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Circular dichroism and aggregation studies of amyloid β (11–28) fragment and its variants✪

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Aggregation of Aβ peptides is a seminal event in Alzheimer's disease. Detailed understanding of Aβ assembly would facilitate the targeting and design of fibrillogenesis inhibitors. Here comparative conformational and aggregation studies using CD spectroscopy and thioflavine T fluorescence assay are presented. As a model peptide, the 11–28 fragment of Aβ was used. This model peptide is known to contain the core region responsible for Aβ aggregation. The structural and aggregational behaviour of the peptide was compared with the properties of its variants corresponding to natural, clinically relevant mutants at positions 21-23 (A21G, E22K, E22G, E22Q and D23N). In HFIP (hexafluoro-2-propanol), a strong α-helix inducer, the CD spectra revealed an unexpectedly high amount of β-sheet conformation. The aggregation process of Aβ(11–28) variants provoked by water addition to HFIP was found to be consistent with a model of an α-helix-containing intermediate. The aggregation propensity of all Aβ(11–28) variants was also compared and discussed.

Keywords: Alzheimer's disease, amyloid β, thioflavine T assay, circular dichroism (CD), secondary structure studies, aggregation studies.

Alzheimer's disease (AD) is the most common form of senile dementia. This syndrome is becoming of paramount importance due to the increased life expectancy in developed countries. AD is characterized by the accumulation of extracellular amyloid plaques in the cerebral neuropil and vasculature, and of intracellular neurofibrillary tangles (for reviews, see Serpell, 2000; Selkoe, 2001). The major component of amyloid deposits is a 40–42 residue peptide called β-amyloid peptide (Aβ), which is produced by endoproteolytic cleavage of the amyloid precursor protein, APP (Selkoe, 1993). In the monomeric form Aβ is benign, but by an unknown mechanism this endogenous peptide becomes aggregated and neurotoxic (Lorenzo & Yanker, 1994).

A number of single-nucleotide mutations in the β-amyloid peptide-encoding region of the β-amyloid precursor protein have been reported which cause single amino-acid substitutions clustering around codon 693 of APP (close to the α–secretase site) that can cause early-onset familial forms of Alzheimer's disease (FAD). The resultant Aβ peptides are: E22Q (the Dutch variant; Levy *et al.*, 1990), E22K (Italian; Rossi *et al.*, 1998), E22G (Arctic; Nilsberth, 2001), A21G (Flemish; Hendriks *et al.*, 1992) and D23N (Iowa; Grabowski *et al.*, 2001). The pathological expressions of the clinically relevant Aβ variants share common features and show distinct clinical characteristics, in which cerebral amyloid angiopathy is a common clinical denominator.

The mutations are thought to cause AD-like diseases through alteration of Aβ production (Stenh, 2002), proteolytic susceptibility (Tsubuki, 2003), toxicity (Wang, 2000; Melchor, 2000; Murakami 2003), and clearance rates (Monro, 2002).

In all five mutant cases the fibrillogenic properties of the Aβ peptides have been found to be changed (Miravalle, 2000; Nilsberth, 2001; Van Nordstrand, 2001; Paivio, 2004). The central role of aggregated forms of Aβ in AD pathogenesis has stimulated the development of therapeutic approaches designed to prevent fibril formation (for a review,

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Abbreviations: Aβ, amyloid β peptide; AD, Alzheimer's disease; APP, β-amyloid precursor protein; CD, circular dichroism; DMF, *N*,*N*-dimethylformamide; FAD, familial AD; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; HOBt, 1-hydroxybenzotriazol; HPLC, high performance liquid chromatography; RC, random coil; TFA, trifluoroacetic acid; ThT, thioflavin T; Fmoc, 9-fluorenylmethoxycarbonyl; Wt, wild type.

