructs by purified 20S proteasomes. Wild-type RNase Sa and each mutant were incubated with purified 20S proteasome as described in Materials and Methods. The half-life of each construct was empirically determined by quantifying the amount of full-length RNase Sa remaining at each time point on Coomassie stained gels and fitting the data to a single exponential decay function.

Wild-type RNase Sa and the stabilizing D79Y mutation are degraded much less rapidly by the 20S proteasome than mutants with lower thermal stability, as a decrease in the amount of full-length protein was not detectable over the time course tested (180 minutes). The results reveal a general trend in which the half-life of a protein decreases as thermal stability decreases. A similar approach was taken with the protein titin (Figure 4-2), with the V13P mutant and wild-type

carboxymethylated titin selected as variants with decreased thermodynamic stability (Kenniston et al. 2003).

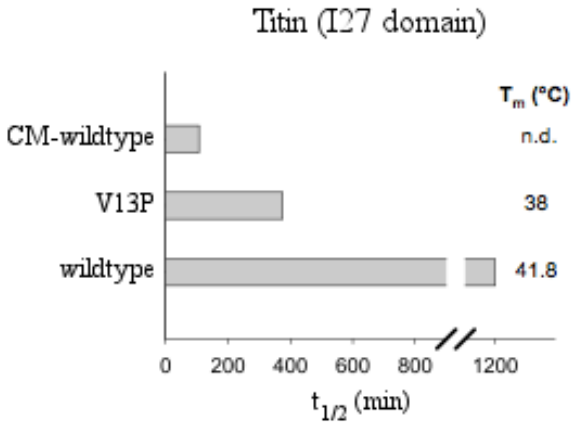

Figure 4-2. Degradation of titin constructs by purified 20S proteasomes. Each ^{14}C-labeled titin construct was incubated with purified 20S proteasome and the amount of degradation product was measured by quantifying soluble ^{14}C levels. The half-life of each construct was calculated by plotting soluble ^{14}C levels and fitting the data to a single exponential decay function.

The destabilizing V13P mutation and carboxymethylated form of titin exhibit a decrease in half-life as compared to wild-type titin. These results suggest that unfolded proteins or proteins with lower thermodynamic stability are more readily degraded by the 20S proteasome, consistent with the idea that unfolded proteins are better substrates for proteolysis by the 20S proteasome than well-folded, globular proteins.

Degradation of alpha-synuclein is dependent on its structural conformation.

Alpha-synuclein is a member of a growing class of proteins collectively referred to as intrinsically disordered proteins (IDPs). Alpha-synuclein lacks secondary structure when unbound to membranes (Eliezer et al. 2001), however, upon membrane association, it adopts a predominantly alpha-helical structure. Upon the addition of SDS or synthetic liposomes, alpha-synuclein adopts a conformation with high alpha-helical content (Eliezer et al. 2001; Davidson et al. 1998), that can be observed by circular dichroism. The degradation propensity of alpha-synuclein decreases with an increase in alpha-helical content of the protein (Figure 4-3), as monitored by calculating the half-life in the presence of various amounts of SDS.

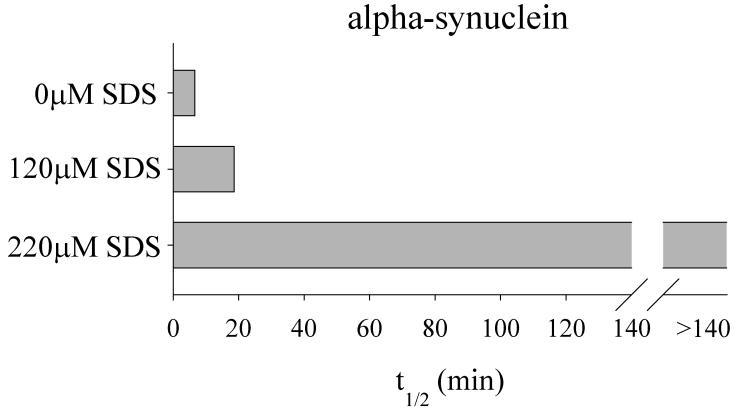

Figure 4-3. Degradation of alpha-synuclein with various amounts of SDS. The stability of alpha-synuclein in the presence of 20S proteasomes is increased with increasing amounts of SDS, revealing that secondary structure formation correlates with increased stability. The half-life of alpha-synuclein under each condition was determined as described in Figure 4-1.

To account for any effect of SDS on the 20S proteasome itself, the activity of 20S against fluorogenic peptide substrates was monitored at these SDS concentrations (data not shown). SDS increases the activity of the 20S proteasome against peptides up to 220μM, revealing that the observed effect of SDS on degradation propensity of alpha-synuclein is due to a change in the substrate and not the enzyme.

Disorder is not sufficient for susceptibility to degradation by the 20S proteasome.

To test whether all natively-disordered proteins are substrates for the 20S proteasome, various artificial constructs were generated (Figure 4-4) in which the sequence elements of alpha-synuclein were swapped while the length of the construct was not changed.

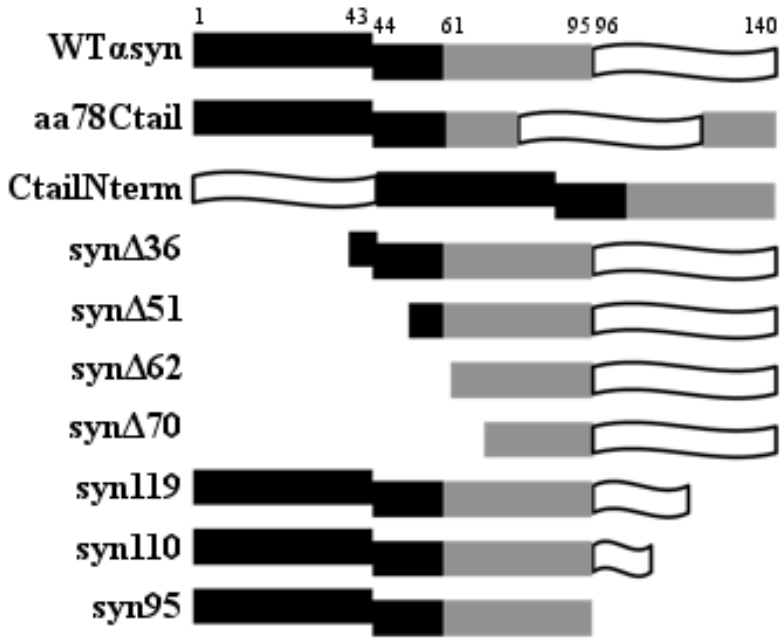

Figure 4-4. Schematic of all alpha-synuclein constructs utilized in this study. All constructs were generated and expressed in *E. coli* from the pET28a bacterial expression vector. Identical purification protocols were utilized for each construct and final purity of each was similar, as estimated by Coomassie-stained gels of the purified protein (not shown).

The CtailNterm construct contains the 45 residue acidic C-tail translocated to the N-terminus of alpha-synuclein, while the aa78Ctail construct contains the acidic C-tail inserted between residues 78 and 79, the center of the highly amyloidogenic region of the protein. Circular dichroism spectroscopy was utilized to qualitatively assess the degree of secondary structural elements in these artificial constructs both in the absence and presence of synthetic lipid vesicles (POPA/ POPC) (Figure 4-5).

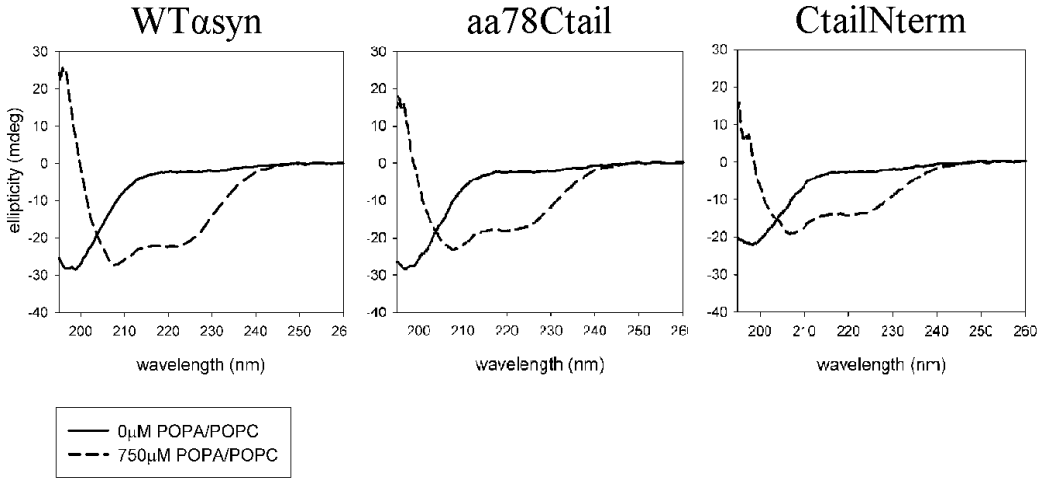

Figure 4-5. Circular dichroism spectroscopy of wild-type alpha-synuclein and the two artificial constructs in the absence and presence of synthetic lipid vesicles. POPA/POPC lipid vesicles were prepared as described in Materials and Methods and allowed to incubate with purified alpha-synuclein. The proteins were monitored by circular dichroism to qualitatively assess secondary structure.

The lack of regular secondary structure is evident in both full-length alpha-synuclein and the two artificial constructs in the absence of synthetic lipid vesicles. Both artificial constructs adopt alpha-helical conformations, similar to full-length alpha-synuclein, when bound to synthetic lipid vesicles. While all of these constructs lack secondary structure, the rates of degradation by the 20S proteasome are different (Figure 4-6).

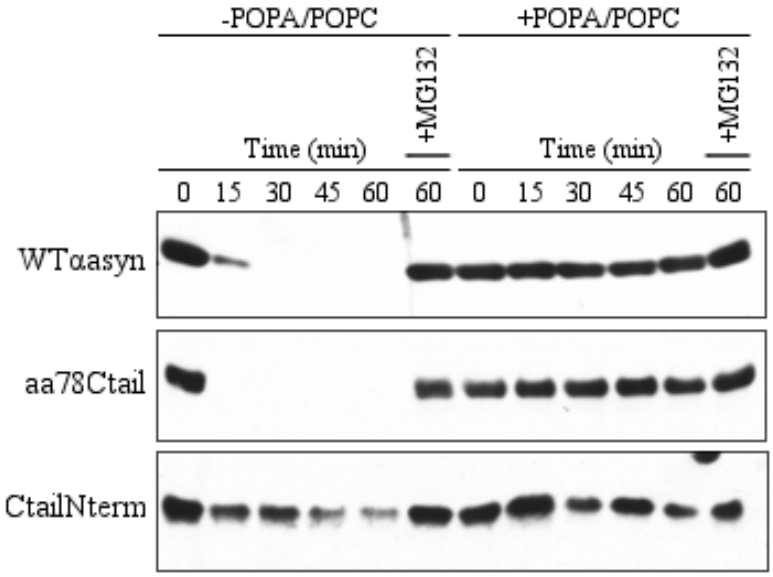

Figure 4-6. Degradation of artificial alpha-synuclein constructs by purified 20S proteasomes. Alpha-synuclein was incubated with purified 20S proteasomes as described in Materials and Methods. Protein was detected by Western blot with an antibody specific for the N-terminus of alpha-synuclein. Degradation can be monitored by the decrease in intensity of the full-length protein. A pre-incubation of the 20S proteasome with the inhibitor MG132, blocks degradation of each alpha-synuclein construct.

These results reveal that the 20S proteasome does not recognize and degrade all natively-disordered proteins with the same propensity, and there are features beyond disorder that influence susceptibility to degradation by the 20S proteasome.

Elements in the N-terminus of alpha-synuclein affect its degradation propensity.

Constructs were generated in which alpha-synuclein was truncated from the N-terminus to assess the role of N-terminal residues on degradation propensity. These constructs begin at residues V37, V52, V63, and V71 (see schematic in Figure 4-4). Each of these proteins was shown to lack secondary structure by circular dichroism spectroscopy (Figure 4-7). Degradation of each protein by the 20S proteasome was monitored at 37°C over one hour (Figure 4-7) and aliquots were removed at the timepoints indicated and added to SDS sample buffer to stop the reaction. Aliquots were analyzed by SDS-PAGE and protein detected by Coomassie blue staining. The band shown is the designated alpha-synuclein construct. A pre-incubation of the 20S proteasome with β-lactacystin blocks alpha-synuclein degradation.

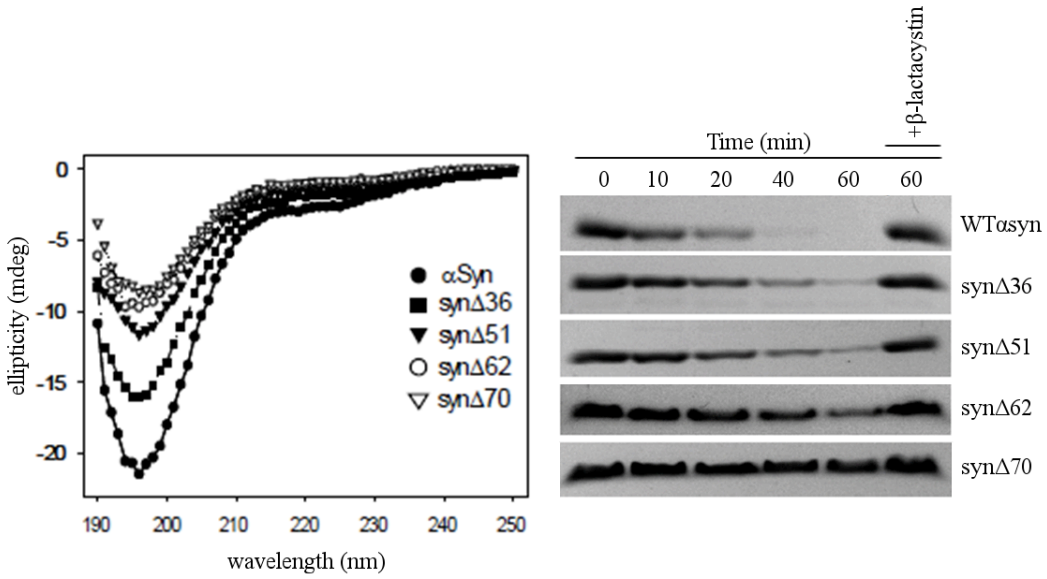

Figure 4-7. Circular dichroism and degradation of alpha-synuclein constructs truncated at the N-terminus. N-terminally-truncated forms of alpha-synuclein were expressed and purified from *E. coli*. Secondary structure propensity was analyzed by circular dichroism, and the degradation propensity of each was measured. Gels were stained with Coomassie and the band shown is the designated protein.

Degradation kinetics of these constructs vary widely; while WTαsyn is almost completely degraded within 40 minutes, the synΔ70 construct is resistant to proteolysis under the conditions tested, although both lack a defined structure as indicated by circular dichroism spectra. These results reveal that some, but not all, intrinsically-disordered proteins are degraded by the 20S proteasome and that the N-terminus plays a role in the ability of the 20S proteasome to recognize and degrade alpha-synuclein.

Elements in the C-terminus of alpha-synuclein affect its degradation propensity.

To determine whether elements in the C-terminus of alpha-synuclein are required for its degradation, C-terminal truncations of alpha-synuclein were generated, including constructs that end at residues 119, 110, and 95 (see Figure 4-4). Alpha-synuclein species similar to syn119 and syn110 have been identified in Lewy bodies and appear to accumulate in both brain tissue of human patients (C-W. Liu et al. 2005; Lewis et al. 2010) and the A53T transgenic mouse model of Parkinson's disease (C-W. Liu et al. 2005). The syn95 construct was generated to investigate the effect of removing the entire acidic C-tail of alpha-synuclein on its degradation propensity. Each of these constructs lacks regular, ordered secondary structure, as indicated by circular dichroism spectra (Figure 4-8).

