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Accelerated formation of α -synuclein oligomers by concerted action of the 20S proteasome and familial Parkinson mutations

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Abstract

A hallmark of Parkinson disease (PD) is the formation of intracellular protein inclusions called Lewy bodies that also contain mitochondria. α -Synuclein (α Syn) is a major protein component of Lewy bodies, where it is in an amyloid conformation and a significant fraction is truncated by poorly understood proteolytic events. Previously, we demonstrated that the 20S proteasome cleaves α Syn in vitro to produce fragments like those observed in Lewy bodies and that the fragments accelerate the formation of amyloid fibrils from full-length α Syn. Three point mutations in α Syn are associated with early-onset familial PD: A30P, E46K, and A53T. However, these mutations have very different effects on the amyloidogenicity and vesicle-binding activity of α Syn, suggesting neither of these processes directly correlate with neurodegeneration. Here, we evaluate the effect of the disease-associated mutations on the fragmentation, conformation, and association reactions of α Syn in the presence of the 20S proteasome and liposomes. The 20S proteasome produced the C-terminal fragments from both the mutant and wildtype α Syn. These truncations accelerated fibrillization of all α -synucleins, but again there was no clear correlation between the PD-associated mutations and amyloid formation in the presence of liposomes. Recent data suggests that cellular toxicity is caused by a soluble oligomeric species, which is a precursor to the amyloid form and is immunologically distinguishable from both soluble monomeric and amyloid forms of α Syn. Notably, the rate of formation of the soluble, presumptively cytotoxic oligomers correlated with the disease-associated mutations when both 20S proteasome and liposomes were present. Under these conditions, the wildtype protein was also cleaved and formed the oligomeric structures, albeit at a slower rate, suggesting that 20S-mediated truncation of α Syn

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may play a role in sporadic PD as well. Evaluation of the biochemical reactions of the PD-associated α -synuclein mutants in our *in vitro* system provides insight into the possible pathogenetic mechanism of both familial and sporadic PD.

Keywords

20S proteasome; Endoproteolysis; Parkinson disease; α Synuclein; Amyloid; Soluble oligomers; Cytotoxicity; Liposomes

Background

Parkinson disease (PD) is characterized by the selective loss of dopaminergic neurons in the *substantia nigra pars compacta* of the brain and is associated with the presence of intracellular inclusion bodies termed Lewy bodies (LBs). In addition to amorphous protein aggregates that stain positive for ubiquitin, LBs contain amyloid fibrils of the protein α -synuclein (α Syn) as well as mitochondria (Bedford et al. 2008). A significant fraction of the α Syn in these structures is proteolytically processed to produce specific fragments (Baba et al. 1998; Campbell et al. 2001). While only a small fraction of PD cases have a genetic basis (Hardy et al. 2006; Lansbury and Brice 2002), several gene products have been implicated in early-onset PD, including α Syn, parkin, DJ-1, UCH-L1, PINK-1, and LRRK2 (Hague et al. 2003; Kitada et al. 1998; Leroy et al. 1998; Paisan-Ruiz et al. 2004; Polymeropoulos et al. 1997; Singleton et al. 2003; van Duijn et al. 2001). α Syn has been implicated in PD by molecular genetic data, including three point mutations: A53T, A30P, and E46K (Kruger et al. 1998; Polymeropoulos et al. 1997; Zarranz et al. 2004). The involvement of parkin and UCH-L1 suggest a critical role for the ubiquitin-proteasome system in this disease. A recent conditional knockout of the Rpt2 subunit that reduces the amount of the 26S proteasome while increasing the amount of the 20S proteasome produces mitochondrial-rich α Syn inclusions, indicating that decreased UPS or increased 20S activity can lead to Lewy body formation in the absence of mutant α Syn (Bedford et al. 2008). In an animal model expressing the A53T α Syn, increased steady state levels of the α Syn fragments are observed (Li et al. 2004). However, the relationship between the fragments, the familial α Syn mutants, the activity of the 20S proteasome, and cellular toxicity is poorly understood.