see Soto, 1999). However, recent studies have shown that other types of Aβ assemblies, including small oligomers and fibril intermediates (protofibrils) are neurotoxic (Klein, 2001; Walsh, 2002). Oligomeric Aβ have also been found in brains of AD patients (Kayed, 2003). Oligomer toxicity has a practical aspect: therapeutic strategies aimed at fibril dissociation to produce these smaller, yet toxic, species may prove counterproductive. In order to rationally develop efficacious therapeutic agents, a rigorous understanding of Aβ assembly is required. Knowledge of early conformational and associative events would allow the targeting of the critical steps in the fibrillogenesis process, in particular those leading to the formation of toxic prefibrillar structures. In-depth examination of these steps may result in a deeper insight as to how the seemingly subtle changes in the primary sequence of Aβ translate into dramatic changes in Aβ metabolism and into the distinct clinical expression of the disease.

In order to examine and compare conformational changes accompanying the aggregational process of clinically relevant Aβ variants, we have examined the structural behaviour of the 11–28 fragment of the peptides using CD spectroscopy.

Although β-amyloid N- and C-truncation alters structural features of Aβ and the kinetics of fibrillogenesis (Huang, 1997; Paivio, 2004), synthetic peptides have been proven to be valuable tools in the studies of Aβ fibril assembly, in addition improving the reproducibility of the results (Rabanal, 2002; Serpell, 2000). The 11–28 fragment was chosen as a convenient model which comprises residues important in conformational switching to β-structures (Fraser, 1991; Zagorski, 1992) and residues critical to α-secretase, non-amyloidogenic cleavage of APP. The fragment is known to form fibril aggregates similar to those found for the natural protein (Castano, 1986; Fraser, 1991) and exert cellular toxicity *in vitro* (Flood, 1994; Rabanal, 2002).

The peptides were studied using CD spectroscopy in solvent mixtures promoting aggregation. Reference spectra were taken in pure HFIP to ensure disaggregated state of the sample (Vieira, 2003; Stine, 2003). The conformational changes accompanying aggregation were followed by analyzing CD spectra in HFIP/H_2O mixtures with an increasing H_2O content (10%, 50%, and 90%). The results were compared with the aggregation properties of Aβ(11–28) variants, estimated by a thioflavine T (ThT) fluorescence assay.

MATERIALS AND METHODS

Materials. The following spectroscopic and analytical instruments were used: peptide synthesizers, Millipore 9050 Plus PepSynthesizer; HPLC, Varian Vista 55000 (Kromasil C_8 4.6 × 250 mm, 5 μm), Knauer system (Vydac C_{18} 10 × 250 mm, 5 μm); matrix-assisted laser desorption/ionization time-of-flight mass spectrometer, Bruker Biflex III MALDI-TOF Mass Spectrometer (Bruker Daltonics); Perkin Elmer LS 50 B Spectrofluorimeter; CD spectropolarimeter, J-20 (Jasco).

TentaGel R RAM resin was purchased from Rapp Polymer, Fmoc amino acids from Peptides International, HOBt, *N,N*-diisopropylethylamine (DIPEA) from Fluka, *N,N*-diisopropylcarbodiimide (DIPCI), thioflavine T (ThT), and 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) from Sigma, *N,N*-dimethylformamide (DMF), trifluoroacetic acid (TFA), piperidine, and Triton 100-X from Merck, diethyl ether from Polish Chemicals, POCh, acetonitrile from Labscan, Quinolin Yellow and phenol from Aldrich.

Peptide synthesis. The peptides were synthesized according to published methods (Atherton & Sheppard, 1989) using the standard solid-phase synthesis technique with a Millipore synthesizer. The resin, TentaGel R RAM (1 g, capacity 0.18 mmol g^{-1}), was treated with piperidine (20%) in DMF, and all amino acids were coupled with the use of the DIPCI/HOBt methodology. The coupling reaction time was 2 h. Piperidine (20%) in DMF was used to remove the Fmoc group at all steps. After deprotection of the last, N-terminal Fmoc group the resin was washed with methanol and dried *in vacuo*. Then the resin was treated with TFA/water/phenol/triisopropylsilane (reagent B, 8.8:0.5:0.5:0.2) at 10 ml g^{-1} of the resin at room temperature for 2 h. After filtration of the exhausted resin, the solvent was concentrated *in vacuo* and the residue was triturated with diethyl ether. The crude peptide was purified with HPLC using a Vydac C_{18} semi-preparative column (10 × 250 mm, 5 μm) with 16 ml/min elution and a 120 min linear gradient of 10–40% CH_3CN in 0.08% TFA. The fraction containing pure peptide was lyophilized twice and the purity was confirmed by analytical HPLC and by MALDI-TOF analysis.

