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Ubiquitination Can Change the Structure of the α -Synuclein Amyloid Fiber in a Site Selective Fashion

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Abstract

Toxic amyloid aggregates are a feature of many neurodegenerative diseases. A number of biochemical and structural studies have demonstrated that not all amyloids of a given protein are equivalent but rather that an aggregating protein can form different amyloid structures or polymorphisms. Different polymorphisms can also induce different amounts of pathology and toxicity in cells and in mice, suggesting that the structural differences may play important roles in disease. However, the features that cause the formation of polymorphisms *in vivo* are still being uncovered. Posttranslational modifications on several amyloid forming proteins, including the Parkinson's disease causing protein α -synuclein, may be one such cause. Here, we explore whether ubiquitination can induce structural changes in α -synuclein aggregates *in vitro*. We used protein chemistry to first synthesize ubiquitinated analogues at three different positions using disulfide linkages. After aggregation, these linkages can be reversed, allowing us to make relative comparisons between the structures using a proteinase K assay. We find that, while ubiquitination at residue 6, 23, or 96 inhibits α -synuclein aggregation, only modification at residue 96 causes an alteration in the aggregate structure, providing further evidence that posttranslational modifications may be an important feature in amyloid polymorphism formation.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.joc.9b02641>.

Supplemental figures and characterization of DNA plasmids for recombinant protein expression (PDF)

The authors declare no competing financial interest.

INTRODUCTION

Several neurodegenerative diseases are associated with the formation of toxic amyloid protein aggregates. One example is α -synuclein, a 140-amino acid protein found at high concentrations at the presynaptic termini of neurons.^{1–3} In solution, α -synuclein is largely an unstructured monomer but can form an extended α -helix on cellular membranes,^{4–6} where it plays its physiological role regulating vesicle trafficking.⁷ In Parkinson's disease, α -synuclein is found in intracellular aggregates called Lewy bodies that spread throughout the course of the disease and correlate with the loss of dopaminergic neurons.^{8,9} The general structure of α -synuclein found in Lewy bodies is amyloid in nature and resembles the fibers formed when the protein is subjected to aggregation conditions in vitro.¹⁰ Addition of small in vitro generated fibers, or preformed fibers (PFFs), to neurons results in their uptake and the induction of cellular pathology and toxicity, and PFFs injected into mice brains cause the further aggregation of endogenous α -synuclein, progressive spreading of protein aggregates, and disease phenotypes.^{11–17} Together, these and other data suggest that the process of amyloid uptake, seeding of additional aggregation, and formation of Lewy bodies are the causative toxic processes in Parkinson's disease. Complicating this issue, however, structural and biochemical experiments have shown that α -synuclein can form different amyloid structures.^{18,19} For example, aggregation under different pH's or salt concentrations or the incorporation of point-mutations into the primary sequence of α -synuclein can result in the formation of different amyloid subtypes.^{19–24} Notably, different amyloid PFFs cause varying degrees of pathogenicity in culture neurons and mice.^{16,21} This raises the possibility that the various rates of disease progression seen in Parkinson's disease patients could result in part from the formation of different amyloid aggregates that go on to seed further aggregation. Despite this intriguing notion, the factors that could favor the formation of one type of amyloid over another *in vivo* are still murky.

We hypothesize that α -synuclein posttranslational modifications (PTMs) are one factor that could promote the formation of different amyloid fibers, similar to the documented consequences of amino acid mutations. α -Synuclein can bear a wide range of enzymatic and chemical PTMs, including phosphorylation, glycosylation, and ubiquitination.^{25–27} Given that all of these modifications can be found *in vivo*, it is important to systematically understand their effects on α synuclein. Unfortunately, the site-specific installation of homogeneous PTMs is very challenging using biological methods. To overcome this limitation, we and others have deployed synthetic protein chemistry, in particular expressed protein ligation (EPL).^{28,29} EPL involves the use of a protein family termed inteins for the recombinant expression of protein thioesters.³⁰ These thioesters can then undergo native chemical ligation (NCL) reactions with peptides or proteins with N-terminal cysteine residues or cysteine equivalents.³¹

Special Issue:

Modern Peptide and Protein Chemistry—For example, we have used this technique to characterize the consequences of O-GlcNAc modification of α -synuclein, while the Lashuel and Brik groups have performed similar experiments on α -synuclein ubiquitination and phosphorylation.^{32–39} Notably, we have found that at least one O-GlcNAc modification can

indeed alter the structure of α -synuclein amyloids, as ascertained using a proteinase K (PK) assay.³⁹ PK is a nonselective protease that will completely digest monomeric α -synuclein. However, the structure of the amyloid aggregates can protect regions of α -synuclein from PK proteolysis, resulting in a pattern of bands that can be visualized by SDS-PAGE. Using this type of assay, the relative PK digestion patterns of unmodified and posttranslationally modified α -synuclein can be compared, yielding a low resolution but meaningful relative picture of differences between amyloid structures.

Unfortunately, this type of analysis cannot be easily applied to ubiquitinated α -synuclein. Ubiquitin is a 76-amino acid protein that is added to substrates through an isopeptide bond to the side chain of lysine residues. In a PK assay, the presence of ubiquitin on any stabilized α -synuclein region would result in an upward mass shift on SDS-PAGE that would prevent any meaningful comparison to the banding pattern of unmodified α -synuclein. Here, we overcome this roadblock using disulfide-directed ubiquitination.^{40–42} This technique uses EPL to yield a disulfide between a cysteine in α -synuclein and a C-terminal thiol on ubiquitin as a lysine isopeptide analogue (Figure 1a). Importantly, we have used this technique in the past to examine the effects of ubiquitin on α -synuclein and found that, while many of the modification sites block protein aggregation, a subset (e.g., K6, K23, and K96) do not, but the effects on the underlying amyloid structure were not explored.⁴⁰ To investigate this possibility, we first used disulfide-directed ubiquitination to construct α -synuclein modified at K6, K23, or K96. Following aggregation of these proteins, we took advantage of the reversibility of the disulfide linkage to release the ubiquitin from the resulting amyloids. Using a subsequent PK assay, we were then readily able to compare these aggregates to those formed by unmodified α -synuclein. Consistent with our previous results, we find that α -synuclein bearing ubiquitin at K6, K23, or K96 can still form aggregates but with different consequences on the gross structure of the aggregate. Specifically, ubiquitination toward the N-terminus of the protein at K6 or K23, and therefore far outside of the typical amyloid core (residues 61–95),^{23,43–46} inhibits aggregate formation but does not change its structure. In contrast, ubiquitination at K96 both inhibits and alters the aggregate formation. These results further highlight the potential site-specific consequences of PTMs in Parkinson's and related diseases and the unique power of the reversibility of the disulfide-directed ubiquitination approach.

RESULTS

To prepare the site-specifically ubiquitinated proteins (Figure 1b), we first recombinantly expressed ubiquitin as an in-frame fusion to an engineered intein from *Anabaena variabilis*.⁴⁷ After purification, we treated the resulting ubiquitin-intein thioester with cysteamine, resulting in transthioesterification and then rearrangement to yield ubiquitin with a C-terminal thiol. We then treated this protein with 2,2'-dithiobis(5-nitropyridine) (DTNP) to give an activated disulfide and purified it by RP-HPLC. In parallel, we expressed either wild-type α -synuclein or the K6C, K23C, or K96C mutants in *E. coli* and also purified them by RP-HPLC. Upon subsequent incubation of 1 equiv of the α -synuclein K to C mutants with 2 equiv of the activated ubiquitin at pH 6.9 for 1 h, we generated the corresponding disulfide-directed ubiquitinated proteins. We then purified all four final protein products,

unmodified α -synuclein and the three ubiquitinated variants, by RP-HPLC and characterized them by ESI-MS (Figure 2).