The cellular function of α Syn has not been fully elucidated. The protein has been shown to localize to presynaptic vesicles, associate with microtubules, and has been proposed to act as a vesicle-bound chaperone (Alim et al. 2004; Chandra et al. 2005; Kahle et al. 2000). Consistent with a function as a vesicle-associated protein, α Syn lacks ordered secondary structure in aqueous solution but adopts an alpha-helical structure in the presence of negatively-charged lipids or detergents (Chandra et al. 2003; Davidson et al. 1998; Eliezer et al. 2001; Ulmer et al. 2005). The 140-residue protein contains three regions: an N-terminal amphipathic region that forms a discontinuous helix on micellar surfaces (residues 1–98), a central amyloidogenic region located within the amphipathic region (residues 61–95), and a C-terminal acidic region that remains disordered even in the presence of vesicles (residues 99–140). Deletions in the C-terminal region increase the amyloidogenicity of the protein, suggesting that this region is important for maintaining solubility (Hoyer et al. 2004; Li et al. 2005; Liu et al. 2005), and may do so through long-range contacts with the N-terminus (Bernado et al. 2005; Dedmon et al. 2005b).

α Syn forms amyloid fibrils in a nucleation-dependent manner, and differences in fibrillization profiles are observed among the familial mutations. A53T-syn forms amyloid earlier and its fibrils elongate faster than wildtype α Syn (Choi et al. 2004; Conway et al. 1998, 2000b; Greenbaum et al. 2005; Li et al. 2001, 2002; Narhi et al. 1999). Data for

A30P-syn are less conclusive. In general, A30P appears to form soluble higher molecular weight aggregates faster than wildtype α Syn (Conway et al. 1998; Li et al. 2002; Narhi et al. 1999), while the formation of amyloid structure is slower than wildtype (Choi et al. 2004; Conway et al. 2000b; Li et al. 2001). The relative timing of the initiation of amyloid formation by A30P-syn varies, with some methods showing a longer lag phase than wildtype (Conway et al. 2000b; Li et al. 2001) and others showing a reduced lag phase (Li et al. 2001; Narhi et al. 1999). Studies of E46K-syn have shown that amyloid is initiated and elongates more rapidly than wildtype, similar to A53T-syn (Choi et al. 2004; Greenbaum et al. 2005).

Because α Syn appears to be associated with presynaptic membranes *in vivo* (Jensen et al. 1998), the lipid-binding characteristics of wildtype and mutant α Syns have been studied. A30P-syn exhibits weaker binding to lipid vesicles both *in vivo* and *in vitro* (Choi et al. 2004; Cole et al. 2002; Jo et al. 2002; Nuscher et al. 2004). Data for A53T-syn have suggested a similar affinity as wildtype (Choi et al. 2004; Cole et al. 2002), while also perhaps interacting with vesicles differently than the wildtype protein (Jo et al. 2004). Liposome pulldown assays suggest that E46K-syn may bind liposomes more strongly than wildtype (Choi et al. 2004).

In both patients and model systems, the levels of both soluble and insoluble α Syn levels increase with age as well as disease progression (Klucken et al. 2006; Li et al. 2004; Liu et al. 2005), suggesting that the clearance of α Syn is compromised. However, the physiological clearance path-way(s) is controversial, since autophagy *via* the lysosome, the ubiquitin-proteasome system (UPS), and calcium-dependent proteases all degrade α Syn (Bennett et al. 1999; Webb et al. 2003). The relevance of the UPS is highlighted by molecular genetics, implicating both a ubiquitin hydrolase and a putative ubiquitin ligase (UCH-L1/*park5* and parkin/*park2*, respectively) (Kitada et al. 1998; Liu et al. 2002). More recently (Bedford et al. 2008), a conditional knockout of the Rpt2 subunit of the 26S proteasome has been shown to have decreased 26S activity and increased 20S activity and to accumulate protein inclusions. A model has emerged in which the lysosome is responsible for the turnover of membrane-bound α Syn, calpain acts on fibrillar protein, the 26S proteasome completely degrades unfolded and/or misfolded α Syn in a ubiquitin-dependent manner, and the 20S proteasome produces fragments in a ubiquitin-independent manner. It remains unknown which of these systems, if any, act on the nucleating and/or toxic species.

