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Autoproteolytic Fragments are Intermediates in the Oligomerization- Aggregation of 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 ($A\beta$), Tau protein, and alpha-Synuclein (α Syn) the key protein in Parkinson's disease (PD) ^[4-6]. α Syn, a 140-aa protein, occurring 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 α Syn aggregates has been recognized at conditions inducing PD^[8,9]. Although oligomeric intermediates may represent major neurotoxic species^[9], detailed chemical structures of α Syn oligomers and their possible intermediates have not been hitherto identified. The slow rates of formation and the low concentrations of aggregating intermediates^[9] may be major reasons for the failure of conventional methods of mass spectrometry (MS) to detect and identify oligomers.

Although MS (especially with electrospray ionization, ESI) ^[10] is now established in biochemistry and structural biology and is proven highly successful for the structure determination of biopolymers from biological samples^[11], low-concentration intermediates and oligomers in α Syn aggregation have not been identified by HPLC-MS. Nevertheless, oligomeric components were 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]. In applications of IMS-MS to oligomerization-aggregation mixtures of α Syn *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 at the central aggregation domain of α Syn, between residues Val-71 and Thr-72. Aggregation studies of the carboxy-terminal fragment, α Syn(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 α Syn was investigated by incubation at 37 °C in sodium-phosphate (pH 7.5) for up to 25 days using a previously established procedure^[19]. Recombinant α Syn expressed in *E. Coli*^[20] was purified by HPLC to molecular homogeneity, as confirmed by ESI-MS (Figure S1, Supporting Information). The formation of oligomers was monitored by tris-Tricine PAGE (Figure 1) that revealed bands corresponding to monomeric and oligomer-like α Syn having molecular weights of approximately 17, 35, and 48 kDa. In addition, protein bands with molecular weights lower than full length α Syn were observed with slowly increasing abundances within two weeks of incubation, indicating the formation of truncation and/or degradation products. The bands corresponding to α Syn 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, Supporting Information). Sequencing of the tryptic peptides confirmed that the monomers and dimers of α Syn contain full-length sequences, respectively; 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 PVDF membrane, which yielded the first 20 amino acids of intact α Syn. Direct ESI-MS analysis of the α Syn incubation mixture after a short time period (3 h), however, revealed a small amount of N-terminally truncated α Syn lacking the first 6 amino acids (α Syn(7-140); spot **1b** in Figure 1; Table S1 and Figure S2, Supporting Information). 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.

The reaction mixtures upon incubation of α Syn for 4 up to 21 days 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 7 days and showed separation into two peaks corresponding to multiply charged ion series with different drift times (Figure 2A; Figure S3, Supporting Information). 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 α Syn 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 Figure 1 (Figure 2C and Table S2, Supporting Information). In contrast to the intact α Syn proteins, the IMS-MS analysis of peak **1** at a decreased drift time revealed proteolytic products arising by N- and C-terminal truncation, α Syn(14-133) (12162.5 Da) and α Syn(40-140) (10436.4 Da), corresponding to the proteolytic gel bands **1c**, **1e**; Figure 1 and Table S1, Supporting Information). A remarkable fragment was identified in the ion series within peak **1**; it arose by cleavage at residues Val-71 and Thr-72 in the central amyloidogenic domain α Syn(61-93), corresponding to the gel electrophoretic band **1d**

(Figure 1). This carboxy-terminal fragment, α Syn(72-140) was identified by (i), accurate mass determination (7274.412 Da) and tandem-MS sequencing in the IMS-MS experiment (Figure 2C, 2D); (ii), moreover, incubation of α Syn for extended time periods (>14 days) afforded formation of this fragment in amounts sufficient for direct elution of the electrophoretic band and identification by MALDI-MS (Table S1, Supporting Information). Additional characterization of the C-terminal fragments α Syn(40-140) and α Syn(72-140) was obtained by affinity-mass spectrometry^[21] and Western-Blot analysis with a monoclonal α Syn antibody recognizing a C-terminal α Syn epitope, as identified by proteolytic-excision mass spectrometry^[22] (data not shown).