Next, we wanted to ensure that once the ubiquitin is released by disulfide reduction from any α -synuclein aggregates it would not interfere in the PK analysis. Accordingly, we first subjected unmodified α -synuclein to aggregation conditions (100 μ M protein concentration at 37 °C with constant agitation at 1000 rpm) for 1 week to generate typical α -synuclein amyloid fibers. Next, we prepared three different PK digestion reactions: (1) only α -synuclein amyloid fibers, (2) ubiquitin C-terminal thiol in isolation, and (3) a 1:1 mixture of α -synuclein amyloid fibers and ubiquitin C-terminal thiol. We then treated these reactions with either nothing or two different concentrations of PK (1 or 2 μ g mL⁻¹) for 30 min. The resulting products were then separated by SDS-PAGE and visualized by staining with Coomassie Blue stain (Figure 3a). As expected, unmodified α -synuclein produced five stable protein bands that are highly consistent with our own and others' previous work.^{21,39} Ubiquitin is a well-folded and highly stable protein; therefore, it is not surprising that digestion with PK resulted in at most slight degradation of this protein. Importantly, ubiquitin C-terminal thiol runs at a molecular weight well below any of the α -synuclein protein bands that are diagnostic of the aggregate structure. Moreover, the 1:1 mixture of α -synuclein and ubiquitin C-terminal thiol did not change the five stable protein bands or prevent the cleavage of α -synuclein fibers by PK. These results indicate that the ubiquitin-release strategy will not prevent the PK analysis of the aggregates formed by ubiquitinated α -synuclein.

To determine the consequences of ubiquitination on the structure of α -synuclein aggregates, we initiated four aggregation reactions containing unmodified α -synuclein or one of the three ubiquitinated variants (100 μ M protein concentration at 37 °C with constant agitation at 1000 rpm) for 4 weeks. The length of these aggregation reactions is notably longer than those we previously performed⁴⁰ to examine the inhibitory effects of ubiquitin on α -synuclein fiber formation. In this prior publication, we used small Teflon stirbars to agitate the reactions. However, studies have shown that additional hydrophobic surfaces, like Teflon spheres, serve as secondary nucleation sites to increase the levels and kinetics of α -synuclein aggregation.^{48,49} Therefore, in our more recent work and the reactions here, we agitate the reactions using a thermomixer. In fact, we have previously shown that disulfide-directed ubiquitination at K23, K23C-Ub, inhibits α -synuclein aggregation more strongly under the thermomixer conditions.⁴² At the end of the 4 weeks of aggregation, we first measured the relative amounts of fibers formed using thioflavin T (ThT) fluorescence. ThT is only weakly fluorescent in solution, but this property increases dramatically when ThT intercalates into hydrophobic grooves that form along the surface of amyloid fibers. We observed notable ThT fluorescence from all four aggregation reactions but significantly less from the ubiquitinated proteins compared to unmodified α -synuclein (Figure 4a). These same reactions were also analyzed by transmission electron microscopy (TEM) to visualize the aggregates that formed (Figure 4b and Figure S1 in the Supporting Information). As predicted from the ThT signal, we observed α -synuclein fibers from all four of the different proteins. α -Synuclein and K6C-Ub formed long and relatively uniform fibers, while those formed by K23C-Ub appeared more heterogeneous and potentially surrounded by amorphous aggregates. Finally, K96C-Ub formed shorter fibers. These results are consistent

with our previous work showing that ubiquitination at K6, K23, or K96 inhibits but does not completely block α -synuclein fiber formation.