Recently, N-terminal fragments of α Syn were identified in both PD patient brain samples and mouse models (Li et al. 2005; Liu et al. 2005). Fragments of α Syn similar to those observed in PD patients are produced by calpain I (Greenbaum et al. 2005; Mishizen-Eberz et al. 2003) and by the 20S core particle of the proteasome (Liu et al. 2005), but not the 26S proteasome (Liu et al. 2006). *In vitro*, similar N-terminal fragments of α Syn form amyloid fibrils earlier and faster than wildtype and are able to seed the fibrillization of the full-length protein at sub-stoichiometric concentrations (Liu et al. 2005; Murray et al. 2003), most likely by releasing long-range interactions within α Syn to reveal highly amyloidogenic regions (Dedmon et al. 2005a; Pawar et al. 2005). The importance of the fragments to disease is further highlighted by the fact that transgenic mice expressing N-terminal fragments of human α Syn display the characteristics of PD, including selective loss of substantia nigral neurons and progressive behavioral deficits (Daher et al. 2009; Tofaris et al. 2006; Wakamatsu et al. 2006).

These data led to a model in which N-terminal fragments of α Syn contribute to early steps in PD pathology. The protease responsible for cleavage remains elusive, although several enzymes have been proposed to cleave α Syn *in vivo*. The 20S proteasome is capable of degrading proteins in a ubiquitin- and ATP-independent manner (Amici et al. 2004; Bennett

et al. 1999; David et al. 2002; Di Noto et al. 2005; Liu et al. 2003; Shringarpure et al. 2003; Tofaris et al. 2001; Touitou et al. 2001). Studies have suggested that 20S particles outnumber both 26S and free regulatory particles *in vivo*, suggesting an independent role for 20S (Brooks et al. 2000; Tanahashi et al. 2000). Previous work from our lab showed that the 20S proteasome endoproteolytically cleaves α Syn *in vitro* to form truncations that are similar to those observed in tissue (Liu et al. 2005).

We set out to test the hypothesis that the action of the 20S proteasome on α Syn produces N-terminal fragments that enhance the formation of the presumptive toxic species. To this end, we evaluated all known reactions of α Syn as well as the three familial PD mutations in the presence of the 20S proteasome. Although assessment of either α Syn amyloidogenicity or liposome binding has not provided a cohesive model for the role of α Syn in PD pathogenesis, these reactions compete with fragmentation and oligomerization reactions for the substrate. In this study, we evaluated the effect of proteasomal cleavage of α Syn proteins on self-association in a system that includes synthetic liposomes and allows for amyloid formation. With this *in vitro* system, we find that the reactions of α Syn degradation, self-association, and liposome binding compete with one another, but that the presence of liposomes and 20S favors the formation of the oligomeric conformation that is thought to be cytotoxic. This system therefore provides a more thorough biophysical picture of the association between α Syn familial PD point mutations and aberrant metabolism of the protein.

Results

Effects of the 20S proteasome on α Syn mutants

Degradation of full-length α Syn by the 20S proteasome *in vitro* produces several N-terminal fragments (Liu et al. 2005). At a ratio of 200:1 (α Syn:20S), three prominent N-terminal fragments are produced: 1–110, 1–83, and 1–73 (Fig. 1, top panel). Under these conditions, there are no dramatic differences in the rate of degradation of full-length wild-type, A53T-syn, and A30P-syn. Full-length E46K-syn is turned over rapidly and produces a different fragmentation pattern. While wildtype α Syn primarily produces fragments consisting of residues 1–110 and 1–83 (Liu et al. 2005), E46K-syn produces the 1–73 fragment, which appears early in the timecourse, and all three fragments are clearly observed by 10 min. Consistent with previously published work (Greenbaum et al. 2005), the 1–73 fragment of E46K-syn migrates faster on the gel than the wildtype 1–73. The identity of these fragments was confirmed by mass spectrometric analysis (data not shown).

Since several sets of data suggest that the mutations can alter the affinity of α Syn for vesicles (Choi et al. 2004; Cole et al. 2002; Jo et al. 2004), and vesicle-bound α Syn is resistant to 20S degradation (Liu et al. 2005), fragmentation in the presence of liposomes was assessed. At 25 and 50 μ M lipids, the conformations of all four proteins are statistically indistinguishable by circular dichroism (data not shown). The fragmentation pattern produced by the 20S proteasome was determined at the lower liposome concentration, where the majority of α Syn is unbound. At 25 μ M lipids, the 20S proteasome continues to produce fragments from the full-length proteins (Fig. 1, bottom panel). Control experiments with fluorogenic peptides showed that the activity of 20S is not affected by liposomes at these concentrations (data not shown). The most prominent and long-lived fragment at both lipid concentrations, regardless of mutation, is syn(1–83). Thus, fragments that are similar to those associated with the disease are produced from all four α Syn proteins by the 20S proteasome in the presence of low concentrations of liposomes.