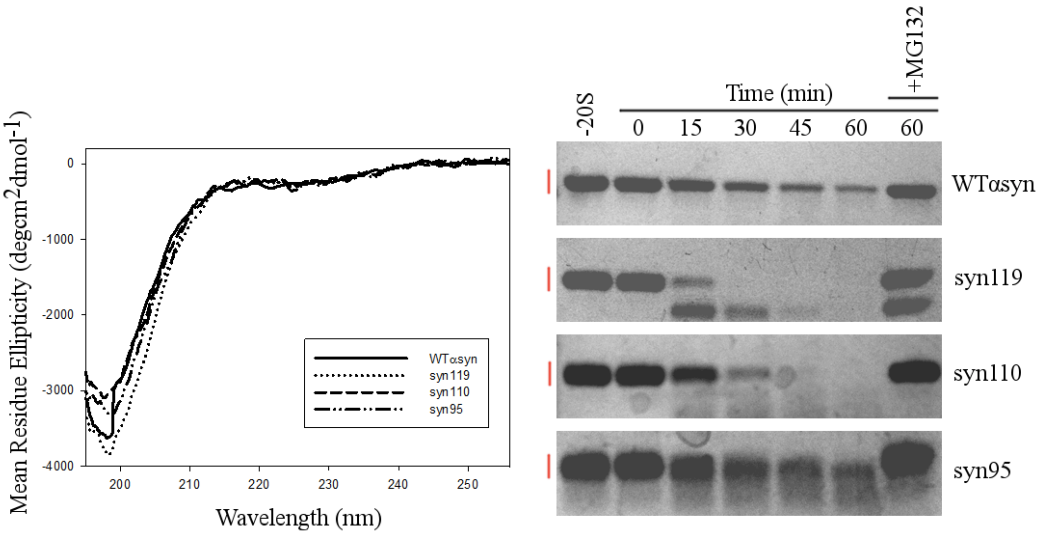

Figure 4-8. Circular dichroism and degradation of alpha-synuclein truncated at the C-terminus. C-terminally-truncated forms of alpha-synuclein were expressed and purified from *E. coli*. The purified proteins were analyzed for secondary structure propensity by circular dichroism and were monitored for degradation propensity by the 20S proteasome. Gels were stained with Coomassie, and the migration of the designated alpha-synuclein construct is indicated by the red, vertical line.

Degradation assays reveal that removing residues from the C-terminus impact the ability of alpha-synuclein to be recognized and degraded by the 20S proteasome (Figure 4-8). Interestingly, while most 20S-produced cleavages within alpha-synuclein occur in the acidic C-tail, the syn95 protein that completely lacks the C-tail is still partially degraded, but at a slower rate than constructs retaining a portion of the C-tail. These results reveal that the acidic C-terminus of alpha-synuclein is not required for its degradation by the 20S proteasome, but its presence can influence the kinetics by which the protein is degraded.

Substrate length alone does not account for susceptibility to degradation by the 20S proteasome.

As shown above, alpha-synuclein constructs truncated from both the N- and C-termini have altered degradation kinetics as compared to the full-length protein. To establish whether there is a correlation between substrate length and degradation propensity, the half-life of each construct was calculated relative to the half-life of the wild-type protein from the same assay. The relative half-life of each protein was plotted as a function of substrate length (Figure 4-9).

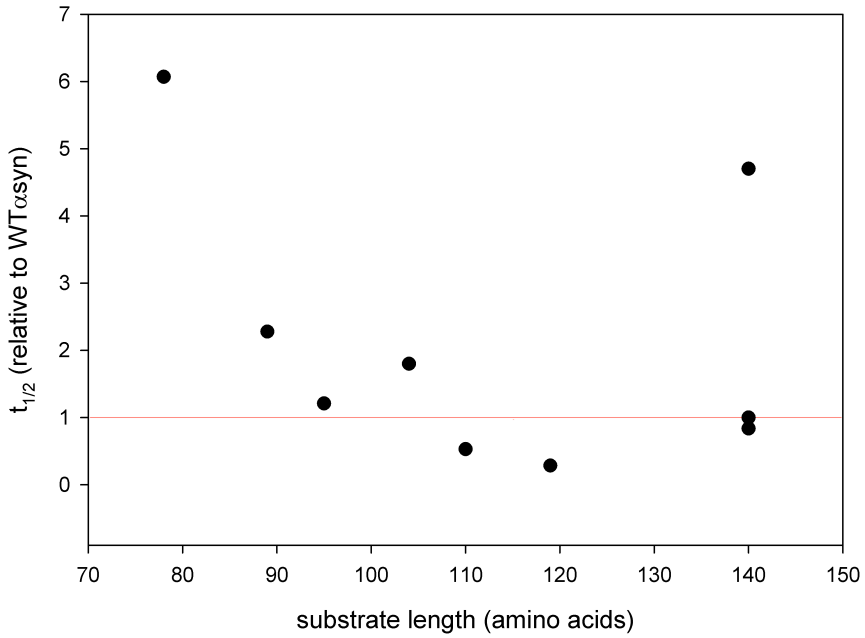

Figure 4-9. Relative half-life of alpha-synuclein constructs in the presence of purified 20S proteasome relative to wild-type alpha-synuclein. The half-life of each protein was calculated by fitting the degradation assay data from Figures 4-6 through 4-8 to a single exponential decay function and normalized to wild-type. The half-life of each construct was plotted relative to the half-life of wild-type alpha-synuclein calculated from the same assay.

While the constructs of intermediate length show a general trend of slower degradation with shorter length, several of the constructs generated are outliers of this trend. Degradation of the synΔ70 construct was not apparent under the conditions tested and, thus, a half-life could not be calculated. Degradation of the synΔ62 construct is dramatically slower than the synΔ51 construct, while other more significant reductions in substrate length have less of an effect on the half-life. The most significant outlier is the CtailNterm construct. While the length of

this construct is the same (140 amino acids) as the wild-type and artificial aa78Ctail proteins, the half-life of the CtailNterm protein is nearly five times longer than the other 140 residue, intrinsically-disordered proteins. These results reveal that protein length alone does not determine the rate of degradation by the 20S proteasome.

Alpha-synuclein may open the 20S gate to activate its own degradation

In order to further understand how substrates are recognized and selectively degraded by the 20S proteasome, degradation of WTαsyn was monitored in the presence of an activator. As discussed previously, binding of the 19S or PA700 regulatory particle to the 20S proteasome can open the 20S gate and lead to degradation of polyubiquitinated proteins. It has been shown that the C-terminal 10-residue peptide of the Rpt5 subunit of PA700 is sufficient in opening the 20S gate, leading to activation of the enzyme against fluorogenic peptide substrates (Gillette et al. 2008). The PGPH-like activity of the M57 20S proteasome was monitored in the absence and presence of the Rpt5 peptide (Figure 4-10).

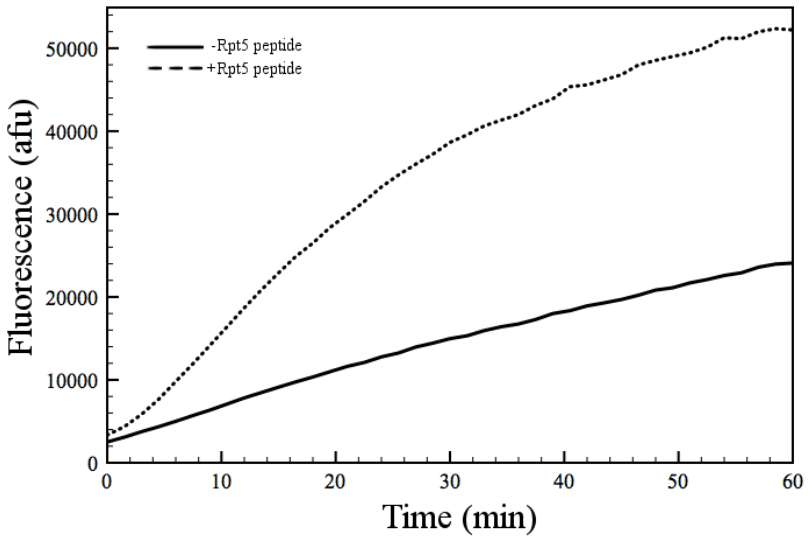

Figure 4-10. Enhancement of peptidase activity of 20S proteasomes by Rpt5 peptide dependent gate opening. The M57 proteasome preparation was pre-incubated with a 5000-fold molar excess of the Rpt5 peptide. After the pre-incubation, a solution containing a fluorogenic peptide specific for the PGPH-like activity of the 20S proteasome (Z-LLE-AMC) was added and the fluorescence was measured as a function of reaction time.

This result suggests that the 20S gate is closed in the 20S proteasome preparations. Interestingly, pre-incubation of M57 20S proteasomes with the Rpt5 peptide neither enhances nor inhibits activity of the enzyme against alpha-synuclein (Figure 4-11), revealing a mechanism for degradation kinetics beyond simply status of the 20S gate.

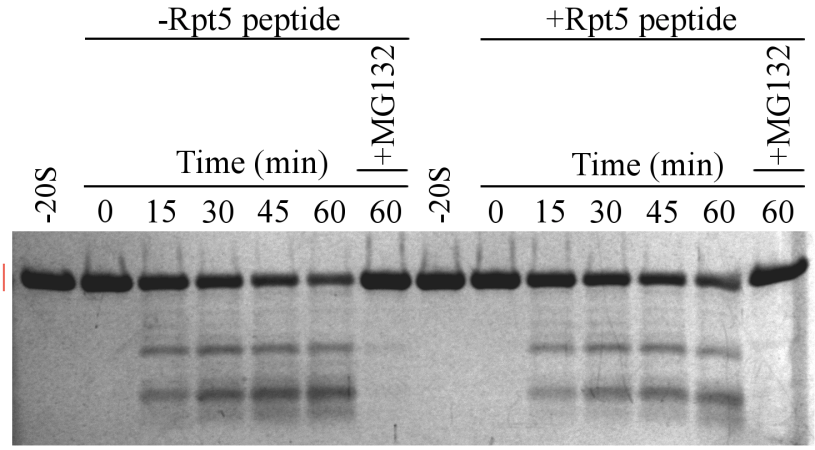

Figure 4-11. The Rpt5 gating peptide has no effect on activity of the M57 20S preparation against alpha-synuclein. M57 20S proteasomes were pre-incubated on ice either with or without a 5000-fold molar excess of the Rpt5 peptide. Following the pre-incubation, an alpha-synuclein degradation assay was performed as described in Materials and Methods.

These results reveal that, while the 20S gate is not completely open, the complex is still able to degrade alpha-synuclein to some extent. This suggests that alpha-synuclein may play a role in opening the 20S gate to activate its own degradation. Because the degradation of alpha-synuclein was not inhibited by the Rpt5 peptide, it is unlikely that the lack of synuclein-stimulating activity is due to competition between alpha-synuclein and the Rpt5 peptide for a binding site on the exterior of the complex or entry into the catalytic chamber. The ability for alpha-synuclein to be degraded by 20S proteasomes with a closed gate and the lack of stimulation when the gate is artificially opened suggest that substrate recognition, gate opening, and substrate degradation are coupled events.

Mechanism of substrate recognition and degradation may differ between mammalian and yeast 20S proteasomes.

Modulation of the alpha subunits of the yeast 20S proteasome has been shown to lead to an open gate conformation. Deletion of the seven N-terminal residues of the alpha-3 subunit removes the interaction between the N-termini such that they no longer occlude access to the catalytic sites (Groll et al. 2000). The accessibility of the active sites can be deduced from the solved structure of the gateless mutant and shown experimentally by an enhancement in activity against fluorogenic peptide substrates (Groll et al. 2000). Results from the previous section led to the hypothesis that alpha-synuclein must be recognized by the 20S proteasome to open the gate for itself, allowing entry into the lumen and its subsequent degradation, thereby providing a mechanism for substrate selection without ubiquitination or other modification. To test whether alpha-synuclein must open the 20S gate itself in order to be degraded, degradation of alpha-synuclein was performed by the yeast wild-type and gateless (α3ΔN) 20S proteasomes (Figure 4-12).

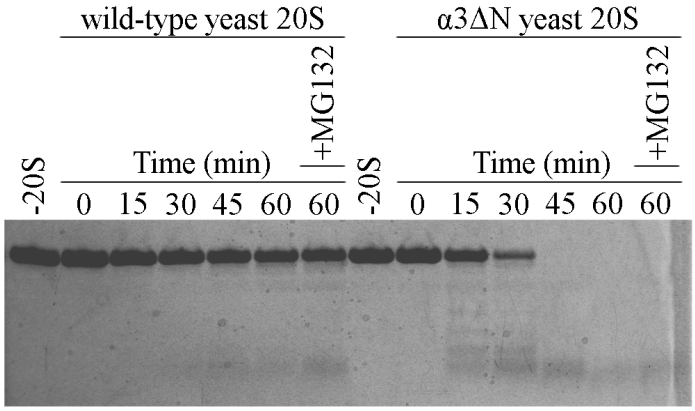

Figure 4-12. Degradation of wild-type alpha-synuclein by the yeast wild-type and gateless forms of the 20S proteasome. Purified yeast 20S proteasomes were generously provided by Dr. Yifan Cheng (Department of Biochemistry & Biophysics, University of California San Francisco). Degradation assays were performed in the same manner as the assays with mammalian 20S proteasome. MG132 does not inhibit yeast 20S proteasomes.

The wild-type yeast 20S proteasome does not significantly degrade alpha-synuclein under the conditions tested, while the α3ΔN mutant completely degrades the protein within 45 minutes. These results reveal a difference between the mammalian and yeast proteasomes, in that an open yeast 20S gate might be sufficient for degradation of alpha-synuclein, while a closed mammalian 20S gate is required for recognition and degradation of alpha-synuclein.

DISCUSSION

It has been assumed that that the 20S core particle, in the absence of any regulatory complex, is latent and, only upon binding to PA700, does the gate open, allowing accessibility to the active sites and degradation of polyubiquitinated protein substrates (Groll 2003). Recently, however, it has been shown that some proteins can be degraded by the 20S proteasome in a ubiquitin-independent manner. The mechanism by which these substrates are recognized, open the 20S gate, and are degraded is still unknown. Crystallographic studies of the yeast 20S proteasome in which the gate is artificially opened by truncation of the N-terminus of the alpha-3 subunit reveal that the complex has a pore diameter of only 13Å (Groll et al. 2000). These structures suggest that a well-folded, globular protein cannot access the catalytic sites within the pore unless they are first unfolded. Studies have proposed that the presence of unfolded or disordered regions within a protein render it a substrate for degradation by the 20S proteasome (Liu et al. 2003; Prakash et al. 2004; Baugh et al. 2009) and that proteins can even be assessed for regions of disorder by their propensity toward 20S-mediated degradation (Tsvetkov et al. 2008; Tsvetkov & Shaul 2012). The results shown here, however, indicate that not all IDPs can be degraded by the 20S proteasome. These results suggest that while the presence of disordered regions within a protein is required for 20S-mediated degradation, it is not

sufficient. Therefore, other requirements must be met for a protein to be a substrate for ubiquitin-independent degradation by the 20S proteasome.

This work aimed to further understand the features of alpha-synuclein that render it a substrate for degradation by the 20S proteasome. Results show that degradation propensity does not correlate with substrate length and that elements in both the N- and C-terminus of alpha-synuclein influence its degradation kinetics. Degradation of some truncated forms of alpha-synuclein is much slower than the full-length protein, supporting a role for the incomplete metabolism of alpha-synuclein in protein accumulation and Lewy body formation. The differential degradation rates of various alpha-synuclein truncations may provide insight into the mechanism by which some truncated species accumulate in pathological aggregates while others do not. The increased propensity of C-terminally truncated forms of alpha-synuclein to aggregate may lead to accumulation that can out-compete the further metabolism of these degradation intermediates. Other proteases that have been shown to cleave alpha-synuclein, in addition to the 20S proteasome, may also play a role in the production and accumulation of aggregation-prone truncated species in the cell. In light of recent studies showing the intercellular transmission of alpha-synuclein (Borghi et al. 2000; El-Agnaf et al. 2003; Sung et al. 2005; H.-J. Lee et al. 2005), the role of extracellular proteases in disease progression must also be taken into account.