We next treated the individual aggregation mixtures with PK, 1 or 2 $\mu\text{g mL}^{-1}$, for 30 min before separation of the products by SDS-PAGE and analysis by Coomassie Blue staining (Figure 4c and d). PK digestions are typically performed on the entire aggregation reaction mixture, which can contain a mixture of remaining α -synuclein monomers and fibers. Therefore, PK proteolysis can provide two pieces of information. First, the intensity of the overall bands in the digestion gives a qualitative measure of the amount of aggregates and their overall stability. Second, the pattern of the remaining stable bands gives a low resolution picture of the fiber structure. Importantly, any monomeric α -synuclein remaining in the mixture can be completely degraded by PK (Figure S2). In terms of aggregate stability, the intensity of the bands after PK cleavage tracked well with our ThT and TEM data. All of the ubiquitinated proteins had less intense bands than the unmodified α -synuclein, consistent with the lower amounts of aggregation for these proteins as determined by ThT. Additionally, the bands from K23C-Ub were dimmer than the other ubiquitinated proteins, confirming our TEM data that showed more heterogeneous aggregates formed by this protein. Identical to our analysis above in Figure 3, we observed a five-band pattern from PK digestion of unmodified α -synuclein. Essentially, the same pattern is readily visible in the case of K6C-Ub. We believe these five bands are also present from the digestion of K23C-Ub; however, due to the relative instability of these aggregates, we cannot say this definitively. It is certainly possible that K23C-Ub does have an effect on the fiber structure that cannot be readily detected by PK analysis. In contrast, PK cleavage of K96C-Ub resulted in the formation of a three-band pattern that is very distinct from the other proteins. To support these results, we used atomic force microscopy (AFM) to measure the cross-sectional heights of the fibers formed by either unmodified or the different ubiquitinated α -synuclein proteins (Figure 4e). Consistent with our PK data, we found no statistically significant difference between the fiber heights from unmodified, K6C-Ub, or K23C-Ub α -synuclein. In contrast, the fibers formed by K96C-Ub were slightly shorter, consistent with an alteration in the aggregate structure. These data demonstrate that α -synuclein ubiquitination can not only have site-specific effects on the kinetics and overall amounts of aggregate formation but also alter the structure of the underlying fibers that form.

DISCUSSION

In summary, we examined the potential for α -synuclein ubiquitination to alter the structure of the associated amyloid aggregates. Specifically, we used a disulfide-directed ubiquitination strategy to test this possibility with three out of the nine identified modification sites (K6, K23, and K96) on α -synuclein, which we previously showed did not completely prevent the formation of α -synuclein fibers. The disulfide linkage used to install the ubiquitin was both a facile method for its installation and also a critical feature that allowed us to reverse the modification and directly compare α -synuclein aggregate structures using PK digestion. We confirm that ubiquitination at K6 and K23 inhibits the formation of amyloid fibers but that the fibers that do form are likely very similar in structure to those formed by unmodified α -synuclein. In the case of α -synuclein ubiquitinated at K23, we attribute the lack of visible bands corresponding to the typical PK

digestion pattern to be due to the low amount of aggregates that form in this reaction. However, we cannot completely rule out the possibility that modification at this position does indeed have a structural effect. In contrast, K96 ubiquitination inhibits aggregation and also alters the aggregation structure. These PK results are consistent with our subsequent analysis of the fibers using AFM, where we found that ubiquitination at K96 forms fibers of shorter height than unmodified α -synuclein. Again, it is possible that the other ubiquitination sites could cause a more subtle effect on the size of the fibers, which might be masked by the heterogeneity of our AFM measurements. However, published analyses using AFM to distinguish small differences have required aggregation reactions with higher protein concentrations to increase the uniformity of the fibers and significantly more height measurements.⁵⁰ Unfortunately, the amounts of protein needed for these types of experiments are more challenging to obtain using a semisynthetic strategy. The differences in these results can be rationalized by where they are located in the primary sequence of α -synuclein, in particular their physical distance from the portion of α -synuclein responsible for the formation of amyloid fibers. Ubiquitination at K6 or K23 is located at least ~40 amino acids away from the N-terminal boundary of the typical α -synuclein fiber, while K96 is found very close to the C-terminal boundary. Interestingly, other modifications like O-GlcNAcylation or certain amino acid mutations also near this boundary at S87 also alter the structure of the amyloid fiber, resulting in a three-band pattern upon PK digestion.^{21,39} A mechanism that explains these similarities is still lacking, but the similarities may suggest that interactions in this region of α -synuclein are key for the formation of the typical amyloid fiber. Finally, there is increasing interest in the structure–toxicity relationships between different α -synuclein fibers or polymorphs, as they can template their structure onto additional protein monomers and can result in differential pathogenicity in mouse models.²¹ Therefore, it is possible that posttranslational modifications may have important roles in the subtypes of α -synuclein aggregates that form *in vivo* and in the progression of Parkinson's disease.

EXPERIMENTAL SECTION

General Methods.

