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Autoproteolytic Fragments Are Intermediates in the Oligomerization/ Aggregation of the Parkinson's Disease Protein Alpha-Synuclein as Revealed by Ion Mobility Mass Spectrometry

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Several neurodegenerative diseases are characterized by the formation and accumulation of "misfolded" polymeric protein aggregates.[1–3] The formation of neurotoxic oligomers is generally thought to precede aggregation, as shown for β-amyloid polypeptide (Aβ), tau protein, and alpha-synuclein (αSyn) the key protein in Parkinson's disease (PD).[4–6] αSyn, a 140-aa protein that occurs in the presynaptic terminals of neurons is natively unfolded but adopts an α-helical structure when it binds to lipid vesicles, and forms a β-sheet that facilitates the formation of aggregated morphologies.[7,8] Intracellular accumulation of αSyn aggregates has been recognized as a condition that induces PD.[8,9] Although oligomeric intermediates represent major neurotoxic species,[9] no detailed chemical structures of αSyn oligomers and their possible intermediates have been identified. The slow rates of formation and the low concentrations of aggregating intermediates[9] could be major reasons for the failure of conventional mass spectrometry methods to detect and identify oligomers.

Although MS (especially with electrospray ionization, ESI)[10] is now established in biochemistry and structural biology and has proved to be highly successful for the structure determination of biopolymers from biological samples,[11] the low-concentration intermediates and oligomers in αSyn aggregation have not been identified by HPLC-MS. Nevertheless, oligomeric components have been observed by gel electrophoresis, atomic force microscopy (AFM), and electron paramagnetic resonance (EPR).[12–14]

Ion mobility mass spectrometry (IMS-MS) is now emerging as a new tool for the analysis of molecular assemblies of proteins.[15–18] The ability of IMS-MS to separate ions as they pass through an electric drift field allows components in protein mixtures to be separated and differentiated according to their size, charge, and conformation-dependent topography, as determined by differences in their collisional cross-sections.[16–18] After applying IMS-MS to oligomerization-aggregation mixtures of αSyn in vitro, we report here the first identification of specific autoproteolytic truncation and degradation products that were previously observed by gel electrophoresis, but not identified. In particular, a highly aggregating fragment was identified by cleavage between Val71 and Thr72 in the central aggregation domain of αSyn,. Aggregation studies of the carboxy-terminal fragment, αSyn(72–140) prepared by both chemical synthesis and recombinant expression, showed a substantially faster fibrillization compared to the intact protein.

The in vitro oligomerization of αSyn was investigated by incubating it at 37 °C in sodium phosphate buffer (pH 7.5) for up to 25 days according to a previously established procedure.[19] Recombinant αSyn expressed in *E. coli*[20] was purified by HPLC to molecular homogeneity, as confirmed by ESI-MS (Figure S1 in the Supporting Information). The formation of oligomers was monitored by tris-tricine PAGE (Figure 1); bands were revealed that corresponded to monomeric and oligomer-like αSyn with molecular weights of approximately 17, 35, and 48 kDa. In addition, protein bands with molecular weights lower than that of full-length αSyn were observed, with slowly increasing abundances over two weeks of incubation, thus indicating the formation of truncation and/or degradation products. The bands corresponding to αSyn monomer (**1a**) at 17 kDa and dimer (**1a'**) at 35 kDa were excised from the gel, digested with trypsin, and analyzed by HPLC-ESI-MS (Table S2). Sequencing of the tryptic peptides confirmed that the monomers and dimers of αSyn contain full-length sequences; indeed, we found all the expected tryptic peptides and no truncated sequences. Further structural characterization was obtained by N-terminal Edman sequencing after transfer of the proteins onto a polyvinylidene fluoride (PVDF) membrane, which yielded the first 20 amino acids of intact αSyn. Direct ESI-MS analysis of the αSyn incubation mixture after a short time period (3 h), however, revealed a small amount of N-terminally truncated αSyn lacking the first six amino acids (αSyn(7–140); spot **1b** in Figures 1 and S2, and Table S1). In contrast to these results, attempts to identify the degradation products (bands **1c**, **1d**, **1e** in Figure 1) by direct ESI-MS and HPLC-MS were unsuccessful, presumably owing to their low concentrations.

