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Exploring the Role of Methionine Residues on the Oligomerization and Neurotoxic Properties of DOPAL-Modified -Synuclein

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Abstract

The dopamine metabolite 3,4-dihydroxyphenylacetaldehyde (DOPAL) is believed to play a central role in Parkinson's disease neurodegeneration by stabilizing potentially toxic oligomers of the presynaptic protein α -Synuclein (aSyn). Besides the formation of covalent DOPAL-Lys adducts, DOPAL treatment promotes the oxidation of Met residues of aSyn, which is also a common oxidative post-translational modification found in the protein *in vivo*. Herein we set out to address the role of Met residues into the oligomerization and neurotoxic properties of DOPAL-modified aSyn. Our data indicate that DOPAL promotes the formation of two distinct types of aSyn oligomers: large and small (dimer and trimers) oligomers, which seem to be generated by independent mechanisms and cannot be interconverted by using denaturing agents. Interestingly, H₂O₂-treated aSyn monomer, which exhibits all-four Met residues oxidized to Met-sulfoxide, exhibited a reduced ability to form large oligomers upon treatment with DOPAL, with no effect on the population of small oligomers. In this context, triple Met-Val mutant M5V/M116V/M127V exhibited an increased population of large aSyn-DOPAL oligomers in comparison with the wild-type protein. Interestingly, the stabilization of large rather than small oligomers seems to be associated with an enhanced toxicity of DOPAL-aSyn adducts. Collectively, these findings indicate that Met residues may play an important role in modulating both the oligomerization and the neurotoxic properties of DOPAL-derived aSyn species.

Graphical Abstract:

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Keywords

DOPAL; α-synuclein; oligomerization; methionine-sulfoxide; oxidation; Parkinson's disease

1. Introduction

The hallmark of Parkinson's disease (PD) is the presence of intraneuronal deposits containing fibrillar aggregates of the presynaptic protein α-synuclein (aSyn) (called Lewy bodies and Lewy neurites) in *substantia nigra* (SN) neurons as well as other brain regions [1,2]. Although the precise contribution of aSyn to neuronal dysfunction and death is still elusive, important evidences suggest that oligomers rather than fibrils are the toxic species generated from aSyn aggregation [3].

The highly cytotoxic aldehyde 3,4-dihydroxyphenylacetaldehyde (DOPAL) [4], levels of which were found to be enhanced in *post mortem* brains of PD patients [5], is capable of causing loss of dopaminergic neurons accompanied by the accumulation of aSyn oligomers in rat brains [6]. In this context, our group proposed a mechanism for DOPAL-induced aSyn oligomerization in which the formation of covalent adducts between DOPAL and N-terminal Lys residues of aSyn is associated with the stabilization of the protein oligomers, likely by cross-linking aSyn molecules [7]. In addition, the oxidation of DOPAL in solution, which results in the production of reactive oxygen species (ROS), leads to the oxidation of all-four Met residues of aSyn [7,9]. aSyn contains four Met residues: two located at N-terminal (Met1 and Met5) and two at C-terminal (Met16 and Met127). The oxidation of Met residues is an important post-translational modification found in aSyn *in vivo* and it also has been pointed out to favor the protein oligomerization *in vitro* [10]. However, the impact of this naturally occurring oxidative modification of aSyn on the oligomerization of the protein induced by DOPAL is unknown.

In this work, we demonstrated that the oxidation of all-four Met residues of aSyn monomer, upon the treatment with H₂O₂, reduces the ability of the protein to form large oligomers in the presence of DOPAL, which is reversed by triple Met-Val mutations (M5V/M116V/M127V) in the protein. The toxicity of DOPAL-treated aSyn on primary neurons is enhanced by increasing the population of large rather than small oligomers, which suggests that large oligomers of aSyn-DOPAL exhibit a higher toxicity. Collectively, our findings

indicate that Met residues may have an important role in driving the oligomerization and the neurotoxic properties of DOPAL-derived aSyn species.

2. Material and Methods

2.1 Expression and purification of aSyn.

The expression and purification of recombinant aSyn was performed as previously described [11]. To evaluate the role of Met residues on DOPAL-induced aSyn oligomerization, Met residues of the protein were replaced by Val, both displaying similar side-chain sizes. Considering that mutations at the initiator methionine ATG may completely abolish translation of the protein, only Met5, Met116 and Met127V have been mutated to valine. The triple Met-Val mutation was performed in steps by using an Agilent/Stratagene QuikChange site-directed mutagenesis kit according to manufacturer's instructions. DNA sequencing confirmed that the PCR product contained the desired nucleotide sequences. Mutations were checked by mass spectrometry (MS) analysis.