While several proteases may work concertedly in the formation of truncated alpha-synuclein species, previous work establishes a strong correlation between the specific alpha-synuclein species that can be generated by the 20S proteasome and pathology associated with Lewy body diseases (C-W. Liu et al. 2005; Daher et al. 2009; Lewis et al. 2010).

The results shown here reveal that the 20S proteasome exhibits substrate selectivity through a mechanism that is more complex than previously appreciated. These results combined with recent results from others reveal that the 20S core particle can degrade many proteins; however, future studies are needed to further establish the ubiquitin-independent degradation of proteins as a normal cellular process. It is currently unknown whether the 20S proteasome plays a role in the cell beyond serving as the catalytic core of the 26S proteasome; however, several observations support a cellular role of the 20S proteasome independent from any known activator complex. First, studies have shown that free 20S proteasome exists in eukaryotic cells (Peters et al. 1994; Tanahashi, Murakami & Minami 2000; Shibatani et al. 2006). Second, results from several studies suggest that substrate recognition and degradation may be a regulated process (Asher et al. 2005; Moorthy et al. 2006). Finally, we have shown previously that truncated alpha-synuclein species similar to those produced by the 20S proteasome *in vitro* are found in brain tissue of transgenic mice (C-W. Liu et al. 2005) and patients

with Lewy body diseases (C-W. Liu et al. 2005; Lewis et al. 2010). Together, these observations suggest a role for the 20S proteasome that must be further studied to fully elucidate its physiological role in the cell, substrate preference, and relationship to disease pathogenesis.

CHAPTER FIVE
A Modification of the 20S Proteasome Correlated With Activity Against Alpha-Synuclein

INTRODUCTION

The ubiquitin-proteasome system serves a wide variety of functions within the cell, and the proper collaboration between every component of this system is required for the survival of organisms--from archaea to humans. The UPS must also be able to respond quickly under conditions of stress or in response to a changing cellular environment. Because of the broad and indispensable role of the UPS in the physiology of organisms, the function of this enzyme is likely subjected to extensive regulation. Activity of the UPS could be modulated in many different ways, including altered expression levels of subunits, a change in cellular localization, and post-translational modifications that affect the structure and function of the complex. Several studies have shown that subunits of the UPS can be subject to extensive post-translational modification in a variety of organisms. Table 5-1 summarizes the known set of post-translational modifications of eukaryotic proteasomal subunits.

Type of Modification	Subunit	Source
acetylation	α2, α4, α5, α6, α7, β3, β4	(Wang et al. 2007; Tokunaga et al. 1990)
	Rpt3, Rpt4, Rpt5, Rpt6, Rpn1, Rpn2, Rpn6, Rpn13	(Wang et al. 2007)
myristoylation	Rpt2	(Wang et al. 2007)
O-linked glycosylation	Rpt2	(F. Zhang et al. 2003)
	α1, α4, α5, α6, β6	(Overath et al. 2012)
phosphorylation	α3, α4, α5, α6, α7	(Horiguchi et al. 2005; Wang et al. 2007; P. F. Murray et al. 2002)
	β7	(Eang et al. 2009)
	Rpt5, Rpn1, Rpn2, Rpn6, Rpn8, Rpn9, Rpn10, Rpn11	(Wang et al. 2007)
truncation	α6	(Weitman & Etlinger 1992; Arribas et al. 1994; Hori et al. 1999; Yokota et al. 2011)
	β1, β2, β5	(Chen & Hochstrasser 1996; Seemuller et al. 1996; Heinemeyer et al. 1997)
ubiquitination	α1, α2, α4, β3, β4, β5, β7	(Ventadour et al. 2007)

Table 5-1. Post-translational modification of proteasome subunits observed in eukaryotic tissues. The subunits are specified using the Baumeister nomenclature (Baumeister et al. 1998).

While many large-scale proteomic studies have aimed to identify post-translational modifications on proteasomal subunits, the consequences of these modifications on proteasomal activity have not been well established. One modification that has been shown to affect proteasomal activity is O-linked glycosylation of the Rpt2 subunit (F. Zhang et al. 2003). The attachment of an O-GlcNAC moiety to the Rpt2 subunit leads to a decrease in the chymotrypsin-like activity of the proteasome, through a mechanism involving reduction in ATPase activity (F. Zhang et al. 2003). Another modification with known functional consequences is removal of the N-terminal propeptide from the β1, β2, and β5 catalytic subunits. These subunits are each translated with a leader sequence that prevents catalytic activity until the 20S proteasome is assembled and sites are sequestered within the lumen (Chen & Hochstrasser 1996). During assembly, the propeptide is removed from the N-terminus through an intermolecular cleavage event, leading to the exposure of an N-terminal threonine residue that serves as the nucleophile required for proteolytic activity (Seemuller et al. 1996). While many other modifications of 20S and PA700 subunits have been identified, they have yet to be tightly correlated to any functional change in activity of the complex. Additionally, the physiological mechanism by which these modifications occur has not been elucidated.

The alpha-6 subunit is the largest of the fourteen gene products that make up the 20S proteasome in eukaryotic organisms, and is roughly 30 kilodaltons. While the alpha-6 subunit is a component of the alpha ring that controls access to the catalytic sites within the 20S lumen, its N-terminus has not been shown to traverse the pore, as has been shown for the alpha-2, alpha-3, and alpha-4 subunits (Groll et al. 2000). The specific role that the alpha-6 subunit plays in gate formation and substrate accessibility is unknown. The eukaryotic alpha-6 subunit has been found to exist with a variety of modifications, suggesting that this subunit may play a key role in the regulation of proteasomal activity. These modifications include acetylation (Wang et al. 2007), phosphorylation (P. F. Murray et al. 2002), O-linked glycosylation (Overath et al. 2012), and truncation (Weitman & Etlinger 1992; Arribas et al. 1994; Hori et al. 1999; Yokota et al. 2011). Several reports also showed that the C-terminus of the alpha-6 subunit can be truncated, as observed in human erythrocytes (Weitman & Etlinger 1992), rat liver (Arribas et al. 1994; Hori et al. 1999), and *Halocynthia roretzi* sperm (Yokota et al. 2011).

In the first report, two forms of the 20S proteasome, an active form and a latent form, were purified from human erythrocytes (Weitman & Etlinger 1992). The purified proteasome was used as an antigen for the creation of monoclonal antibodies. An alpha-6 subunit-specific antibody was generated that could detect a

32 kilodalton band in the latent proteasome preparations and a 28 kilodalton band in the active proteasome preparations. This study revealed a correlation between the 28 kilodalton alpha-6 band and activity of the 20S proteasome against casein. Both of the bands were separated into three distinct spots by two-dimensional SDS-PAGE, indicating additional post-translational modifications on each form of alpha-6. It was shown that, during the purification of 20S proteasomes from human erythrocytes, the 32 kilodalton band was partially converted to the 28 kilodalton band, and dialysis-mediated activation of the latent proteasome did not affect the electrophoretic mobility of the 32 kilodalton alpha-6 band.

In the second study, 20S proteasomes were purified from rat liver and found to exist in two forms, one of which that was not recognized by an alpha-6-specific antibody of unknown epitope. Truncated forms of the mouse alpha-6 subunit were generated and expressed in *E. coli* to map the epitope of the antibody. The epitope was found to be located in the C-terminal nine to eleven residues, indicating that this C-terminal region of the protein is only present in the latent form of the rat liver 20S proteasome. The authors then recapitulated the C-terminal truncation to the alpha-6 subunit *in vitro* by incubating the latent 20S proteasome with elastase. Incubation of the purified 20S proteasome with elastase led to a time-dependent cleavage of the alpha-6 subunit from a 32 kilodalton form to a 28 kilodalton form. Cleavage of the alpha-6 subunit did not affect the activity

of the 20S proteasome against peptide substrates specific for each catalytic activity, myelin-basic protein, or casein.

In the third study, a similar truncation was observed in the alpha-6 subunit isolated from microsomal fractions of the 20S proteasome (Hori et al. 1999). The authors of this study observed that all 20S proteasomes had similar peptidase acitivities, regardless of the amount of full-length alpha-6 detected. However, proteasomes with a lesser amount of the full-length alpha-6 were shown to be more active against the protein substrates reduced and carboxymethylated (RCM) lysozyme and β-casein. In the fourth study, a C-terminally truncated form of the alpha-6 subunit was identified in sperm cells of *H. roretzi* (Yokota et al. 2011). This subunit was found to be full-length (285 amino acids) in egg and muscle cells, but only in a truncated form (residues 1-269) in sperm cells; however, the mechanism and effect of this modification were not studied.

The observations described above demonstrate that a similar C-terminal truncation of the alpha-6 subunit occurs in multiple species and tissues. Therefore, truncation of this subunit is likely a common and specific event that could play a role in altering the activity of the 20S or 26S proteasome. In addition, because this truncation appears to be tissue-specific in *H. roretzi*, the mechanism of C-terminal truncation is likely subjected to some form of regulation that is necessary for its proper function in certain cell types. The observation that a post-translational

modification can affect activity against some substrates but not others challenges the notion that the 20S proteasome only degrades substrates “by default” (Asher et al. 2006; Tsvetkov & Shaul 2012). Addtionally, these results reveal that the 20S proteasome may be subject to regulatory mechanisms that allow it to recognize and degrade substrates through a far more complex process than previously understood.

Figure 5-1. Alignment of the alpha-6 subunits of the 20S proteasome core particle from various species. Accession numbers are as follows: *H. sapiens* (AAH22372.1), *B. taurus* (AA102217.1), *M. musculus* (NP_036095.1), *R. norvegicus* (AAH62233), *G. gallus* (042265.2), *D. melanogaster* (P12881.1), *C. elegans* (044156.1), *A. thaliana* (P34066.3), *H. roretzi* (P34066.3), and *S. cerivisiae* (P40302.1). Alignments were generated using the PRALINE multiple sequence alignment tool through the Centre for Integrative Bioinformatics VU. Black background indicates identical residues and gray background indicates similar residues.

The 20S proteasome is a large, multi-subunit complex, and, therefore, the study of eukaryotic proteasomes is often accomplished by purifying the endogenous complex from tissues (DeMartino & Goldberg 1979; McGuire &

DeMartino 1986; Saitoh et al. 1989; Hendil & Uerkvitz 1991; Fujinami et al. 1994) or mammalian cells in culture (Scanlon et al. 2009). Because many of the subunits can be post-translationally modified, it is reasonable to assume that there are differences in each preparation of the enzyme that are beyond experimental control, including the care of the animal from which the tissues were harvested. Studies aimed to understand the role of specific post-translational modifications in proteasomal activity are difficult due to the inability to characterize each and every difference between proteasome preparations. In the studies presented below, endogenous 20S proteasomes were purified from bovine red blood cells. Red blood cells have been found to express high levels of the proteasome (McGuire & DeMartino 1986), the proteasome from *B. taurus* is very similar to that of humans, and this source material is easy to obtain in large quantities. Studies were conducted to characterize the activity of various preparations of bovine 20S proteasomes against alpha-synuclein and to correlate any differences with modifications of the enzyme.

RESULTS

Preparations of bovine 20S proteasomes have different activities against alpha-synuclein.

20S proteasomes were purified from bovine red blood cells as described in Experimental Procedures and named “M39”, “M57”, “M58”, “M60”, “M61”, “M64”, based on the chronological order of their purification. Each preparation was initiated from a different source of bovine red blood cells (i.e. a different animal or animals) with the blood obtained on a different date. The activity of each 20S proteasome preparation against alpha-synuclein was monitored over 60 minutes in the presence or absence of MG132, a proteasomal inhibitor. Aliquots of the reaction were removed at various time points, added to SDS sample buffer to stop the reaction, and placed on ice. Aliquots were analyzed by SDS-PAGE with Coomassie staining. The relative activities of the 20S preparations against alpha-synuclein can be monitored by a decrease in the intensity of the band corresponding to the full-length substrate or, alternatively, the appearance of lower molecular weight species over time. Figure 5-2 shows a representative Coomassie-stained gel of degradation samples performed by each proteasome preparation analyzed in this study. Under the conditions tested, M39 and M57 appear to degrade alpha-synuclein, and are referred to as “alpha-synuclein-active”

preparations. M58, M60, M61, and M64 do not readily degrade alpha-synuclein, and are referred to as “alpha-synuclein-inactive” preparations.

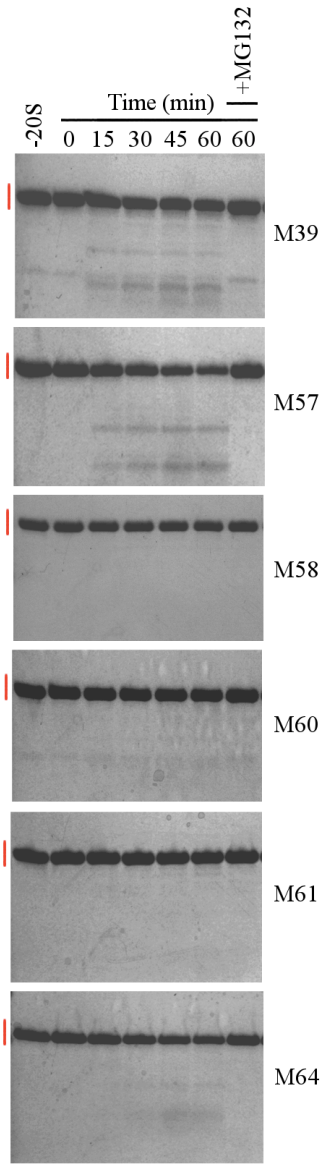

Figure 5-2. Degradation of alpha-synuclein by purified mammalian 20S proteasome preparations. Purified 20S proteasome preparations were allowed to incubate with purifed alpha-synuclein as described in Materials and Methods. Samples were analyzed by SDS-PAGE and Coomassie staining. Gels shown are representative of many repetitions of the assay, and reveal that there are differences in activity against alpha-synuclein of the various 20S preparations. The vertical, red line represents the mobility of full-length alpha-synuclein (residues 1-140).

At much higher enzyme-to-substrate ratios, the alpha-synuclein-inactive 20S preparations can cleave alpha-synuclein at detectable levels, as shown in Figure 5-3 with the M61 20S preparation. These results suggest that either a small percentage of alpha-synuclein-inactive proteasomes are active or the entire population of proteasomes has reduced activity compared to the alpha-synuclein-active 20S preparations.

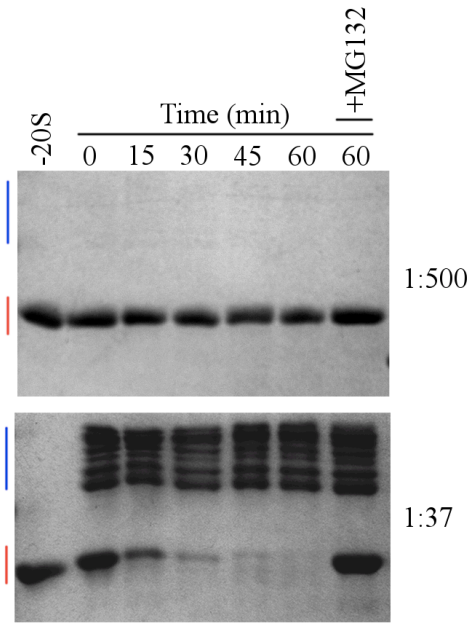

Figure 5-3. Activity of alpha-synuclein-inactive 20S at a high enzyme-to-substrate ratio. The activity of M61 alpha-synuclein-inactive 20S proteasomes against alpha-synuclein at 1:500 and 1:37 enzyme-to-substrate ratios was qualitatively assessed. Degradation assays were performed as described in Materials and Methods and samples were analyzed by SDS-PAGE and Coomassie staining. Blue, vertical lines indicate migration of the 20S proteasomeal subunits and the red, vertical lines represent mobility of full-length alpha-synuclein. Similar results were observed for M60 (not shown). The activities of M58 and M64 proteasome preparations against alpha-synuclein were not tested at higher enzyme-to-substrate ratios.