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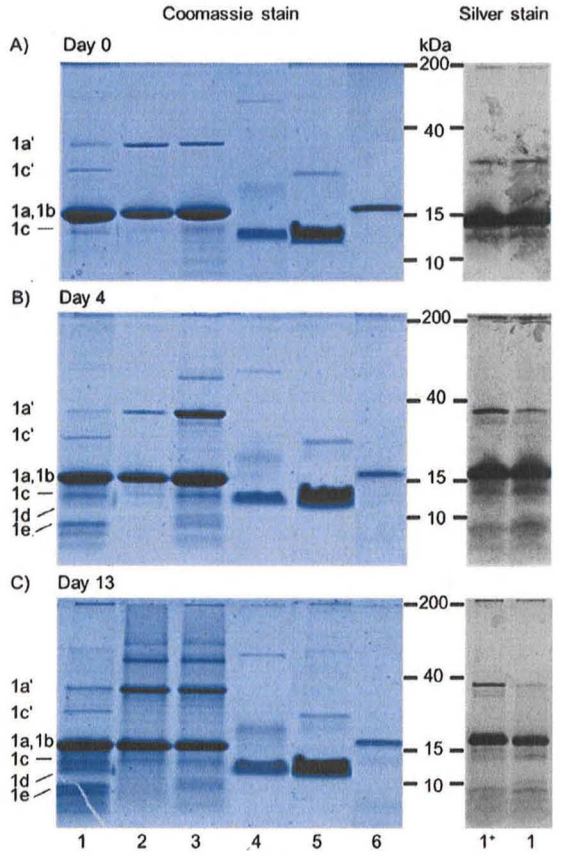

Figure 1. Time-dependent autoproteolytic degradation of αSyn and modified αSyn polypeptides visualized by Coomassie and silver staining A) at the beginning of incubation, B) after four days, and C) after 13 days. Lanes 1: wt-αSyn, 2: recombinant αSyn(Ala_6(70–75)), 3: recombinant αSyn(Gly_6(70–75)), 4: αSyn(72–140) synthesized by SPPS; 5: recombinant αSyn(72–140), 6: recombinant βSyn; 1+ and 1: incubation of wt-αSyn with and without the addition of protease inhibitors (serine-, cysteine-proteases; metalloproteases). Full-length αSyn monomer and dimer are denoted **1a** and **1a'**, respectively; N-terminally truncated αSyn(7–140) is denoted **1b**. αSyn fragments are denoted **1c**, **1d**, **1e**; **1c'** is the dimer of fragment **1c**. β-Synuclein **6** lacks the (72–83) sequence.

After incubation of αSyn for four to 21 days, the reaction mixtures were subjected to ion mobility-MS analysis, which provides inherently three-dimensional data consisting of ion mobility-dependent drift time and separation according to differences in protein collisional cross sections,[15,16] mass/charge, and relative abundances. Broad-band admittance to the drift region afforded an ion-mobility plot of the incubation mixture after seven days and showed separation into two peaks corresponding to multiply charged ion series with different drift times (Figures 2A and S3). These proteins were identified by molecular-mass determinations and ESI-tandem-MS sequencing (Figure 2B–D). Deconvolution of the multiply charged ion series to singly charged ions provided identification of full-length αSyn monomer (14459.4 Da) and dimer (28919.6 Da) in peak 2, in agreement with the mass spectrometric identification of the gel electrophoretic bands **1a** and **1a'** in Figures 1 (also, Figure 2C and Table S2). In contrast, IMS-MS analysis of peak 1 at a decreased drift time revealed proteolytic products arising from N- and C-terminal truncation, αSyn(14–133) (12162.5 Da) and αSyn(40–140) (10436.4 Da); these correspond to the proteolytic gel bands **1c** and **1e** (Figure 1 and Table S1). A remarkable fragment was identified in the ion series within peak 1; it arose from cleavage between residues Val71 and Thr72 in the central amyloidogenic domain αSyn(61–93) and corresponded to the gel electrophoresis band **1d** (Figure 1). This C-terminal fragment, αSyn(72–140) was identified by 1) accurate mass determination (7274.412 Da) and tandem-MS sequencing in the IMS-MS experiment (Figure 2C, D); 2) incubation of αSyn for extended time periods (>14 days), which afforded this fragment in amounts sufficient for direct elution of the electrophoretic band and identification by MALDI-MS (Table S1). Additional characterization of the C-terminal fragments αSyn(40–140) and αSyn(72–140) was obtained by affinity-mass spectrometry[21] and Western blot analysis with a monoclonal αSyn antibody that recognizes a C-terminal αSyn epitope, as identified by proteolytic-excision mass spectrometry[22] (data not shown).

