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Semi-Synthetic, Site-Specific Ubiquitin Modification of α-Synuclein Reveals Differential Effects on Aggregation

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Abstract

The process of neurodegeneration in Parkinson's Disease is intimately associated with the aggregation of the protein α-synuclein into toxic oligomers and fibrils. Interestingly, many of these protein aggregates are found to be posttranslationally modified by ubiquitin at several different lysine residues. However, the inability to generate homogeneously ubiquitin modified α-synuclein at each site has prevented the understanding of the specific biochemical consequences. We have used protein semi-synthesis to generate nine site-specifically ubiquitin modified α-synuclein derivatives and have demonstrated that different ubiquitination sites have differential effects on α-synuclein aggregation.

Parkinson's Disease (PD) is the second most common neurodegenerative disease, characterized by the chronic and progressive loss of dopaminergic neurons from the substantia nigra, leading to muscle tremors, stiffness, and slowing of movement.[1] There is no cure for PD, and current treatments are simply palliative. Although the variety of exact mechanisms involved in the progression of PD are still being uncovered, the presence of intracellular protein aggregates (Lewy bodies and Lewy neurites) are characteristic of the disease.[2] The major component of these aggregates is the protein α-synuclein (α-syn),[3] a 140 amino acid protein that is normally localized to neuronal presynaptic terminals. In addition, a number of specific missense mutations in α-syn and short chromosomal duplications and triplications that include the α-syn gene have been identified in familial forms of PD, further supporting α-syn's role in PD. Continued biochemical analysis of α-syn has illuminated the characteristics that result in the formation of the toxic protein species associated with PD. α-Syn alone has little secondary structure but will readily assemble into β-sheet oligomers and fibrils *in vitro,* closely resembling the disease state. Taken together with data demonstrating

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ASSOCIATED CONTENT

Supporting Information. Supporting figures and detailed experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

Author Contributions

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that these species are toxic to cells in culture and model organisms *in vivo*, it is widely accepted that α-syn is a large contributing factor to PD.[4]

Complicating the issue of α-syn oligomerization and toxicity, the protein is a substrate for several posttranslational modifications, including phosphorylation, C-terminal proteolysis, and ubiquitination[5–8]. All of these modifications have been identified within Lewy bodies isolated from human patients, and therefore understanding their contributions to α-syn aggregation and toxicity is key for a complete picture of PD. Analysis of the ubiquitin modification[9,10] in Lewy bodies has revealed that the majority of α-syn is mono- or diubiquitinated at several lysine residues.[6,11] This result has been confirmed by both coexpression of ubiquitin ligases with α-syn in cell culture and *in vitro* modification of recombinant protein, resulting in the characterization of α-syn ubiquitinated at nine different lysine residues (K6, K10, K12, K21, K23, K32, K34, K46, and K96), some of which (K6, K10, and K12) can be modified after the formation of protein fibrils.[12–14] Despite the information gained from these experiments, they also demonstrated that it is not possible to generate homogeneously ubiquitinated α-syn at distinct lysine residues with enzymatic modification. This limitation has been recently addressed with the generation of K6 ubiquitinated (K6-Ub) α-syn using an elegant semi-synthetic strategy based on expressed protein ligation (EPL).[15] Using this approach, monomeric K6-Ub α-syn was shown to resist fibril formation when compared to unmodified protein; however, the heroic nature of the chemistry involved makes the facile generation of all the potential ubiquitinated α-syn derivatives difficult.

To overcome this roadblock, we took advantage of a complementary strategy, termed disulfide-directed ubiquitination. [16,17] This method results in a ubiquitin modified lysine analog, resulting from a disulfide forming reaction between a cysteine residue on the target protein and a ubiquitin molecule bearing a C-terminal thiol. Importantly, this analog has been shown to be functionally equivalent to the native ubiquitin linkage,[16] and α-syn is particularly amenable to this chemistry, as it contains no native cysteine residues. Herein, we describe the use of disulfide-directed ubiquitination to generate site specifically ubiquitinated analogs representing all nine known α-syn modification sites (Figure 1). Subsequent characterization demonstrated differential effects on fibril formation, highlighting the potentially unique properties of individual ubiquitin modification sites.