Effect of mutations, liposomes, and 20S fragmentation on amyloidogenesis

Liposome binding slows the degradation of α Syn by the 20S proteasome (Liu et al. 2005), and the disease-associated mutations have been demonstrated to alter the affinity of α Syn for vesicle surfaces (Choi et al. 2004). To understand how the liposome binding together with 20S-mediated fragmentation affects amyloid formation, the fibrillization of α Syn into amyloid in the presence of liposomes and/or 20S proteasome was monitored by Thioflavin T fluorescence (Fig. 2). In samples with only α Syn protein, the lag phase of polymerization of the A30P mutant prior to fibril initiation is slightly longer than that of wildtype α Syn. Both E46K-syn and A53T-syn form amyloid fibrils earlier and elongate faster than wildtype, consistent with previous reports (Choi et al. 2004; Conway et al. 1998, 2000b; Greenbaum et al. 2005; Li et al. 2001, 2002; Narhi et al. 1999). The addition of liposomes lengthened the lag phase and slowed the rate of fibrillization of both E46K-syn and A53T-syn, but did not appear to measurably affect A30P-syn at a ratio of 2.5:1 lipids: α Syn (Fig. 2a). These results indicate that binding to liposomes counteracts the accelerating affect of the E46K and A53T mutations on amyloid formation.

Previously, we established that the 20S proteasome cleaves wildtype α Syn into amyloidogenic fragments that seed the aggregation of full-length protein (Liu et al. 2005). In this study, 20S proteasome was added to amyloid formation reactions to create sub-stoichiometric amounts of amyloidogenic fragments (less than 1% of the total synuclein in the reaction, less than that reported to be present in Lewy bodies, data not shown). As previously observed for wildtype α Syn, proteasome-produced fragments also accelerated amyloid formation of the full-length mutant proteins as measured by the lag phase before amyloid fibrils were detected by Thioflavin T fluorescence (Fig. 2c). This confirms that the fragments produced by proteasomal cleavage of the mutant α Syn proteins are also amyloidogenic.

The α Syn reactions of lipid-binding and proteasomal degradation are coupled as binding to membranes induces structural changes in α Syn that alter 20S degradation. Therefore, we evaluated the effects of the mutations on fibrillization under conditions where the competing lipid binding and 20S degradation reactions occur. In the presence of both liposomes and 20S proteasome, all four α Syn proteins began forming amyloid fibrils at earlier times than when only liposomes were present, although the lag times are still longer than when only 20S was present (Fig. 2c). Importantly, there was no observed correlation between the fibrillization kinetics and any of the three disease-associated mutants under any condition. If there is a common mechanism of cytotoxicity exerted by the disease-associated mutations, it must be reflected by a biochemical characteristic other than lipid binding or amyloid fibril formation.

Evaluation of oligomer formation in the *in vitro* system

A growing body of evidence indicates that the cytotoxic conformation of α Syn is not amyloid fibrils, but is instead a pre-amyloid oligomeric conformation (Kayed et al. 2003; Nuclea et al. 2007). Antibodies that specifically recognize the soluble oligomeric species of α Syn and other aggregation-prone proteins, but not the monomeric or the amyloid forms of α -synuclein, have been used to establish a correlation between the presence of the oligomers and cytotoxicity (Kayed et al. 2007; Kostka et al. 2008). Although the basis of this toxicity still remains unclear, several studies suggest the oligomers perturb membranes (Kayed et al. 2004; Sokolov et al. 2006). To test the hypothesis that the 20S proteasome and liposomes promote the formation of the potentially cytotoxic oligomeric species from the mutant α Syn proteins, a dot-blot analysis of oligomer formation was performed using the oligomer specific antibodies. Thus, for this analysis, an "oligomer" was defined as any species that was immunoreactive with the anti-oligomer antibody I-11 (Kayed et al. 2007). To validate