The proteolytic fragment αSyn(72–140) was synthesized by 1) solid-phase peptide synthesis on a semiautomated peptide synthesizer by using the Fmoc strategy with double coupling and capping in the final 30 cycles (band **4**), and 2) recombinant expression in *E. coli*[20] (band **5**; Figure 1). Polypeptides prepared by both methods were purified to homogeneity by semipreparative HPLC; their molecular masses and sequences agreed with expected values as determined by ESI-MS and tandem-MS sequencing (Figure S4). Tris-tricine PAGE of **4** and **5** showed identical major bands corresponding to MWs of approximately 12 kDa; this is in agreement with the band of the autoproteolytic fragment **1d** of intact αSyn; remarkably, and in contrast to PAGE of intact αSyn, fragment **5** revealed the rapid formation of oligomers (Figure 3A). The in vitro aggregation of αSyn(72–140) **5** was analyzed in comparison with full-length αSyn **1** for up to five days at concentrations of 7–30 μM by using thioflavin T (ThT) fluorescence[23] as an established fibrillization assay, and the results were compared with those of the oligomerization analysis by using tris-tricine PAGE (Figure 3). Although exact monitoring of fibrillization kinetics was hampered by the increasing insolubility of **5** at longer aggregation times, the results showed a substantially faster aggregation of αSyn(72–140). In contrast to full-length αSyn, the fragment **5** showed the typical sigmoidal curve characteristic of reactive aggregating polypeptides.

The autoproteolytic degradation of αSyn and the significance of the fragment αSyn(72–140) were ascertained from a number of additional mass spectrometric and gel electrophoresis data, and from control experiments with several synuclein polypeptides. Comparative in vitro studies of the nonaggregating brain protein, β-synuclein (βSyn), which lacks the central amyloidogenic domain (72–83), showed neither oligomerization-aggregation nor any autoproteolytic cleavage within 21 days of incubation (Figure 1, lane 6); identical expression systems and HPLC purification procedures were employed for βSyn and αSyn, thus excluding αSyn degradation by an external protease. Further results provided evidence to exclude a