Our semisynthetic strategy is generalized in Figure 1A and began with the recombinant expression of ubiquitin as a linear fusion with the Gyr A intein (**1**). Incubation of this construct with cysteamine for 16 h yielded the known free ubiquitin bearing a C-terminal aminoethanethiol linker (**2**, Figure S1 in Supporting Information). This resulting thiol was then activated by reaction with 2,2'-dithiobis(5-nitropyridine) (DTNP) to generate the mixed disulfide reagent **3** (Figure 1 in Supporting Information).[18] Next wild-type α-synuclein and the nine lysine to cysteine mutants (K6C, K10C, K12C, K21C, K23C, K32C, K34C, K46C, and K96C) (**4**, Figure 1B and Figure S2) were expressed heterologously in *Escherichia coli*. To create the disulfide-directed ubiquitinated α-synuclein conjugates, two equivalents of **3** were incubated with **4** at pH 6.9 for one hour. Essentially complete product formation was observed for each α-synuclein mutant (Figure S3), consistent with previous reports. All ten proteins, wild-type and the nine ubiquitinated mutants, were purified by reverse-phase (RP) HPLC, characterized by mass spectrometry (Figure S4), and readily obtained in multimilligram quantities. SDS-PAGE analysis of the same ubiquitination reactions revealed a shift from the parent α-synuclein mutants at 15kDa to a slower-migrating band at approximately 22kDa, consistent with the addition of one ubiquitin molecule and our mass spectrometry data (Figure 2A).

Having synthesized and characterized the disulfide-directed ubiquitinated mutants, we next analyzed the effect of ubiquitin on protein structure and aggregation of α-synuclein using circular dichroism (CD), thioflavin T (ThT) fluorescence, and transmission electron microscopy (TEM). Consistent with previous data, the CD spectrum of unmodified, wild-type α-synuclein (α-Syn) corresponded with a random-coil structure, while the ubiquitin C-terminal thiol (**2**) displayed the appropriate mixture of random coils, α-helices, and β-sheets found in the correctly folded ubiquitin structure (Figure 2B).[19] The CD data of all nine ubiquitin modified α-synuclein molecules were all very similar, and as expected, resembled a combination of the individual α-synuclein and ubiquitin C-terminal thiol spectra. Importantly, this is completely consistent with the K6-linked ubiquitinated α-synuclein and with an equimolar mixture of free ubiquitin and α-synuclein (α-Syn + Ub, Figure 2B), suggesting that the native secondary structures of ubiquitin and α-synuclein are preserved despite their covalent conjugation. To ensure that the purified proteins were monomeric and did not contain oligomeric structures that were undetectable by SDS-PAGE and CD, we used a combination of dynamic light scattering and dot-blot analysis. Analysis by dynamic light scattering showed that all proteins had Stokes radii of 3 nm, except for K21C-Ub and K43-Ub (~6 nm), both consistent with monomeric structures, and no evidence of oligomeric structures at ~10–50 nm (Figure S5).[20,21] Next, the ten proteins were subjected to dot-blotting using an antibody (A11),[22] which specifically recognizes oligomers from a variety of amyloid-forming proteins. Consistent with the data above, none of the proteins contained detectable oligomeric structures (Figure S6A), further suggesting that they were purified in the monomeric state.

