Protein Aggregation | Hot Paper

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Co-existence of Two Different α-Synuclein Oligomers with Different Core Structures Determined by Hydrogen/Deuterium Exchange Mass Spectrometry**

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Abstract: *Neurodegenerative disorders are characterized by the formation of protein oligomers and amyloid fibrils, which in the case of Parkinson's disease involves the protein α-synuclein (αSN). Cytotoxicity is mainly associated with the oligomeric species, but we still know little about their assembly and structure. Hydrogen/deuterium exchange (HDX) monitored by mass spectrometry is used to analyze oligomers formed by wild-type (wt) αSN and also three familial αSN mutants (A30P, E46K, and A53T). All four variants show co-existence of two different oligomers. The backbone amides of oligomer type I are protected from exchange with D_2O until they dissociate into monomeric αSN by EX1 exchange kinetics. Fewer residues are protected against exchange in oligomer type II, but this type does not revert to αSN monomers. Both oligomers are protected in the core sequence Y39–A89. Based on incubation studies, oligomer type I appears to form straight fibrils, while oligomer type II forms amorphous clusters that do not directly contribute to the fibrillation process.*

Many human diseases arises from protein misfolding,[1] which in neurodegenerative diseases such as Parkinson's disease (PD)[2] can lead to fibrillar aggregates called amyloids.[3] In PD there is progressive loss of dopaminergic neurons in the brain's *substantia nigra* (SN) and appearance of Lewy bodies (LB) and Lewy neurites (LN) in surviving cells.[4] The main component of LB and LN is the pre-synaptic protein α-synuclein (αSN).[5] αSN has no persistent structure in the monomeric state under physiological conditions.[6] However, it can self-associate into oligomeric species[7] and amyloid fibrils.[8] αSN oligomers disrupt membranes and kill nerve cells,[7b,9] making them promising targets for therapies against PD.[8a,10] Recent advances in solid-state nuclear magnetic resonance spectrometry (NMR) and cryo-electron microscopy have provided structural insights into fibrillar forms of β-amyloid,[11] human islet amyloid polypeptide (hIAPP),[12] transthyretin,[13] and α-synuclein.[14] Furthermore, ion mobility mass spectrometry has provided structural information for amyloidogneic systems, such as β-amyloid,[15] beta-2 microglobulin (β_2m),[16] hIAPP,[17] transthyretin,[18] and also αSN.[15b,19] However, knowledge about oligomeric species remains limited owing to their transient nature, and in several cases intrinsic structure polydispersity.[20] Recently, the dynamics of metastable αSN oligomers were characterized by hydrogen/deuterium exchange monitored by mass spectrometry (HDX-MS),[21] which is also used to resolve dynamics of other proteins[22] and the core of αSN fibrils.[23] Herein we resolve the co-existence of two species of αSN oligomers using HDX-MS. HDX-MS monitors the exchange rate of backbone amide hydrogens ($^1H/^2H$), providing information on the dynamics and hydrogen bonding of the backbone.[24]

Global exchange analysis of αSN monomers revealed one unprotected population, corresponding to the fully deuterated monomer (Figure 1; Supporting Information, Figures S1, S2), consistent with the absence of organized secondary structural elements in αSN (Supporting Information, Figure S3). Very rapid (< 0.1 min) isotopic exchange of the backbone amides was also observed at peptide level (Supporting Information, Figure S4).

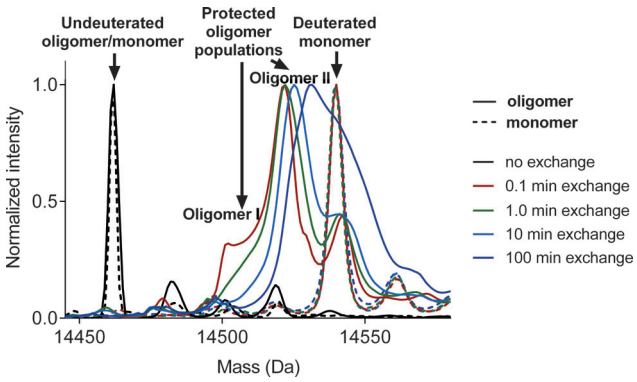

Figure 1. Deconvoluted mass spectra of αSN wt monomers and oligomers before and after 0.1, 1, 10, and 100 min of incubation in deuterated buffer. For each sample one of three replicates is shown. The two oligomeric populations are marked. Mass errors between replicates are less than 1 Da.