2.2 Modification of aSyn by H₂O₂ or DOPAL.

For preparation of Met-oxidized aSyn monomer, 200 µM of monomeric wild-type (WT) aSyn in 20 mM sodium phosphate buffer, pH 7.4, was incubated with 8 mM H₂O₂ at 37 °C for 24 hours, and then lyophilized for H₂O₂ removal. The oxidation of Met residues was evaluated by MS. Unmodified-aSyn (unmod-aSyn) monomer and H₂O₂-treated aSyn monomer were incubated in the absence or the presence of 10-fold molar excess of DOPAL at 37 °C for 24 hours. For kinetic experiments, aliquots were withdrawn at different incubation times and the oligomeric state of the protein was evaluated by using both 15% SDS-PAGE and size exclusion chromatography (SEC-HPLC) using a Superdex 200 10/300 GL column (void volume: 7.8 mL) (GE Healthcare, Little Chalfont, UK). The percentage of oligomers formed was determined by the relative areas in SEC-HPLC. Far-UV circular dichroism (CD) analysis was performed in a Chirascan Spectropolarimeter (Applied Photophysics, UK).

2.3 Mass Spectrometry (MS).

Recombinant aSyn (200 µM in 20 mM sodium phosphate), in the absence or the presence of either 8 mM H₂O₂ or 2 mM DOPAL, was incubated at 37 °C, no agitation, for 72 hours. aSyn monomers were purified by SEC-HPLC using a Superdex 200 10/300 GL column equilibrated with the volatile buffer ammonium bicarbonate, and then lyophilized. Protein samples were submitted to digestion with Trypsin or Glu-C and the peptides analyzed using an ESI-q-TOF (Impact Maxis, Bruker) at the *Centro de Espectrometria de Massas de Biomoléculas at the Instituto de Biofísica Carlos Chagas Filho*, Federal University of Rio de Janeiro. The resulting data were evaluated using the free software Mmass version 5.5.0 [12] or the *in silico* analysis software Mascot (Matrix Science, England) [13].

2.4 Paramagnetic Relaxation Enhancement (PRE).

NMR experiments were performed at the Weill Cornell Medical College, Cornell University, New York City, USA, using a 600 MHz Bruker Avance spectrometer equipped with cryogenic probes. Phase-sensitive two-dimensional ¹H-¹⁵N Heteronuclear Single Quantum

Coherence (^1H - ^{15}N -HSQC) spectra were recorded using Echo-anti Echo gradient selection. TopSpin 3.2 was used for data acquisition. Considering that aSyn has no Cys residues, protein variants G31C, A85C and P120C were generated by using site-directed mutagenesis. The purified mutant proteins were labeled with 1-oxy-2,2,5,5-tetramethylpyrrolidine-3-methyl-methanethiosulfonate (MTSL) (Toronto Research Chemicals, Canada) as described [14]. PRE experiments were done as described by Follmer *et al.* [7].

2.5 Neurotoxicity assays.

Neurons from the midbrain of 14-day-old Swiss mice (E14) were prepared as previously described [15]. All animals were kept under standard laboratory conditions according to National Institute of Health guidelines and using the protocols approved by the Animal Use Ethics Committee of the Federal University of Rio de Janeiro (Protocol No 025/15). For the preparation of aSyn samples for the toxicity assays, 100 μM of either wild-type (WT)- or M5V/M116V/M127V-aSyn were incubated in the absence or the presence of 0.5 or 1 mM DOPAL at 37 °C for 24 hours, and then the mixture was divided in two different fractions: 1) TOTAL sample, containing DOPAL-modified aSyn plus non-bound DOPAL; 2) Non-bound DOPAL that was obtained by filtering the TOTAL sample using a 10 KDa cut-off Amicon Ultra Filter (Merck Millipore, Burlington, USA). Note that the pass-through sample contains exactly the same concentration of non-bound DOPAL found in the TOTAL sample. aSyn samples were added to *in vitro* cultures of mesencephalic neurons (7 days old) at a final concentration of 10 μM (monomer concentration), and then incubated at 37 °C in a humidified 5% CO_2 and 95% air chamber for 24 hours. Viable cells were quantified by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay. Buffer alone was used as the control.