the assay, we evaluated oligomer formation from two recombinant C-terminal truncations of α Syn, α Syn119 and α Syn110, that were previously demonstrated to exert cytotoxicity in cell culture (Liu et al. 2005). Aliquots were removed at regular intervals from a fibrillization assay plate for dot-blot analysis with I-11 (Fig. 3). The truncated α Syn proteins formed oligomers more quickly than the wildtype protein. Assays were then performed on the disease-associated mutants under the same conditions as used for the fibrillization assessment above (Fig. 4). In samples that contained only α Syn (Fig. 4, top panel), oligomers were only observed in E46K-syn and A53T-syn over the course of the experiment. These two mutant proteins also readily produce oligomers in the presence of 20S, liposomes, or both 20S and liposomes (Fig. 4, bottom panel). Notably, immunoreactive oligomers of the third disease-associated mutant, A30P-syn, are only observed when both 20S and liposomes are present (Fig. 4, bottom panel). Therefore, a correlation exists between the rate formation of the putative cytotoxic oligomeric species and the presence of the three disease-associated mutations. It should be noted that wild-type α Syn produced small amounts of oligomers when incubated with both 20S proteasome and liposomes (Fig. 4, bottom panel), conditions that most closely relate to those in the cell. These results demonstrate for all three disease-associated α Syn proteins, oligomer formation is promoted by the fragments produced by the 20S proteasome in the presence of liposomes.

Discussion

As observed in our previous studies, α Syn is fragmented by the 20S proteasome *in vitro*. The N-terminal fragments of α Syn produced by 20S accelerate amyloid fibril formation (Liu et al. 2003, 2005). These data led to a model of sporadic PD pathogenesis in which a misprocessing of α Syn by the 20S proteasome accelerates the formation of a cytotoxic conformer (Fig. 5). In this study, we produced simple, reconstituted system for evaluation of the biochemical effects of the three point mutations in α Syn associated with early-onset familial PD. The *in vitro* system is comprised of α Syn, the 20S proteasome, and synthetic liposomes to mimic the lipid vesicles with which α Syn associates *in vivo*. The model predicted that the formation of the cytotoxic species would be accelerated in this simple reconstituted system by the early-onset mutations PD mutations, by contrast to the lack of correlation observed when the reactions were examined in isolation.

The individual reactions composing the *in vitro* system were first evaluated independently. The point mutations did not have a significant effect on either endoproteolysis or turnover by 20S (Fig. 5, “endoproteolysis” and “turnover”) nor did they have a correlated effect on either liposomal binding or amyloid formation. However, the binding of α Syn to a liposomal surface affects the rate of degradation by 20S (Liu et al. 2005). Therefore, a variant that affects liposome binding affinity or orientation may also affect 20S degradation by altering the availability of α Syn substrate. Liposomes were added to the degradation reaction to evaluate the effect of mutations when the reactions of liposome binding and 20S proteolysis are in direct competition (Fig. 5, “liposome binding”, “endoproteolysis”, and “degradation”). Even when liposomes are present, the 20S proteasome produces the similar fragments, which are similar to those previously identified in patients, from all four α Syn proteins (Fig. 1, bottom panel) (Liu et al. 2005).

The most frequently studied biochemical characteristic of α Syn is its propensity to fibrillize into amyloid (Fig. 5, “fibrillization”). As shown in Fig. 2, this property is affected by liposomes (“liposome binding” + “fibrillization”) and by fragments produced by 20S (“endo” + “degradation” + “fibrillization”). However, again none of these reactions reveal a clear correlation to the presence of disease-associated mutations. Liposomes considerably slow the initiation of amyloid formation of E46K- and A53T-syn, but only slightly delay that of the A30P mutant. There is a slight increase in fibrillization of wildtype α Syn, which

may be due to a local concentration effect on vesicle surfaces (Necula et al. 2003). As expected, the presence of 20S-produced fragments promotes the formation of amyloid and fibril elongation for all four proteins (Fig. 5). This indicates that the three mutant α Syn proteins are susceptible to seeding by those amyloidogenic fragments, as observed for the wildtype protein (Liu et al. 2005). By measuring fibrillization in the presence of both liposomes and 20S proteasome, we evaluated the end result of multiple competing reactions: binding, endoproteolysis, fibril initiation, and fibril elongation. Each of these reactions is affected by the PD-associated mutations in different ways when evaluated independently. However, when these reactions were combined in a system with both 20S and liposomes, still no correlation was found between the disease-associated mutations and amyloid fibril formation, despite the fact that the presence of amyloidogenic fragments dramatically accelerates both the initiation of amyloid formation and fibril elongation.