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

The autoproteolytic degradation of α Syn and the significance of the fragment α Syn(72-140) were ascertained by a number of additional mass spectrometric and gel electrophoretic data, and by control experiments with several synuclein polypeptides. Comparative *in vitro* studies of the non-aggregating brain protein, β -synuclein (β Syn) that lacks the central amyloidogenic domain (72-83), showed neither oligomerization-aggregation, nor any autoproteolytic cleavage within 21 days of incubation (Figure 1, **6**); identical expression systems and HPLC purification procedures were employed for β Syn and α Syn, thus excluding α Syn degradation by an external protease. Further results provided evidence to exclude a proteolytic cleavage of α Syn by contaminating proteases; : (i), incubation of α Syn with and without addition of a broad spectrum of protease inhibitors to yield identical autoproteolytic degradation (Figure 1; **1+** and **1**, right lane); (ii), boiling of samples prior to incubation and use of sterile buffers to show no change of α Syn autoproteolysis; and (iii), observation to show autoproteolytic degradation for α Syn mutants **2** and **3** in which the six residues (70-75) were exchanged against Ala and Gly residues (s. Figure 1). Moreover, a recent study on the interaction of α Syn 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 α Syn upon depletion of GCase is abolished in a mutant α Syn lacking the sequence (71-82)^[24].

Further support for the importance of the central domain for the autoproteolysis of α Syn was obtained from initial hydrogen-deuterium exchange mass spectrometry (HDX-MS)^[25] studies using high resolution ESI-MS. HDX-MS of full length α Syn **1** showed rapid exchange (<3 hrs) for 115 of 134 backbone hydrogens while 19 aa remained resistant to exchange for >14 days (data not shown). Remarkably, an identical number of 19 aa in the

α Syn(72-140) fragment **5** was found resistant to HDX (exact determination of the shielded domain is currently 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 α Syn and its autoproteolytic products even though the concentration of the latter is small, enabled the characterization of intermediate fragments in the *in vitro* oligomerization-aggregation; particularly, IMS-MS revealed a possible key fragment, α Syn(72-140). The yet unknown mechanism of autoproteolysis, possibly proceeding via initial N-terminal truncation products observed, may be amenable to examination of specific functional α Syn residues. Thus, modification of full length α Syn **1** by amino-succinylation^[26] provided a stable derivative succinylated at all Lys residues and at His50 as confirmed by ESI-MS; this succinylated α Syn did not show any aggregation or fragmentation, but did form a dimer (Figure S5, Supporting Information). The detailed biochemical evaluation of autoproteolytic products, presently carried out in our laboratories, may provide a key to the elucidation of the oligomerization-aggregation mechanism of α Syn 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 α Syn.

Experimental Section

Gel electrophoresis of α Syn oligomerization in vitro

α Syn oligomers were prepared by incubation of several batches in triplicate using 5 - 30 μ M 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 containing 4 % SDS, 25 % glycerol, 50 mM *tris*-buffer and 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 Mini-Protean-3 cell (BioRad, München, Germany) using 12-15 % PAGE, and protein bands visualized by Coomassie Blue and silver staining (90 $\times$ 60 $\times$ 1 mm gels).

For the protease inhibitor mixture (Complete-Mini; Roche Applied Science, Mannheim, Germany) ca. 50 μ M was dissolved in 1.5 mL 20 mM phosphate buffer 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 comprising a T-Wave trap unit for ion accumulation; stored ions were gated (500 μ sec) 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; Supporting Information). Pressures in the T-Wave trap and transfer regions were 7e-2 mbar (Ar) and 0.5 mbar (nitrogen), respectively; sample injection volume was 5 μ L. IMS acquisition was performed at 350 - 4000 *m/z* range, 25 V cone voltage, 0.45 bar IMS pressure, and 5-15 V wave height. Sample injection was performed by using an Advion Triversa Nanomate, as previously described^[18a,b].