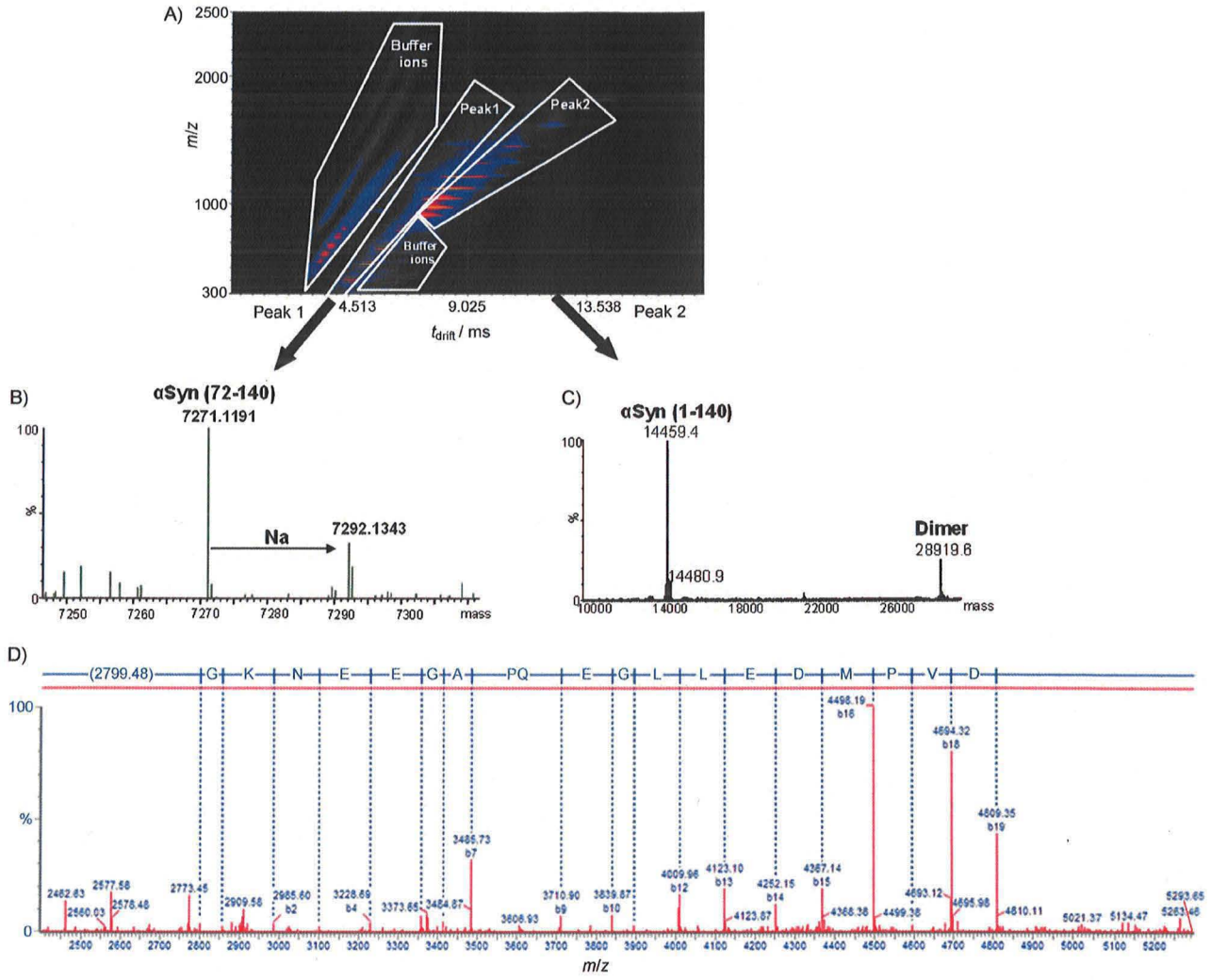

Figure 2. ESI-IMS-MS identification of full-length αSyn and the αSyn(72–140) fragment in the incubation mixture of wt-αSyn after seven days at 37 °C. A) IMS-MS drift time vs *m/z* of the αSyn incubation mixture in PBS buffer (pH 7.5). B) Deconvoluted mass spectrum of ions corresponding to peak 1, proteolytic fragment αSyn(72–140). C) Deconvoluted mass spectrum of ions corresponding to peak 2 showing full-length αSyn monomer and dimer. D) Tandem-MS sequence determination of αSyn(72–140), peak 1, showing the partial sequence (101–119).

proteolytic cleavage of αSyn by contaminating proteases: 1) Incubation of αSyn with and without addition of a broad spectrum of protease inhibitors yielded identical autoproteolytic degradation (Figure 1 right, lanes 1+ and 1); 2) Boiling samples prior to incubation and the use of sterile buffers did not lead to a change in αSyn autoproteolysis; and 3) Observation showed autoproteolytic degradation for αSyn mutants **2** and **3** in which the six residues (70–75) were exchanged against Ala and Gly (Figure 1). Moreover, a recent study on the interaction of αSyn with β-glucocerebrosidase (GCase), the target enzyme of Gaucher's disease, in cortical neurons lends additional support to the validity of our results, showing that the neurotoxicity-enhancing effect of αSyn upon depletion of GCase is abolished in a mutant αSyn that lacks the sequence 71–82.[24]

