Spin-label EPR on Disordered and Amyloid Proteins

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CHAPTER 4

THE AGGREGATION POTENTIAL OF 1-15 AND 1-16 FRAGMENTS OF THE AMYLOID β PEPTIDE AND THEIR INFLUENCE ON THE AGGREGATION OF Aβ40

The aggregation of amyloid β (Aβ) peptide is important in Alzheimer's disease. Shorter Aβ fragments may reduce Aβ's cytotoxicity and are used in diagnostics. The aggregation of Aβ16 is controversial; Liu et al. (*J. Neurosci. Res.* **2004**, 72, 162-171) and Liao et al. (*FEBS Lett.* **2007**, 581, 1161-1165) find that Aβ16 does not aggregate and reduces Aβ's cytotoxicity, Du et al. (*J. Alzheimers Dis.* **2011**, 27, 401-413) reports that Aβ16 aggregates and that Aβ16 oligomers are toxic to cells. Here the aggregation potential of two shorter fragments, Aβ15 and Aβ16, and their influence on Aβ40 is measured by Electron Paramagnetic Resonance (EPR) spectroscopy and the Thioflavin T fluorescence assay (ThioT). Continuous wave, 9 GHz EPR measurements and ThioT results reveal that neither Aβ15 nor Aβ16 aggregate by themselves and that they do not affect Aβ40 aggregation.

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4.1 Introduction

Fibrillar plaques and aggregates of the 39 to 42 amino-acid residue amyloid β (Aβ) peptide in the brain have been recognized as major characteristics of Alzheimer's disease [1-4]. The Aβ peptide originates from a proteolytic cleavage of the amyloid precursor protein (APP), a human transmembrane protein crucial for memory [3,5]. Recently, more than about 20 shorter Aβ fragments, comprising residues 1-15, 4-15, 5-15, 14-15, 1-13, 1-14, etc. of the N-terminus of the full-length Aβ (table 4.1), have been reported in the cerebrospinal fluid (CSF), in addition to the previously reported Aβ1-16 fragment (Aβ16) [6-9]. Some of these fragments are up-regulated in Alzheimer's disease [6,7]. The soluble Aβ16 fragment in the brain results from the cleavage between amino acids K_{16} and L_{17}, the proposed α-secretase cleavage site in the Aβ sequence. Amongst the 20 Aβ fragments that are upregulated, 11 fragments with different length end at amino acid 15, one amino acid before the α-secretase cleavage site, which suggests a novel metabolic pathway for APP [6,7]. The short Aβ fragments draw a lot of attention especially in the search for peptide or peptidomimetic inhibitors of Aβ aggregation in the pathological context [8,10,11]. For example the Aβ1-15 fragment (Aβ15) can be used as a vaccine [12,13]. However, the precise sequence of events through which these short fragments form and their role in aggregation and toxicity of the full-length Aβ are still under debate. Reports about the aggregation potential of the N-terminal Aβ fragments by themselves are discrepant. According to Liu et al. [14] and Liao et al. [15], Aβ16 does not aggregate and reduces Aβ's cytotoxicity in neuronal cells, whereas Du et al. [16] report that Aβ16 aggregates and that Aβ16 oligomers are toxic to cells. Du et al. [16] also show that Aβ15 forms aggregates, which are not toxic to cells. These conflicting results, obtained by NMR [17-19], FTIR, AFM, and CD [14-16] prompted us to investigate the behavior of these N-terminal Aβ fragments by a different technique. We used spin-label EPR and ThioflavinT fluorescence assay to study the fragments Aβ15 and Aβ16 (for sequence see table 4.1). Earlier, it was shown that signatures of the oligomeric Aβ peptide can be detected by the spin-label EPR methodology, which suggests this technique as a possible tool to detect the early stages of aggregation of the Aβ peptide [20]. Here we employ spin-label EPR to investigate and compare the aggregation potential of Aβ15 and of Aβ16, and their influence on the aggregation of Aβ40. We combined spin-label EPR with diamagnetic dilution, in which the spin-labeled Aβ peptide (SL-Aβ) is diluted with unlabeled Aβ peptide (wild type Aβ peptide) to avoid line broadening by spin-spin interaction [20-22].

Table 4.1 The amino-acid sequence of the full-length Aβ peptide (Aβ40) and the two fragments, Aβ15 and Aβ16 studied here. Residues 15 and 16 of Aβ peptide are glutamine and lysine, respectively. The cysteine variants (Cys-Aβ) of each of the three peptides with an additional cysteine at the N-terminus are used for spin labeling.