Recent work by others has strongly implicated a pre-amyloid conformation as being responsible for cytotoxicity (Danzer et al. 2009). To that end, the acceleration of oligomerization, rather than amyloid formation, has been suggested as the common link between the mutants (Conway et al. 2000a, b). To directly test this hypothesis, the rate of oligomer formation in the presence of 20S-produced fragments and liposome binding was assessed for the wildtype and the three PD-associated mutations. Here the “oligomer” is operationally defined as any conformation of α Syn that was detected by the anti-oligomer antibody I-11 (Kayed et al. 2003). When the reaction contained only α Syn protein, oligomers were observed only for E46K-syn and A53T-syn (Fig. 4, top panel). The addition of either 20S or liposomes failed to induce appreciable oligomer formation in either A30P-syn or wildtype (Fig. 4, middle panels). By contrast, the presence of both liposomes and 20S produced considerable amounts of oligomers from all three disease-associated mutant proteins: A30P-syn, E46K-syn, and A53T-syn (Fig. 4, bottom panel). Therefore, all of the known disease-causing mutations in α Syn accelerate the formation of oligomers only when liposomes and the 20S proteasome are present. Interestingly, under the same conditions (both 20S and liposomes), low levels of oligomers formed from the wildtype protein (Fig. 4, bottom panel). Presumably, gene duplication or other defects that lead to increased levels of wildtype α Syn, such as the Rpt2 knockout (Bedford et al. 2008), would accelerate the concentration-dependent oligomerization. Therefore, these results also suggest a mechanism for α Syn-mediated pathogenesis in the absence of familial mutations, as observed in the majority of PD patients.

Methods

Antibodies

Syn303 is a mouse monoclonal antibody specific for amino acids 2–4 of human α Syn (Duda et al. 2002). The secondary antibody used was horseradish peroxidase-conjugated AffiniPure goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA).

Purification of proteins

20S proteasome was purified from bovine red blood cells essentially as described (McGuire and DeMartino 1986). α Syn proteins were expressed and purified as described (Liu et al. 2005).

Preparation of liposomes

Liposomes were prepared essentially as described (Eliezer et al. 2001). Equimolar amounts of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (monosodium salt) (POPA) in chloroform (Avanti Polar Lipids, Inc., Alabaster, AL, USA) were combined, evaporated under nitrogen, and lyophilized for a

minimum of 3 h. Following resuspension to a final concentration of 2.5 mM in lipid buffer (100 mM NaCl, 10 mM Na₂HPO₄, pH 7.4), the solution was sonicated for 4 cycles of 2 min each (power 3, 40% duty cycle).

***In vitro* α Syn degradation assay**

20S proteasomal degradation of α Syn was carried out as previously described (Liu et al. 2005).

α Syn fibrillization assays

500 μ L reactions of 100 μ M α Syn were prepared as previously described, with some modification (Liu et al. 2005). Samples also contained either lipid buffer or 250 μ M lipids in the form of vesicles. Where present, 20S proteasome was added at a final concentration of 20 nM. Samples were incubated at 37°C for 10 min followed by the addition of the proteasomal inhibitor MG132 at a final concentration of 100 μ M. After mixing, the sample was centrifuged at 16,000 $\times g$ for 30 sec at room temperature prior to initiating the ThioflavinT assay. The aggregation reaction was performed in triplicate in microtiter plates with a Teflon bead, essentially as described (Liu et al. 2005). The plates were sealed with an ABI-PRISM optical adhesive cover (Applied Biosystems, Carlsbad, CA, USA), and shaken continuously for 8 min 20 sec of every 10 min at 37°C. Thioflavin T fluorescence was monitored at 450 nm excitation/482 nm emission on a Molecular Devices fluorescence plate reader. An aliquot of the reaction mix was reserved for analysis by western blot. Lag times for initiation of fibril formation were determined by the time at which the Thioflavin T fluorescence signal exceeded twice the noise in the data acquired to that point.