Further support for the importance of the central domain to the autoproteolysis of αSyn was obtained from initial hydrogen–deuterium exchange mass spectrometry (HDX-MS)[25] studies with high-resolution ESI-MS. HDX-MS of full-length αSyn **1** showed rapid exchange (< 3 h) for 115 of the 134 backbone hydrogens, whereas 19 amino acids remained resistant to exchange for > 14 days (data not shown). Remarkably, an identical number of 19 amino acids in the αSyn(72–140) fragment **5** were found to be resistant to HDX (exact determination of the shielded domain is currently being performed). These results suggest shielding of a C-terminal part of the hydrophobic amyloidogenic domain within residues 73/74 and 93, and are consistent with autoproteolytic accessibility at residues 71/72.

In conclusion, ion-mobility MS, owing to its capability to separate αSyn and its autoproteolytic products even though the concentration of the latter is small, enabled the characterization of intermediate fragments in in vitro oligomerization-aggregation. In particular, IMS-MS revealed a possible key fragment, αSyn(72–140). The as yet unknown mechanism of autoproteolysis, possibly proceeding via initial N-terminal trunca-

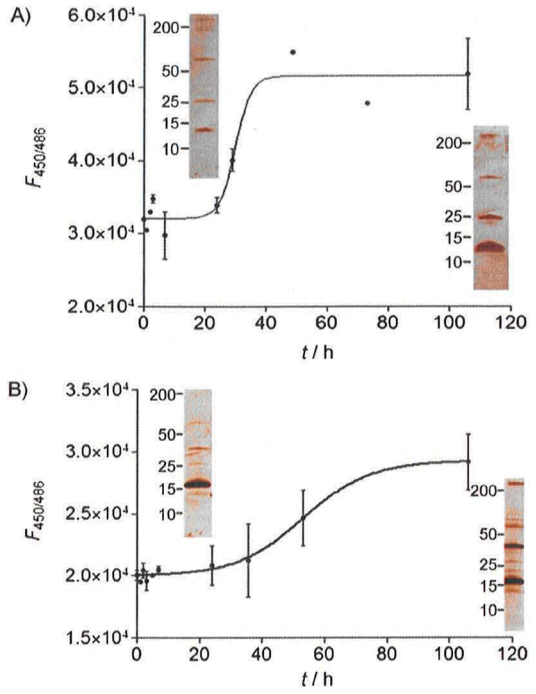

Figure 3. Thioflavin T fibrillization of A) the αSyn(72–140) fragment (**5**) compared to B) intact wt αSyn **1**. Proteins (30 μM) were incubated for five days in Na_2HPO_4 (20 mM, pH 7.5) at 37 °C, and ThT fluorescence was determined at $\lambda_{ex} = 450$ nm and $\lambda_{em} = 486$ nm ($F_{450/486}$). The final assay volume (100 μL) contained 25 μM ThT and 7 μM αSyn sample. The inserts show Tris-tricine-PAGE at the beginning of incubation (left) and after four days (right) with silver nitrate staining.

tion products, might be amenable to examination by using specific functional αSyn residues. Thus, modification of full-length αSyn **1** by amino-succinylation[26] provided a stable derivative succinylated at all Lys residues and at His50, as confirmed by ESI-MS; this succinylated αSyn did not show any aggregation or fragmentation, but did form a dimer (Figure S5). The detailed biochemical evaluation of autoproteolytic products, presently being carried out in our laboratories, might provide a key to the elucidation of the oligomerization-aggregation mechanism of αSyn underlying its neurotoxicity. Moreover, the application of IMS-MS is expected to be highly valuable for identifying proteolytic products in the in vivo aggregation of αSyn.

Experimental Section

Gel electrophoresis of αSyn oligomerization in vitro: αSyn oligomers were prepared by incubation of several batches in triplicate using 5–30 μM solutions for up to 25 days in sodium phosphate buffer (pH 7.5), as previously described.[19] Proteins were solubilized and denatured by using stock solutions of sample buffer (4% SDS, 25% glycerol, 50 mM Tris buffer, 6 M urea; pH 6.8). Gels were run at 100 V until the tracking dye reached the bottom. Separations were performed by tris-tricine PAGE on a MiniProtean-3 cell (BioRad) with 12–15% PAGE, and protein bands were visualized by Coomassie Blue and silver staining (90×60×1 mm gels).