Aβ peptide	**amino-acid sequence**
Cys-Aβ15	$CD_1A_2E_3F_4R_5H_6D_7S_8G_9Y_{10}E_{11}V_{12}H_{13}H_{14}Q_{15}$
Cys-Aβ16	$CD_1A_2E_3F_4R_5H_6D_7S_8G_9Y_{10}E_{11}V_{12}H_{13}H_{14}Q_{15}K_{16}$
Cys-Aβ40	$CD_1A_2E_3F_4R_5H_6D_7S_8G_9Y_{10}E_{11}V_{12}H_{13}H_{14}Q_{15}K_{16}L_{17}V_{18}F_{19}F_{20}A_{21}E_{22}D_{23}V_{24}G_{25}S_{26}$ $N_{27}K_{28}G_{29}A_{30}I_{31}I_{32}G_{33}L_{34}M_{35}V_{36}G_{37}G_{38}V_{39}V_{40}$

By spin-label EPR, the mobility of the peptide is directly monitored enabling the detection of even small amounts (< 10 %) of aggregate, which would be difficult by the methods currently employed [23,24]. We demonstrate that under the conditions of our experiments neither Aβ15 nor Aβ16 aggregate and that they do not seem to affect full-length Aβ aggregation.

4.2 Materials and methods

The Aβ40 peptide and its cysteine-Aβ variant (H-Cys-Asp-Ala-…-Val-OH) were purchased from AnaSpec (purity > 95 %). The Aβ15 and Aβ16 and their cysteine-Aβ variants were purchased from Peptide 2.0 Inc, Chantilly, VA (purity > 95 %), the solvent DMSO was purchased from Biosolve (purity 99.8 %). the MTS spin label ((1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl) methanethiosulfonate) was purchased from Toronto Research Chemicals Inc. (Brisbane Rd., North York, Ontario, Canada, M3J 2J8). Spin labeling was performed and the purified spin-labeled Aβ was analyzed by liquid chromatography as described previously [20]. The peptides were lyophilized and stored in the freezer (-20° C) until used.

4.2.1 Sample preparation protocol

The Aβ peptide samples in phosphate-buffered saline (PBS, 12 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4 at pH 7.4) were prepared as diamagnetically diluted (dd) samples according to the protocol reported before [20]. Typical samples contained a mixture of 86 % wild type Aβ and 14 % SL-Aβ. Two peptide concentrations (0.55 mM and 1.1 mM) were investigated. For the shorter peptides, the spin-labeled shorter peptides were mixed either with the respective wild type peptide or with the wild type Aβ40. A typical sample of SL-Aβ15 with wild type Aβ40 had the following concentrations: 0.077 mM SL-Aβ15, 0.47 mM wild type Aβ15 (dd-SL-Aβ15) and 0.55 mM wild type Aβ40. To investigate the

effect of shorter peptides on the Aβ40 peptide, either of the wild type shorter peptides was added to the SL-Aβ40 sample.

4.2.2 EPR experiments

Samples of 10-15 µl peptide solution were drawn into Blaubrand 50 µl capillaries. The X-band continuous wave EPR measurements were performed using an ELEXSYS E680 spectrometer (Bruker, Rheinstetten, Germany) equipped with a rectangular cavity. A modulation frequency of 100 kHz was used for all measurements; the accumulation time for the spectra was 40 minutes per spectrum. Samples were measured at room temperature (20° C) using 6.331 mW microwave power and a modulation amplitude of 1.4 G. The large modulation amplitude ensured a better signal-to-noise ratio for broad lines. The measurements were made immediately after dissolving in PBS. Samples were kept at room temperature in these capillaries for 10 days without agitation and measured again after two, seven, and 10 days to monitor time dependent effects. In all cases, the spectra were identical to those measured initially (data not shown).

4.2.3 Simulations of EPR spectra

The spectra were simulated using Matlab and the EasySpin package [25]. For the simulation the following parameters were used: g = [2.00906, 2.00687, 2.00300] [20,26] and A_{xx}= A_{yy} = 12 and 13 MHz in DMSO and buffer, respectively. Over-modulation effects were taken into account in EasySpin. According to the simulation, the EPR spectra were composed of three components of different mobilities. These components were referred to as fast, medium, and slow according to their rotation correlation times (τ_r) as reported before [20].