3. Results and Discussion

3.1 Met oxidation reduces the formation of large oligomers from DOPAL-treated aSyn.

Fig. 1A shows that the incubation of aSyn with DOPAL leads to a decrease of the population of monomer (eluting at 14.3 mL) concomitant with the formation of two distinct populations of oligomers accordingly to SEC-HPLC analysis: a population of large oligomers eluting at ~8.0 mL (very close of the void volume of the column) and a population of small oligomers (dimer and trimers) eluting at 11–13 mL (Fig. 1A). Only small aSyn-DOPAL oligomers were visualized by using SDS-PAGE. The intrinsically disordered nature of aSyn monomer is responsible for the premature elution of the protein in SEC (volume of elution corresponding to a 60–70 kDa protein) (Fig. 1A, upper panel). CD spectra of purified DOPAL-modified aSyn oligomers indicate that both small and large oligomers exhibit a disordered conformation similarly to the unmodified (unmod)-aSyn monomer (Fig. 1B), instead of a beta-sheet rich structure that is characteristic of amyloid fibrils. This finding suggests that large aSyn-DOPAL oligomers are not on-pathway intermediates in fibril growth.

A recently study indicated that the formation of large oligomers of DOPAL-modified aSyn occurs via reversible association of the small oligomeric species (dimers and trimers) [9]. Our data results pointed out that the denaturing treatments with urea, guanidine-HCl or

guanidine-HCl/boiling were incapable of promoting an extensive dissociation of large oligomers (Fig. 1C). Indeed, only a slight increase of the area of the peaks corresponding to dimer/trimers and monomer was observed, which suggest that only a minor population of these species interacts reversibly during the formation of large aSyn-DOPAL oligomers. Importantly, the long-term incubation of purified small oligomers seems do not produce large oligomers of DOPAL-derived aSyn (data not shown).

Our next objective was to evaluate the role of the oxidation of Met residues on the oligomerization of aSyn promoted by DOPAL. Accordingly to MS data (Table 1), the treatment of WT-aSyn with either H₂O₂ or DOPAL causes to the oxidation of all-four Met residues to Met-sulfoxide, which is evidenced by an increase of mass of ~16 kDa for each Met residue. Fig. 2A,B shows that the oxidation of Met residues of aSyn significantly reduces the formation of large oligomers of the protein in the presence of DOPAL, with no effect on the population of dimers and trimers. The inspection of the kinetic of oligomerization suggests that the formation of both large and small oligomers of aSyn-DOPAL occurs simultaneously (Fig. 2C). For unmod-aSyn, the rate of formation of the large oligomers was higher than that for the small oligomers, which suggests that the formation of large aSyn-DOPAL oligomers likely occurs via a mechanism different from that involved in the formation of small oligomers. On the other hand, H₂O₂-treated M5V/M116V/M127V-aSyn displayed an oligomerization profile similar to the untreated protein upon incubation with DOPAL (Fig. 2D), which reinforces the idea that the lower propensity of H₂O₂-treated WT-aSyn to form large oligomers in the presence of DOPAL is caused by the oxidation of Met residues.

3.2 Met oxidation alters the conformational ensemble of aSyn, possibly accounting for decreased DOPAL-mediated oligomerization.

To better understand the impact of the oxidation of Met residues on the conformation of aSyn, the transient long-range contacts of unmod- and H₂O₂-treated aSyn was studied by using PRE [14]. aSyn is divided into three distinct domains: an amphipathic N-terminal domain, a central hydrophobic domain (denoted as Non-Amyloid- β Component: NAC), and an acidic C-terminal domain. As previously described for aSyn monomer [16], the presence of MTSL near the N-terminus (G31C), or in the NAC region (A85C) leads to a broadening of signals originating from residues in the C-terminal domain in the proximity of residue 128, whereas a spin-label at P120C lead to broadening in the NAC region as well as in the N-terminal domain of aSyn (Fig. 3, upper panels). On the other hand, H₂O₂-treated aSyn monomer presented a much more dramatic reduction in NMR signal intensities that extends over a much larger region of the protein (Fig. 3, lower panels). The oxidation of all-four Met residues to Met-sulfoxide in H₂O₂-treated aSyn variants (G31C, A85C and P120C) was confirmed by MS (data not shown). Spinlabeled at G31C position produced a much stronger broadening throughout the NAC domain in H₂O₂-treated aSyn monomer than that observed for the unmodified protein. Similarly, remarkable effects were observed for H₂O₂-aSyn spin-labeled at A85C and P120C, with the latter indicating a much enhanced interaction between the C- and N-terminal domains. It is worth noting that similar broadening of NMR signals at the spin-labeled site was observed in untreated MTSL-aSyn in comparison with H₂O₂-treated MTSL-aSyn, which suggest that H₂O₂ treatment does not affect significantly the