Synthesis of α Syn polypeptides

Solid-phase synthesis of α Syn(72-140) **4** was performed on a semi-automated peptide synthesizer EPS-221 (Intavis, Köln, Germany) using 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 (Laufelfingen, Switzerland). 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 PyBOP and *N*-methylmorpholine (NMM) in DMF; the resin was then washed with DMF, ethanol and dried under vacuum^[27]. Final deprotection of side chains and cleavage from the resin was performed with 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 submitted to HPLC purification.

Recombinant expression of full length α Syn **1** and α Syn(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, resuspended in PBS and heated for 2 min to 100 °C; the cell suspension was centrifuged for 15 min at 4300 rcf and the protein redissolved in PBS buffer. HPLC purification of synthetic and recombinant α Syn polypeptides was performed with a BioRad-3000 semipreparative system (BioRad, Richmond, CA) on a Vydac-C4 column (250 × 4.6 mm) with a linear gradient (0.1 % TFA/0.1 % TFA/80% acetonitrile) at a flow rate of 1 mL/min (Figure S4; Supporting Information).

Thioflavin-T (ThT) aggregation

Purified α Syn **1** and α Syn(72-140) **5** were dissolved in 20 mM Na₂HPO₄ containing 0.03% NaN₃ (pH 7.5) at 7 up to 30 μ M in microcentrifuge tubes, and triplicates incubated at 37 °C with agitation. For ThT assay a 23 μ L sample was withdrawn at regular time intervals. The final assay volume (100 μ L) contained 25 μ M ThT/7 μ M protein; a blank of 25 μ M ThT was used as a control. Determinations were performed in 96-well MTP with a Victor-2 fluorescence plate reader (Perkin-Elmer, Überlingen, Germany) at 450 nm excitation and 486 nm emission. Data were analyzed with Graph Pad Prism wherein the sigmoidal increase of the ThT fluorescence was analyzed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Time-dependent autoproteolytic degradation of α Syn and modified α Syn polypeptides visualized by Coomassie (left) and silver staining (right), at the beginning of incubation (A), 4 days (B) and 13 days (C). **1**, wt- α Syn; **2**, recombinant α Syn(Ala₆(70-75)); **3**, recombinant α Syn(Gly₆(70-75)); **4**, α Syn(72-140) synthesized by SPPS; **5**, recombinant α Syn(72-140); **6**, recombinant β Syn. Full length α Syn monomer and dimer are denoted **1a** and **1a'**, respectively; N-terminally truncated α Syn(7-140) is denoted **1b**. α Syn fragments are denoted **1c**, **1d**, **1e**; **1c'** is the dimer of fragment **1c**. β -Synuclein **6** lacks the (72-83) sequence. **1** and **1+** denote incubation of wt- α Syn without and with addition of protease inhibitors (serine-, cysteine-proteases; metalloproteases).

Figure 2. ESI-IMS- MS identification of full length α Syn and fragment α Syn(72-140) in the incubation mixture of wt- α Syn after 7 days. (A), IMS-MS Drift time vs. m/z of α Syn incubation mixture in PBS buffer (pH 7.5) after seven days at 37 °C. (B), Deconvoluted mass spectrum of ions corresponding to peak 1, proteolytic fragment α Syn(72-140); (C), Deconvoluted mass spectrum of ions corresponding to peak 2 showing full length α Syn monomer and dimer; (D), Tandem-MS sequence determination of α Syn(72-140), peak 1, showing the partial sequence (101-119).

Figure 3.

Thioflavin T fibrillization of the αSyn(72-140) fragment **5** (A) compared to intact wt- αSyn **1** (B). Proteins were incubated at 30 μM concentrations for 5 days in 20 mM Na₂HPO₄ pH 7.5 at 37°C, and ThT fluorescence determined 450 nm excitation and 486 nm emission ($F_{450/486}$). The final assay volume (100 μL) contained 25 μM ThT and 7 μM αSyn sample. The inserts show *tris*-Tricine-PAGE at the begin of incubation (left) and after 4 days (right) using silver nitrate staining.