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Unlike the monomers, HDX-MS analysis of oligomers comprised of wt or mutant αSN revealed three populations with distinct protection levels (Figure 1; Supporting Information, Figure S5a,b). One population shows no protection against deuterium uptake, matching the exchange behavior of the monomeric protein. This population was therefore assigned to monomeric αSN in equilibrium with the aggregated species (Supporting Information, Figure S5c,d). We use this population as internal reference to obtain the relative deuteration level of the oligomers (that is, the ratio of the deuterium content of the oligomer to that of the monomer; see the Supporting Information). Two populations, which exhibited protection from isotopic exchange, could be observed. These were termed oligomer I and II, with oligomer I being the most protected and least abundant of the two. Interestingly, oligomer I also slowly decreases in abundance with prolonged labeling, a phenomenon termed EX1 exchange kinetics, which is usually related to slow refolding or dissociation reactions during labeling.[25] Based on these observations, oligomer II is stable on the timescale of the labeling (100 min), while oligomer I is an unstable assembly which primarily undergoes isotopic exchange through dissociation. This could occur either as multimers of oligomers in equilibrium with individual oligomers, or as a single oligomer in equilibrium with αSN monomers.

Labeled samples were digested and subjected to local exchange analysis to identify protected regions. Using online pepsin digestion and LC/MS/MS, a 97% sequence coverage could be achieved for all three states of αSN (Supporting Information, Figure S1). Local exchange analysis revealed two distinct protected populations for some peptides (Supporting Information, Figure S6). These populations were assigned to either oligomer I or II, based on whether they matched the characteristics of the global exchange profiles (that is, more/less protected, EX1/no EX1 kinetics; Supporting Information, Figure S7). In peptides with a single protected population, the deuterium uptake was taken to be identical for both of the oligomer populations in this region. Oligomer I was highly resistant to isotopic exchange in the Y39–A89 region, which we assign to the rigid core of the oligomer[26] (Figure 2a–c). The C-terminus was unprotected (Figure 2f). The N-terminal part is also protected, as seen by a population with a low deuterium content at short exchange times. This population disappears on prolonged exchange (>1 min) through EX-1 type exchange kinetics, suggesting a structured region that undergoes unfolding (Figure 2d,e). The protection pattern for oligomer I (Figure 3a; Supporting Information, Figures S9–S12) shares the same protected regions as αSN fibrils (Figure 3b; Supporting Information, Figure S8).

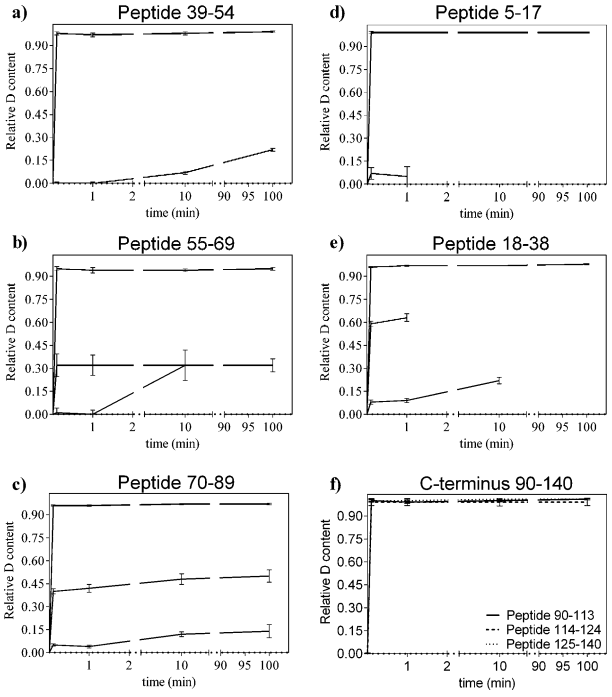

Figure 2. Relative deuterium incorporation for the a)–c) core, d), e) N-terminal, and f) C-terminal fragment of wt αSN oligomers. All of the data points are averaged triplicates with standard deviation indicated.