4.2.4 Ratio of the intensity of the "fast" and "slow" components in each spectrum

Another way to define the mobility of the spin label, rather than by the relatively time-consuming simulations, is to measure amplitude ratios directly from the spectra [20,27,28]. Here we use the amplitude ratio of the mobile component to the strongly immobilized component. The contribution of these two components was obtained by selecting specific positions in the EPR spectra (B_{0slow} = 334.3 ± 0.1 mT; B_{0fast} = 337.3 ± 0.1 mT) at which one component has a large amplitude and the other a small one. The ratio of the fast to slow component, i.e., the ratio of the amplitudes of the two selected spectral positions (see results) gives an indication of the aggregation state of the peptide. The larger the ratio, the smaller is the degree of aggregation in the sample. The values and the sample to sample variation of this ratio (standard deviation) were determined for three independent sets of samples.

4.2.5 Thioflavin T fluorescence assay

As there is a stoichiometric and saturable interaction between ThioT and amyloid fibrils, fluorescence from the amyloid-ThioT complex provides accurate quantification of amyloid fibril formation as a function of amyloid fibril number [29,30]. For the ThioT fluorescence assay, six different Aβ peptide samples, differing in Aβ peptide content were investigated. For the full-length peptide two peptide concentrations (0.55 mM and 1.1 mM) and for the shorter peptides one concentration (0.55 mM) was investigated. To examine the effect of shorter peptides on the Aβ40 peptide, 1:1 mixtures of the shorter peptides with the full-length peptide (total peptide concentration of 1.1 mM) were prepared. For ThioT readings, the peptide was diluted with 10 μM ThioT in 50 mM glycine/NaOH buffer, pH 8.6. The final concentration of peptide in the ThioT wells was 11 μM or 27.5 μM. Fluorescence was measured with the Fluostar Galaxy fluorometer, 96 well, Black, uClear–Plate Ref. 655090 Greiner (fluor plate). The settings were: excitation λ: 450 nm emission λ: 485 nm, 1 cycle, 10 flashes, and gain: 75. All samples were prepared and measured at least three times.

4.3 Results

Figure 4.1 shows the continuous-wave EPR spectra of SL-Aβ15 in DMSO and in PBS measured at room temperature. The spectrum of the SL-Aβ15 peptide under aggregation conditions (in PBS) is similar to the spectrum of the peptide in DMSO, a solvent in which amyloid peptides are in the monomeric form [31-33]. The same is true for the EPR spectra of SL-Aβ16 in PBS and in DMSO (spectra not shown), which indicates that Aβ15 and Aβ16 are monomeric in PBS. The spectrum of the SL-Aβ40 peptide under aggregation conditions has multiple components and broadened lines (figure 4.2) [20]. In figure 4.2, the spectra of the spin-labeled shorter peptides, SL-Aβ15 and SL-Aβ16, in PBS, are compared to that of the SL-Aβ40 peptide at the same peptide concentration. The differences of aggregation in SL-Aβ40 (broad lines, extra signals) are absent in the spectra of shorter peptides further emphasizing the absence of aggregation in SL-Aβ15 and SL-Aβ16.

