The conformation of Alzheimer's β peptide determines the rate of amyloid formation and its resistance to proteolysis

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Amyloid β-peptide (Aβ) is found in an aggregated poorly soluble form in senile or neuritic plaques deposited in the brain of individuals affected by Alzheimer's disease (AD). In addition soluble Aβ (sAβ) is identified normally circulating in human body fluids. In this study we report that synthetic peptides containing the sequences 1–40 and 1–42 of Aβ, and Aβ analogues bearing amino acid substitutions can adopt two major conformational states in solution: (1) an amyloidogenic conformer (Aβac) with a high content of β-sheet and partly resistant to proteases and (2) a non-amyloidogenic conformer (Aβnac) with a random coil conformation and protease-sensitive. The differences in the fibrillogenesis rate and in the protease resistance among the several Aβ peptides studied depend mainly on the relative propensity for adopting the amyloidogenic conformation, which in the absence of external factors is largely conditioned by the primary structure of the peptide. Aβnac containing the sequence 1–40, 1–42 or bearing amino acid substitutions (Dutch variant of Aβ) was protease-sensitive and unable to form a significant amount of amyloid even at high concentrations or after long incubations. The finding of the simultaneous existence of different Aβ conformers with distinct abilities to form amyloid may help to explain why Aβ is found in both soluble and fibrillar forms *in vivo*.

INTRODUCTION

Two of the main pathological features in Alzheimer's disease (AD) are the presence of amyloid neuritic plaques and the formation of neurofibrillary tangles. The main component of amyloid deposits is the 39–44 amino acid amyloid β-peptide (Aβ), an internal proteolytic product of a much larger precursor protein (βPP) coded in chromosome 21 [1]. In addition to amyloid deposits [2,3] Aβ is found in a soluble form (sAβ) in human cerebrospinal fluid and plasma [4,5]. Both the soluble and insoluble species of Aβ consist of peptides of different lengths, showing heterogenicity in N- and C-termini with the sequences 1–40 and 1–42 representing the major forms. A relevant feature of amyloid deposits is that they cannot be removed by the proteolytic mechanisms catalysing protein turnover and preventing the abnormal accumulation of aged polypeptides in the tissues [6]. Since the discovery of sAβ as a normal cellular product, the mechanism and the factors involved in the modulation of the fibrillogenesis and protease resistance of sAβ seem to be the most important questions in AD.

Synthetic peptides identical with Aβ have been extensively used to study fibril formation *in vitro* (for review see [7]). Peptides containing the sequence 1–40 or 1–42 and shorter derivatives form amyloid-like fibrils *in vitro* exhibiting a β-pleated sheet conformation [8–17]. A fruitful approach in the study of the structure and solubility of Aβ has been to alter the primary structure of the Aβ fragments and study their properties. The substitution of Glu^{22} for Gln found in the Dutch-type amyloidosis cerebrovascular haemorrhagic disease [18] yields an Aβ analogue with an increased ability to form amyloid fibrils [15,19–22]. The replacement of hydrophobic with hydrophilic residues in the internal Aβ hydrophobic domain (residues 17–21) can impair fibril formation [23], suggesting that Aβ assembly is driven partly by hydrophobic interactions. Conversely, substitution of charged residues in the N-terminal domain of Aβ showed that the absence of the centrally charged region (His^{13}-His^{14}-Gln^{15}-Lys^{16}) arrested amyloid assembly [24]. The idea that ionic interactions could modulate amyloid formation is consistent with studies showing that Aβ secondary structure and its ability to aggregate is pH-dependent [12,13,25,26]. The picture emerging from studies with synthetic peptides is that ionic and hydrophobic interactions in the Aβ N-terminal fragment contribute to modulate the adoption of a β-sheet conformation and assembly of amyloid fibres. Conversely, on the basis of indirect measurement by turbidity of Aβ aggregation, it has been suggested that the length of the C-terminal domain of Aβ can influence the rate of Aβ assembly by accelerating nucleus formation [16]. In this view, amyloid fibril formation is rate-limited by nucleation, in a similar way to the mechanism proposed previously for the formation of glucagon fibrils *in vitro* [27].