All solvents and reagents were obtained from commercial sources and used without further purification. LuriaBertani-Miller (LB) and Terrific broth (TB) culture media was purchased from EMD and prepared, sterilized, stocked, and used as directed by the manufacturer. Antibiotic working solutions were prepared at 100 mg/mL ampicillin sodium salt (EMD) and 50 mg/mL kanamycin sulfate (EMD) and stored at -20°C . Reverse-phase high performance liquid chromatography (RP-HPLC) was performed with either an Agilent Technologies 1200 Series HPLC with Diode Array Detector (buffer A, 0.1% TFA in water; Buffer B, 0.1% TFA and 90% acetonitrile in water) or a Biotage Isolera Spektra FLASH system (buffer A, 0.1% TFA in water; buffer B, 0.1% TFA in acetonitrile). An API 150EX (Applied Biosystems/MDS SCIEX) was used to analyze mass spectra.

Plasmid Construction.

The generation of the plasmids used in this research was described previously.^{40,42}

Ubiquitin-Thiol Expression and Purification.

E. coli BL21 (DE3) cells were transformed with the Ub-AvaE-6xHis plasmid via a 30 s heat shock at 42 °C and grown overnight on a LB agar ampicillin plate (100 µg/mL) at 37 °C prior to storage at 4 °C. Single colonies were used to inoculate 50 mL LB cultures (100 µg/mL ampicillin), which were grown with shaking at 250 rpm overnight at 37 °C. Overnight cultures were used to inoculate 3 L of TB broth (100 µg/mL ampicillin), and TB cultures were grown with shaking at 250 rpm at 37 °C until mid log phase was reached (0.6–0.8 OD_{600nm}). IPTG was added to the cultures at 0.5 mM to induce Ub-AvaE-6xHis overexpression, and the cells were incubated with shaking at 25 °C overnight before centrifugal harvesting at 6000g, 4 °C for 15 min. Cell pellets were resuspended in lysis buffer (50 mM Na₂HPO₄, 300 mM NaCl, 5 mM imidazole, 2 mM PMSF, 2 mM TCEP-HCl, pH 7.4) prior to probe sonication (70% amplitude, 30 s on/30 s off, 12 min total, on ice). Insoluble lysate components were cleared by centrifugation at 7000g for 1 h at 4 °C, and the remaining soluble fraction was incubated with pre-equilibrated Co-NTA affinity resin for 1 h at 4 °C with agitation. The resin was rinsed thoroughly with wash buffer (50 mM Na₂HPO₄, 300 mM NaCl, 20 mM imidazole, 2 mM TCEP-HCl, pH 7.4) to remove endogenous proteins before elution of the Ub-AvaE-6xHis with elution buffer (50 mM Na₂HPO₄, 300 mM NaCl, 250 mM imidazole, 2 mM TCEP-HCl, pH 7.4). The eluted protein was concentrated and buffer exchanged into water using Amicon Ultra 15 mL centrifugal filters (3K MWCO) before the ubiquitin was cleaved from the AvaE-6xHis fusion with 100 mM aminoethanethiol (TCI), 50 mM TCEP-HCl, pH 7.75 for 16 h. The ubiquitin-thiol was then purified from the intein fusion by C4 reverse-phase purification using a Biotage Isolera Spektra FLASH system. Characterization of the ubiquitin thiol was performed using C4 analytical RP-HPLC and ESI-MS.

Disulfide-Activated Ubiquitin Preparation.

Lyophilized Ubthiol was resuspended to 0.5 mM in a 3:1 acetic acid:water solution containing 10 mM DTNP. The reaction was agitated at 25 °C for 48 h. The activated ubiquitin thiol was purified by an initial C4 reverse-phase purification using a Biotage Isolera Spektra FLASH system followed by a more stringent C4 RP-HPLC purification to fully remove unreacted DNTP. Characterization of the disulfide-activated ubiquitin thiol was performed using C4 analytical RP-HPLC and ESI-MS.

α-Synuclein K#C Expression and Purification.