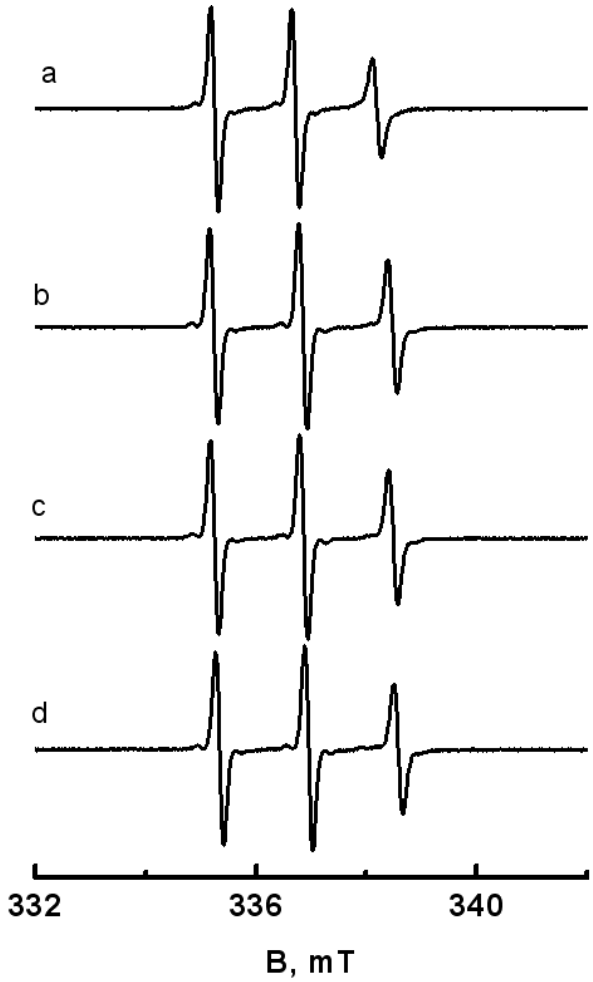

Figure 4.1 Room temperature EPR spectra of SL-Aβ15 under different conditions. a: SL-Aβ15 in DMSO. b: Mixture of the diamagnetically diluted SL-Aβ15 (diamagnetically diluted SL-Aβ15 (dd-SL-Aβ15): a mixture of 86 % wild type Aβ15 and 14 % SL-Aβ15) with wild type Aβ15 in PBS (dd-SL-Aβ15: wild type Aβ15, 1:1). Total peptide concentration is 1.1 mM. c: Mixture of dd-SL-Aβ15 with wild type Aβ40 in PBS (dd-SL-Aβ15: wild type Aβ40, 1:1). Total peptide concentration is 1.1 mM. d: Mixture of dd-SL-Aβ15 with wild type Aβ40 in PBS (dd-SL-Aβ15: wild type Aβ40, 1:2). Total peptide concentration is 1.65 mM.

The EPR spectra of the shorter peptides in the presence of Aβ40 are similar to the spectra of the shorter peptides alone. Furthermore, the spectral line-shape of the shorter peptides did not change for the two concentrations of the Aβ40 peptide used (0.55 mM and 1.1 mM), showing the absence of interaction of Aβ15 and Aβ16 with Aβ40 (figure 4.1). These observations are confirmed by the similarity of the simulation parameters given in table 4.2.

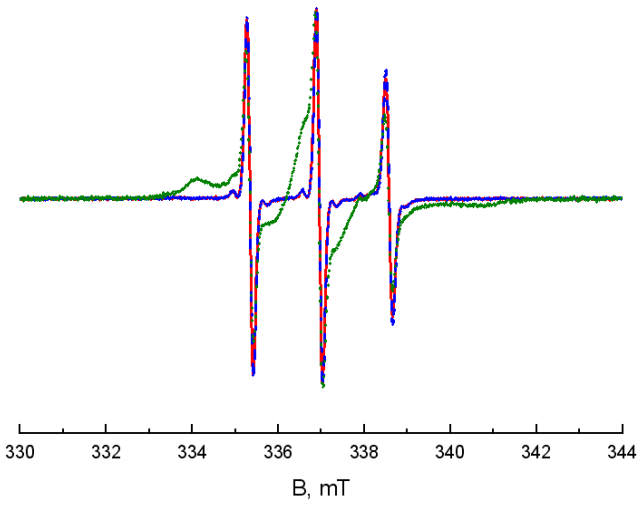

Figure 4.2 Room temperature EPR spectra of SL-Aβ15, SL-Aβ16, and SL-Aβ40 under conditions, where Aβ40 aggregates. Red line: diamagnetically diluted SL-Aβ15, i.e., SL-Aβ15: wild type Aβ15 (14 % SL-Aβ15: 86 % wild type Aβ15). Blue line: diamagnetically diluted SL-Aβ16, i.e., SL-Aβ16: wild type Aβ16 (14 % SL-Aβ16: 86 % wild type Aβ16). Green line: diamagnetically diluted SL-Aβ40, i.e., SL-Aβ40: wild type Aβ40 (14 % SL-Aβ40: 86 % wild type Aβ40). Total peptide concentration is 0.55 mM for all samples.

