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Extent of Inhibition of α-Synuclein Aggregation in Vitro by SUMOylation Is Conjugation Site- and SUMO Isoform-Selective

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Supporting Information

ABSTRACT: α-Synuclein, the major aggregating protein in Parkinson's disease, can be modified by the small protein SUMO, indicating a potential role in disease. However, the effects of SUMOylation on α-synuclein aggregation remain controversial due to heterogeneous nature of the proteins previously investigated. Here we used protein semisynthesis to obtain homogeneously SUMOylated α-synuclein and discovered site- and isoform-dependent effects of SUMOylation on α-synuclein aggregation. Our results indicate that SUMOylation at K102 is a better inhibitor of aggregation than corresponding modification at K96 and SUMO1 modification, a better inhibitor than SUMO3.

The pathology of Parkinson's disease is intimately associated with the formation toxic, β-sheet-rich aggregates of the protein α-synuclein.[1−3] The precise mechanisms underlying the transformation of soluble α-synuclein into aggregates are still being uncovered, and this is complicated by post-translational modifications (PTMs),[4] including several (e.g., ubiquitination, phosphorylation, and C-terminal truncations) that are associated with pathogenesis. In vivo and cell-culture experiments have found that α-synuclein is covalently modified by small ubiquitin-related modifier proteins (SUMOylation),[5,6] a reversible post-translational modification that regulates a wide range of cellular processes.[7] SUMO proteins resemble ubiquitin in protein tertiary structure and enzymatic addition and removal. There are three SUMO isoforms in mammals, SUMO1 and SUMO2/3, which are added to lysine residues within a consensus motif (ψ-K-x-D/E; ψ = hydrophobic, x = any amino acid) in a vast majority of the substrates.[8,9] α-Synuclein has one perfect SUMO acceptor site at K96 and one imperfect site at K102 that are the major α-synuclein SUMO modification sites in cell culture.[5,6,10] Notably, α-synuclein aggregates from Parkinson's disease and dementia with Lewy bodies affected brains are immunoreactive to SUMO1, and two studies found that SUMOylation is associated with α-synuclein aggregation during proteasome inhibition.[11,12] However, in vitro aggregation studies performed on SUMO1-modified α-synuclein obtained from a bacterial coexpression system showed that SUMOylation completely blocked aggregation and mutation of the SUMOylation sites promoted α-synuclein aggregation in cell culture[6] and yeast.[13] These contradictory consequences of SUMOylation on α-synuclein aggregation could be attributed to the heterogeneous nature of the protein populations under investigation. For instance, SUMOylation in a bacterial system results in a mixture of SUMOylation at multiple substrate sites, and SUMO1/α-synuclein coexpression in mammalian cell culture does not exclude the modification by SUMO2/3. All three SUMO proteins adopt the same overall 3D structure. SUMO3 and SUMO2 share 97% sequence identity despite having only 47% sequence identity to SUMO1,[7] and these proteins display both overlapping and distinct cellular functions.[14,15] Moreover, our previous work has shown that ubiquitination affects α-synuclein aggregation and proteasome mediated turnover in a site-specific manner.[16,17]

To examine the site- and isoform-specific effects of SUMO, we generated site-specifically SUMOylated α-synuclein at K96 and K102 with either SUMO1 or SUMO3, utilizing a disulfide-directed protein semisynthesis strategy (Figure 1 and Supporting Information, Figures S1−S4).[18−20] Circular dichroism (CD) spectra of SUMO-α-synuclein conjugates resembled a combination of the individual α-synuclein and SUMO1/3 C-terminal thiol spectra (Figure S5, Supporting Information), indicating that the disulfide-linkage has no effect on the native secondary structure of either protein. Additionally, analysis of the purified SUMO-α-synuclein conjugates by dynamic light scattering (DLS) showed that all the proteins contained no preformed oligomers or aggregates (Figure S6, Supporting Information). We next tested the site-specific effects of SUMOylation on α-synuclein aggregation by incubating each protein at a concentration of 50 μM with constant agitation at 1000 rpm for 84 h at 37 °C. Every 12 h the extent of fiber