MTSL-spin labeling of the protein. Thereby, the oxidation of Met residues may result in an enhancement of the transient long-range interactions in aSyn monomer. The unexpected high broadening at both N- and NAC domains caused by spin-labeled at P120 position in oxidized-aSyn could be associated to conformational changes associated with the lost of proline, with its unique backbone geometry, at very close proximity of oxidized methionines (Met116- and Met127-sulfoxide). This result constitutes direct evidence corroborating an earlier report in which the effects of pH the aggregation of Met-oxidized aSyn are interpreted to indicate that Met-oxidation strengthens long-range interactions in aSyn [17].

For DOPAL-modified aSyn monomer, the formation of Lys-DOPAL adducts causes the reduction of positive charges at N-terminal as well as an increase in its hydrophobicity, which together lead to an increase in intramolecular interactions between the N-terminus and the NAC-domain [7]. Interestingly, a decrease in long-range interactions between the N- and C-terminal regions was observed for aSyn-DOPAL monomer [7], whereas an increase the interaction between N- and C-terminal domains was observed in H₂O₂-treated aSyn monomer. These conformational alterations in Met-oxidized aSyn might be associated to a reduction of the formation of large DOPAL-mediated aSyn oligomers by different mechanisms. For instance, an enhanced interaction of the C-terminal domain with the N-terminal domain of aSyn, caused by Met oxidation, could potentially interfere in the formation of either Lys-DOPAL adducts at N-terminal or cross-links between aSyn-DOPAL monomers. In particular, the oxidation of Met5 could perturb the reactivity of Lys residues at the proximity of Met5 such as Lys6 and Lys10. Overall, these conformational changes seem to affect more the formation of large rather than small oligomers.

3.3 Met residues play a pivotal role into the formation of neurotoxic DOPAL-mediated aSyn adducts.

The toxicity on primary neurons of *in vitro*-produced large *versus* small oligomers from DOPAL-treated aSyn was investigated. The comparison of the toxicities of the purified oligomers requires that the same molar concentrations be assayed, which implies in the determination of exact molecular masses of these species. In addition, the effect of the purification of aSyn-DOPAL adducts on their toxicities is unclear. For these reasons, we decided not to isolate aSyn-DOPAL oligomers but instead to determine the correlation between the toxicity and the relative populations of monomer, small and large oligomers generated by the treatment of aSyn with different concentrations of DOPAL. In addition, the effect of Met residues on the toxicity of aSyn-DOPAL adducts was evaluated by comparing the toxicity of WT- *versus* M5V/M116V/M127V-aSyn upon the treatment with DOPAL.

Fig. 4A shows that M5V/M116V/M127V-aSyn behaves as a monomeric and unfolded protein similarly to the WT protein. However, upon incubation with DOPAL, M5V/M116V/M127V-aSyn forms a higher content of large oligomers in comparison with the WT protein (Fig. 4B). While for M5V/M116V/M127V-aSyn the population of large oligomers rises to ~20 % with 1 mM DOPAL, for WT-aSyn less than 10% of the total protein is found as large oligomers under this condition. This result reinforces the hypothesis of an inhibitory role of Met-oxidation on the formation of large DOPAL-aSyn oligomers. Interestingly, M5V/M116V/M127V-aSyn exhibits a higher toxicity on neurons, with the increase of DOPAL