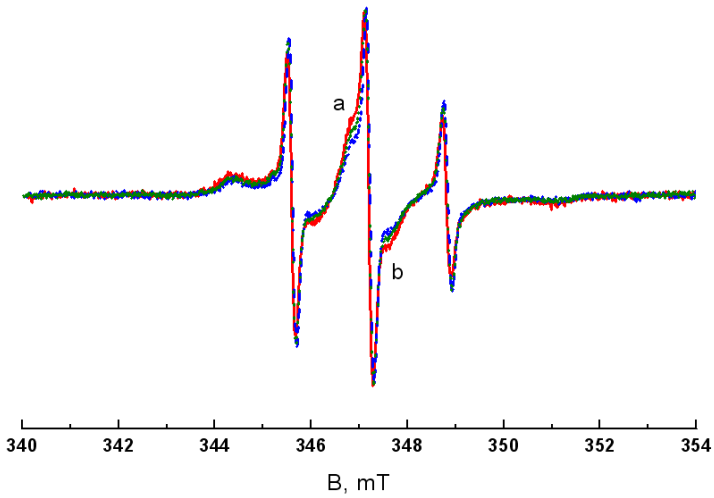

Figure 4.3 Room temperature EPR spectra showing the effect of Aβ15 and Aβ16 on Aβ40. Red line: dd-SL-Aβ15 (14 % SL-Aβ15: 86 % wild type Aβ15): wild type Aβ40 (1:1). Blue line: dd-SL-Aβ16 (14 % SL-Aβ16: 86 % wild type Aβ16): wild type Aβ40 (1:1). Green line: dd-SL-Aβ40 (14 % SL-Aβ40: 86 % wild type Aβ40): wild type Aβ40 (1:1). Total peptide concentration is 1.1 mM for all samples. There is a small difference among the three spectra in regions a and b.

Table 4.2 EPR parameters obtained from simulation for the SL-Aβ15 peptide and the SL-Aβ16 peptide in PBS and in DMSO. Given are: τ_r, rotation-correlation time, A_{zz} is the hyperfine splitting along the z-direction, lw is the component line-width of the simulation.

sample	solvent	[total peptide] (mM)	τ_r (ns)	A_{ZZ} (MHz)	lw (mT)
SL-Aβ15	DMSO	0.55	0.17 ± 0.02	100	0.09
SL-Aβ15 + Aβ15	PBS	0.55	0.10 ± 0.02	110	0.11
SL-Aβ15 + Aβ40	PBS	0.55	0.11 ± 0.02	110	0.10
SL-Aβ15 + Aβ40	PBS	1.10	0.15 ± 0.02	110	0.10
SL-Aβ16	DMSO	0.55	0.18 ± 0.02	100	0.09
SL-Aβ16 + Aβ16	PBS	0.55	0.10 ± 0.02	110	0.11
SL-Aβ16 + Aβ40	PBS	0.55	0.11 ± 0.02	110	0.10
SL-Aβ16 + Aβ40	PBS	1.10	0.15 ± 0.02	110	0.10

The spectra of the SL-Aβ40 peptide alone and of the SL-Aβ40 peptide in the presence of the unlabeled shorter peptides are shown in figure 4.3. By means of simulation [20], we determined the τ_r values of each mobility component (see materials and methods) and their contribution to the total spectra (table 4.3). The τ_r values of the SL-Aβ40 peptide in the presence or absence of shorter peptides as well as the amount of each mobility component are given in table 4.3. There is a small difference among the three spectra in regions "a" and "b", also reflected in small differences of the τ_r values and their relative contributions (figure 4.3).

Table 4.3 EPR parameters obtained from simulation for the Aβ40 peptide alone as well as in the presence of shorter peptides: Aβ15 and Aβ16. Given are: τ_r, rotation-correlation time, A_{zz} is the hyperfine splitting along the z-direction, lw is the component line-width of the simulation and % stands for the contribution of the component to the total spectrum.

	fast				medium				slow			
sample	τ_r (ns)	A_{ZZ} (MHz)	lw (mT)	%	τ_r (ns)	A_{ZZ} (MHz)	lw (mT)	%	τ_r (ns)	A_{ZZ} (MHz)	lw (mT)	%
SL-Aβ40	0.19 ± 0.04	110	0.14	12 ± 2	2.50 ± 0.35	110	0.32	52 ± 2	> 50	94	0.50	36 ± 2
SL-Aβ40 + Aβ15	0.19 ± 0.04	110	0.14	11 ± 2	2.50 ± 0.35	110	0.32	53 ± 2	> 50	94	0.50	36 ± 2
SL-Aβ40 + Aβ16	0.19 ± 0.04	110	0.14	15 ± 2	2.50 ± 0.35	110	0.32	47 ± 2	> 50	94	0.50	37 ± 2

To determine whether these differences are significant with respect to the sample-to-sample variation we analyzed the ratios of the intensities of the fast to the slow component in each spectrum (figure 4.4) for a large set of samples. Although there is a difference in the intensity ratios of the fast to the slow component of the samples of the SL-Aβ40 peptide alone and those containing SL-Aβ and the shorter

peptides, the differences are not significant, in view of the sample-to-sample variation.