Thioflavine T (ThT) fluorescence assay. All peptides were first dissolved in HFIP in order to have a disaggregated sample. Then the solvent was removed by centrifugation *in vacuo*. Next, the peptides were dissolved in 50 mM phosphate buffer (pH 7.1) at 5 mg/ml and were incubated at 37°C for 30 min to 72 h. After the incubation period, 15 μl of the peptide solution was added to 15 μl of a ThT solution (0.3 mg ThT in 1 ml of the phosphate buffer), then 0.970 ml of 50 mM phosphate buffer (pH 7.1) was added and the fluorescence was measured at an excitation wavelength of 430 nm and an emission wavelength of 482 nm (slit widths 5 nm).

CD spectroscopy. The spectra were recorded with a Jasco J-20 spectropolarimeter with a 2 cm/min scan speed and data points were collected from 260 to 190 nm at room temperature with a 1 mm path

length quartz cell. All peptides were first dissolved in HFIP in order to have monomeric sample. Then the solvent was removed by centrifugation *in vacuo* and the peptides were dissolved in HFIP or mixed solvents: 90% HFIP/10% H_2O, 50% HFIP/50% H_2O, 10% HFIP/90% H_2O (the second solvent was phosphate buffer, pH 7.1). The final concentration of all samples was 40 μM. Spectra were also registered after 13 days of incubation of the solutions at room temperature. The secondary structure content was calculated from the CD spectra using the selfconsistent method (SELCON3 program, Sreerama, 2000). Raw CD data were converted to molar ellipticity and transferred into input files as outlined in the DicroPro documentation. The data presented in Table 1 are obtained from the program and expressed in percentage.

RESULTS AND DISCUSSION

Peptide models

Since both N- and C-terminally truncated versions of amyloid β peptide were shown to retain the aggregation properties of full-length Aβ in terms of tinctorial properties, secondary structure, pH dependence, fibril morphology and cytotoxicity, we have selected the Aβ(11–28) fragment as a model peptide to facilitate the synthesis and ensure reproducibility of results. Our conformational and aggregation studies were carried out on Aβ fragments corresponding to clinically relevant Aβ alloforms. Their sequences are presented in Fig. 1.

All the peptides studied were synthesized using solid-phase Fmoc-amino acid chemistry and purified by reversed-phase HPLC using a Vydac C_{18} semi-preparative column. The products were identified by MS MALDI-TOF analysis.

CD spectroscopy studies

In our studies we followed the conformational changes as a function of the composition of the solvent mixtures (HFIP — water). The Aβ(11–28) variants were at first dissolved in pure HFIP to have a disaggregated sample (Stine, 2003). Hexafluoroisopropanol is well known for its ability to stabilize local hydrogen bonds between residues close in the amino-acid sequence, particularly those forming α-helices (Buck, 1998; Vieira, 2003). Addition of water provokes aggregation, with different rates depending on the composition of the solvent mixtures and the natural propensity of a particular peptide for aggregation.

E V H H Q K L V F F A E D V G S N K Aβ(11-28) Wt

E V H H Q K L V F F A **K** D V G S N K Aβ(11-28) E22K, Italian mutant

E V H H Q K L V F F A **G** D V G S N K Aβ(11-28) E22G, Arctic mutant

E V H H Q K L V F F A **Q** D V G S N K Aβ(11-28) E22Q, Dutch mutant

E V H H Q K L V F F **G** E D V G S N K Aβ(11-28) A21G, Flemish mutant

E V H H Q K L V F F A E **N** V G S N K Aβ(11-28) D23N, Iowa mutant

Figure 1. Structures of Aβ(11–28) peptides.