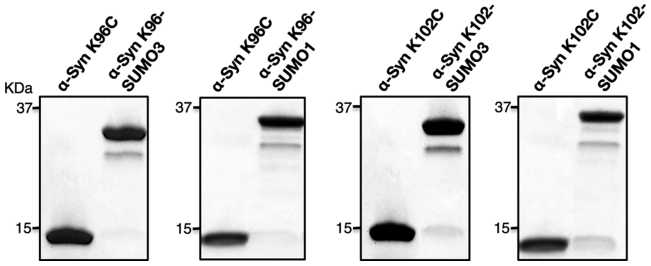

Figure 1. Disulfide-directed α-synuclein SUMOylation was carried out on the corresponding KtoC mutants and analyzed by SDS-PAGE.

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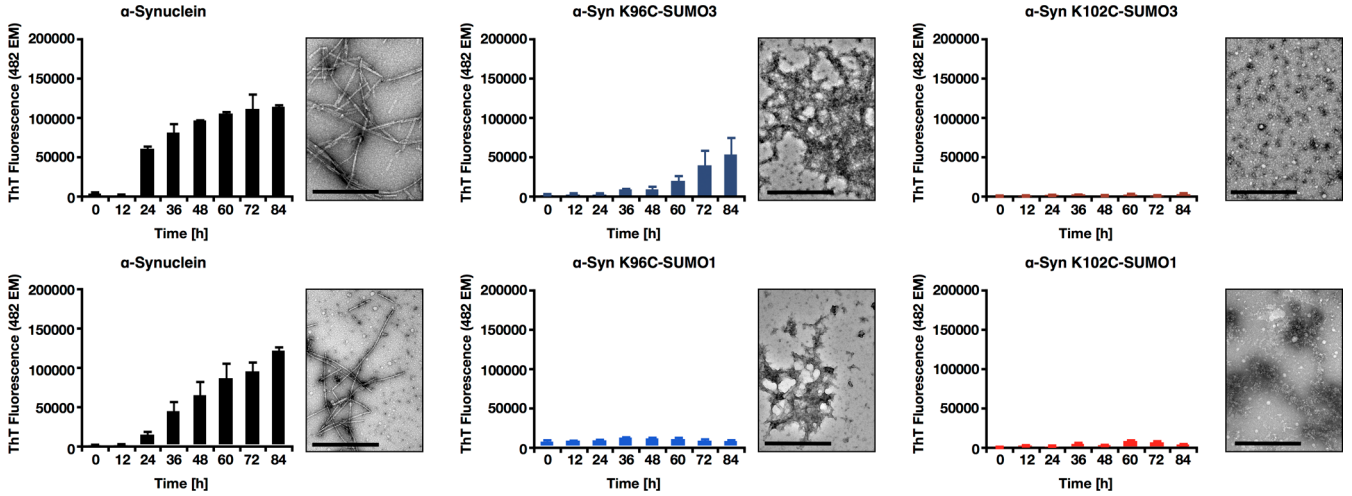

Figure 2. Aggregation of SUMOylated α-synuclein. Purified α-synuclein and the disulfide-directed SUMOylated derivatives (K#C-SUMO1/SUMO3) at a concentration of 50 μM were incubated at 37 °C before analysis by ThT fluorescence at the indicated time points. The same protein samples at day 4 were analyzed by TEM; scale bar: 500 nm. The experiments were performed in triplicate, and error bars represent standard deviation.

