Structural characterization of β -amyloid oligomer-aggregates by ion mobility mass spectrometry and electron spin resonance spectroscopy

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Abstract Formation and accumulation of fibrillar plaques and aggregates of β -amyloid peptide (A β) in brain have been recognized as characteristics of Alzheimer's disease (AD). Oligomeric aggregates of A β are considered critical intermediates leading to progressive neurodegeneration; however, molecular details of the oligomerization and aggregation pathway and the structures of A β -oligomers are hitherto unclear. Using an *in vitro* fibril formation procedure of A β (1–40), β -amyloid aggregates were prepared and insoluble aggregates separated from soluble products by centrifugation. In this study, ion mobility mass

spectrometry (IM-MS) was applied in combination with electron paramagnetic resonance spectroscopy (EPR) to the identification of the components of A β -oligomers, and to their structural and topographical characterization. The formation of A β -oligomers and aggregates was monitored by gel electrophoresis, and A β -oligomer bands were identified by *in-gel* tryptic digestion and matrix-assisted laser desorption ionization–mass spectrometry (MALDI-MS) to consist predominantly of A β (1–40) peptide. First, ion mobility-MS studies of soluble A β -aggregates prepared by incubation for 5 days were performed on a quadrupole time-of-flight mass spectrometer and revealed (1) the presence of at least two different conformational states, and (2), the formation of Met-35 oxidized products. For estimation of the size of A β -aggregates using EPR spectroscopy, a modified A β (1–40) peptide containing an additional N-terminal cysteine residue was prepared, and a 3-(2-iodoacetamido)-2,2,5,5-tetramethyl-1-pyrrolidinyloxy radical spin label derivative (IPSL) was coupled by S-alkylation. The EPR spectra of the spin-labeled Cys-A β (1–40) oligomers were matched with spectra simulations using a multi-component simulation strategy, resulting in complete agreement with the gel electrophoresis results.

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Introduction

A significant proportion of elderly population is affected by progressive neurodegenerative disorders. Formation and accumulation of fibrillar plaques and aggregates of

β -amyloid peptide (A β) and α -synuclein (Syn) in brain have been recognized as characteristics of Alzheimer's disease (AD) and Parkinson's disease (PD) [1–3]. A β is a polypeptide containing 39–43 amino acid residues derived from proteolytic cleavage of the transmembrane A β precursor protein (APP). Recently, the formation of A β -oligomers has become of particular interest, since oligomers have been suggested to be key neurotoxic species for progressive neurodegeneration [2, 4, 5]; however, molecular details of the pathophysiological degradation of APP, and of A β -aggregation pathways are hitherto unclear [6]. Despite the lack of molecular mechanism(s), studies towards the development of immunotherapeutic approaches for AD [7] have shown initial success in the production of therapeutic antibodies that disaggregate A β -fibrils and improve the memory impairment in transgenic AD mice [8–11]. The identification of the epitopes recognized by both A β -plaque specific and aggregation-preventing antibodies, and the discovery of their A β -oligomer specificity are presently causing enhanced interest in the elucidation of A β -oligomeric structures [12–16].

Ion mobility mass spectrometry (IM-MS) is a new MS method that has received increasing attention as a tool for the characterization of molecular assemblies according to their conformation and/or topography [17–20]. The IM-MS instrument employed in this study consists of two parts, (a) an ion mobility drift cell where ions are separated within an electric field according to their collisional cross-section, and (b) a quadrupole time-of-flight mass spectrometer (Synapt-QTOF-MS) [21–23]. Thus, the IM-MS implements a new mode of separation that allows the differentiation of protein conformational states.