Alpha-synuclein-inactive 20S preparations are functional enzymes.

One plausible explanation of the difference in activity between various 20S preparations is that the alpha-synuclein-inactive enzymes are less functional, due to differences in the original source of bovine red blood cells or as a consequence of the purification. To test this possibility, the peptidase activities of several alpha-synuclein-inactive 20S preparations against a fluorogenic peptide substrate were monitored. The PGPH-like activity of the 20S proteasome was monitored in the absence and presence of the Rpt5 peptide (Figure 5-4). Both M60 and M61 preparations have a low, but measurable, PGPH-like peptidase activity in the absence of the Rpt5 peptide, as revealed by an increase in fluorescence intensity as a function of reaction time. In the presence of the Rpt5 peptide, the activity of the M60 and M61 20S preparations is enhanced several-fold, demonstrating that the gate is closed in the absence of the peptide.

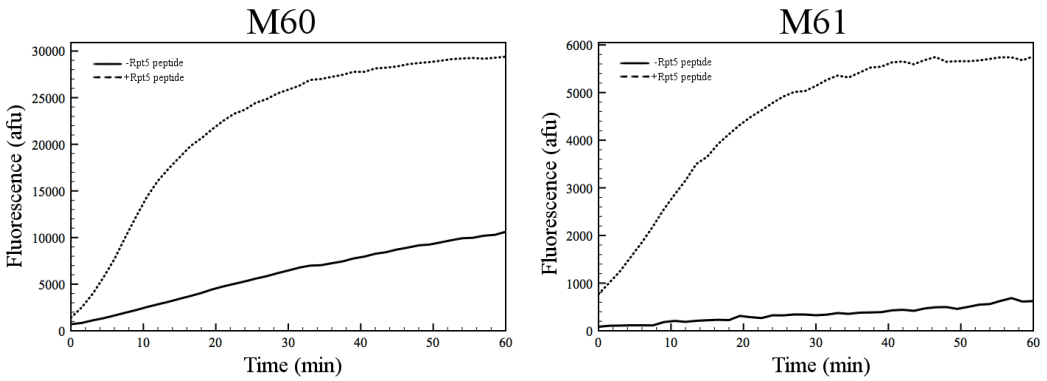

Figure 5-4. Enhancement of peptidase activity of M60 and M61 alpha-synuclein-inactive 20S preparations by the Rpt5 peptide. M60 and M61 proteasome preparations were pre-incubated with a 5000-fold molar excess of the Rpt5 peptide on ice for at least 30 minutes. After the pre-incubation, a solution containing a fluorogenic peptide specific for the PGPH-like activity of the 20S proteasome (Z-LLE-AMC) was added and the fluorescence signal was monitored as a function of reaction time.

The activities of the M60 and M61 alpha-synuclein-inactive 20S preparations against alpha-synuclein were then monitored in the presence and absence of the Rpt5 peptide. As shown previously, in the absence of the Rpt5 peptide, the M60 and M61 proteasome preparations lack activity against alpha-synuclein under the conditions tested. In contrast to activation against the fluorogenic peptide substrate, there was no stimulation of activity against alpha-synuclein when these 20S preparations were pre-incubated with the Rpt5 peptide (Figure 5-5).

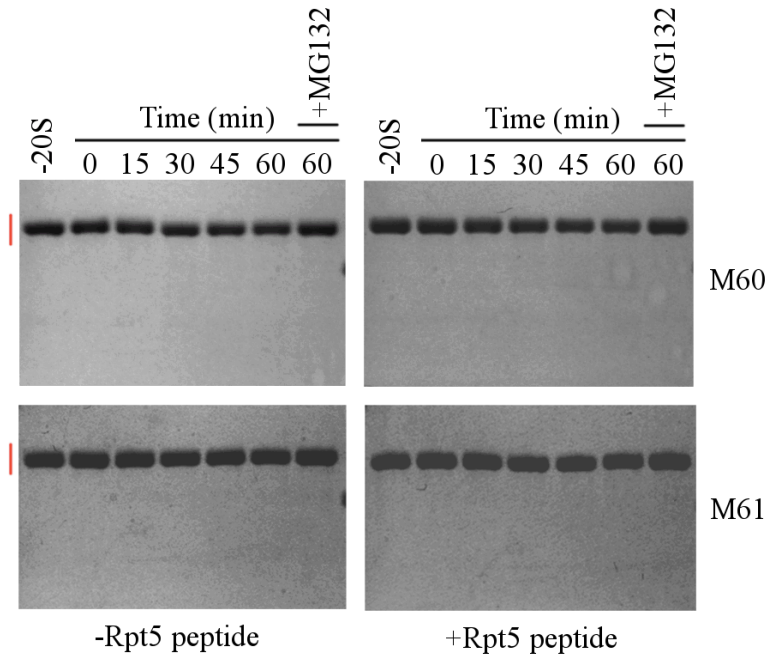

Figure 5-5. Lack of activation of M60 and M61 alpha-synuclein-inactive 20S preparations by the Rpt5 peptide. M60 and M61 proteasome preparations were pre-incubated with a 5000-fold molar excess of the Rpt5 peptide for at least 30 minutes on ice. Alpha-synuclein degradation assays were then performed as described in Materials and Methods and analyzed by SDS-PAGE and Coomassie staining. The vertical, red line indicates the migration of full-length alpha-synuclein.

An interpretation of this result is that the Rpt5 peptide and alpha-synuclein are competing either for the same binding site on the 20S proteasome or for access to the catalytic sites within the 20S lumen. Identical experiments performed with the M57 alpha-synuclein-active preparation (Figures 4-10 and 4-11) demonstrated that this is not the case, as pre-incubation of the Rpt5 peptide with the M57 proteasome preparation did not lead to any inhibition of activity against alpha-

synuclein. Together, the results from Figures 5-4 and 5-5 reveal that simply opening of the 20S gate does not activate the alpha-synuclein-inactive preparations against alpha-synuclein, and this is not due to competition between the Rpt5 peptide and alpha-synuclein for either a binding site or the active sites within the 20S lumen. The results shown thus far suggest that there is a difference in 20S preparations that can and cannot degrade alpha-synuclein, and this difference is not due exclusively to a general enzymatic ability of the various 20S preparations.

A 427 Dalton modification of the alpha-6 subunit correlates with alpha-synuclein-inactive 20S preparations.

To determine if there is a molecular difference between alpha-synuclein-active and alpha-synuclein-inactive 20S preparations, two-dimensional gel electrophoresis was utilized to separate subunits of the purified 20S preparations. Gels were stained with Coomassie to reveal a set of spots that had different mobility in alpha-synuclein-active versus alpha-synuclein-inactive 20S preparations (data not shown). The subunits were assigned based on previous reports in the literature (Claverol et al. 2002; Hayter et al. 2005; Uttenweiler-Joseph et al. 2008), and the assignments confirmed by immunoblotting with subunit-specific antibodies. The most notable difference between the alpha-

synuclein-active and alpha-synuclein-inactive preparations was altered electrophoretic mobility of the alpha-6 subunit (Figure 5-6).

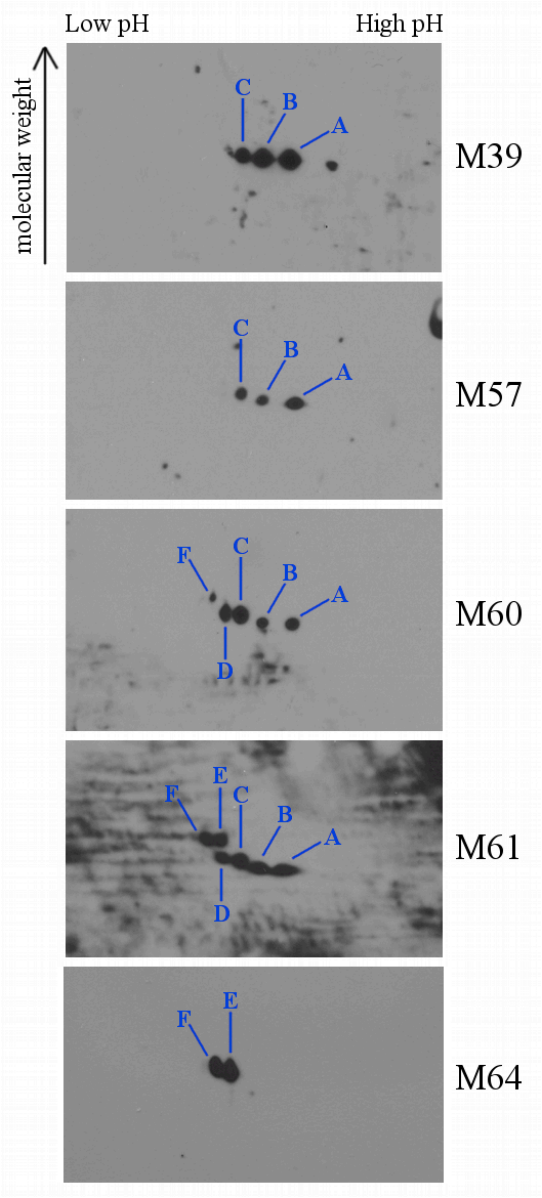

Figure 5-6. Alpha-6 subunit composition of purified 20S proteasome preparations. Purified 20S proteasome preparations were loaded onto an isoelectric focusing strip with a pH range of 3.0 to 10.0. After isoelectric focusing, the strip was run in the second dimension such that maximum separation in the 25 to 30 kilodalton range was achieved. The alpha-6 subunit was detected with the alpha-6_sc antibody.

Multiple immunoreactive spots were observed in both the alpha-synuclein-active and alpha-synuclein-inactive 20S preparations, indicating several forms of the protein. Alpha-synuclein-inactive 20S preparations contained signal corresponding to proteins of higher molecular weight and lower isoelectric point either instead of or in addition to the signal that was also observed in the alpha-synuclein-active preparations. To determine the mass difference of these forms of the protein, the alpha-6 subunit was isolated from various 20S preparations by reversed-phase chromatography (Figure 5-7).

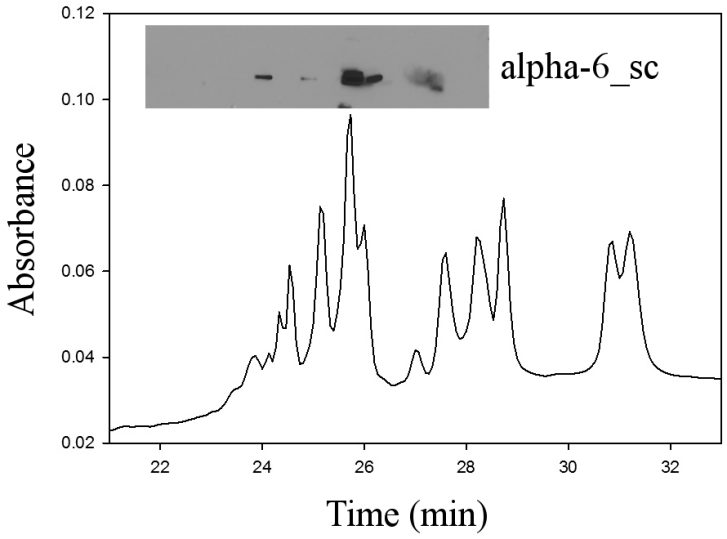

Figure 5-7. Isolation of the alpha-6 subunit by reversed-phase chromatography. The M60 alpha-synuclein-inactive 20S proteasome preparation was subjected to reversed-phase chromatography to isolate the alpha-6 subunit as described in Materials and Methods. Fractions were collected manually and the fractions containing alpha-6 were determined by SDS-PAGE and Western blotting of each fraction.

Fractions containing the alpha-6 subunit were then subjected to electrospray ionization mass spectrometry for whole mass analysis (Figure 5-8).

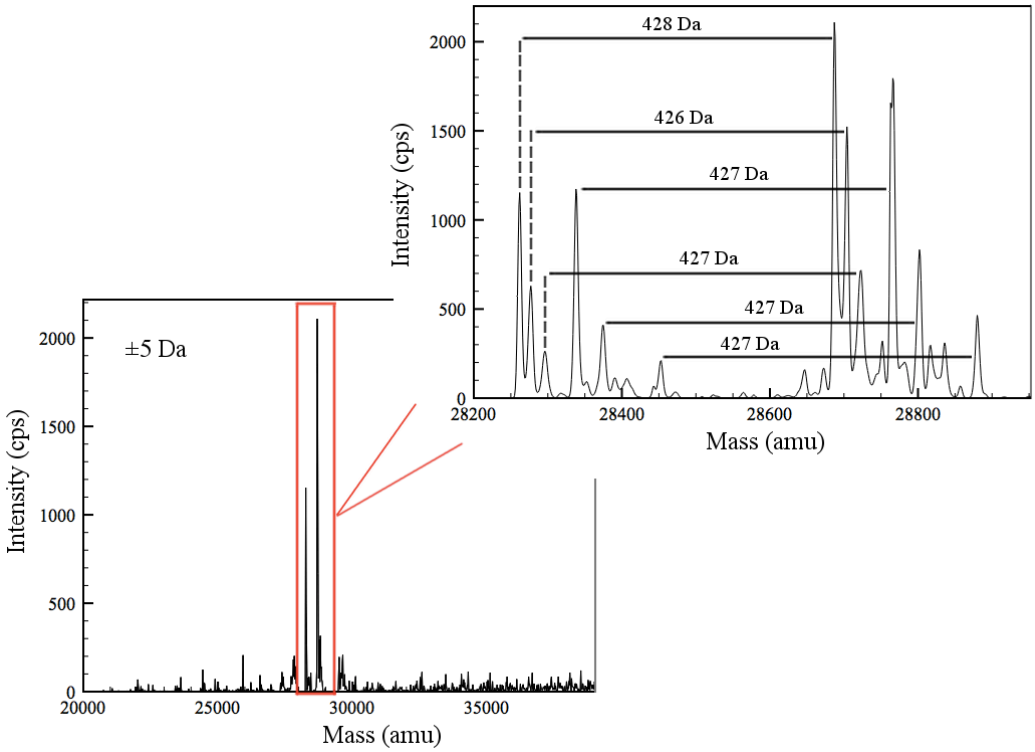

Figure 5-8. Electrospray ionization mass spectrometry of the fraction containing the alpha-6 subunit. The fraction from reversed-phase chromatography of the M60 proteasome preparation containing the most alpha-6 signal was analyzed by electrospray ionization mass spectrometry (ESI-MS) for whole mass analysis.

The mass spectrum of the purified alpha-6 subunit from the M60 alpha-synuclein-inactive 20S preparation revealed two sets of similar peaks. The difference in mass between the two sets of peaks is approximately 427 Daltons, revealing that a

modification of this mass may correlate with activity of the 20S proteasome against alpha-synuclein. Isolation of the alpha-6 subunit from the M39 alpha-synuclein-active preparation and whole mass analysis revealed a peak corresponding to the lower set of peaks from M60, but no peaks at the +427 Dalton mass (data not shown).

Modification is a C-terminal truncation of the alpha-6 subunit.