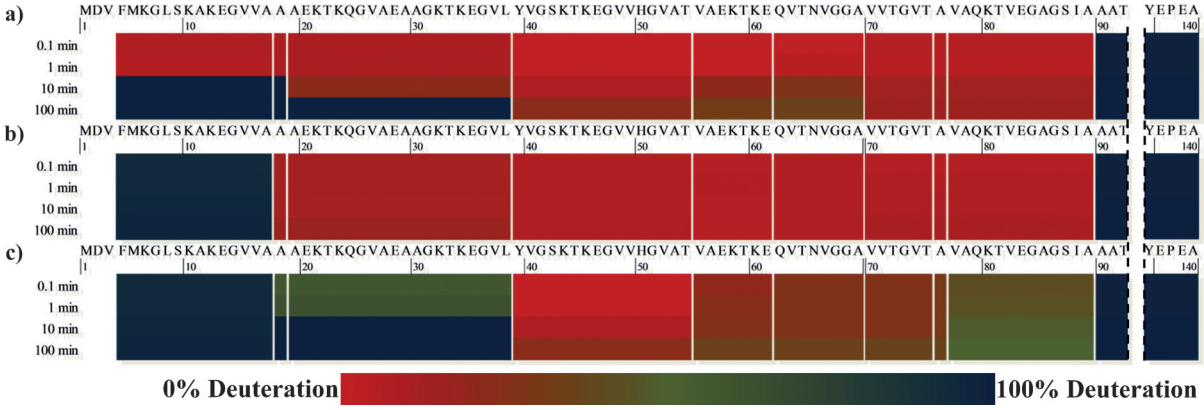

Figure 3. Deuterium exchange profile of αSN wt a) oligomer I population, b) fibrils, and c) oligomer II population. The deuteration level at each time point is indicated below the amino acid sequence. Deuterium incorporation is given relative to that of the monomeric protein (inset shows color scale). The exchange times are specified on the left side of the sequence. Vertical lines specify the ends of overlapping peptides used to calculate deuteration levels. The C-terminal sequence is truncated for the sake of clarity. For peptides exhibiting EX1 exchange kinetics (that is, N-terminal region (3-38) of oligomer I and 18-38 of oligomer II), the deuteration level of the protected population is shown until it is depleted; in the subsequent time points the peptides are shown as completely exchanged.

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Oligomer II exhibits backbone amide structuring in a smaller region than the fibrils and oligomer I (Figure 3c). Residues Y39-T75 are most protected, consistent with a structured core, while the two small fragments, A18–L38 and A76–A89, may represent dynamic flanking regions of the core. There are small variations for mutant αSN oligomers: in A30P and E46K oligomers, the A18 L38 segment shows no protection, while A53T oligomers show the same lack of protection in the A76–A89 segment (Supporting Information, Figures S9–S11, S13). At first glance it may seem counter-intuitive that oligomer I, with a higher number of protected backbone amides, is less stable with respect to dissociation than oligomer II, which has fewer protected backbone amides. We speculate that the lower stability of oligomer I (with respect to dissociation) could be due to fewer intermolecular hydrogen bonds in oligomer I, rendering it prone to dissociation. Although they lead to protection against exchange, the intramolecular hydrogen bonds are less important for the stability of the assembly.

Electron microscopy reveals that the oligomers are uniform round structures. The populations that give rise to the oligomer I and II protection patterns therefore likely have very similar surface structures. When incubated for 3 days at 37 °C, two different structures appear: A large number of worm like clusters, formed by concatenation of round oligomers, and a small number of short, straight fibrils (Supporting Information, Figure S14).