For the protease inhibitor mixture (Complete-Mini; Roche Applied Science) about 50 μM was dissolved in phosphate buffer (1.5 mL, 20 mM; pH 7.5).

Ion mobility mass spectrometry: Ion mobility mass spectrometry was performed on a Synapt-G1 QTOF-mass spectrometer (Waters, Manchester, UK) equipped with an electrospray ionization source. Ions were passed through a quadrupole, either set to transmit a substantial mass range or to select a particular *m/z* ion, before entering the Triwave ion mobility unit—a T-Wave trap unit for ion accumulation. Stored ions were gated (500 μs) into the IMS T-Wave unit for separation according to their mobilities and passed to the T-Wave unit for transfer into the orthogonal-TOF analyzer[15a] (Figure S3 A). The pressures in the T-Wave trap and transfer regions were 7×10^{-2} mbar (argon) and 0.5 mbar (nitrogen), respectively; the sample injection volume was 5 μL. IMS acquisition was performed over the 350–4000 *m/z* range, with 25 V cone voltage, 0.45 bar IMS pressure, and 5–15 V wave height. Sample were injected by using an Advion Triversa Nanomate, as previously described.[18a,b]

Synthesis of αSyn polypeptides: Solid-phase synthesis of αSyn(72–140) **4** was performed on a semiautomated peptide synthesizer EPS-221 (Intavis, Köln, Germany) with a NovaSyn TGR resin; Fmoc protection was employed with double coupling and capping within residues (73–98), followed by mass spectrometric monitoring of intermediate crude products.[21a,27] Fmoc amino acids, NovaSyn TGR resin, and other reagents for activation and protection were obtained from Novabiochem. The general protocol applied was DMF washing followed by deprotection with 20% piperidine in DMF; 8 min deprotection, 30 min coupling. Coupling was carried out in a mixture solution containing benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) and *N*-methylmorpholine (NMM) in DMF; the resin was then washed with DMF and ethanol, and dried under vacuum.[27] Final deprotection of side chains and cleavage from the resin was performed in trifluoroacetic acid/triethylsilane/water (95: 2.5: 2.5, *v/v/v*) for 2 h. Resin and crude peptide were separated by filtration, and the crude peptide was submitted to HPLC purification.

Recombinant expression of full-length αSyn **1** and αSyn(72–140) **4** was performed by using the *E. coli* expression system BL 21 (DE3) [pLys] strain and the T7 RNA polymerase system. Harvested cells were centrifuged (4000 *g*), resuspended in PBS, and heated for 2 min to 100 °C; the cell suspension was centrifuged for 15 min at 4300 *g*, and the protein was redissolved in PBS. HPLC purification of synthetic and recombinant αSyn polypeptides was performed with a BioRad-3000 semipreparative system on a Vydac-C4 column (250×4.6 mm) with a linear gradient (0.1% trifluoroacetic acid with 0→80% acetonitrile) at a flow rate of 1 mL min^{-1} (Figure S4).

Thioflavin-T (ThT) aggregation: Purified αSyn **1** and αSyn(72–140) **5** (7–30 μM) were dissolved in Na_2HPO_4 (20 mM) containing 0.03% NaN_3 (pH 7.5) in microcentrifuge tubes, and triplicate solutions were incubated at 37 °C with agitation. For ThT assays, a sample (23 μL) was withdrawn at regular time intervals. The final assay volume (100 μL) contained ThT (25 μM) and protein (7 μM); a blank of ThT (25 μM) was used as a control. Determinations were performed in 96-well microtiter plates with a Victor-2 fluorescence plate reader (Perkin–Elmer) at $\lambda_{ex} = 450$ nm and $\lambda_{em} = 486$ nm. Data were analyzed with Graph Pad Prism wherein the sigmoidal increase of the ThT fluorescence was analyzed.

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