To determine whether the 427 Dalton modification is being added (through the addition of a moiety or functional group) or subtracted (by truncation or removal of a post-translational modification), the status of the alpha-6 modification was monitored throughout the early and intermediate stages of the 20S purification scheme (Figure 5-9). Detection of the alpha-6 subunit could not be achieved until after the DE52 cation exchanger due to high background and, therefore, the lysed red blood cells could not be analyzed prior to any manipulation. Eluate from the DE52 cation exchange resin was analyzed both before and after dialysis into a buffer with low ionic strength. 20S proteasomes from both steps contained only the alpha-6 upper band. The dialyzed proteasome was then subjected to an ammonium sulfate precipitation, followed by centrifugation. 20S proteasomes from the resulting supernatant were analyzed and found to contain alpha-6 of entirely upper band species. This supernatant was then

subjected to exhaustive dialysis, and the dialyzed protein was analyzed in a similar manner. After dialysis, a lower molecular weight alpha-6 band was present that had similar electrophoretic mobility to the purified M60 lower molecular weight alpha-6 band.

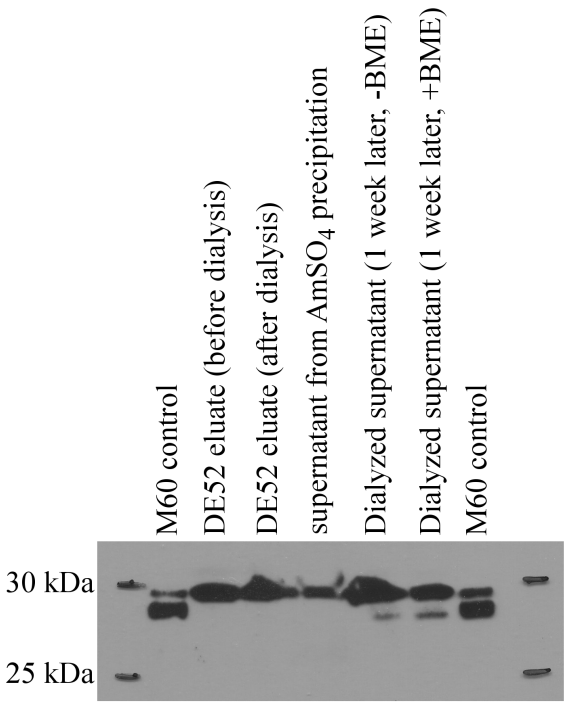

Figure 5-9. The lower molecular weight alpha-6 species is produced over time during early steps of the purification scheme. 20S proteasomes were partially purified from bovine red blood cells according to the purification scheme described in Materials and Methods. Samples from several steps early in the purification scheme were analyzed by SDS-PAGE and detection of the alpha-6 subunit using the alpha-6_sc antibody.

These results reveal that the 427 Dalton molecular difference between 20S proteasome preparations is due to the removal of this mass, either by a N- or C-

terminal truncation or removal of one or more post-translational modifications. The purified alpha-6 subunit from the M60 alpha-synuclein-inactive preparation was extracted from a gel and digested with trypsin, followed by LC/MS/MS analysis (Figure 5-10). Based on sequence coverage and mass difference between the bands, it was suggested that the 427 Dalton cleavage was a removal of residues 252-255 of the subunit.

M60 alpha-6:

Sequence coverage: 66%

```
  1 MFRNQYDNDV TVWSPQGRIH QIEYAMEAVK QGSATVGLKS KTHAVLVALK
 51 RAQSELAAHQ KKILHVDNHI GISIAGLTAD ARLLCNFMRQ ECLDSRFVFD
101 RPLPVSRLVS LIGSKTQIPT QRYGRRPYGV GLLIAGYDDM GPHIFQTCPS
151 ANYFDCRAMS IGARSQSART YLERHMSEFM ECNLNELVKH GLRALRETLP
201 AEQDLTTKNV SIGIVGKDLE FTIYDDDDVS PFLEGLEERP QRKAQPTQPA
251 DEPAEKADEP MEH
```

M60 bottom alpha-6 band (1-255):

Sequence coverage: 69%

```
  1 MFRNQYDNDV TVWSPQGRIH QIEYAMEAVK QGSATVGLKS KTHAVLVALK
 51 RAQSELAAHQ KKILHVDNHI GISIAGLTAD ARLLCNFMRQ ECLDSRFVFD
101 RPLPVSRLVS LIGSKTQIPT QRYGRRPYGV GLLIAGYDDM GPHIFQTCPS
151 ANYFDCRAMS IGARSQSART YLERHMSEFM ECNLNELVKH GLRALRETLP
201 AEQDLTTKNV SIGIVGKDLE FTIYDDDDVS PFLEGLEERP QRKAQPTQPA
251 DEPAE
```

M60 bottom alpha-6 band (1-251):