To determine the effect of ubiquitination on α-synuclein fibril formation, we next used ThT fluorescence. Towards this end, wild-type α-synuclein or each disulfide-directed ubiquitinated mutant was incubated at a concentration of 100 μM with agitation at 37 °C for 5 days. Every 12 h a reaction aliquot was removed and added to a solution of ThT before analysis by fluorescence (Figure 3). While unmodified α-synuclein showed a rapid increase in fibril formation, ubiquitination displayed differential effects on ThT fluorescence dependent upon the location of the modification. Some ubiquitin modification sites (K10C-Ub and K23C-Ub) displayed similar levels of fibrils when compared to wild-type protein, despite having somewhat altered kinetics of formation. In contrast, when other sites (K6C-Ub, K12C-Ub, and K21C-Ub) where modified by ubiquitin, the formation of fibrils was inhibited compared to α-synuclein. Finally, the ubiquitination sites located in the middle of α-synuclein, including K32C-Ub, K34C-Ub, K43C-Ub, and K96C-Ub, showed a strong inhibition of fibrils. In addition, after 5 days of incubation the same samples were analyzed by CD spectroscopy (Figure S7). Importantly, both unmodified α-synuclein and K23C-Ub displayed absorption spectra consistent with the formation of β-sheets, while the remaining ubiquitin-modified derivatives showed different levels of absorption shifts that are consistent with the levels of fibers observed by ThT. To further visualize any α-synuclein aggregation, after 5 days of incubation samples were analyzed by TEM (Figure 3 and Figure S8). Importantly, these results were very consistent with the ThT fluorescence data. Unmodified α-synuclein, as well as K10C-Ub and K23C-Ub, formed extensive mature fibrillar structures, while other modification sites (K6C-Ub, K12C-Ub, and K21C-Ub) displayed only short fibrils and smaller structures consistent with amorphous aggregates and potentially protein protofibrils/oligomers. Lastly, the ubiquitinated derivatives in the middle of the protein (K32C-Ub, K34C-Ub, K43C-Ub, and K96C-Ub) contain absolutely no fibrils, consistent with their low ThT fluorescence. Closer examination of the small aggregates formed by the ubiquitin-modified derivative that did not form fibers (e.g. KC43-Ub and KC96-Ub), revealed gross differences in their structures. To examine whether these aggregates were oligomeric, dot blotting was performed with the A11 antibody (Figure S6B). Interestingly, while unmodified α-synuclein and KC43-Ub did not form oligomers,

dot blotting revealed that K96C-Ub could form oligomeric structures, suggesting that ubiquitin can not only inhibit fiber formation but also promote oligomerization.

Finally, to ensure that our disulfide-directed ubiquitin modifications were still intact at the conclusion of our fibrilization experiment, we resuspended the remaining samples in 4% SDS containing buffer with boiling and analyzed them by SDS-PAGE (Figure S9). Importantly, no ubiquitin was removed from any of the α-synuclein mutants during the course of the experiment, confirming that the results are reflective of modified protein.

Taken together these data demonstrate that ubiquitin modification has differential and site-dependent effects on α-synuclein aggregation. The first set of ubiquitinated forms of α-synuclein includes modification at K10 and K23. These proteins readily form fibrils, with a moderate inhibition of the formation kinetics. This supports previous results, where the ubiquitin ligase seven in absentia homolog (SIAH), which ubiquitinates α-synuclein at a variety of lysine residues including K10 and K23, promotes the formation of inclusions in cell culture, although the specific modification site(s) responsible is unknown.[12,13] Ubiquitination at K6, K12, and K21 represents the next set of modified α-synuclein proteins, which moderately inhibit the formation of fibrils. Importantly, this is consistent with the investigation of α-synuclein homogeneously ubiquitinated at K6, where incubation of this protein at a far lower concentration (14 μM) resulted in the formation of no fibrils.[15] Finally, the third set of α-synuclein ubiquitination sites at K32, K34, K43, and K96 displayed no fibril formation, suggesting a strong inhibitory effect. Interestingly, K96 modification might additionally promote the formation of oligomers. Together these data are very consistent with the region of α-synuclein (residues 22–36 to 90–98, depending on the analytic method) making up the core of the fiber.[23–25]. For example, any ubiquitin modifications that occur well within the core region (K32, K34, and K43) completely block fiber formation, while modifications at the N-terminus (K6, K10, and K12) do not completely prevent fibrillizaiton. The inhibitory effect of these modifications could be a result of steric interference of ubiquitin on important aggregation intermediates, including long- range interactions with the N-terminus near ubiquitination sites,[26,27] or masking of N-terminal lysine charges that may be involved in interactions with the highly negatively charged C-terminus or ions in solution.[28–33] Finally, ubiquitination sites located near the boundaries of the fiber core have very site-specific fiber forming properties (e.g. K21 vs. K23) or can promote the formation of oligomers (K96). We are currently using site-directed spin labeling and electron paramagnetic resonance to investigate the structure of unmodified and ubiquitin-modified α-synuclein fibers.