EPR spectroscopy is a technique for studying chemical species with unpaired electrons that has recently shown to be an efficient bioanalytical tool for determination of molecular distances and molecular size estimations from spin motion measurements [24–30]. This technique has recently shown high sensitivity compared to the traditional techniques for protein structure determination, such as NMR spectroscopy [31]. In this study, ion mobility-MS in combination with EPR was applied to the study of A β -oligomers prepared *in vitro* from A β (1–40). For EPR studies of A β -oligomers and aggregates, *N*-cysteinyl-A β (1–40) peptides were synthesized by solid phase peptide synthesis (SPPS) and an 3-(2-iodoacetamido)-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (iodo-proxyl) spin label derivative (IPSL) was attached to the N-terminal cysteine residue by alkylation. The complementary application of IM-MS and EPR provided first molecular details of A β -oligomers by the identification of oxidative structure modifications, and an estimation of the size and shape of A β -oligomers.

Materials and methods

Synthesis and purification of spin-labeled A β -peptide derivatives

A β (1–40) (H-DAEFRHDSGYEVHHQKLVFFAEDVGSNK GAIIGLMVGGVV-NH₂) and Cys-A β (1–40) were synthesized by SPPS on a semiautomatic peptide synthesizer EPS 221 (Abimed/Intavis, Langenfeld, Germany), according to the Fmoc strategy. The synthesis was carried out on a NovaSyn® TGR resin, with a loading capacity 0.33 mmol/g. The spin label used was 3-(2-iodoacetamido)-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (Iodo-Proxyl; IPSL; Sigma-Aldrich, München, Germany). The labeling reaction was performed using a tenfold molar excess of IPSL in 25 mM Tris buffer, pH 9, by incubation for 12 h at 4°C [32]. The synthetic A β peptides and the labeling reaction mixture were purified by reverse-phase high-performance liquid chromatography (RP-HPLC; Fig. 1) on a semi-preparative Vydac C4 column. The mobile phases were: 0.1% TFA in milliQ water (eluent A) and 80% acetonitrile in eluent A (eluent B); a linear gradient of 20–80% eluent B was used in 30 min at a flow rate of 4 mL/min.

Mass spectrometry

Synthetic A β -peptides were separated by HPLC and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) on a Bruker Daltonik Biflex ITM linear TOF mass spectrometer (Bruker Daltonik, Bremen, Germany). Aliquots of 0.8 μ L of peptide sample were mixed with 0.8 μ L 4-hydroxy α -cyano cinnamic acid (HCCA) in AcCN:0.1% TFA in water 2:1 (v:v). Upon lyophilization, peptide samples were redissolved in 0.2% formic acid at a concentration of 0.1 μ g/ μ L. HPLC was performed on an Agilent 1100 instrument (Agilent Technologies, Waldbronn, Germany) equipped with a C8 column (Vydac 150 $\times$ 1 mm, 5 μ m), using a flow rate of 50 μ L/min. The following gradient was used: equilibration step, 2% B for 5 min; linear gradient from 2% B to 65% B in 63 min; from 65% B to 98% B in 10 min; 10 min washing step 98% B; 10 min to return to the initial concentration of 2% B (solvent A, 0.2% formic acid in water, solvent B, 0.2% formic acid in acetonitrile). Electrospray mass spectrometry was performed on an Esquire 3000+ ion trap spectrometer (Bruker Daltonik, Bremen, Germany) at the following conditions: capillary temperature 250°C, nebulizer gas 20 psi (Ar), drying gas 9 l/min (N₂), potential difference 4 kV (positive-ion mode), endplate offset 500 V, skimmer 40 V, and capillary exit 136 V. The ion trap was locked on automatic gain control, and six microscans were collected for

Fig. 1 HPLC profiles (crude products) and MALDI-TOF mass spectra of HPLC-purified **a** Cys-A β (1–40) and **b** IPSL-Cys-A β (1–40) peptides

each full MS scan with a maximum accumulation time of 200 ms for each ion.

Preparation of A β -fibrils

For A β -oligomerization and fibril formation, A β (1–40) and PSL-Cys-A β (1–40) peptides were solubilized at a concentration of 1 μ g/ μ L in a buffer containing 50 mM Na₃PO₄, 150 mM NaCl, 0.02% NaN₃ at pH 7.5 [30]. Because of the low solubility of A β -fibrils, several sonication/vortex cycles were performed to ensure complete solubilization. Each solution was incubated for 5 days at 37°C, yielding a white precipitate at the end of the incubation period. The precipitate was briefly sonicated, centrifuged 15 min at 13,000 rpm, and the supernatant removed and replaced with an equal volume of deionized water (MilliQ). Both the

supernatant and the resuspended precipitate were subjected to gel electrophoresis and EPR spectroscopy experiments.