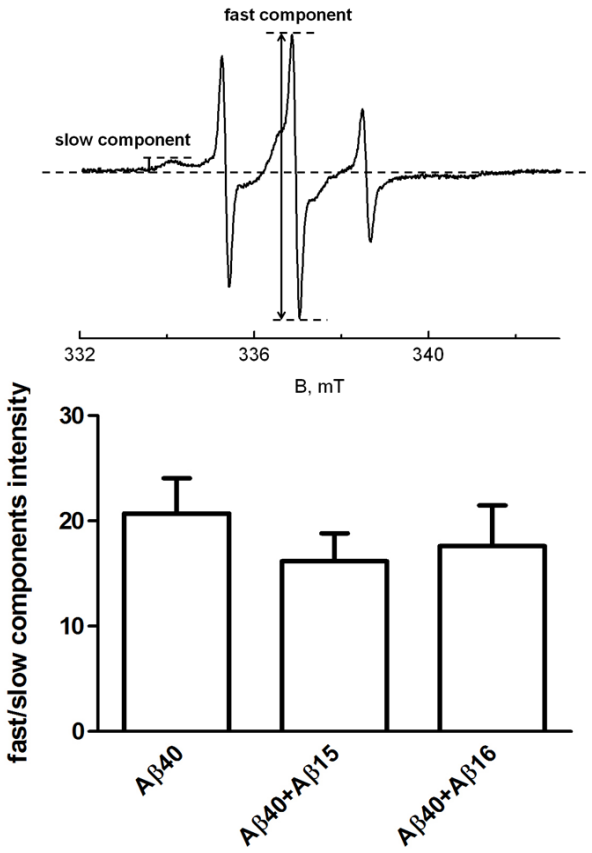

Figure 4.4 Effect of short Aβ peptides (Aβ15 and Aβ16) on the aggregation of Aβ40. Intensity ratios of the fast/slow components of three Aβ samples; Aβ40, Aβ40 + Aβ15 and Aβ40 + Aβ16 are represented as mean ± SEM of three sets of samples, i.e., each Aβ sample was fibrillized and measured under the same conditions three times.

An increase in fluorescence of the fibril-specific dye ThioT, compared to the fluorescence of the free ThioT in PBS buffer, is a marker for fibril formation. Relative fluorescence levels for the samples are shown in figure 4.5. The fluorescence level increased in the samples with the full-length peptide, while no fluorescence increase is observed in the samples which contained only the shorter peptides. Samples containing an equivalent amount of shorter peptides and the full-length Aβ (0.55 mM: 0.55 mM), have a fluorescence level similar to that of pure 0.55 mM full-length Aβ samples, whereas the fluorescence in a sample with 1.1

mM full-length Aβ is significantly higher compared to that of 0.55 mM full-length Aβ.