It is well documented in the literature that in the assembly of oligomers a partially folded, helix–containing intermediate may be involved (Kirkitadze, 2001; Fezoui & Teplow, 2002). Simulation studies of Aβ(16–22) have found, however, that α-helical intermediates are not necessary during amyloid-β assembly (Santini, 2004). Our conformational studies were aimed at checking whether the conformational changes of Aβ(11–28) peptides follow the scheme with an α-helical intermediate. If this was the case, we should expect a continuous decline of unstructured conformation content, a transient rise of α-helix content, and then a gradual increase of β-structure content. Our analysis of CD spectra might have been, however, slightly distorted, as the observed changes of the spectra were the result of conformational changes and simultaneous aggregation with formation of higher, partially insoluble aggregates. Very fast aggregation of the Aβ(11–28) peptides with the Dutch (E22Q) and Iowa (D23N) mutations rendered this type of conformational studies impossible.

CD spectra in HFIP

There are some similarities between the CD spectra of the native peptide fragment and the fragment with the Italian (E22K) mutation, and between the spectra of the Arctic (E22G) and Flemish (A21G) mutant fragments (Fig. 2A). For the two first peptides (WT, Italian) the highest α-helix content was observed (20% and 16%, respectively — see Table 1). Nevertheless, even in this strong α-helix inducer (Vieira, 2003) this conformation was not the prevailing one. For Aβ(11–28) E22K a high content of random coil and β-sheet conformations was observed (32% and 31%, respectively), and for the wild peptide fragment there was a negligible percentage of unstructured conformations and the β-sheet conformation was dominant. This observation seems surprising, as it has been shown that HFIP is not only an α-helix inducing solvent, but it can also disrupt and refold the β-conformation. It is possible that HFIP, by destabilizing hydrophobic interactions, promotes not only intramolecular hydrogen bonding (α-helix, β-turns), but in some cases facilitates β-sheet formation.

For the peptides with the Flemish and Arctic mutations, the CD spectra were consistent with dominant random coil and β-sheet conformations