Sequence coverage: 68%

```
  1 MFRNQYDNDV TVWSPQGRIH QIEYAMEAVK QGSATVGLKS KTHAVLVALK
 51 RAQSELAAHQ KKILHVDNHI GISIAGLTAD ARLLCNFMRQ ECLDSRFVFD
101 RPLPVSRLVS LIGSKTQIPT QRYGRRPYGV GLLIAGYDDM GPHIFQTCPS
151 ANYFDCRAMS IGARSQSART YLERHMSEFM ECNLNELVKH GLRALRETLP
201 AEQDLTTKNV SIGIVGKDLE FTIYDDDDVS PFLEGLEERP QRKAQPTQPA
251 D
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Figure 5-10. Peptide coverage of alpha-6 bands by LC/MS/MS for detection of post-translational modifications. The alpha-6 containing fraction was run on a gel to separate the upper and lower alpha-6 bands. The lower alpha-6 band was excised and digested with trypsin and analyzed by LC/MS/MS. The resulting peptides were searched against a home-built database. Coverage was observed for residues shown in red.

The peptide NH2-EPAE-COOH is 444.45 Daltons and the removal of this peptide from the subunit would account for a 426.44 Dalton difference in mass. These results reveal that there are two C-terminally-truncated alpha-6 species present in these 20S proteasome preparations. The full-length, 263-amino acid, alpha-6 subunit can be truncated after residue 251 and after residue 255.

Construct	Molecular Weight (Daltons)	Theoretical pI
1-263 (full-length)	29585.6	6.15
1-255	28647.6	6.45
1-251	28221.2	7.08

Table 5-2. Forms of the alpha-6 subunit identified in 20S proteasomes purified from bovine red blood cells. The protein sequence of the full-length alpha-6 subunit from *B. taurus* (GenBank: AAI02217.1) was analyzed with the ExPASy Bioinformatics Resource Portal to determine the molecular weight and the theoretical isoelectric point in the absence of any additional modifications.

To determine the molecular identities of alpha-6 species observed by one-dimensional and two-dimensional gel electrophoresis, fractions from a single alpha-6 purification of the M61 alpha-synuclein-inactive preparation were collected such that one fraction contained only the lower molecular weight alpha-6 band (Fraction A) by one-dimensional SDS-PAGE, while the next fraction

collected (Fraction B) contained both the upper and lower molecular weight alpha-6 bands.

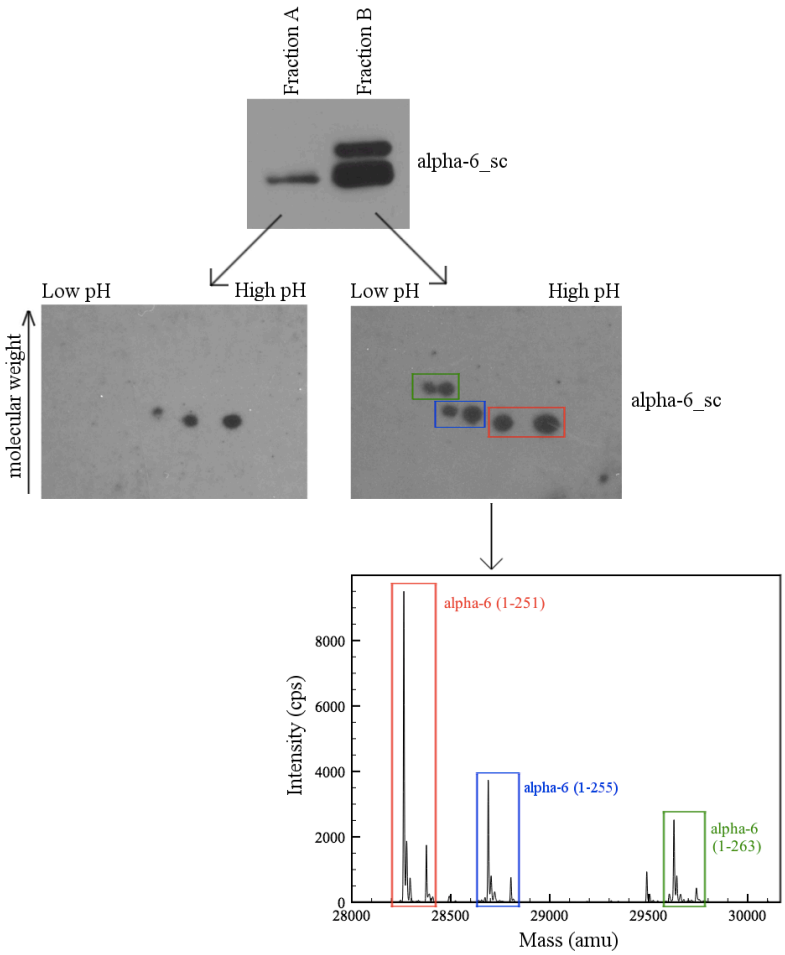

Figure 5-11. Isolation and characterization of M61 alpha-synuclein-inactive proteasomes. The M61 proteasome preparation was subjected to reversed-phase chromatography to isolate the alpha-6 subunit. Fractions were collected such that two consecutive fractions contained either the lower alpha-6 band or both the upper and lower alpha-6 bands by one-dimensional SDS-PAGE. Both fractions were then analyzed by two-dimensional SDS-PAGE, and Fraction B was analyzed by ESI-MS.

Both fractions were then analyzed by two-dimensional gel electrophoresis and electrospray ionization mass spectrometry (Figure 5-11). The spots produced by two-dimensional SDS-PAGE can be assigned to the full-length and C-terminally-truncated forms of the protein according to the mass spectrometry results.

Truncation-specific antibodies were generated against the alpha-6 subunit.

Polyclonal antibodies were generated to recognize the full-length and truncated forms of the human alpha-6 subunit. Antigenic peptides were designed based on a previous report (Liang et al. 1996) and our own previous success in generating antibodies specific to the C-terminus of proteins (Lewis et al. 2010). These peptides contained the seven C-terminal residues from the desired epitope with a cysteine residue on the N-terminus of the peptide for conjugation to a carrier protein, as shown in Table 5-3. The alignment of the human and bovine forms of the alpha-6 subunit reveals only one amino acid difference in the entire subunit, and this difference occurs near the C-terminus. The antigenic peptide for the alpha-6_251 antibody contained the residue for the human sequence, to ensure that these antibodies could potentially be used in human tissue. Rabbits were injected with the peptide conjugated to the keyhole limpet hemocyanin (KLH) carrier protein.

Antibody Name	Epitope	Antigen	Source
alpha-6_sc	residues 131-225	purified proteasome	Santa Cruz (sc-67046)
alpha-6_263	C-terminus at residue 263	KLH-CADEPMEH-COO	these studies
alpha-6_255	C-terminus at residue 255	KLH-CPADEPAE-COO	these studies
alpha-6_251	C-terminus at residue 251	KLH-CQPAQPAD-COO	these studies

Table 5-3. Commercial and non-commercial anti-alpha-6 antibodies used in these studies. Truncation-specific antibodies were generated by Proteintech Group, Inc. In the alpha-6_251 antigen, the alanine residue in red (*H. sapiens* sequence) is a threonine in *B. taurus*.

Crude sera was collected and affinity-purified and the specificity of each antibody to various forms of truncated human alpha-6 was assessed. Controls were generated by expressing the full-length and truncated forms of human alpha-6 in *E. coli*, and expression of each protein was achieved to similar levels. In addition, other truncated forms of alpha-6, with residues ending at 248 through 262, were generated and expressed by a similar method. The concentration of supernatant from lysed cells was normalized and these lysates were used as controls to

determine antibody sensitivity and specificity (Figure 5-12). A truncated version of alpha-synuclein (residues 1-95) was expressed in *E. coli* in a similar manner and utilized as a negative control to ensure that these antibodies do not recognize a general *E. coli* protein. The antibody generated to recognize the C-terminus of the full-length human alpha-6 subunit is very specific, reacting only to alpha-6 (1-263). The antibody generated against alpha-6 (1-255) exhibits minimal reactivity against alpha-6 (1-254), but primarily recognizes its intended target. Although alpha-6_251 also strongly reacts with alpha-6 (1-250) and alpha-6 (1-252), its set of ligands does not overlap with that of alpha-6_255. This allows these two antibodies to be used to distinguish an alpha-6 subunit truncated at residue 255 or 254 from an alpha-6 subunit truncated at residue 250-252.

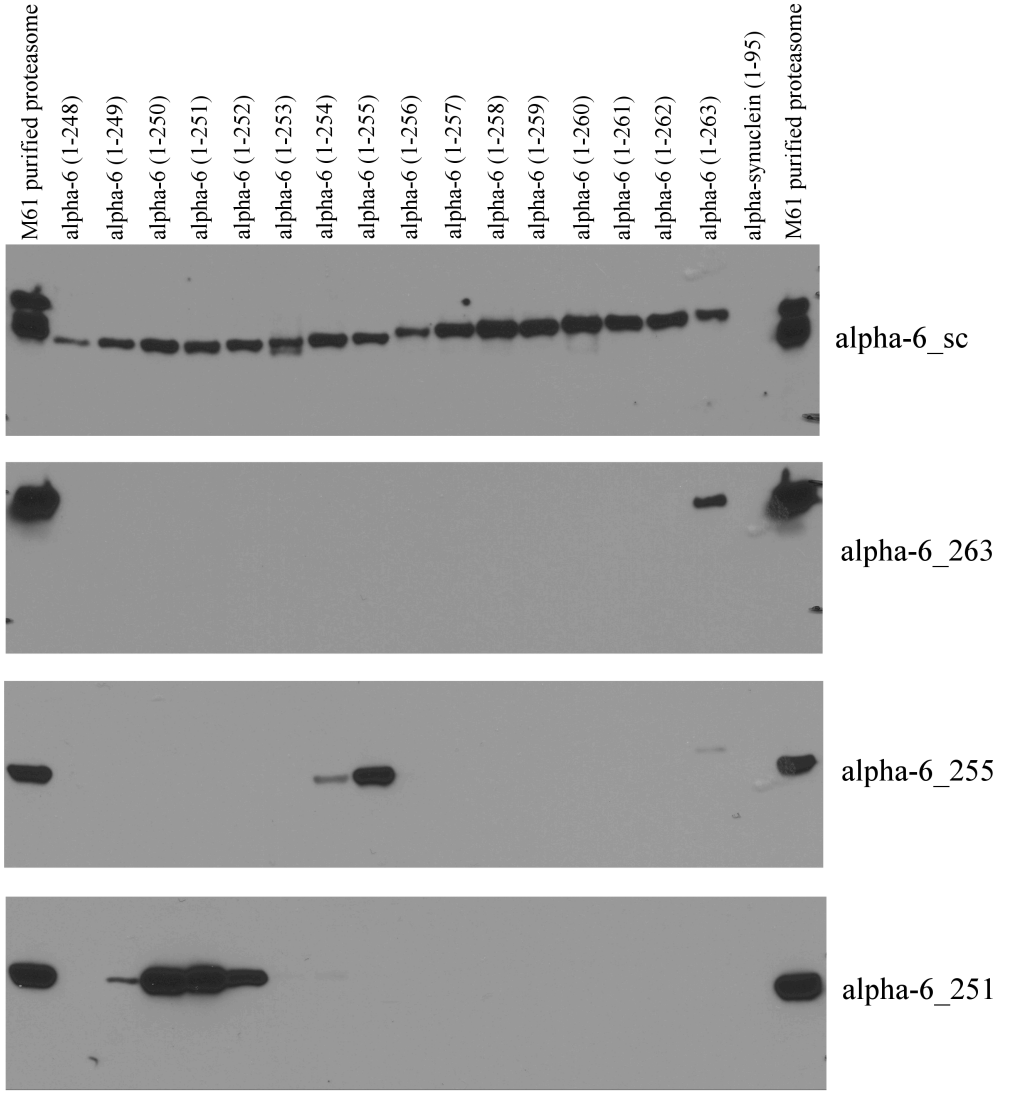

Figure 5-12. Specificity of C-terminus-directed alpha-6 antibodies. Full-length and C-terminally-truncated forms of alpha-6 were expressed in *E. coli* and the supernatant from these cells was used as experimental controls. The specificity of the C-terminus-specific alpha-6 antibodies were tested by running supernatant from *E. coli* expressing each alpha-6 construct and a truncated form of alpha-synuclein.

The purified 20S preparations were then analyzed with these truncation-specific antibodies to confirm the identities of the alpha-6 bands that were identified by mass spectrometry (Figure 5-13).

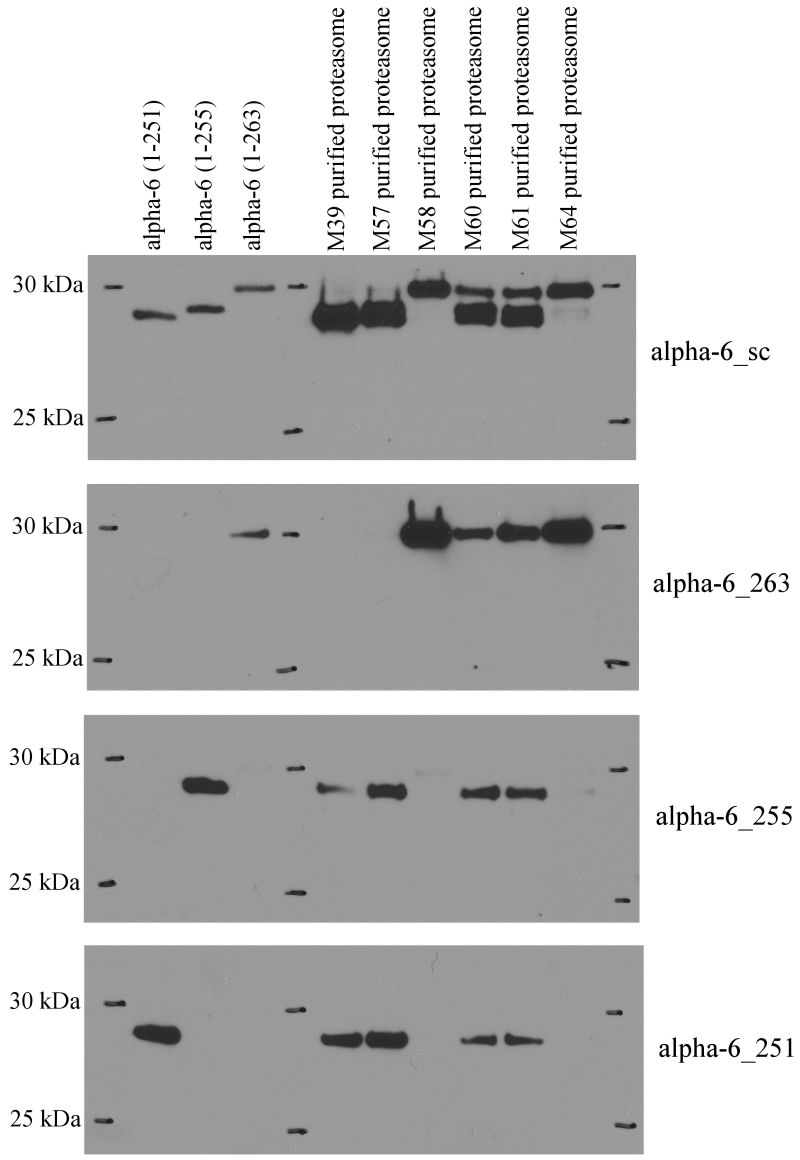

Figure 5-13. Analysis of purified 20S proteasome preparations with C-terminal-specific alpha-6 antibodies. Equal amounts of purified 20S proteasome preparations were analyzed by SDS-PAGE and Western blotting with the alpha-6_sc and C-terminus-specific antibodies. Supernatant from *E. coli* expressing the full-length (1-263) and C-terminally truncated (1-255 and 1-251) proteins were run on each gel as controls.

The upper alpha-6 band contained in preps M58, M60, M61, and M64 are recognized by the alpha-6_263 antibody, revealing that the upper band is the full-length alpha-6 subunit. The lower band of preps M39, M57, M60, and M61 are recognized by the alpha-6_251 antibody, confirming that this species is truncated near residue 251. Preps M57, M60, M61, and M39 (to a lesser extent) also contain a band recognized by the alpha-6_255 antibody that is at the apparent molecular weight of the subunit truncated near residue 255.

Association of C-terminal peptide may inhibit activity of the 20S proteasome against alpha-synuclein.

As shown previously, proteasome preparations containing any detectable levels of the full-length alpha-6 are inactive against alpha-synuclein under the conditions tested. Activity of these proteasome preparations did not change according to the ratio of the upper molecular weight band to the lower molecular weight bands. This observation led to the hypothesis that in preparations containing upper and lower alpha-6 bands (M60 and M61), the C-terminal peptide has been partially cleaved, but can still remain associated and act *in trans* to inhibit activity of the enzyme against alpha-synuclein. This hypothesis will be referred to as the "association hypothesis". Previous reports in the literature have revealed an activation of the 20S proteasome against peptide substrates upon

dialysis of the enzyme against water (McGuire et al. 1989; B. Yu et al. 1993), and the hypothesis proposed above could explain these earlier observations. Proteasome preparations that contained both the upper and lower alpha-6 bands (M60 and M61) were subjected to exhaustive dialysis against water as performed previously in the literature (McGuire et al. 1989), and the activity of the enzyme against alpha-synuclein was tested before and after dialysis (Figure 5-14). These preparations showed a significant increase in activity against alpha-synuclein after dialysis against water. Alternatively, preps in which the C-terminal peptide is still covalently attached to the alpha-6 subunit (M64), did not show an appreciable change in activity upon dialysis.

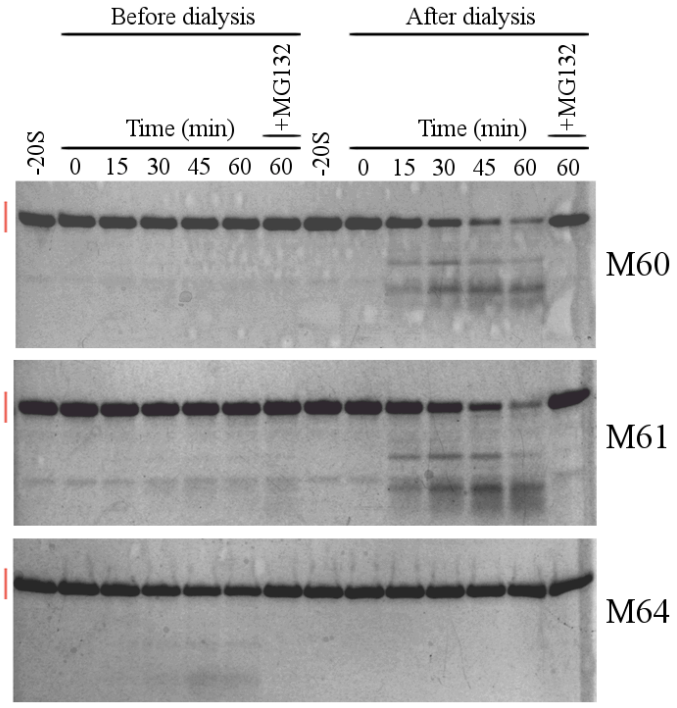

Figure 5-14. Dialysis-mediated activation of the M60 and M61 alpha-synuclein-inactive 20S preparations. Alpha-synuclein-inactive 20S preparations were dialyzed against water. Activity of 20S before and after dialysis was compared by performing alpha-synuclein degradation assays as described in Materials and Methods. The concentration of 20S before and after dialysis was determined by Bradford assay to ensure that the enzyme-to-substrate ratio was identical under each condition. The vertical, red line indicates the position of full-length alpha-synuclein on the gel.

These results are consistent with the hypothesis that the cleaved C-terminal peptide may remain associated with the 20S proteasome to inhibit its activity against alpha-synuclein. To ascertain whether these results reveal a general mechanism of 20S proteasomal activation unrelated to the hypothesis outlined above, M57 (an alpha-synuclein-active proteasome preparation) was dialyzed

against water, and activity against alpha-synuclein was monitored before and after dialysis (Figure 5-15). The association hypothesis would predict no change in activity of the enzyme against alpha-synuclein after exhaustive dialysis against water, as this 20S proteasome preparation appears to lack the alpha-6 C-terminal peptide.

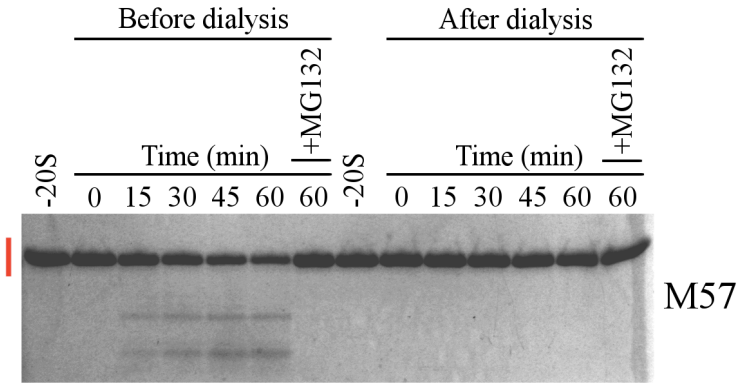

Figure 5-15. Dialysis-mediated inactivation of the M57 alpha-synuclein-active 20S preparation. The M57 alpha-synuclein-active 20S preparation was dialyzed in a similar manner as shown in Figure 5-14. The concentration of M57 before and after dialysis was normalized and alpha-synuclein degradation assays were performed as described in Materials and Methods. The vertical, red line indicates the position of full-length alpha-synuclein on the gel.

There was a decrease in activity of M57 proteasomes against alpha-synuclein after dialysis against water. These results prove that not all 20S proteasomes can be activated against alpha-synuclein by dialysis against water and suggest that a mechanism exists for activation that is specific to the proteasome preparations containing both full-length and truncated forms of the

alpha-6 subunit. There was no change in the electrophoretic mobility of the alpha-6 subunit upon dialysis, as monitored by one-dimensional SDS-PAGE analysis with the alpha-6_sc antibody (data not shown).

Dialysis may result in altered accessibility of the alpha-6 C-terminus.

To gain a further understanding of the full-length and truncated forms of alpha-6 within the 20S complex, proteasomes were analyzed by native PAGE with the C-terminus-specific alpha-6 antibodies. M60, M61, and M64 20S proteasome preparations were dialyzed against water as described previously. An equal concentration of each proteasome preparation, both before and after dialysis against water, was analyzed by native PAGE and Western blotting, using both the alpha-6_sc antibody and the three C-terminus-specific antibodies (Figure 5-16). The signal obtained with the antibodies for the undialyzed samples are consistent with earlier results observed by one-dimensional SDS-PAGE analysis. In M64 proteasome, there is much greater signal with the alpha-6_263 antibody than observed for the M60 and M61 proteasomes. In addition, the signal obtained with the alpha-6_255 and alpha-6_251 antibodies in M64 proteasomes is weaker than observed in M60 and M61 proteasomes. The hypothesis that the non-covalently associated C-terminal peptide is being removed from the 20S complex upon dialysis into water would predict that the signal corresponding to the alpha-6_263

antibody would decrease after dialysis, with an increase in signal corresponding to the alpha-6_251 or alpha-6_255 antibodies, in both the M60 and M61 20S preparations. Additionally, no change would be expected in the signal corresponding to the C-terminus-specific alpha-6 antibodies comparing M64 before and after dialysis.

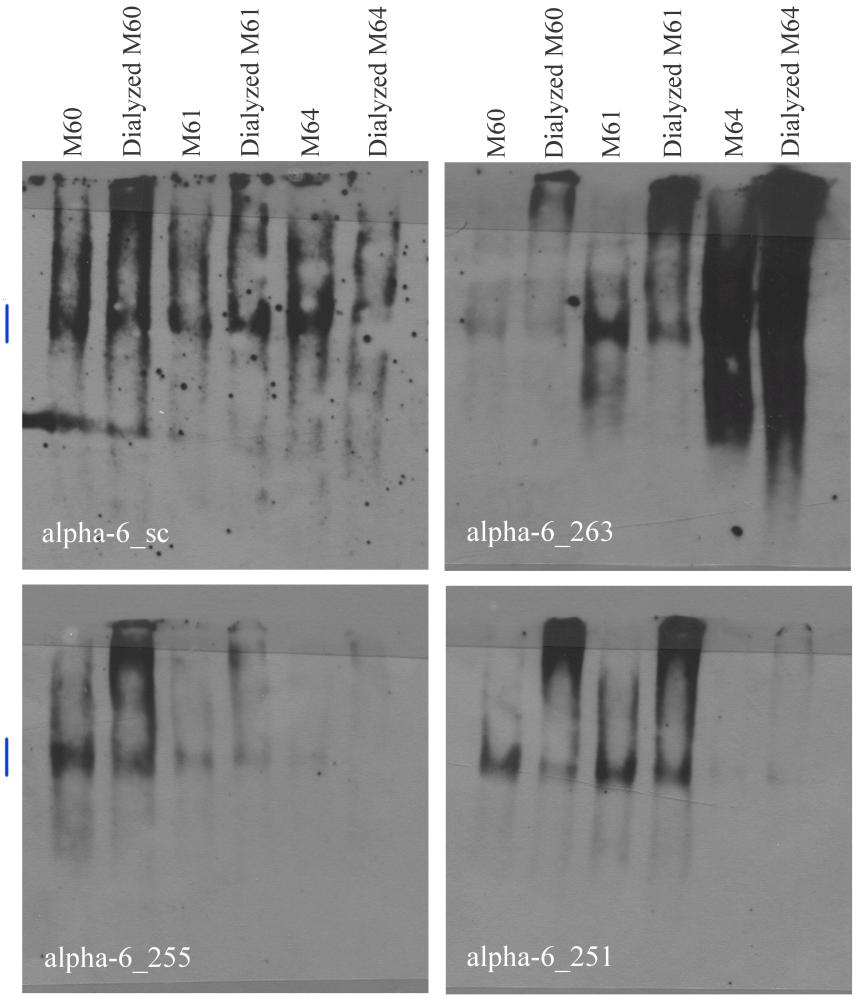

Figure 5-16. Native-PAGE of proteasome preparations before and after dialysis. An equal amount of 20S was loaded in each lane and signal corresponding to the full-length and two C-terminally truncated forms of alpha-6 was compared by Western blotting. The vertical, blue lines to the left indicate the position of 20S proteasome complex as revealed by fluorescence overlay assays (data not shown).

A slight decrease in the intensities of the alpha_6-263 signal after dialysis of the M60 and M61 20S preparations was observed at the expected migration of the 20S complex; however, a corresponding increase in signal with either the alpha-6_255 or alpha-6_251 antibody was not observed. Additionally, the relative signals from the truncation-specific antibodies comparing M60 and M61 proteasomes are not consistent with results observed by one-dimensional SDS-PAGE and Western blotting. This inconsistency suggests that, while these two proteasome preparations are similar in activity and activation potential, their conformations are not identical. The alpha-6 C-terminus from the M60 and M61 proteasome preparations is not equally accessible in the native state of the enzyme. After dialysis, all three proteasome preparations exhibited a noticeable increase in signal of higher molecular weight species and, in some instances, a smear of signal was observed. These observations could be indicative of protein aggregation occurring during dialysis; however, filtration of the samples prior to gel loading did not reduce signal of these upper molecular weight species (data not shown).

Other approaches to support the association hypothesis were attempted.

Other attempts aimed to support a role for the C-terminal alpha-6 peptide in modulating 20S proteasomal activity against alpha-synuclein *in trans* were

unsuccessful. Peptides corresponding to the possible C-terminal cleavage products (residues 252-263, residues 256-263, and residues 252-255) were generated and were added back to dialyzed M60 and M61 proteasomes. Peptides were pre-incubated with the dialyzed proteasomes at various molar ratios, and the activity of the 20S proteasome against alpha-synuclein was monitored after different incubation conditions. No conditions produced any apparent inhibition of proteasome activity against alpha-synuclein. While the association hypothesis would predict that the C-terminal peptide should inhibit proteasomal activity in trans, negative results could be caused by a conformational change occurring upon dialysis that prevents the peptide from re-binding to the complex. Dialyzing into other solutions did not affect activity of M60 or M61 proteasomes against alpha synuclein. Exhaustive dialysis into proteasome storage buffer, proteasome storage buffer without glycerol, or proteasome storage buffer with high salt did not yield the results observed with dialysis into water. Previous reports of dialysis-mediated activation of 20S proteasomes indicate that the activation is due to removal of EDTA, however the mechanism of activation is unknown (B. Yu et al. 1993).