α Syn oligomerization assays

650 μ L reactions containing 100 μ M α Syn were prepared as for the fibrillization reactions described above. At appointed times, a 10 μ L aliquot was removed and spotted 2 μ L at a time onto a 0.45 μ m nitrocellulose membrane. The membrane was blotted in TBS with 0.05% Tween-20 (TBS-LowTween) with a 1:8,000 dilution of I-11 anti-oligomer antibody ((Kayed et al. 2007), generous gift of R. Kayed, UTMB), and a 1:10,000 dilution of anti-rabbit secondary (Jackson Immuno-Research, #111-035-144; resuspended in water and stored in aliquots at -20°C). Detection was performed with SuperSignal West Dura Extended Duration Substrate (Pierce, #34075). Data from each of two (α Syn truncations) or three (α Syn point mutants) independent experiments were quantitated by densitometry using ImageQuantTL (GE Biosciences) and normalized to a range of 0–1, following which the mean and SEM (truncations) or standard deviation (mutants) were determined.

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Abbreviations

20S	20S proteasome
α Syn	α -synuclein

CD	circular dichroism
EDTA	ethylenediaminetetracetic acid
β-ME	beta-mercaptoethanol
PD	Parkinson disease
PBS	phosphate-buffered saline
POPC	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphocholine
POPA	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphate

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Fig. 1.

Wildtype and mutant α Syn degradation by the 20S proteasome *in vitro*. *Top panel:* α Syn proteins were incubated with purified latent 20S proteasome for the times indicated (in minutes). *Bottom panel:* 20S degradation of α Syn proteins was carried out in the presence of 25 μ M liposomes. For both panels, MG indicates reactions that were performed with 20S proteasome that had been preincubated with the inhibitor MG132. Specific N-terminal fragments were produced in a time-dependent manner and were identified by mass spectrometry (1–110, white triangle; 1–83, gray triangle; and 1–73, black triangle)

Fig. 2. Fibrillization of α Syn proteins. Representative traces of α Syn fibrillization. 100 μ M α Syn protein in 20 mM phosphate buffer (pH 7.2) was measured utilizing Thioflavin T fluorescence to monitor amyloid fibril formation. **a** Samples were prepared with either protein alone (solid lines) or preincubated for 10 min with 20 nM purified latent 20S proteasome (broken lines). **b** As in **a**, except all samples also contained POPA/POPC liposomes. **c** The average length of the lag phase for amyloid formation for each protein. Error bars indicate the standard deviation. Abscissa labels: syn, α Syn protein alone; +20S, α Syn protein and 20S; +lip, α Syn protein and liposomes; +20S + lip, α Syn protein with both liposomes and 20S

Fig. 3. Oligomer formation of truncated α Syn proteins. 100 μ M recombinant α Syn protein (full-length, α Syn119, or α Syn110) was incubated in 20 mM phosphate buffer (pH 7.2) at 37°C with shaking. Aliquots were removed at the indicated time points and assayed for oligomer content using the anti-oligomer antibody I-11, which does not cross-react with either monomeric or amyloid α Syn (Kayed et al. 2007). **a** Representative blot. **b** The data from two independent experiments was first quantitated, and then normalized to a range of 0 to 1 before being averaged. The height of the gray bars indicates the mean and error bars show the SEM

Fig. 4.

Oligomer formation of mutant α Syn proteins. α Syn proteins were incubated in 20 mM phosphate buffer (pH 7.2) at 37°C with shaking. Where indicated, 20S proteasome and/or liposomes were also included in the incubation mixture. Aliquots were removed at the indicated times, and assayed for oligomer content using the anti-oligomer antibody I-11, which does not cross-react with either monomeric or amyloid α Syn (Kayed et al. 2007). The data from three independent experiments were quantitated, normalized to a range of 0 to 1, and averaged. The Y-axis has a height of 1. Gray bars indicate the mean, and error bars show the standard deviation

Fig. 5.

A dynamic model of Parkinson disease pathogenesis. The point mutations in α Syn that are associated with early-onset Parkinson disease have variable effects on independent reactions which exist within the cell as an interconnected pathway. The *in vitro* system of this study evaluated the effects of the disease-associated mutations on each of these reactions independently and in combination. A correlation was found between all three disease-associated mutations and formation of the presumptive toxic oligomeric species only in the presence of both liposomes and 20S