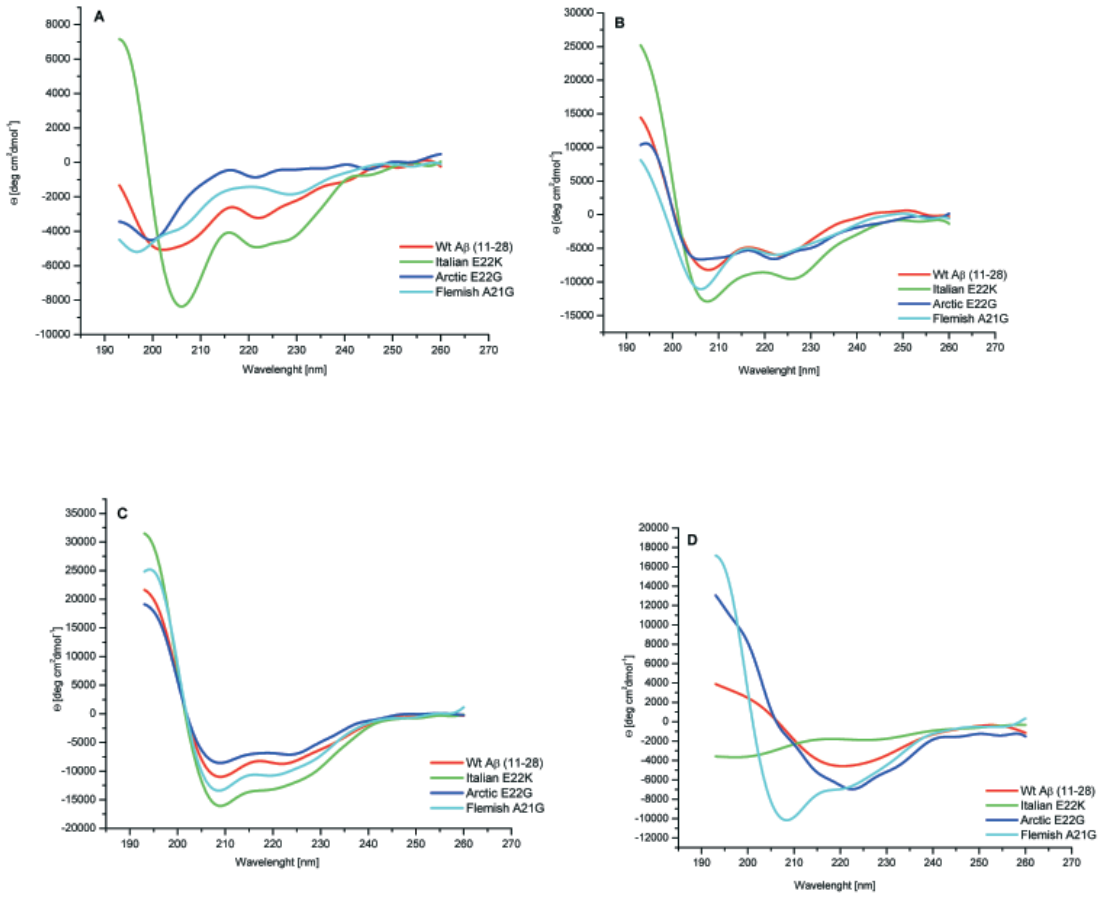

Figure 2. CD spectra for the Aβ(11–28) variants.

in Medium: (A) HFIP, (B) 90% HFIP/10% H_2O, (C) 50% HFIP/50% H_2O, (D) 10% HFIP/90% H_2O.

(Fig. 2A, Table 1). The minimal content of α-helix conformation in the case of these peptides may be related to the mutation type, in which the glycine residue, introduced during mutation, increased the flexibility of the peptides and promoted unstructured conformations. According to CD spectra analysis the peptide with the Arctic mutation is the most unstructured (66% of random coil). After a 13-day incubation at room temperature the random coil content dropped (66% → 55%), while the β-sheet conformation content increased (0 → 25%). For all other Aβ peptides the incubation resulted in a decrease of β-sheet content, as it was found in the literature (Vieira, 2003).

CD spectra in HFIP–water mixtures

The spectra in a mixture of HFIP and water (10%) assumed shapes closer to those characteristic for α-helix (minima at 208 nm and 222 nm, Fig. 2B). Deconvolution of the spectra revealed a rise of the helical conformation content with the highest α-helix content for the Italian mutant fragment (39%, Table 1). This rise slightly increased during the 13-day incubation period. The addition of water also resulted in a transient decrease of β-sheet conformation content. Only in the case of the Arctic mutant fragment a significant rise in β-sheet content was observed. The spectra recorded after the incubation period were characteristic for a further fall of unstructured conformation content and a small rise in α-helix content (highest for the Arctic mutant peptide, Table 1).

The spectra recorded in the mixture of 50% HFIP and 50% water were the most similar to a spectrum characteristic for the α-helix conformation (Fig. 2C). A quantitative analysis revealed that increasing the water content in the solvent mixtures resulted in a further rise in α-helix content (maximal for the E22K peptide — 53%), accompanied by a fall of random coil and β-sheet content (only for the Arctic mutant the rise in β-sheet was observed).

Further increase of water content in the solvent mixture (90% H_2O/10% HFIP) brought about a significant differentiation of CD spectra reflecting various speeds of aggregation (Fig. 2D). For all the peptides studied, a substantial decrease of α-helical conformation content with a simultaneous increase of β-sheet content was observed.

The CD curve for the Flemish mutant fragment resembles the spectrum characteristic for α-

Table 1. Secondary structure content in Aβ(11–28) variants in HFIP–H_2O mixtures containing 40 μM of peptides.

The spectra were registered approximately 15 min after dissolution or after 13 days of incubation at room temperature the values are as obtained from the SELCON3 program, expressed in percentage — see Materials and Methods.