concentration, than WT, where the former was almost 2-fold more toxic at a DOPAL/aSyn ratio of 10 (Fig. 4C). An elevation of toxicity of DOPAL-treated M5V/M116V/M127V-aSyn might be attributed to a higher content of large oligomers in comparison with the WT protein. For both WT- and M5V/M116V/M127V-aSyn, it was observed an increase of the toxicity with the increase of the populations of both small and large oligomers induced by DOPAL (Fig. 4D,E). However, the toxicity seems to increase more readily with the increase of the population of large than small oligomers, being the degree of dependence of the toxicity with the population of large oligomers nearly the same for WT- and M5V/M116V/M127V-aSyn (Fig. 4F). These data suggest that large soluble oligomers from DOPAL-derived aSyn exhibit a higher toxicity than the monomer or small oligomers. Plotez et al have proposed a possible mechanism for the toxic action of aSyn-DOPAL oligomers for the first time [9]. In this study, they demonstrated that the formation of aSyn-DOPAL oligomers in neurons is associated to an impairment of synaptic vesicles homeostasis likely via a pore-forming ability of these oligomers on cholesterol-containing lipid membranes. Whether this ability to damage synaptic vesicle is attributed to a specific type of DOPAL-derived aSyn oligomer or it is shared by all aSyn-DOPAL oligomers is a question that remains to be elucidated. Unfortunately, the unexpected high toxicity observed for H₂O₂-treated WT-aSyn on neurons (data not shown) did not allow us to estimate the effect of DOPAL on the toxicity of the Met-oxidized protein.

Although the formation of fibrillar aSyn in Lewy bodies is a common hallmark of PD, it is widely believed that soluble aSyn oligomers are the toxic species formed during aSyn aggregation. Interestingly, most of these studies indicate that large aSyn oligomers are potentially more toxic to cells than small oligomers, which is in accordance with our results for aSyn-DOPAL oligomers. For instance, in a study using aSyn variants engineered to promote oligomer formation, it was observed that the ability of aSyn to form *in vitro* large oligomers (but not fibrils) is correlated to an increased *in vivo* toxicity [3]. Dopamine-stabilized large aSyn oligomers (~250 kDa) are capable of inhibiting neuronal SNARE-mediated lipid mixing via binding to the N-terminal domain of the protein synaptobrevin-2, which results in blocking of the docking between vesicles [18]. Larger aSyn oligomers have been shown to impair protein degradation as well as the function of several organelles, such as the mitochondria and the endoplasmic reticulum [19,20]. The PD-linked aSyn mutations A30P and A53T were shown to increase the population of large soluble oligomers [21]. Overall, therefore, it is not a surprise that large aSyn-DOPAL oligomers exhibited a higher toxicity than small oligomers. Additional studies are necessary to understand the molecular basis behind these distinct toxic properties of aSyn-DOPAL oligomers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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4. References