Gel electrophoresis

For in-gel proteolytic digestion and mass spectrometry, the resuspended precipitate fraction was subjected to SDS-PAGE separation. The fibril fraction (10 μ L) was mixed with 10 μ L sample buffer, 4% SDS, 25% glycerol, and bromophenol blue staining reagent. After loading on a 15% gel, electrophoresis was developed for 15 min at 60 V, and subsequently for 1–2 h at 100 V.

Gel electrophoresis of the spin-labeled IPSL-Cys-A β (1–40) fibril preparation was carried out with a freshly prepared solution in fibril growth buffer, and a sample after incubation for 5 days (supernatant and

resuspended precipitate). 1D Tris-tricine polyacrylamide gel electrophoresis (15%) was run as described above and visualized by Coomassie blue staining.

In-gel tryptic digestion and peptide extraction

Protein spots were cut from the gel (Fig. 2a, bands 1–4), washed with MilliQ water, and the gel pieces shaken for 30 min at 25°C with 60% acetonitrile to dehydrate the gel, and then dried in a Speed Vac centrifuge (30 min). Protein spots were destained by addition of 50 mM NH_4HCO_3 (15 min), dehydrated with 60% acetonitrile solution (15 min) and dried in a Speed Vac centrifuge (30 min).

The gel pieces were then swollen in digestion buffer (12.5 ng trypsin/ μl 50 mM NH_4HCO_3) at 4°C (on ice) for 45 min, and incubated at 37°C overnight (12 h). After removal of supernatant, peptide extraction was performed at 25°C with a solution of 60% acetonitrile, 0.1% TFA in MilliQ water (three steps of 1 h each). The tryptic digestion mixtures were analyzed by MALDI-TOF MS, and data were searched against the NCBI protein database by means of the Mascot MS/MS ion search engine.

Ion mobility mass spectrometry

Ion mobility mass spectrometry was performed with a Waters SYNAPT quadrupole time-of-flight (QTOF) mass

Fig. 2 ESI-mass spectra (LC-MS) of **a** A β (1–40), **b** Cys-A β (1–40), and **c** IPSL-Cys-A β (1–40)

Fig. 3 1D-Gel electrophoresis (Coomassie blue staining) of A β -aggregates in vitro. **a** SDS-PAGE of A β (1–40) after 5 days incubation at 37°C; *band 1* A β (1–40) monomer, *band 2* A β -oligomer, *bands 3 and 4* high-molecular-weight aggregates (“soluble protofibrils”); *bands 3 and 4* being only partially separated. **b** Tris-tricine PAGE of IPSL-Cys-A β (1–40) after 5 days incubation at 37°C. *Lane 1* a freshly prepared IPSL-Cys-A β (1–40) solution (in fibril growth buffer), *lane 2* precipitate after 5 days incubation; only the soluble part entered the separation gel, the rest remaining in the stacking gel, *lane 3* supernatant after 5 days incubation. In *band 1* the monomeric IPSL-Cys-A β (1–40) is present, in *band 2* the dimer, in *band 3* the trimer, and in *band 4* high-molecular-weight aggregates (“soluble protofibrils”)

Table 1 Identification of A β -fragments in oligomer bands separated by gel electrophoresis (cf. Fig. 3a) by MALDI-TOF-MS