E. coli BL21 (DE3) cells were transformed with the α-synuclein K#C plasmid via a 30 s heat shock at 42 °C and grown on an LB agar kanamycin plate (100 µg/mL) at 37 °C prior to storage at 4 °C. Single colonies were used to inoculate 50 mL LB cultures (100 µg/mL kanamycin) which were grown with shaking at 250 rpm overnight at 37 °C. Overnight cultures were used to inoculate 6 L of TB broth (50 µg/mL kan), and TB cultures were grown with shaking at 250 rpm at 37 °C until mid log phase was reached (0.6–0.8 OD_{600nm}). IPTG was added to the cultures at 0.5 M to induce α-synuclein K#C overexpression, and the cells were incubated with shaking at 25 °C overnight before centrifugal harvesting at 6000 × g, 4 °C for 15 min. Cell pellets were flash frozen in liquid nitrogen followed by a 15 min incubation at 37 °C. This freeze–thaw process was performed

a total of three times before resuspending the cells in lysis buffer (100 mM Tris, 500 mM NaCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, pH 8). The cells were then lysed by boiling at 80 °C for 10 min, agitating the resuspension briefly every 1 min. The lysed cells were incubated at room temperature for 30 min before the addition of 2 mM PMSF and another 30 min incubation on ice. The insoluble lysate fraction was cleared by centrifugation at 7000 × g, 4 °C for 20 min, and the remaining soluble fraction was acidified to pH 3.5. This solution was incubated on ice for 30 min before centrifugal clearing at 7000 × g, 4 °C for 20 min. The remaining supernatant was dialyzed overnight into a degassed, 1% acetic acid solution using SnakeSkin dialysis tubing (3.5K MWCO). The dialyzed solution was cleared again by centrifugation at 7000 × g, 4 °C for 20 min, and the α -synuclein K#C was then purified by C4 reverse-phase purification using a Biotage Isolera Spektra FLASH system. Characterization of the α -synuclein K#C variants was performed using C4 analytical RP-HPLC and ESI-MS.

Synthesis of Disulfide-Linked, Ubiquitin-Conjugated α -Synuclein.

Lyophilized α -synuclein K#C was resuspended with 2 molar equiv of disulfide-activated ubiquitin in coupling buffer (6 M Gu·HCl, 1 M HEPES, pH 6.93) at an α -synuclein K#C concentration of 0.2 mM. The reaction was incubated at 25 °C with inversion for 1 h, and the resulting α -synuclein K#C-Ub was purified by C4 RP-HPLC purification. Characterization of the α -synuclein K#C-Ub variants was performed using C4 analytical RP-HPLC and ESI-MS.

α -Synuclein K#C-Ub Aggregation.

Lyophilized α -synuclein K#C-Ub variants were resuspended in filter-sterilized aggregation buffer (10 mM phosphate, 0.05% NaN₃, pH 7.4), bath sonicated for 10 min, then centrifuged at 15,000 × g, 4 °C for 10 min to remove any preformed aggregates. The supernatants were transferred to new tubes, and concentration was determined by BCA assay. Samples were diluted to 100 μ M with additional 1× DPBS, 0.05% NaN₃, and then aliquoted into triplicate 150 μ L samples. These samples were aggregated for 28 days in a thermoshaker at 1000 rpm at 37 °C. Aliquots were removed at indicated times for ThT analysis.

Thioflavin T (ThT) Analysis.

Samples from aggregation reactions were diluted to 2.5 mM in either filter-sterilized aggregation buffer containing 10 μ M ThT in separate wells of a 96-well plate. Samples were incubated at room temperature for 3 min prior to collecting fluorescence data ($\lambda_{\text{ex}} = 450$, $\lambda_{\text{em}} = 482$) using a Biotek Synergy H4 Hybrid Reader.

Transmission Electron Microscopy.

From aggregation assays, 1 μ L of each sample was deposited on a Formvar coated copper grid (150 mesh, Electron Microscopy Sciences) for 5 min. Excess liquid was dried with filter paper before negative staining with 1% uranyl acetate for 2 min. Grids were washed three times with 1% uranyl acetate and dried each time with filter paper. The grids were then dried for 24 h in a vacuum desiccator before imaging at 200 kV, 60,000× magnification using a JEOL JEM-2100F transmission electron microscope and a Prius Pre-GIF CCD.

Pilot Proteinase K Assay.