Despite differences in the dynamics and aggregation properties, the oligomer I and oligomer II populations displayed similar deuterium uptake patterns in the Y39–T75 region. They differ in flanking regions, which are more protected in the oligomer I population. These results are confirmed by limited trypsin cleavage, which leaves a protected hydrophobic core sequence that slowly precipitates (Supporting Information, Figure S15). This insoluble part of the oligomer consists of residues E35–K96 (Supporting Information, Figures S16–S18), in good agreement with our HDX-MS data, which shows most protection for residues Y39–A89. The grand average of hydropathicity value (GRAVY) for E35–K96 is 0.31, while for intact αSN it is −0.40. A higher GRAVY value indicates a more hydrophobic species. The increase in GRAVY values in the cleaved oligomer rationalizes how trypsin digestion leads to precipitation and also suggests that this region of the oligomer might be responsible for interactions with the hydrophobic core of lipid membranes, pore formation, and consequent membrane permeabilization.

The global exchange results can be interpreted in two ways. Either oligomer I is a multimer of oligomer II, or the two populations are entirely separate oligomer species. We favor the second view for two reasons. First, the oligomer solution gives rise to two distinct types of aggregates over longer times. Second, if oligomer I is not in direct exchange with monomers, its protected population can only decrease if oligomer II exchanges with monomers (while remaining at a constant overall population level). We can rule this out, as oligomer II is extremely stable and does not revert into monomers (Supporting Information, Figure S19). As oligomer II makes up the majority of the sample, it is likely that the amorphous worm-like structures are formed by this conformer. In contrast, oligomer I may be an on-pathway aggregate that is able to form fibrils. If oligomer I exists in equilibrium with monomeric αSN, we suggest that they are protofibril filaments that are elongated by monomers dissociated from isolated oligomers.

The low levels of oligomer I and its dynamic equilibrium with αSN monomers may potentially mis-identify αSN oligomer samples as homogeneous and on-pathway to fibril formation. We and others have reported on the properties of apparently homogeneous single cytotoxic αSN oligomers formed during fibril formation.[7a,27] However, none of these studies were sensitive enough to detect minor secondary species in the fibrillation process. For example, our SAXS study of αSN fibrillation[7a] only identified one oligomer (most likely oligomer II), probably because the less populated oligomer I was overwhelmed by, as well as structurally similar to, oligomer II. Its abundance makes oligomer II the likely cytotoxic membrane-permeabilizing species isolated by us[7a] and others.

Two distinct oligomers of αSN have previously been reported by Cremades et al. in an elegant FRET study,[28] which proposes that both types of oligomers are formed as intermediate species during fibril formation and the harmless oligomer converts into a toxic form. Our data suggests that this model may be refined further: the two types of oligomers belong to distinct aggregation pathways and only one of them is in dynamic equilibrium with the monomer.

We propose that during fibrillation two distinct αSN oligomers are formed (Figure 4). Oligomer I exists in equilibrium with αSN monomers and forms straight fibrils. Its transient nature, low quantity, and ability to equilibrate with αSN monomers makes it very hard to isolate and characterize. Oligomer II is formed in greater amount, is not in equilibrium with monomers (on the minute-to-one hour time scale), and cannot be elongated by them to form fibrils. However, it can cluster to form worm-like structures after prolonged incubation, which may lead to their eventual disappearance as reported.[7a] The co-existence of two non-interconverting

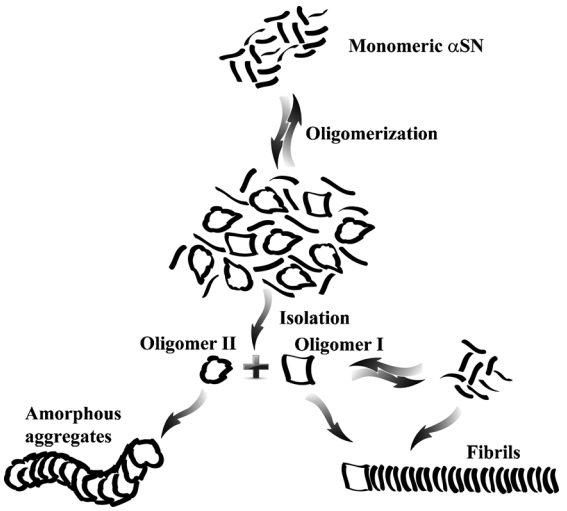

Figure 4. Proposed model for the formation and further aggregation of two distinct αSN oligomers.

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oligomers may have significant implications for the development of therapeutic strategies against Parkinson's disease.

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