Gel band no.	Tryptic peptide masses [M+H] ⁺	A β -peptide sequences
1	1,084.6	[29–40]
	1,326.0	[17–28]
	1,337.0	[6–16]
	2,393.7	[17–40]
	2,643.6	[6–28]
2	1,084.7	[29–40]
	1,326.0	[6–16]
	1,337.0	[17–40]
3	1,085.8	[29–40]
	1,336.3	[6–16]
	2,392.6	[17–40]
4	1,085.6	[29–40]
	1,326.5	[17–28]
	1,337.3	[6–16]
	2,393.4	[17–40]
	3,709.9	[6–40]

spectrometer (Waters Corp., Manchester, UK). A freshly prepared A β (1–40) peptide solution (1 μ g/ μ L), and the supernatant fraction from *in vitro* fibril preparation (5 days incubation at 37°C) were analyzed by injection of 5 μ L (0.5 μ g/ μ L) in a desalting cartridge, and elution for 10 min at 20 μ L/min with a gradient of acetonitrile from 10% to 90%. Ion mobility-MS was performed in the m/z range of 350–4,000 at a pressure of 0.45 bar, cone voltage of 25 V, and a drift voltage (wave height) of 5 to 15 V.

Electron paramagnetic resonance spectroscopy

The freshly prepared IPSL-Cys-A β (1–40) solution (in fibril growth buffer), and a sample incubated for 5 days as described above (precipitate and supernatant) were used for EPR spectroscopy. EPR spectra were obtained on a MiniScope MS200 (Magnetech GmbH, Berlin, Germany) spectrometer at 25°C. An accumulation of 60 scans was carried out at 1 min/scan; the modulation amplitude was set at 400 G and the attenuation at 23 dB. Following acquisition, the spectra were fitted by simulations using the EasySpin package [33] and Matlab (The MathWorks Inc., Natick, Massachusetts, United States) software. From these simulations, multiple fractions featuring significantly

different correlation times were extracted. For all of these fractions, the following simulation parameters were kept constant: g -tensor $g=[2.01\ 2.0068\ 2.0027]$, hyperfine tensor $A=[13\ 13\ 108.4]$ MHz, and intrinsic linewidth 0.14 mT.

Results and discussion

Synthesis and characterization of spin-labeled A β -peptides

A β (1–40) and *N*-cysteiny-A β (1–40) peptides were synthesized by SPPS at the 100 μ M scale according to the Fmoc strategy, and the crude products purified by RP-HPLC (Fig. 1). A β (1–40) was separated at a retention time of 21.3 min at the HPLC conditions employed, while the Cys-A β (1–40) derivative was isolated with a slightly different retention time of 20.5 min (Fig. 1a). For the preparation of the IPSL-spin-labeled peptide derivative (see Fig. 1b), the Cys-A β (1–40) crude product was used

Fig. 4 MALDI-MS of in-gel tryptic digestion mixture of A β (1–40), high-molecular-weight aggregates (*band 4* from Fig. 2a). **a** MALDI-TOF mass spectrum of tryptic digestion mixture; **b** database search result with identified peptide fragments

Fig. 5 Ion mobility-MS analysis of a freshly prepared A β (1–40), **b** supernatant after 5 days of incubation; **c, d** extracted ion mobility profiles for m/z 866.6, $[M+5H]^{5+}$ from freshly prepared A β (1–40) and supernatant

directly for alkylation with IPSL, in order to minimize oxidation and disulfide bridge formation at the N-terminal cysteine residue. To prevent disulfide formation, the IPSL derivative was used at a tenfold molar excess compared to the A β -peptide; at these conditions no dimeric A β -peptide derivatives were observed. The HPLC profile showed the presence of two peaks at 13.6 and 21.5 min, respectively (Fig. 1b); however, MALDI-MS analyses confirmed the presence of only one A β -peptide at 21.5 min due to the IPSL-Cys-A β (1–40), while the compound at a retention time of 13.6 min was found to be unreacted spin-label reagent.

The HPLC-purified peptides, A β (1–40), Cys-A β (1–40), and IPSL-Cys-A β (1–40) were also subjected to LC-MS analysis (Fig. 2) which confirmed the expected molecular masses. In all cases, the multiply charged molecular ions (6+ and 7+) were found to be the most abundant ions, with loss of one water molecule as the only detectable fragmentation.