Unmodified α -synuclein aggregates (35 μ M), ubiquitin-thiol (35 μ M), or both were incubated in 20 μ L of 1 DPBS with 5 mM CaCl₂ and 0, 1, and 2 μ g mL⁻¹ proteinase K for 30 min at 37 °C. SDS loading buffer was added (2% final SDS concentration), and samples were boiled for 10 min at 95 °C to halt the digestion. Products were analyzed by SDS-PAGE using precast 10% Bis-Tris gels (Bio-Rad, Criterion XT) with MES running buffer (Bio-Rad) and stained with Coomassie Brilliant Blue (Bio-Rad).

Proteinase K Digestion.

Ubiquitin-modified α -synuclein aggregates (10 μ g) were incubated in 20 μ L of 1× DPBS, 5 mM CaCl₂, 5 mM DTT, and 0, 1, and 2 μ g mL⁻¹ proteinase K for 30 min at 37 °C. SDS loading buffer was added (2% final SDS concentration), and samples were boiled for 10 min at 95 °C. Products were analyzed by SDS-PAGE using precast 10% Bis-Tris gels (Bio-Rad, Criterion XT) with MES running buffer (Bio-Rad) and stained with Coomassie Brilliant Blue (Bio-Rad).

Atomic Force Microscopy.

Small volumes from aggregation assays of each sample were diluted to 5 μ g/mL using additional aggregation buffer, and 100 μ L of the resulting solutions was added to freshly cleaved mica discs and allowed to evaporate overnight at room temperature. These discs were then imaged using a Bruker Dimension Icon atomic force microscope equipped with a Bruker ScanAsyst-Air silicon nitride probe in peak force tapping mode. Random fibers were selected for measurement of cross-sectional height. Height analysis was performed using ProfilmonOnline's AFM analysis software (profilmonline.com).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Disulfide-directed ubiquitination. (a) Endogenous ubiquitination is an enzymatic modification of substrate proteins through the formation of an isopeptide bond between a lysine side chain and the C-terminus of ubiquitin. Disulfide-directed ubiquitination is a tractable method for the chemical construction of a ubiquitinated analogue. (b) Synthesis of activated ubiquitin. Ubiquitin is expressed as a fusion to an intein, resulting in the formation of a ubiquitin-intein thioester. This thioester can then be thiolyzed with cysteamine and then activated with DTNP.

Figure 2. Characterization of semisynthetic proteins. Unmodified α -synuclein and the disulfide-directed ubiquitinated variants were characterized by RP-HPLC and ESI-MS.

Figure 3. Disulfide-directed ubiquitination does not prevent the analysis of α -synuclein fibers by PK digestion. α -Synuclein fibers, ubiquitin C-terminal thiol, and a 1:1 mixture of the two were subjected to PK digestion for 30 min at 37 °C. The presence of ubiquitin C-terminal thioester does not inhibit or change the digestion of α -synuclein fibers by PK.

Figure 4.

Ubiquitination alters the structure of α -synuclein fibers in a site-specific fashion. (a) Ubiquitination at K6, K23, or K96 inhibits but does not completely block α -synuclein aggregation. Unmodified α -synuclein or the indicated disulfide-directed ubiquitinated proteins ($100 \mu\text{M}$) were subjected to aggregation conditions (agitation at 37°C) for 4 weeks. After this length of time, the reactions were subjected to analysis by ThT fluorescence ($\lambda_{\text{ex}} = 450 \text{ nm}$, $\lambda_{\text{em}} 482 \text{ nm}$). The y-axis shows the fold difference in ThT fluorescence compared to the same conditions at time = 0. Results are means $\pm$ SEM of three experimental replicates ($n = 3$). (b) The same samples were also analyzed by TEM. (c) PK digestion shows site-specific differences in fiber structure induced by ubiquitination. The aggregation reactions were incubated with the indicated amounts of PK for 30 min before analysis by SDS-PAGE separation and staining with Coomassie Blue. (d) Highlights of the digestions with $1 \mu\text{g mL}^{-1}$ from panel c. (e) The cross-sectional heights of fibers were measured using AFM. Randomly selected fibers were measured by AFM, and the results are means $\pm$ SEM of fiber replicates (unmodified $n = 9$, ubiquitinated $n = 6$). Statistical significance was determined using a one-way ANOVA test followed by Dunnett's multiple comparisons test.