DISCUSSION

The work presented in Chapter 5 reveals that a difference in activity of purified 20S proteasome preparations against alpha-synuclein may be explained by a C-terminal truncation of the alpha-6 subunit. Isolation and mass spectrometry of the alpha-6 subunit and gel electrophresis studies, in conjuction with activity assays of various 20S proteasome preparations, strengthen the correlation between alpha-6 truncated at residues 250-255 and activity of the complex against alpha-synuclein. In addition, the molecular weight and theoretical isoelectric point of the full-length and truncated forms of the alpha-6 subunit are consistent with the two-dimensional Western blots utilizing the alpha-6_sc antibody. Finally, the successful development of antibodies that react with the C-termini of truncated forms of the alpha-6 subunit will be a useful tool for the field, as these antibodies exhibit moderate to high degrees of specificity for the intended targets.

This work has confirmed and expanded upon previous reports in the literature describing a truncated form of the alpha-6 subunit. Furthermore, we have for the first time identified a functional consequence of the truncation. This C-terminal truncation of the alpha-6 subunit indicates a mechanism by which a modification of the proteasomal complex distal to either the gate or catalytic sites can affect enzymatic activity. In addition, these results suggest a possible effect of alpha-6 truncation on activity against a physiologically relevant substrate to

produce a cleavage product found in pathological samples. Thus, this work highlights a process that may play a role in the pathogenesis of disease and warrants further study.

All subsequent attempts at directly establishing a causative role of this truncation in activity against alpha-synuclein were unsuccessful. Controlled *in vitro* proteolysis experiments were carried out to truncate the C-terminus of alpha-6 in M58 and M64 proteasome preparations with various proteases and peptidases. However, many technical difficulties were encountered that could not be resolved. It is unlikely that these peptidases or proteases could truncate only the alpha-6 subunit, leading to multiple simultaneous truncation events and data that would be uninterpretable. Non-specific truncation of the N-termini of certain alpha subunits could lead to opening of the 20S gate, and other cleavage events could lead to structural rearrangements within the proteasome complex that might alter substrate recognition or enzymatic activity.

Cell culture experiments that were aimed at supporting a role for C-terminal truncation of alpha-6 in 20S proteasomal activity against alpha-synuclein in an *in vivo* system were also unsuccessful. There were many technical issues that prevented these experiments from being completed. Transient expression of the alpha-6 subunit was very low under all conditions tested. Even if high expression were achieved, it would not guarantee incorporation of the

recombinant subunit into the 20S complex, especially if the truncations themselves have an effect on 20S assembly. If the truncated subunits were highly expressed and incorporated into the 20S complex, the lack of effect on 20S activity would still not be interpretable, as C-terminal truncation of the alpha-6 subunit might be necessary, but not sufficient, for 20S proteasomal activity against alpha-synuclein.

While a causative role for the alpha-6 truncation in activity of the enzyme against alpha-synuclein was not shown, the results suggest a mechanism by which the 20S proteasome may be modified under certain physiological conditions to alter its enzymatic activity. A similar C-terminal truncation has now been shown in human erythrocytes, rat liver, and bovine erythrocytes, revealing a mechanism that is conserved among mammalian species. Together, these independent studies provide evidence for a modification that might be utilized in the regulation of 20S proteasomal activity, which may affect many downstream cellular events and possibly contribute to disease pathogenesis.

CHAPTER SIX
Prospectus for Future Work

Results in Chapter Four disprove the hypothesis that all IDPs are recognized and degraded by the 20S proteasome. While a mechanism for recognition was not clearly established, the results presented suggest that the 20S proteasome can selectively degrade some substrates but not others, and that this mechanism may involve substrate-mediated opening of the 20S gate. Understanding the means by which full-length alpha-synuclein is degraded to produce aggregation-prone products that are less susceptible to further aggregation might provide insight into how this process plays a role in disease pathogenesis. The long-range interactions that have been shown to exist in the membrane-unbound forms of alpha-synuclein might play a role in recognition by the 20S proteasome. Alpha-synuclein mutations could be generated that alter the strength of these long-range interactions, as analyzed by PRE and RDC. *In vitro* degradation assays of these constructs with purified 20S proteasomes could establish a correlation between strength of interactions and degradation propensity. If this hypothesis proves to be valid, therapeutics could be designed to disrupt these long-range interactions. Providing that this disruption does not cause other negative physiological effects, these therapeutics could retard the

incomplete degradation of alpha-synuclein, the generation of aggregation-prone truncated species, and protein accumulation.

Additionally, it would be interesting to understand whether C-terminally truncated forms of alpha-synuclein play a unique role in the cell, and if the intercellular transmission of alpha-synuclein is altered by removal of the C-terminus. A mammalian cell culture model could be employed to study this process, and C-terminally truncated forms of alpha-synuclein can either be transfected into the cells to monitor export, or added to the cell media to monitor import. If these truncated forms of alpha-synuclein have altered transmissibility, this process could be a target for therapeutics aimed at blocking the spread of disease-related pathology.

The results in Chapter Five show that some, but not all, 20S proteasome preparations are capable of recognizing and degrading alpha-synuclein at specific sites within the C-terminus of the protein in an *in vitro* system. A specific C-terminal cleavage of the alpha-6 subunit of the 20S proteasome was identified that correlates with activity against alpha-synuclein in this same *in vitro* system; however, the truncation of the 20S alpha-6 subunit and activity of the enzyme against alpha-synuclein is still merely correlative.

Attempts at establishing a causative role for this modification in enzyme activity both *in vitro* and *in vivo* have been unsuccessful. Negative results

presented here were mostly due to technical issues in enzymatic assays and protein overexpression in a mammalian cell culture model. The half-life of the proteasome has been calculated to be 5 days in HeLa cells (Hendil 1988) and up to 15 days in rat liver (Tanaka & Ichihara 1989), revealing that the complex is very stable. Transient transfection of a single 20S subunit (alpha-6) might not allow for adequate expression levels for the appropriate amount of time, such that the subunit can be incorporated into the 20S complex. It could be possible to induce upregulation of proteasome expression, such that subunits are expressed at higher levels to promote *de novo* proteasome assembly. Alternatively, or in addition to the previous idea, the expression of the endogenous alpha-6 subunit could be knocked down by small-interfering RNA (siRNA), while transiently-transfected alpha-6 constructs could be designed with silent mutations, such that its transcript is not recognized by the siRNA. Utilizing these two methods in tandem could lead to *de novo* proteasome formation, preferentially with incorporation of the transiently-transfected full-length and truncated alpha-6 constructs. This might generate cells with enough of the desired form of the 20S proteasome to observe an effect on alpha-synuclein degradation, if the previous *in vitro* results hold up in an *in vivo* system. However, truncation of the alpha-6 subunit of the 20S proteasome may be necessary but not sufficient for an alteration in enzyme activity against alpha-synuclein. Other factors that might

play a role in this process are unlimited, and could include other modifications to the 20S proteasome itself or other physiological components within the cell.

In addition to establishing a causative role for the C-terminal cleavage of alpha-6 in 20S proteasomal activity against alpha-synuclein, finding the enzyme responsible for generating the cleavage is essential in understanding the mechanism by which this modification occurs in the cell and how it might be regulated. It is still unclear from these results whether C-terminal alpha-6 cleavage is a physiological phenomenon or whether it is merely a consequence of the purification scheme. Biochemistry experiments can be performed to determine at which step in the purification this cleavage event is able to occur over time, and further fractionation of proteins remaining at this step, followed by mass spectrometry, could lead to identification of the responsible enzyme. The role of the identified enzyme could then be tested in a mammalian cell culture model, by overexpressing or knocking down the responsible enzyme, and monitoring the electrophoretic mobility of the alpha-6 proteasomal subunit under different cellular conditions.

Finally, the role that this truncation may play in Lewy body diseases could reveal an unprecedented mechanism for disease progression. More sensitive and specific antibodies need to be generated to analyze human brain tissue for the full-length and truncated forms of the alpha-6 subunit. Detection and quantification of

truncated alpha-6 can be compared to quantification of alpha-synuclein truncations utilizing the antibodies previously developed (Lewis et al. 2010) to determine if a significant correlation exists.

APPENDIX A
Materials and Methods

Alpha-synuclein expression and purification

Alpha-synuclein was inserted into the pET28a vector using NcoI and HindIII restriction sites and expressed in the BL-21 line of *E. coli*. Large scale cultures were grown to a OD_{600} of 0.5, and expression of alpha-synuclein was induced by adding IPTG to a final concentration of 500μM. After the addition of IPTG, growth of the cultures was continued at 37°C (or 20°C, for syn95 protein) for 4 hours with shaking. After 4 hours, cells were harvested by centrifugation at 5000xg for 5 minutes at 4°C in a fixed-angle rotor. Lysis was performed in 20mM Tris, pH=7.6 at 4°C + 20mM NaCl + Roche Complete Protease Inhibitor Cocktail Tablet at 4°C (roughly 10mL lysis buffer for each liter of culture). Cells were either disrupted by sonication at 40-50% output for 2 minutes in an ice water bath or via mechanical force utilizing glass beads (0.1mm diameter) When lysed with glass beads, roughly one-third glass beads was added to the cell lysate (volume per volume) in a 50 mL tube with a screw top. The lysate was vortexed at maximum speed for 5 x 2 minutes with 2 minutes on ice between each vortexing cycle. The cell lysate was spun at 40,000xg for 20 minutes at 4°C in a fixed-angle rotor and the supernatant collected. The supernatant was then heated at 90°C for 10 minutes, followed by centrifugation at 40,000xg for 20 minutes. The

supernatant was brought to a final concentration of 1M $(NH_4)_2SO_4$, and 8-10mL of a 50% slurry of charged phenyl beads (Phenyl Sepharose 6 Fast Flow (high sub), Amersham Pharmacia Biotech, cat#17-0973-05; charged in 20mM Tris, pH=7.6 at 4°C + 20mM NaCl + 1M $(NH_4)_2SO_4$) was added. The supernatant was allowed to incubate with the resin for 30 minutes to 1 hour with rocking at 4°C, and the flowthrough was retrieved through a BioRad glass column. Resin was washed with 10mL wash buffer (20mM Tris, pH=7.6 at 4°C + 20mM NaCl +1M $(NH_4)_2SO_4$). The flowthrough and wash fractions were pooled and brought to a final concentration of 3M $(NH_4)_2SO_4$, and allowed to agitate for 1-2 hours at 4°C. The sample was then centrifuged at 40,000xg for 20 minutes at 4°C in a fixed-angle rotor. The pellet was resuspended in 10-15mL of 20mM Tris, pH=7.6 at 4°C + 20mM NaCl, and dialyzed into 20mM Tris, pH=7.6 at 4°C + 20mM NaCl + 1mM EDTA with Spectra/Por 1 6,000-8,000 Dalton MWCO (cat#132650) dialysis tubing. Dialysis occurred in 3 x 2L buffer over roughly 36 hours. After dialysis, protein was loaded onto a DEAE anion exchange column (DEAE Sepharose Fast Flow, Amersham Pharmacia Biotech, cat#17-0709-01) and eluted with 8-25% gradient of 20mM Tris, pH=7.2 at 4°C + 1M NaCl. Fractions were analyzed by SDS-PAGE and fractions of desired purity were pooled and dialyzed into storage buffer (20mM sodium phosphate, pH=7.6). The purified alpha-synuclein was concentrated using a Millipore Amicon Ultra concentrator,

10,000K MWCO (cat#UFC901024) to a concentration of 100-500μM, aliquotted, snap-frozen in liquid nitrogen, and stored at -80°C.

Alpha-synuclein degradation

10μm alpha-synuclein was incubated with 20nM 20S proteasome in reaction buffer (20mM Tris, pH 7.6 at 4°C + 20mM NaCl + 1mM EDTA) at 37°C. Aliquots were removed at the indicated time points, mixed with 5X SDS sample buffer, and placed on ice. Control reactions were performed with 20S that had been pre-incubated with 100μM MG132 or β-lactocystin for 20 minutes at 37°C. Samples were boiled at 100°C for 5 minutes and loaded onto SDS polyacrylamide gels. Gels were either stained with Coomassie or transferred to 0.45μm nitrocellulose. Where indicated, alpha-synuclein was detected with the monoclonal syn303 antibody (epitope: residues 2-4) provided by Dr. Virginia M.-Y. Lee (Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine). In samples containing lipid vesicles, POPA/ POPC vesicles (prepared as described below) were preincubated with alpha-synuclein at a 100-fold molar excess for 30 minutes at room temperature.

Lipid vesicle preparation

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (monosodium salt) (POPA) were obtained from Avanti Polar Lipids, Inc. Equimolar amounts of the lipids were combined and the chloroform evaporated under nitrogen, followed by lyophilization for a minimum of 3 hours. Following resuspension to a final concentration of 2.5mM in lipid buffer (10mM Na_2HPO_4, pH 7.4 + 100mM NaCl), the solution was sonicated for 4 cycles of 2 min each (power 3, 40% duty cycle).

Circular dichroism spectroscopy

10μM alpha-synuclein was pre-incubated with lipid buffer or 750μM POPA/POPC lipid vesicles in 20mM sodium phosphate buffer, pH=7.2, at room temperature for 10 minutes. Internal temperature was set to 20°C on a Jasco model J-810 spectropolarimeter. Spectra were scanned from 260 to 195 nm at 100nm/min with a 1 second averaging time. The background was automatically subtracted using the background correction algorithm of the JASCO Spectra Analysis program. Spectra shown are the average of 3 repetitions.

Purification of M39, M57, M60, and M61 20S Proteasomes