Gel electrophoresis and mass spectrometric characterization of in vitro A β -oligomer formation

Due to the insolubility of A β -aggregates, fibril formation cannot be studied directly by mass spectrometry. In order to determine the molecular composition of aggregates, in-gel tryptic digestion and mass spectrometric analysis of the A β -oligomeric gel bands was performed. A β (1–40) was solubilized at 1 μ g/ μ L (220 μ M) in fibril growth buffer solution, pH 7.5 and incubated for 5 days at 37°C. At the end of the incubation, fibrils were obtained as a white precipitate, sonicated, and centrifuged for 15 min, as described in the “Materials and methods” section, and the supernatant was separated from the precipitate for ion mobility-MS. The precipitate was separated from the supernatant, resuspended in MilliQ water, and subjected to polyacrylamide gel electrophoresis (Fig. 3). Only the fraction soluble in running buffer entered the gel (Fig. 3a) while the insoluble part remained at the top of the stacking gel (data not shown). This soluble fraction was found by MALDI-MS to contain monomeric A β (Fig. 3a, band 1), A β -oligomers at approximately 20 kDa (pentamer, Fig. 3a—band 2), and high-molecular-weight aggregates at >100 kDa (Fig. 3a, bands 3 and 4). The A β -oligomers in Fig. 3a were not separated by SDS-PAGE, but provided separation into defined A β -oligomers (dimers to pentamers) in Tris-tricine PAGE (data not shown). All gel bands were cut out, digested with Trypsin and analyzed by MALDI-MS. The mass spectrometric data ascertained the presence of A β -peptides in all gel bands by the corresponding peptide fragments (Table 1 and Fig. 4).

The preparation of aggregates of the spin-labeled IPSL-Cys-A β (1–40) peptide was carried out at identical con-

ditions, by incubation of a 200 μ M solution for 5 days at 37°C. Following incubation, supernatant and precipitate were separated by centrifugation and the precipitate suspended in MilliQ water for gel electrophoresis. Tris-tricine PAGE analysis was performed with a freshly prepared IPSL-Cys-A β (1–40) solution in fibril growth buffer, the supernatant and the fibril suspension of the spin-labeled A β -aggregates (Fig. 3b). The gel showed that the IPSL-Cys-A β (1–40) peptide started to aggregate already during the solubilization, as indicated by dimeric and trimeric gel bands (Fig. 3b, lane 1). From the fibril precipitate, only the fraction soluble in running buffer entered the separation gel (Fig. 3b), the insoluble part remaining at the top of the stacking gel (data not shown). In the gel, the presence of monomer, dimer, and trimer, and some high-molecular-weight aggregates with a molecular

Fig. 6 Drift-time profile of the A β (1–40) supernatant after 5 days of incubation. **a** The ion mobility profile for the $[M+5H]^{5+}$ ion; **b** the drift scope and **c** deconvoluted spectrum showing the presence of the Met-35 sulfoxide oxidation product

Fig. 7 EPR spectra of **a** freshly prepared A β (1–40), **b** the precipitate, and **c** the soluble fraction (supernatant) after 5 days of incubation (gray) and corresponding simulations (black; see Table 2 and “Materials and methods” section for parameters)

weight beyond 200 kDa were observed (Fig. 3b, lane 2). In contrast, the supernatant showed only the band of the A β monomer (Fig. 3b, lane 3).

Characterization of A β -oligomers by ion mobility mass spectrometry

The soluble fraction of the A β (1–40) fibril preparation, obtained by A β -incubation for 5 days at 37°C, and a freshly prepared A β (1–40) peptide solution (220 μ M) were subjected to comparative analysis by ion mobility-MS (Fig. 5a and b). In the freshly prepared A β (1–40) solution, the $[M+5H]^{5+}$ ion was found predominant, while in the fibril preparation the $[M+6H]^{6+}$ ion was most abundant. The signal/noise ratio of the $[M+5H]^{5+}$ and $[M+6H]^{6+}$ ions was lower in the fibril preparation sample than in the freshly prepared A β (1–40) sample, suggesting a lower amount of A β (1–40) monomer due to the aggregate formation. The extracted ion mobility profiles for the $[M+5H]^{5+}$ ion, of the freshly prepared A β (1–40; Fig. 5c) and the fibril preparation (Fig. 5d) indicate the presence of two conformational states (A and B), with different ion mobilities, which may be indicative of the oligomerization process.