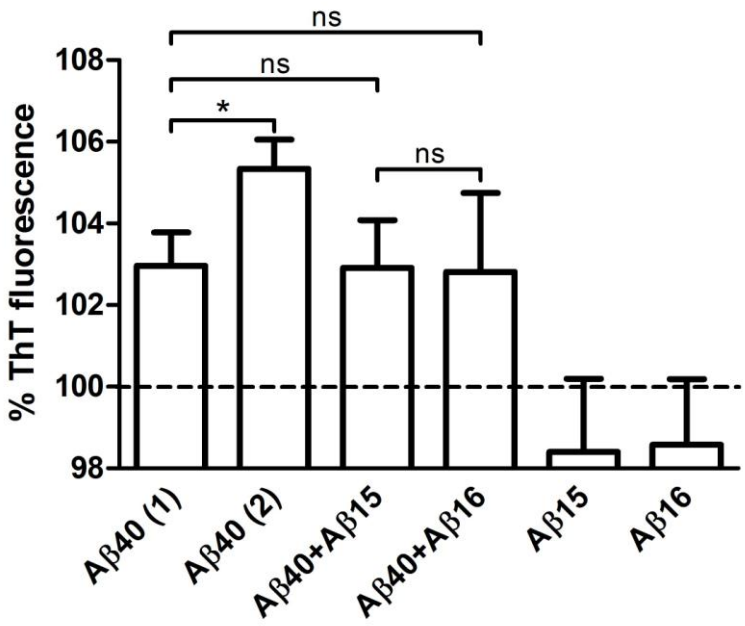

Figure 4.5 ThioT fluorescence (%) of six Aβ samples; Aβ40 (1) (0.55 mM), Aβ40 (2) (1.1 mM), Aβ40 + Aβ15 (0.55 mM), Aβ40 + Aβ16 (0.55 mM), Aβ15 (0.55 mM), Aβ16 (0.55 mM). For ThioT readings the peptide was diluted to concentrations of 11 or 27.5 μM. Values for ThioT fluorescence of ThioT alone were set to 100 % and the ThioT fluorescence of all samples is reported relative to this value (mean ± SEM of three experiments, i.e., each Aβ sample was fibrillized and measured under the same conditions three times; *$p < 0.05$ unpaired t test, ns: not significant).

4.4 Discussion

The aim of this study was to investigate and compare the aggregation potential of the two shorter N-terminal Aβ peptides Aβ15 and Aβ16 and to determine if the shorter peptides had an effect on the aggregation of the Aβ40 peptide. To monitor the aggregation we used spin-label EPR by which, previously, the aggregation of the Aβ40 peptide was studied [20,34]. In the present study either the short peptides (SL-Aβ15 or SL-Aβ16) or the Aβ40 (SL-Aβ40) peptide was spin labeled. By measuring the spin-label mobility of the SL-Aβ15 and the SL-Aβ16 in the absence/presence of the wild type Aβ40 or measuring the spin-label mobility of SL-Aβ40 in the presence of either wild type short peptides, the properties of each of the components of the aggregating samples was monitored individually.

For the short Aβ fragments, Aβ15 and Aβ16, the ThioT results show the absence of fibril formation. The same is true for the EPR results, where an observed trend towards larger τ_r values at higher concentrations of Aβ (given in table 4.2 and results) most likely is not a sign of aggregation, but an effect of the increasing viscosity of the solution.

The behavior of Aβ15 and Aβ16 is not affected by the presence of Aβ40, which reveals that the full-length Aβ does not induce aggregation in Aβ15 or Aβ16. Furthermore, no evidence for interaction of Aβ15 or Aβ16 with Aβ40 is observed. The Aβ40, on the other hand does aggregate under these conditions as is demonstrated in the experiments where SL-Aβ40 was monitored in the presence of unlabeled Aβ15 and Aβ16. In these experiments the difference between the EPR spectra is not significant (figure 4.3), revealing that the shorter peptides also do not inhibit or promote aggregation of full-length Aβ (table 4.3).

Previous reports about the aggregation of Aβ15 and Aβ16 had differing outcomes. As in the present study, NMR investigations at high concentrations of Aβ16 showed the absence of aggregation [17-19]. Similarly, Liu [14] and Liao [15] conclude from CD, AFM, and ThioT that Aβ16 does not aggregate. In contrast, a recent study [16] proposes that Aβ16 can assemble into a novel type of toxic oligomers and fibrils. These findings cannot be reconciled with the present study unless only a small fraction of the peptide (< 10 %) was in the oligomeric state in the AFM samples.

In summary, we find that the short peptides Aβ15 or Aβ16 do not directly influence the aggregation of Aβ40. This reveals that the effects that these short peptides show in neurotoxicity essays cannot derive from a direct influence of Aβ15 and Aβ16 on the aggregation of full-length Aβ. An alternative pathway for their action could be related to the metal-binding site in the N-terminus of Aβ [27,35-49]. The fragments Aβ15 and Aβ16 contain all ligands that were proposed for binding Cu (II) and Zn (II) ions that were suggested to increase Aβ aggregation. The short fragments could reduce Aβ aggregation, because they scavenge the metal ions making the full-length Aβ less prone to aggregation.

Overall, the present EPR investigation suggests that the short peptides Aβ15 or Aβ16 do not influence the aggregation of Aβ40 directly, supporting the view that physiological effects of these shorter fragments occur via a different route, possibly via metal-ion interactions. Therefore, examining the effect of metal ions on the aggregation of Aβ40 in the presence of the short peptides Aβ15 or Aβ16 is suggested as a future line of investigation.

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