Bovine erythrocytes were washed in PBS (137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 14.7mM KH_2PO_4, pH 7.4) several times and frozen at -80˚C. Cells were thawed at 4˚C, and lysed in 50mM Tris, pH=7.6 at 4˚C + 5mM β-mercaptoethanol (β-ME). The cells were the centrifuged at 11,500×g for 1 hr at 4˚C, and the supernatant was incubated batchwise with DE52 anion exchange resin and eluted with 50mM Tris, pH=7.6 at 4˚C + 5mM β-ME + 400mM NaCl. The eluate was brought to 40% ammonium sulfate, and the supernatant was separated from precipitate by centrifugation. The supernatant was extensively dialyzed against 50 mM Tris, pH=7.6 at 4˚C + 5mM β-ME + 50mM NaCl and purified over a DEAE Fractogel anion exchange column. The elution peak was determined based on chymotrypsin-like peptidase activity. The pooled fractions were concentrated on an XM-300 filter (BioRad) and separated on a Sephacryl S300 size exclusion column. Eluted fractions containing peptidase activity were collected and dialyzed against 20mM potassium phosphate buffer (5mM KH_2PO_4/ K_2HPO_4, pH=7.6 with HCl + 5mM β-ME + 20% glycerol). Following separation over hydroxyapatite resin, the 20S-containing fractions were determined by peptidase and protease (^{14}C-casein) activities and by gel electrophoresis. The pooled fractions were dialyzed overnight against proteasome storage buffer (20mM Tris, pH=7.6 at 4˚C + 20mM NaCl + 1mM $MgCl_2$ + 0.1mM EDTA +

0.5mM DTT + 20% glycerol), concentrated on an XM-300 filter, and snap-frozen in liquid nitrogen prior to storage at -80˚C.

Purification of M58 and M64 20S Proteasomes

Bovine erythrocytes were washed in PBS (137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 14.7mM KH_2PO_4, pH=7.4) and lysed in 5.5 volumes of Buffer H without glycerol (20mM Tris-HCl, pH=7.6 at 4°C + 20mM NaCl + 0.1mM EDTA + 5mM β-ME). The lysate was centrifuged (11,500×g, 1 hr, 4˚C) to pellet the insoluble material and the resulting supernatant was incubated batchwise with DE52 anion exchange resin in Buffer X (20 mM Tris, pH=7.6 at 4˚C + 20mM NaCl + 1mM $MgCl_2$ + 0.1mM EDTA + 0.5mM DTT) and eluted with Buffer X + 400mM NaCl. The eluate was dialyzed against Buffer X, then separated over a DEAE-Fractogel anion exchange column using a gradient of Buffer X + 50mM NaCl and Buffer X + 450mM NaCl. The 20S peak was collected and concentrated on an XM-300 filter (BioRad) before size exclusion chromatography over a Sephacryl S300 column in Buffer X + 120mM NaCl. The elution peak was again collected and dialyzed against Buffer K (20mM KH_2PO_4/K_2HPO_4, pH=7.6 with HCl + 0.1mM EDTA (pH=7.6) + 1mM $MgCl_2$ + 5mM β-ME + 20% glycerol). The dialysate was bound to a HAP column (2.5cmx9cm; 10 g total resin) in Buffer K and eluted over a 500 mL gradient of Buffer K from 20mM KH_2PO_4/

K_2HPO_4 to 250mM KH_2PO_4/K_2HPO_4. The 20S peak was collected and dialyzed against Buffer X with 120mM NaCl, then concentrated on a PM-10 filter. The concentrated protein was then again subjected to size exclusion chromatography over the S300 column in Buffer X + 120mM NaCl, and the eluted 20S was dialyzed against Buffer H + 20% glycerol. The protein was then concentrated, aliquoted, and snap-frozen in liquid nitrogen prior to storage at -80°C.

One-dimensional SDS-PAGE of proteasomes

Proteasomes were diluted in proteasome storage buffer (20mM Tris pH=7.6 at 4°C + 20mM NaCl + 1mM $MgCl_2$ + 0.1mM EDTA + 0.5mM DTT), and added to SDS sample buffer. Samples were boiled in sample buffer for 5 minutes, spun down to remove condensation, and loaded onto a 12.5% Tricine polyacrylamide gel. Gels were run for approximately 3.5 hours at 120V to ensure adequate separation between the various alpha-6 bands. Gels were either stained with Coomassie or transferred to 0.45μm nitrocellulose for Western blotting.

Western blotting of proteasomes

After transferring to 0.45μm nitrocellulose at 90V for 1.5 hours or 40V for 16 hours at 4°C, samples were blocked in 5% milk (in TBS-Tween) for at least 2 hours at room temperature or overnight at 4°C. The membrane was then rinsed in

TBS-Tween and allowed to incubate (continuous gentle agitation) with primary antibody (1:1000 for alpha-6_sc; 1:10000 for C-terminus-specific alpha-6 antibodies) diluted in TBS-Tween for 1 hour at room temperature. After incubation with the primary antibody, the membranes were washed (continuous gentle agitation) four times with TBS-Tween for 5 minutes each. HRP-conjugated goat anti-rabbit secondary antibody was added at a 1:10000 dilution in 5% milk (in TBS-Tween), and allowed to incubate (continuous gentle agitation) for 50 minutes at room temperature, followed by four 5-minute washes with TBS-Tween. Liquid was poured off of membranes, and signal was developed with either ECL+ or Thermo-Scientific SuperSignal West Dura chemiluminescent substrate for 5 minutes at room temperature.

Alpha-6 expression in *E. coli*

Human alpha-6 was amplified from a human brain cDNA library and inserted into a pET28a cloning vector for expression in *E. coli*. Stop codons were introduced into the vector to produce the desired truncated constructs. Alpha-6 was expressed in the BL-21 (DE3) strain of *E. coli*. At OD_{600}=0.5, expression was induced with a final IPTG concentration of 500uM. Upon induction, cultures were moved to a 20°C shaker, and were harvested 24 hours post-induction. Lysis was performed in 20mM Tris, pH=7.6 at 4°C + 20mM NaCl + Roche Complete

Protease Inhibitor Cocktail Tablet at 4°C (500µL lysis buffer for each 25mL of culture). Cells were disrupted via mechanical force utilizing glass beads (0.1mm diameter) by filling 1.5mL microtubes to between the 100µL and 200µL mark with beads before the lysate was added. The lysate was vortexed with the glass beads at maximum speed for 5 x 2 minutes with 2 minutes on ice between each vortexing cycle. After the final vortexing cycle, the tubes were placed on ice for at least 5 minutes to allow the glass beads to settle, after which 350µL of the lysate was transferred to a new microtube and centrifuged at 15,000xg for 15 minutes at 4°C. The total protein concentration of the supernatant was determined by Bradford assay and the alpha-6-containing supernatant was stored at -20°C.

Peptide synthesis

Peptides were synthesized using Fmoc (N-[9-fluorenyl]methoxycarbonyl) chemistry and purified using HPLC by the Protein Core Facility at UT Southwestern Medical Center. Sequences of all peptides were verified by mass spectrometry. Table A1-1 indicates the peptides that were generated and utilized throughout the studies presented in this document.

Peptide Name	Peptide Sequence	Purpose
Rpt5	N'-KKKANLQYYA-C'	proteasome activation
alpha-6_252-255	N'-EPAE-C'	peptide add-back
alpha-6_252-263	N'-EPAEKADEPMEH-C'	peptide add-back
alpha-6_256-263	N'-KADEPMEH-C'	peptide add-back

Table A1-1. Peptides utilized throughout these studies. Peptides were supplied as a lyophilized powder and were brought into solution in either Tris buffer (for proteasome activation assays) or water (for the peptide add-back experiments). The concentration of the peptide solutions was determined by tyrosine fluorescence (for the Rpt5 peptide) or backbone fluorescence using a Qubit fluorometer (for the alpha-6 peptides).

Activation of proteasomes by dialysis

Purified 20S proteasomes were transferred into a pre-wet Thermo Scientific Slide-A-Lyzer Dialysis Cassette (Extra Strength) with a 10,000K MWCO (cat#66383) or a Thermo Scientific Slide-A-Lyzer MINI Dialysis Unit with a 10,000K MWCO (cat#69570). Proteasomes were dialyzed against 1L deionized water at 4°C with rapid stirring. Dialysate was exchanged at least three times over a 24 hour period. After 24 hours, the water was exchanged with 20S proteasome storage buffer (20mM Tris pH=7.6 at 4°C + 20mM NaCl + 1mM $MgCl_2$ + 0.1mM EDTA + 0.5mM DTT) and allowed to dialyze in a similar manner with a minimum of 3 exchanges over a 24 hour period. Dialyzed 20S

proteasomes were removed from the dialysis cassette and the final concentration was determined by Bradford assay.

Rpt5 peptide-mediated activation of 20S proteasomes

The Rpt5 peptide (in 50mM Tris, pH=7.6 at 25°C + 5mM DTT) was pre-incubated with 20S proteasomes at a 5000-fold molar ratio (20nM 20S + 100μM Rpt5 peptide) on ice for at least 30 minutes. Samples were then added to alpha-synuclein or fluorogenic peptide substrates to monitor activity.

20S peptidase activity assay

20nM purified 20S proteasomes pre-incubated with the Rpt5 peptide or buffer was added to 200μM peptide substrate in 45mM Tris, pH=8.0 at 37°C + 5mM β-ME that had been pre-heated to 37°C. Samples were added to wells in triplicate of a 96-well plate (Costar #3631), the plate was covered with clear polyolefin sealing tape (VWR International), and fluorescence (λ_{ex}=355nm, λ_{em}=460nm) was monitored every 1.5 minutes for a total of 60 minutes on a Molecular Devices Spectra Max Gemini fluorescence plate reader set to 37°C.

Activity	Peptide
PGPH-like	Z-LLE-AMC
chymotrypsin-like	Suc-LLVY-AMC

Table A1-2. Fluorogenic peptide substrates used to assess proteasomal activity. Peptides were ordered from Enzo Life Sciences and were resuspended in DMSO to 50mM and stored at -20°C.

Two-dimensional SDS-PAGE

5µg or 15µg of purified 20S proteasomes was pre-incubated with rehydration buffer (7M urea + 2M thiourea + 4% CHAPS + 65mM DTT + 0.02% Pharmolytes (pH 3-10) + a trace amount of bromophenol blue) for two hours with occasional mixing followed by absorption onto a pH=3.0-10.0 isoelectric focusing gel strip (ReadyStrip IPG Strip, 7 cm, linear gradient, pH 3-10 BioRad #163-2000) overnight at room temperature. The following day, the strip was subjected to isoelectric focusing in a BioRad Protean IEF Cell using a six-step program at 20°C: 50V, 10hr; 500V, 250 Vhr; 1000V, 500Vhr; 5000V, 7500 Vhr; 50V, 12 hr; 50 V, 24 hr. The isoelectric focusing strip was then incubated in 5mL equilibration buffer (50 mM Tris-HCl, pH=8.8 at 25°C, + 6M urea + 30% glycerol + 2% SDS + 65mM DTT + a trace amount of bromophenol blue) for 15-30 minutes at room temperature with constant agitation. The strip was then placed on top of a 12.5% Tricine gel (no stacking gel) and overlaid in 0.5%

agarose solution (in cathode buffer). Gels were electrophoresed at 30V for 30 minutes followed by approximately 5 hours at 90V. Gels were either stained with Coomassie or transferred to nitrocellulose for Western blotting.

Reversed-phase chromatography

10μg or 20μg purified 20S proteasomes were injected onto a Jupiter C4 reversed-phase chromatography column and run by high-performance liquid chromatography. Column was equilibrated and washed with 5% acetonitrile + 0.1% formic acid. The sample was eluted along a gradient from 55% to 95% acetonitrile with a flow rate of 1.0 mL/min. Fractions were collected manually based on absorbance at 221nm.

Electrospray ionization mass spectrometry (ESI-MS)

HPLC samples were dehydrated by rotovapping and then resuspended in DMSO. Samples were injected into a QStar XL Q-141TOF mass spectrometer (Applied Biosystems, Framingham, MA). Spectra were acquired with mass range m/z 500-2000. The molecular weights of proteins were calculated with the Baysesian Protein Reconstruct tool of the Analyst QS1.1 software.

Tandem mass spectrometry for PTM identification

HPLC samples were dehydrated and resuspended in SDS sample buffer. The samples were fractionated by one-dimensional SDS-PAGE and the desired bands were excised from the gel, subjected to iodoacetamide alkylation, and digested with trypsin or chymotrypsin. Dionex LC-Packings HPLC (Sunnyvale, CA) was coupled with a QStar XL Q-142TOF mass spectrometer (Applied Biosystems, Framingham, MA). The mass of resulting peptides was determined and searched against a home-built database.

Native-gel electrophoresis

4% non-denaturing acrylamide gels were cast under conditions to promote 26S assembly (with 0.5mM ATP and 2.5mM $MgCl_2$) in a Tris-borate buffering system. Gels for 20S and 26S proteasomes were run for 4-4.5 hours at 70V at 4°C. For fluorescence overlay assays, the gel (attached to the spacer plate but with the short glass plate removed) was incubated in a solution containing 50μM Suc-LLVY-AMC fluorogenic peptide substrate in 50mM Tris, pH=8.0 at 37°C + 5mM β-ME at 37°C for up to an hour with rocking. Images were acquired on a fluorescence gel imager with λ_{ex}=360nm. For Western blots, transfer and Western blotting procedure were carried out as for SDS-PAGE.

APPENDIX B
Chapter Collaborations

Chapter Four

A portion of the work in Chapter Four was conducted by Dr. Karen Lewis, a former graduate student in the lab and current postdoctoral fellow at the University of Colorado in Boulder and Dr. Changwei Liu, a former postdoctoral fellow in the lab and current faculty member in the Department of Molecular Biology at the University of Colorado Health Science Center in Denver. Dr. Changwei Liu assisted in the purification of the M39 proteasome preparation and, with the help of Dr. Karen Lewis, performed the M57 proteasome purification. Dr. Changwei Liu performed the degradation assays of RNase Sa. He also generated, purified, and performed assays with the N-terminally truncated forms of alpha-synuclein. Dr. Karen Lewis generated cloning vectors for the C-terminally truncated forms of alpha-synuclein and initiated the circular dichroism and degradation experiments with these constructs. Dung Mac, a former graduate student from Dr. George Demartino's laboratory, performed the degradation assays of titin and its mutants. The RNase Sa proteins were generated and their thermodynamic stabilities determined by Dr. Nick Pace, current faculty member in the Department of Biochemistry and Biophysics at Texas A&M University. Yeast proteasomes were provided by Dr. Yifan Cheng, Assistant Professor in the

Department of Biochemistry & Biophysics at the University of California San Francisco.

Chapter Five

Dr. Karen Lewis initiated the work shown in Chapter Five. During her time as a graduate student, she observed a difference in activity of several 20S proteasome preparations against alpha-synuclein. She utilized two-dimensional SDS-PAGE and Western blotting with subunit-specific antibodies to show that the major distinction between alpha-synuclein-active and alpha-synuclein-inactive 20S preparations is a molecular difference in the alpha-6 subunit. Linda Millen, a member of Dr. Philip Thomas' laboratory, optimized the reverse-phase chromatography protocol that allowed for isolation of the alpha-6 subunit. Yan Li, Ph.D. and Hayden Ball, Ph.D., at the University of Texas Southwestern Medical Center Protein Chemistry Technology Center, performed the mass spectrometry experiments and synthesized the peptides shown in Table A1-1. David Thompson, a member of Dr. George DeMartino's laboratory, purified several of the proteasome preparations used in this study, and his expertise in proteasome purification and assays was critical in the completion of this work. Dr. Brajesh Kumar and Dr. Young-Chan Kim, former postdoctoral fellows in Dr. George DeMartino's laboratory, provided assistance and expertise in several of the

proteasomal assays utilized for this work. Several undergraduate students also contributed to this project. Elizabeth Vinson, an undergraduate student from University of Dallas, assisted in early proteasome work by characterizing the activities of the M60 and M61 proteasome preparations at different enzyme to substrate ratios. Margaux Schwartzstein, an undergraduate from University of Oklahoma, generated several of the alpha-6 truncation plasmids for expression in bacteria and qualitatively analyzed the specificity of the affinity-purified alpha-6 C-terminus-specific antibodies. Katie Matlock, another undergraduate from University of Oklahoma, worked alongside Margaux to gain a more quantitative understanding of the specificity of these antibodies.

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