The extracted ion mobility plot for the $[M+5H]^{5+}$ ion of the fibril preparation is presented in Fig. 6a, b together with the drift scope of the 5+, 6+, and 7+ ions. The deconvoluted spectrum of the fibril preparation is shown in Fig. 6c. From the mass spectrometric data of the ion mobility profiles, modifications of A β by oxidation and loss of one water molecule could be identified, in addition to the intact A β (1–40) monomer. The oxidation at the Met³⁵ residue of A β was confirmed by MS/MS sequence determination (data not shown).

Characterization of spin-labeled A β -aggregates by electron paramagnetic resonance spectroscopy

First EPR spectroscopic determinations of the spin-labeled A β monomer peptide, in comparison with the A β -fibril preparations, were performed with the aim to obtain information about the size of A β -aggregates monitored by the local spin-label mobilities. EPR spectra of the freshly

Table 2 Fractions contributing to the EPR spectra as derived from simulation parameters (cf. Fig. 7)

	Component 1: 0.036 ns	Component 2: 0.394 ns	Component 3: 0.803 ns	Component 4: 5.46 ns
Freshly prepared (7a)	0.19	0.30	0.51	0.00
Precipitate (7b)	0.01	0.02	0.21	0.76
Supernatant (7c)	1.00	0.00	0.00	0.00

For each fraction the rotational correlation time is given

prepared solution of IPSL-Cys-A β (1–40), the precipitate residue and the soluble A β -fibril preparation are compared in Fig. 7a–c). All spectra were quantitatively analyzed by spectral-lineshape-simulations. The supernatant spectrum (Fig. 7c) could be described by a single component, the other spectra consist of at least two additional components which were significantly broadened relative to the supernatant spectrum. This broadening reflects the reduced mobility of the spin-label-side group due to oligomerization and/or aggregation.

Using a multi-component simulating strategy a number of spectral components was chosen in accordance with the gel electrophoretic results in order to simulate the spectra. The fractions of the contributing components as well as their rotational correlation times could be identified (Table 2). The supernatant of the fibril preparation sample was found to contain only one component which could be attributed to the A β -monomer, again in agreement with the gel electrophoresis data (Fig. 3b, lane 3). In the freshly prepared IPSL-Cys-A β (1–40) solution, the corresponding, A β -monomer component was found only with a relative amount of approximately 19%. In addition, two further contributions presumably representing oligomers were found. In the fibril precipitate, the identical mixture of components was found, but the main component (76%) featured a very slow rotational correlation time suggesting that this component can be attributed to fibrils. Thus, the EPR data were in complete agreement with the gel electrophoretic separation of A β -aggregates, suggesting that aggregation starts rapidly upon solubilization of monomeric A β -peptide in the fibril growth buffer. In the precipitate, almost exclusively high-molecular-weight compounds were found.

Conclusions

In the present study, ion mobility-MS in combination with electron paramagnetic resonance spectroscopy was explored for the characterization of the oligomerization and fibril formation reaction of β -amyloid in vitro. The results obtained here indicate the combination of both methods as an efficient tool to characterize the composition of oligomerization products, and the molecular dimensions of aggregate products. The EPR spectra and gel electrophoresis data were in complete agreement to show that (a) A β -aggregation in vitro begins rapidly upon solubilization of the A β -monomer, and (b) the insoluble fibril product contains large aggregates; while in the supernatant, the soluble A β -monomer is predominant. The ion mobility-MS data provide evidence of (a) the presence of at least two different conformational forms involved in A β -aggregation, and (b) the formation of a Met³⁵ oxidized A β peptide

during the oligomerization and aggregation. Hence, the complementarity of both methods appears promising for the characterization of the A β -oligomerization and aggregation pathway. Corresponding studies using suitably modified A β -peptides is presently carried out in our laboratory.

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