- [1]. Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M, Alpha-Synuclein in Lewy bodies, *Nature* 388 (1997) 839–840. [PubMed: 9278044]
- [2]. Spillantini MG, Crowther RA, Jakes R, Hasegawa M, Goedert M, α -Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with Lewy bodies, *Proc. Natl. Acad. Sci. USA* 95 (1998) 6469–6473. [PubMed: 9600990]
- [3]. Winner B, Jappelli R, Maji SK, Desplats PA, Boyer L, Aigner S, Hetzer C, Loher T, Vilar M, Campioni S, Tzitzilonis C, Soragni A, Jessberger S, Mira H, Consiglio A, Pham E, Masliah E, Gage FH, Riek R, *In vivo* demonstration that alpha-synuclein oligomers are toxic, *Proc. Natl. Acad. Sci. USA* 108 (2011) 4194–4199. [PubMed: 21325059]
- [4]. Burke WJ, Li SW, Williams EA, Nonneman R, Zahm DS, 3,4-Dihydroxyphenylacetaldehyde is the toxic dopamine metabolite in vivo: implications for Parkinson's disease pathogenesis, *Brain Res.* 989 (2003) 205–213. [PubMed: 14556942]
- [5]. Goldstein DS, Sullivan P, Holmes C, Kopin IJ, Basile MJ, Mash DC, Catechols in post-mortem brain of patients with Parkinson disease, *Eur. J. Neurol* 18 (2011) 703–710. [PubMed: 21073636]
- [6]. Burke WJ, Kumar VB, Pandey N, Panneton WM, Gan Q, Franko MW, O'Dell M, Li SW, Pan Y, Chung HD, Galvin JE, Aggregation of alpha-synuclein by DOPAL, the monoamine oxidase metabolite of dopamine, *Acta Neuropathol.* 115 (2008) 193–203. [PubMed: 17965867]
- [7]. Follmer C, Coelho-Cerqueira E, Yatabe-Franco D, Araujo GD, Pinheiro AS, Domont GB, Eliezer D, Oligomerization and membrane-binding properties of covalent adducts formed by the interaction of α -synuclein with the toxic dopamine metabolite 3,4-dihydroxyphenylacetaldehyde (DOPAL), *J. Biol. Chem* 290 (2015) 27660–27679. [PubMed: 26381411]
- [8]. Werner-Allen JW, DuMond JF, Levine RL, Bax A, Toxic Dopamine Metabolite DOPAL Forms an Unexpected Dicatechol Pyrrole Adduct with Lysines of α -Synuclein, *Angew. Chem. Int. Ed. Engl* 20 (2016) 7374–7378.
- [9]. Plotegher N, Berti G, Ferrari E, Tessari I, Zanetti M, Lunelli L, Greggio E, Bisaglia M, Veronesi M, Girotto S, Dalla-Serra M, Perego C, Casella L, Bubacco L, DOPAL derived alpha-synuclein oligomers impair synaptic vesicles physiological function, *Sci. Rep* 7 (2017) 40699. [PubMed: 28084443]
- [10]. Glaser CB, Yamin G, Uversky VN, Fink AL, Methionine oxidation, alpha-synuclein and Parkinson's disease. *Biochim. Biophys. Acta* 1703 (2005) 157–169. [PubMed: 15680224]
- [11]. Coelho-Cerqueira E, Carmo-Gonçalves P, Pinheiro AS, Cortines J, Follmer C, α -Synuclein as an intrinsically disordered monomer – fact or artefact? *FEBS J.* 280 (2013) 4915–4927. [PubMed: 23927048]
- [12]. Niedermeyer THJ, Strohalm M, mMass as a Software Tool for the Annotation of Cyclic Peptide Tandem Mass Spectra, *PLoS One* 7 (2012) e44913. [PubMed: 23028676]
- [13]. Koenig T, Menze BH, Kirchner M, Robust prediction of the MASCOT score for an improved quality assessment in mass spectrometric proteomics, *J. Proteome Res* 7 (2008) 3708–3717. [PubMed: 18707158]
- [14]. Eliezer D, Distance information for disordered proteins from NMR and ESR measurements using paramagnetic spin labels, *Methods Mol. Biol* 895 (2012) 127–138. [PubMed: 22760317]
- [15]. Braga CA, Follmer C, Palhano FL, Khattar E, Freitas MS, Romão L, Di Giovanni S, Lashuel HA, Silva JL, Foguel D, The anti-Parkinsonian drug selegiline delays the nucleation phase of α -synuclein aggregation leading to the formation of nontoxic species, *J. Mol. Biol* 405 (2011) 254–273. [PubMed: 21050861]
- [16]. Sung YH, Eliezer D, Residual structure, backbone dynamics, and interactions within the synuclein family, *J. Mol. Biol* 372 (2007) 689–707. [PubMed: 17681534]
- [17]. Zhou W, Long C, Reaney SH, Di-Monte DA, Fink AL, Uversky VN, Methionine oxidation stabilizes non-toxic oligomers of alpha-synuclein through strengthening auto-inhibitory intramolecular long-range interactions, *Biochem. Biophys. Acta* 1802 (2010) 322330.
- [18]. Choi BK, Choi MG, Kim JK, Yang Y, Lai Y, Kweon DH, Lee NK, Shin YK, Large α -synuclein oligomers inhibit neuronal SNARE-mediated vesicle docking, *Proc. Nat. Acad. Sci. USA* 110 (2013) 4087–4092. [PubMed: 23431141]

- [19]. Parihar M, Parihar A, Fujita M, Hashimoto M, Ghafourifar P, Alpha-synuclein overexpression and aggregation exacerbates impairment of mitochondrial functions by augmenting oxidative stress in human neuroblastoma cells, *Int. J. Biochem. Cell Biol* 41 (2009) 2015–2024. [PubMed: 19460457]
- [20]. Colla E, Jensen PH, Pletnikova O, Troncoso JC, Glabe C, Lee MK, Accumulation of toxic alpha-synuclein oligomer within endoplasmic reticulum occurs in alpha-synucleinopathy *in vivo*, *J. Neurosci* 32 (2012) 3301–3305. [PubMed: 22399752]
- [21]. Conway KA, Lee SJ, Rochet JC, Ding TT, Williamson RE, Lansbury PT, Acceleration of oligomerization, not fibrillization, is a shared property of both a-synuclein mutations linked to early-onset Parkinson's disease: Implications for pathogenesis and therapy, *Proc. Nat. Acad. Sci. USA* 97 (2000) 571–576. [PubMed: 10639120]

Figure 1. Formation of DOPAL-induced aSyn oligomers.