α-Helix								
Peptide	Flemish		Italian		Wt		Arctic	
Time (days)	0	13	0	13	0	13	0	13
HFIP	0	11	16	15	20	11	6	3
10% H_2O	20	23	39	42	22	26	14	27
50% H_2O	47	50	53	56	32	32	26	30
90% H_2O	27	33	0	6	2	1	18	0
β-Sheet								
Peptide	Flemish		Italian		Wt		Arctic	
Time (days)	0	13	0	13	0	13	0	13
HFIP	29	17	31	20	41	34	0	25
10% H_2O	16	19	12	9	18	22	32	15
50% H_2O	11	9	6	8	14	14	36	16
90% H_2O	16	13	31	46	30	23	22	39
Random coil								
Peptide	Flemish		Italian		Wt		Arctic	
Time (days)	0	13	0	13	0	13	0	13
HFIP	48	40	32	39	8	6	66	55
10% H_2O	40	37	32	30	39	35	40	33
50% H_2O	27	26	24	22	35	33	36	36
90% H_2O	36	36	41	23	48	48	37	33
Turns								
Peptide	Flemish		Italian		Wt		Arctic	
Time (days)	0	13	0	13	0	13	0	13
HFIP	10	7	3	12	17	8	6	9
10% H_2O	13	12	11	12	12	10	11	13
50% H_2O	12	10	11	11	15	13	13	14
90% H_2O	13	15	13	10	14	9	16	10

helix. Analysis of the spectrum confirmed that the Flemish mutant was the least aggregated one of all the peptides studied: a maximal content of α-helical and minimal of β-sheet conformations. The CD spectrum for Aβ(11–28) Wt in 90% H_2O/10% HFIP mixture is typical of β-sheet secondary structure (minimum at 220 nm, maximum at 195 nm) and indicates advanced aggregation.

In all solvent mixtures the turn content is similar and amounts to approx. 10–15%. The conformational changes observed correspond to an aggregation model with an α-helix-containing intermediate (Fezoui & Teplow, 2002). Analysis of the CD spectra of all Aβ(11–28) variants showed a maximum of helix content during aggregation provoked by water addition to HFIP. To our surprise, we observed a decrease of random coil content only for peptide variants with a high content of this conformation in HFIP (A21G, E22G). For other variants the changes in RC content were not as clear, as they reflected a number of processes: conformational transitions (RC → α-helix → β-sheet), β-sheet aggregation, and conformational changes accompanying the solvent change (HFIP → HFIP/H_2O). Contrary to the results obtained for full-length Aβ in pure HFIP, our data imply that this solvent is not able to disrupt β-sheet conformation of the Aβ(11–28) fragment, as it was observed for Aβ(1–40) and Aβ(1–42) (Stine, 2003).

Aggregation studies using ThT fluorescence assay

The time course of aggregation of Aβ(11–28) variants was monitored with ThT, a fluorophore that shifts excitation and emission maxima upon binding to amyloid fibrils (LeVine, 1993). Fluorescence was measured after 0.5, 1.5, 3, 6, 24, 30, 48 and 72 h of incubation at 37°C as emission at 482 nm. The results are presented in Fig. 3.

All Aβ(11–28) variants displayed a sigmoidal increase in ThT binding, typical of fibrillogenesis processes. Based on the aggregation course, the Aβ(11–28) variants can be divided into two groups: slowly aggregating peptides (Flemish A21G, Italian E22K and wild type) and fast aggregating peptides (Iowa D23N, Arctic E22G and Dutch E22Q). After 30 min of incubation peptides of the first group show only small fluorescence, whereas the peptides of the second group form aggregates which bind ThT much faster. Peptides with the E22G and E22Q substitutions show maximal fluorescence after 24 h. This