In summary, we have described the application of disulfide-directed ubiquitination to the semisynthesis of site-specifically ubiquitinated forms of α-synuclein representing every known modification site. This strategy allowed us to generate these proteins in large quantities that enabled the subsequent characterization of their structure and fibrilization properties. Our results strongly suggest that unique sites of ubiquitin modification have very different effects on α-synuclein oligomerization and fibril formation. We believe that these differences potentially explain the seemingly contradictory results others have obtained using heterogeneously ubiquitinated α-synuclein obtained enzymatically and that they lay a strong foundation for and encourage the targeted investigation of specific ubiquitin modification sites in cellular models of synucleinopathies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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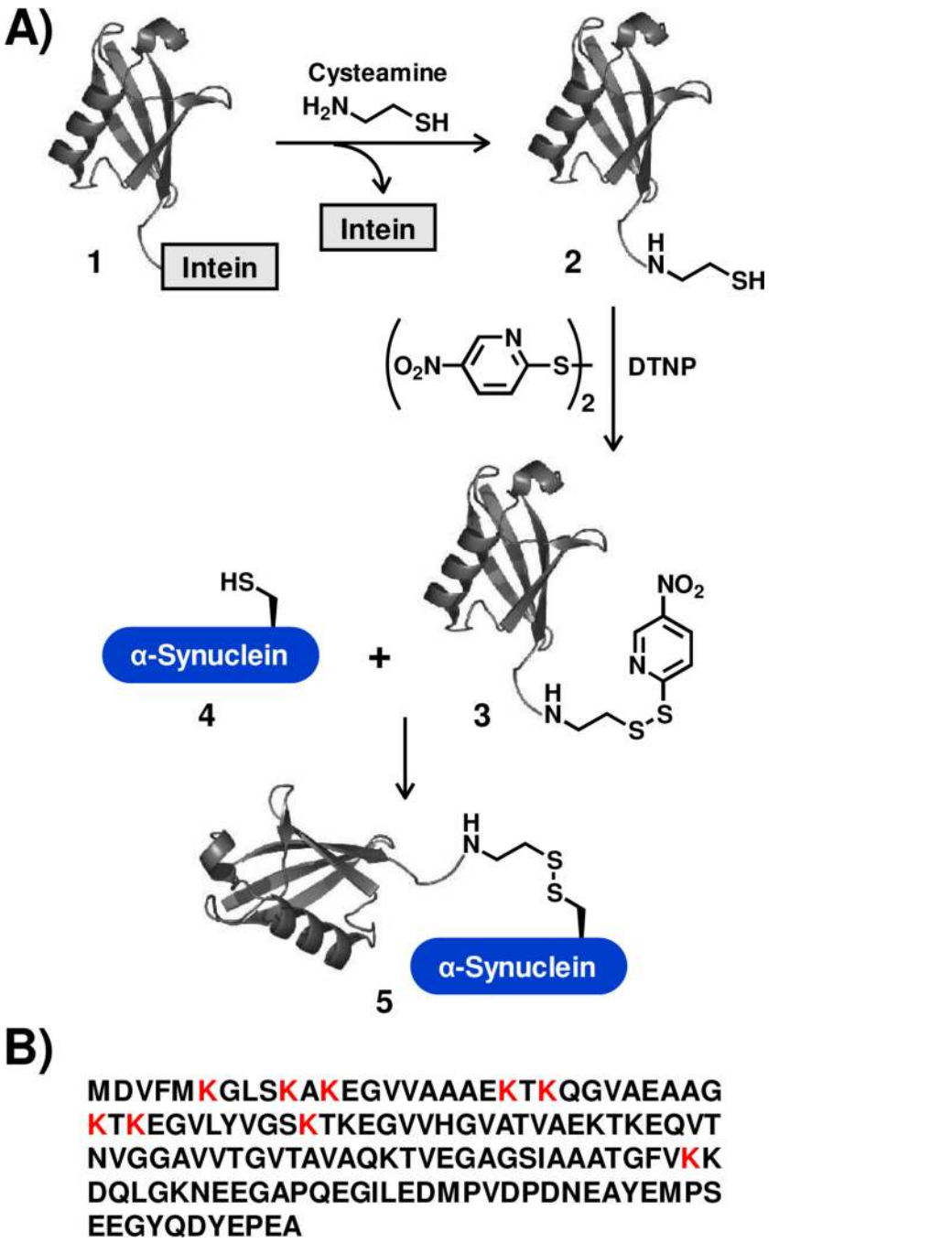