(A) Oligomeric states of WT-aSyn treated or not with 10-fold molar excess of DOPAL, monitored by SEC-HPLC. The elution volumes of the unfolded monomer and dimer were plotted in the upper panel; a standard curve was generated by using the proteins lysozyme (14.4 kDa), carbonic anhydrase (30 kDa), ovalbumin (45 kDa), BSA (66 kDa) and phosphorylase B (97 kDa). Samples of aSyn in the absence (-) or presence (+) of DOPAL were analyzed by 15% SDS-PAGE. M = monomer; D = dimer; T: trimer. (B) Far-UV CD spectra of purified large (elution at ~ 8 mL in SEC-HPLC) and small (elution at 11–13 mL) aSyn-DOPAL oligomers, in comparison with unmod-aSyn monomer. (C) Effect of different denaturing treatments (1 hour of incubation at 37 °C with either 4.0 M urea or 4.0 M guanidine-HCl, or 4.0 M guanidine-HCl/boiling for 5 min) on the oligomeric state of DOPAL-derived aSyn species determined by SEC-HPLC.

Figure 2. Role of the oxidation of Met residues of aSyn on the formation of DOPAL-derived oligomers.

Formation of oligomers of unmod-aSyn (**A**) and H₂O₂-treated aSyn (**B**) incubated in the presence of 10-fold molar excess of DOPAL at 37 °C, no agitation, monitored overtime by using SEC-HPLC. (**C**) The kinetic of formation of large or small oligomers of aSyn incubated in the presence of DOPAL was determined through the relative areas in SEC-HPLC. Data are shown as mean $\pm$ S.D. of three independent experiments. (**D**) DOPAL-induced oligomerization of M5V/M116V/M127V-aSyn, which was pretreated or not with H₂O₂.

Figure 3. Long-range interactions in unmod-aSyn and H₂O₂-treated aSyn monomers monitored by PRE.

Upper panel: aSyn is divided into three distinct domains: an amphipathic N-terminal domain, a central hydrophobic domain (denoted as Non-Amyloid- β Component: NAC), and an acidic C-terminal domain. For PRE, H₂O₂-treated aSyn samples were prepared by incubation of MTSL-treated aSyn (in 20 mM sodium phosphate, pH 7.5) in the presence of 8 mM H₂O₂ at 25 °C overnight. The histograms represent the intensity of cross-peaks of the residues in the ¹H-¹⁵N-HSQC spectra of MTSL spin-labeled aSyn normalized by the corresponding peak intensities in the reduced samples. Reduction of MTSL was achieved by ascorbic acid treatment. Spectra were recorded at 10 °C.

Table 1.

Masses of the peptides generated from Trypsin[#] and Glu-C digestion of untreated- or DOPAL-treated aSyn monomer.

```

      10      20      30      40      50
MDVFMKGLSK AKEGVVAAAE KTKQGVAEAA GKTKEGVLYV GSKTKEGVVH
      60      70      80      90     100
GVATVAEKTG EQVTNVGGAV VTGVTAVAQK TVEGAGSIAA ATGFVKKDQL
      110     120     130     140
GKNEEGAPQE GILEDMPVDP DNEAYEMPSE EGYQDYEPEA

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aS sample	Peptide	Expected mass [*]	Observed mass	Oxidized Met	Adduct
Untreated	1–13	1482.75	1482.75	none	-
	115–123	1030.39	1087.41	none	carbamidomethylation in M116 (+57.0215)
	124–131	954.36	1011.38	none	carbamidomethylation in M127 (+57.0215)
DOPAL-treated	1–6 [#]	770.34	802.37	M1 and M5	+32.03 (Met-sulfoxide)
	98–140 [#]	4285.73	4317.69	M116 and M127	+31.96 (Met-sulfoxide)
H ₂ O ₂ -treated	1–13	1482.76	1514.74	M1 and M5	+ 31.98 (Met-sulfoxide)
	115–123	1030.39	1046.39	M116	+16.00 (Met-sulfoxide)
	124–131	954.36	970.35	M127	+16.01 (Met-sulfoxide)

^{*} Monoisotopic mass calculated using PeptideMass (ExPASy)