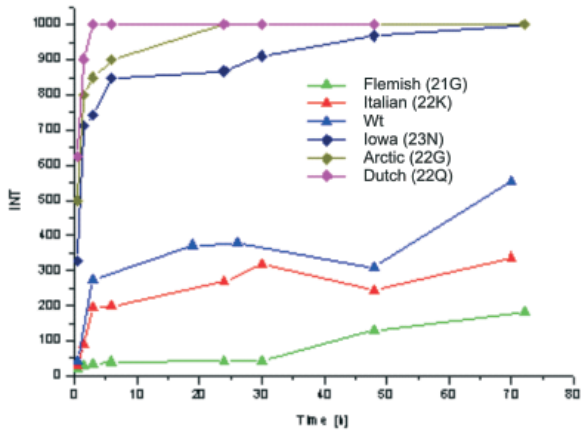

Figure 3. Comparative study of the aggregation of Aβ(11–28) variants at position 21–23 estimated by ThT fluorescence method (INT, relative fluorescence intensity).

very fast aggregation was the cause of the failure of our CD studies in the case of Aβ(11–28) E22Q and D23N variants.

Our results are consistent with the ThT assay results obtained for full-length Aβ(1–40) (Murakami, 2003), proving that our peptides are good models for conformational and aggregation comparative studies. The authors also found the smallest aggregation propensity for the A21G mutant of both Aβ(1–40) and Aβ(1–42). This result was confirmed by a sedimentation assay (HPLC analysis after centrifugation, Murakami, 2003) in spite of many discrepancies between the results of both tests.

In both tests the E22K mutant of Aβ(1–40) and Aβ(1–42) was found to aggregate faster compared to the native peptide (Murakami, 2003), whereas other studies (Miravalle, 2000; Yoshiike, 2003) showed the aggregative potency of Aβ40 E22K to be lower. In our fluorescence studies Aβ(11–28) with the Italian or Flemish mutations have a lower aggregative potency compared to that of Aβ(11–28) WT. The discrepancies between the results of different aggregation studies may be related to the fact that ThT fluorescence can vary depending on the structure and morphology of the fibrils.

The low aggregative potency of the Italian and Flemish variants correlates with the highest content of α-helix conformation for these peptides in CD solvent effect studies. There is no clear correlation between the aggregation propensity of the mutants determined by ThT assay and the β-sheet content obtained in CD studies. It can be noted, however, that in the case of the fastest aggregating Arctic variant, the addition of water results in the highest increase of β-sheet content. It also seems meaningful that slow aggregating peptides (Flemish, Italian, Wt) reveal a significant amount of β-sheet conformation in HFIP, which drops after the addition of water.

Despite some parallelism in the conformational/aggregation behaviour of the Aβ peptides in our CD studies and ThT assay, it is necessary to take into account the different conditions applied in both studies. As is generally known, formation of Aβ oligomers and fibrils is influenced by time, solvent, temperature, pH, ionic strength of the solution, etc. So in the case of our studies the main conclusions should refer to the comparison between the conformational behaviour of the Aβ variants.

CONCLUSIONS

Our CD studies showed that aggregation process of Aβ(11–28) variants provoked by water addition to HFIP is consistent with the model of an α-helix-containing intermediate. The studies disclosed many similarities between the conformational and aggregational behaviour of the native, 11–28 fragment of Aβ and its variant with the E22K mutation. Wild type Aβ(11–28) and its Italian mutant were found as the most α-helical ones from the group, and the Arctic variant as the most unstructured one. CD studies in HFIP, a strong α-helix inducer, revealed an unexpectedly high amount of β-sheet conformation.

The aggregation studies based on ThT fluorescence assay showed the highest aggregation propensity for the Arctic (E22G), Iowa (D23N) and Dutch (E22Q) variants. The lowest aggregative potency was found for the Flemish and Italian variants. However, the smallest propensity of A21G for aggregation may be related to a specific morphology and tinctorial properties of its fibrils. In spite of the fact that the CD studies on water provoked structural changes and the ThT aggregation studies were performed in different environment (HFIP/H_2O *versus* pH 7.1 phosphate buffer) the aggregation propensity of Aβ(11–28) variants in both conditions seems to be similar.

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