Figure 1.

Disulfide-directed ubiquitination of α-synuclein. A) Ubiquitin was expressed in *E. coli* as a fusion to the GyrA intein (**1**). Intein-mediated thiolysis with cysteamine yielded **2** and subsequent reaction with DTNP generated the activated ubiquitin mixed disulfide (**3**). α-Synuclein cysteine point mutants (**4**) were also expressed in *E. coli* and were then reacted with **3** to generate the corresponding disulfide-directed ubiquitinated derivates. B) Primary sequence of α-syn with all sites of ubiquitination noted in red.

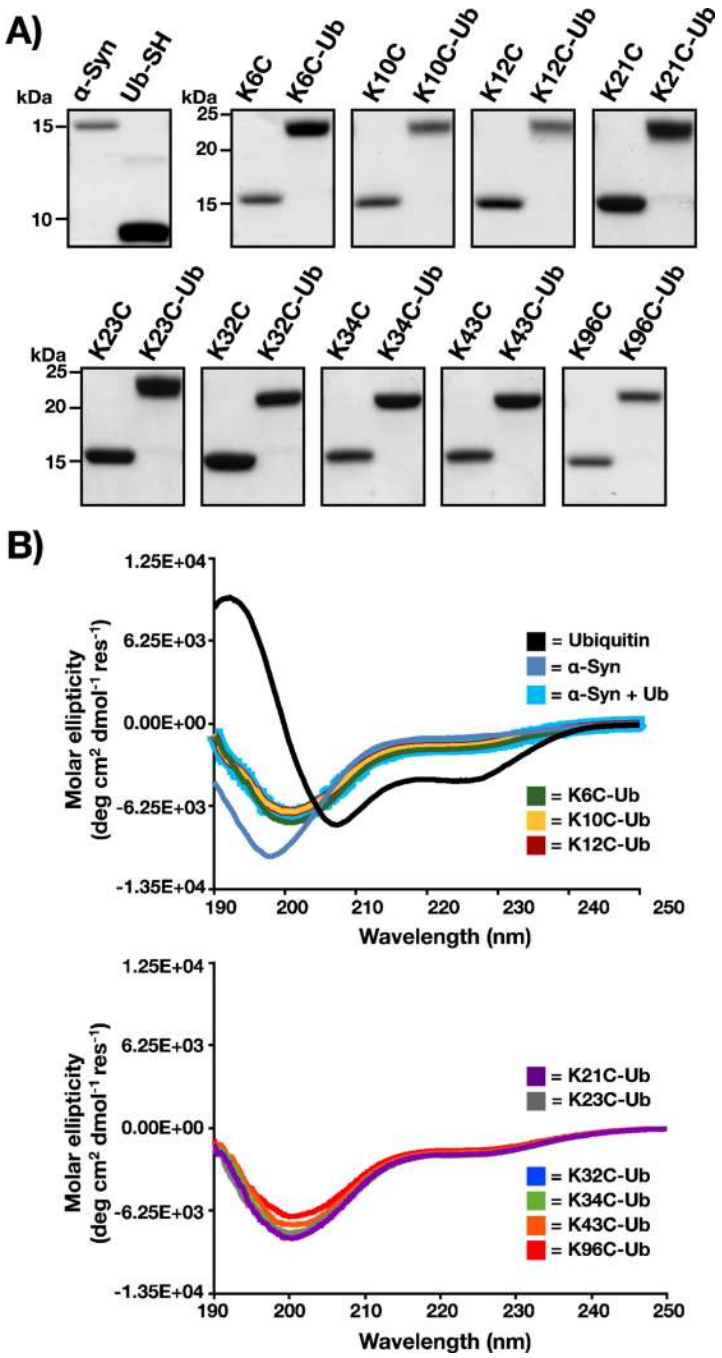

Figure 2.

Characterization of ubiquitinated α-synuclein. A) Purified α-synuclein (α-Syn), ubiquitin C-terminal thiol (Ub-SH, **2**), α-synuclein lysine to cysteine point mutants (K#C), and the corresponding ubiquitinated derivatives (K#C-Ub) were separated by SDS-PAGE and analyzed by Coomassie staining. B) CD spectra of the same proteins.

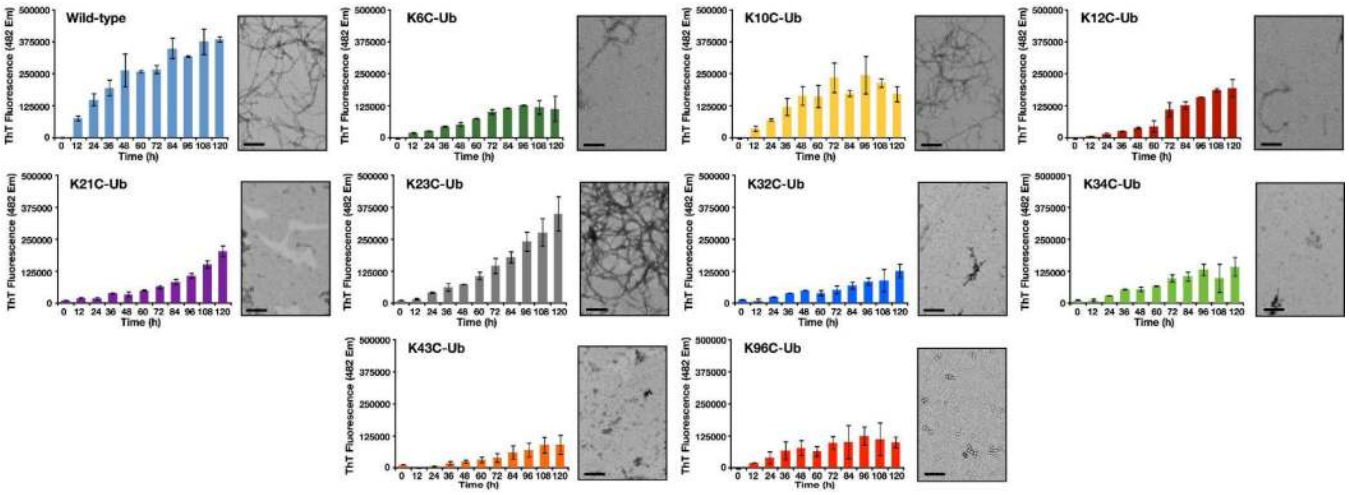

Figure 3.

Aggregation of ubiquitinated α-synuclein. Purified α-synuclein (α-Syn) and the disulfide-directed ubiquitinated derivatives (K#C-Ub) at a concentration of 100 μM were incubated at 37 °C before analysis by ThT fluorescence (450 nm Ex/482 nm Em) at the indicated timepoints. The same protein samples at day 5 were analyzed by TEM; scale bar: 500 nm). The experiments were performed in triplicate and error bars represent standard deviation.