formation was quantitated using thioflavin T (ThT) fluorescence (Figure 2). Wild type protein readily aggregated while modified proteins generally inhibited fibril formation. However, we detected ThT fluorescence for α-synuclein-K96C-SUMO3 beginning at 60 h and increasing until the termination of the reaction. Visualization of any aggregates at the conclusion of the assay (84 h) using transmission electron microscopy (TEM) revealed the formation of long mature fibrils when α-synuclein was unmodified and none of these "mature" fibers for SUMOylated protein (Figure 2). However, SUMO modification at K96C resulted in deposits that are more rigid than the amorphous deposits detected with α-synuclein K102C-SUMO conjugates. α-Synuclein-K96C-SUMO3 aggregation also resulted in more extensive formation of these rigid deposits than the corresponding SUMO1 modification. To explore these differences further, we also employed DLS (Figure S7, Supporting Information). Consistent with the ThT and TEM data, unmodified α-synuclein and α-synuclein-K96C-SUMO3 produced aggregates with similar radii, while the other SUMOylated derivatives showed the presence of only protein monomers and species with very large Stokes radii that we attribute to amorphous aggregates. We next performed an aggregation assay under more aggressive conditions by stir-bar mediated agitation, which catalyzes fibril formation compared to the thermomixer agitation used above and should accentuate any differences in aggregation proclivities.[21] Analysis using ThT fluorescence (Figure S8, Supporting Information) demonstrated that SUMO1/3 modification at K96C only reduced the kinetics of aggregation, while SUMOylation at K102C still significantly inhibited fibril formation. TEM analysis of the aggregates (Figure S8) showed fibrils for α-synuclein-SUMO conjugates at K96C, although these structures appeared irregular when compared to those generated by unmodified protein. Notably, no large fibril structures were observed with SUMOylation at K102C, and the majority of material was amorphous in nature. These data further support that α-synuclein SUMO modifications at K102 are more inhibitory toward fibril formation than SUMOylation at K96. Importantly, the potentially liable, disulfide-directed SUMO modifications were intact at the conclusion of these aggregation reactions (Figure S9, Supporting Information). We next studied how substoichiometric levels of SUMOylated α-synuclein affect its aggregation by mixing site-specifically modified and unmodified α-synuclein at ratios of 1:1 and 1:3 for each SUMO isoform followed by agitation in a thermomixer. Analysis by ThT fluorescence (Figure S10, Supporting Information) demonstrated that SUMOylation at both sites largely blocked α-synuclein aggregation when making up 50% of the total protein. However, at 25% of the total protein concentration, we again observed site-specific effects on aggregation (Figure S10). For both SUMO1 and SUMO3, modifications at K102C had a more pronounced effect on the kinetics of aggregation compared to the respective modifications at K96C. Furthermore, modification by SUMO1 was again generally more inhibitory than SUMO3.

Finally, we sought to investigate any effect of SUMOylation on subsequent α-synuclein phosphorylation at serine 129 (S129) that is closely associated with disease,[22,23] since SUMOylation can both mask interacting surfaces and act as docking sites for downstream effectors. We incubated unmodified α-synuclein and site-specifically SUMO1-modified α-synuclein analogues, as they have a more pronounced effect on aggregation, at 30 °C for 12 h with G-protein-coupled receptor kinase 5 (GRK5), which is known to phosphorylate α-synuclein at S129.[24] Although the majority of these synuclein proteins were at the expected molecular weights (Ponceau staining, Figure S11, Supporting Information), the bulk of the phosphorylated protein was found as higher molecular weight aggregates (Western blotting, Figure S11), indicating that the fraction of phosphorylated material aggregated during the kinase reaction. Notably, SUMO1-modification inhibited this phosphorylation, with modification at K102C again having a larger effect.

In summary, our results provide the first systematic analysis of the consequences of SUMOylation on α-synuclein aggregation in vitro. Using a combination of ThT fluorescence and TEM visualization, we found notable site- and isoform-specific effects of SUMO. In general, SUMOylation at K102 of α-synuclein results in more pronounced inhibition of aggregation than the corresponding modification at K96. Notably, ubiquitination at K96 inhibits the formation of fibers, even under aggressive aggregation conditions,[16] highlighting

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the importance of subtle alterations. Additionally, our results indicate that SUMO1 is a more potent blocker of aggregation than SUMO3. Unique to our chemical approach compared to previous cellular and in vitro methods is the preparation of homogeneous and site-specific SUMOylated proteins instead of complex mixtures. This enabled us to identify SUMO1ylation at K102 as the most attractive potential target to inhibit α-synuclein aggregation, encouraging the investigation of the enzymes that add and remove SUMO1 at K102 of α-synuclein.

■ ASSOCIATED CONTENT

Supporting Information

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

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The authors declare no competing financial interest.

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