By using synthetic Aβ analogues containing single amino acid substitutions, we showed that the conformation adopted by the Aβ N-terminal domain in solution modulated the extension of amyloid formed by the Aβ analogue [21]. Moreover, we recently showed that Aβ(1–40) can adopt two different conformational states in solution (called Aβnac and Aβac), which have distinct abilities to form amyloid [28]. In this study we additionally characterize the physicochemical properties of the Aβ conformers and we analysed the existence of different conformational states in Aβ peptides of different length and containing specific amino acid substitutions. Our results show that two conformational states are also present in all the Aβ variants studied, and that the fibrillogenic potential and the level of protease resistance of each peptide depends mainly on the relative propensity for adopting the amyloidogenic conformation.

MATERIALS AND METHODS

Synthetic peptides

The following synthetic peptides were used: Aβ(1–40) containing the sequence 1–40 of Aβ; Aβ(1–42) corresponding to the sequence

Abbreviations used: Aβ, amyloid β-peptide; Aβac, amyloidogenic conformer of Aβ; Aβnac, non-amyloidogenic conformer of Aβ; AD, Alzheimer's disease; sAβ, soluble Aβ; ThT, thioflavin T.

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1–42 of Aβ; Aβ(1–40Q) bearing the substitution glutamine for glutamic acid at position 22, identical with the Dutch variant of Aβ [18]; Aβ(1–40G) containing glycine for glutamic acid at position 22, found in some cases of familial AD [29] and Aβ(1–40A) with a substitution of alanine for valine at position 18, theoretically designed to diminish the propensity of the N-terminal domain of Aβ to adopt a β-sheet conformation [21]. Aβ peptides were synthesized in W. M. Keck Facility at Yale University by using solid-phase *N*-tert-butyloxycarbonyl chemistry, except Aβ(1–40A), which was obtained from Chiron Corp. Inc. (Emerville, CA, U.S.A.). The peptides were purified by HPLC with a Vydac C18 reverse-phase column and a linear gradient of 0–80 % acetonitrile in 0.05 % trifluoroacetic acid. The purity of the peptides was evaluated by amino acid analysis and by laser desorption mass spectrometry.

Three different stock solutions of Aβ peptides were prepared by dissolving them in 50 % acetonitrile, DMSO or hexafluoropropanol. The concentration was determined by amino acid analysis on a Waters AccQ-Tag amino acid analyser. The above-mentioned solvents did not affect significantly the peptide concentrations as determined by this method. Therefore for the experiments aliquots from Aβ stock solutions in 50 % acetonitrile were freeze-dried and resuspended in the assay buffer at the indicated concentrations.

Separation of the Aβ amyloidogenic (Aβac) and non-amyloidogenic (Aβnac) conformers

The separation of the Aβ species is based on differences in their ability to aggregate [28]. Aβ peptides at 1 mg/ml were incubated in 0.1 M Tris/HCl, pH 7.4, for 10 d at room temperature and the soluble and aggregated Aβ forms were separated by centrifugation at 11400 ***g*** for 15 min in an Eppendorf microfuge at 25 °C. The supernatant containing Aβnac was quantified by UV absorption at 214 nm, and the solution was kept at −70 °C in the presence of a final concentration of 50 % acetonitrile to make the conditions as close as possible to the treatment of Aβac. The pellet containing Aβac was redissolved in 50 % acetonitrile and centrifuged to eliminate the aggregated material that had not redissolved with the acetonitrile treatment. The solubilized Aβac was quantified by amino acid analysis and kept at −70 °C. Immediately before the experiments the appropiate aliquots of both Aβ populations were freeze-dried and resuspended in the experimental buffer. Owing to the presence of Tris in the incubation buffer, measurement of Aβnac by amino acid analysis was done after extensive dialysis (cut-off 3500 Da) of the supernatant against distilled water. Because there was a good correlation between Aβnac concentration determined by amino acid analysis after dialysis and UV absorption at 214 nm using Aβ(1–40) as a standard, the latter method was used in most of the experiments. To ascertain that equivalent amounts of peptides were used, aliquots of Aβnac and Aβac stocks were analysed by HPLC and the respective areas compared. HPLC analysis was also essential to rule out peptide degradation after long incubation times.

Fluorimetric determination of amyloid formation

Aliquots of peptides were incubated for different times at room temperature in 0.1 M Tris/HCl, pH 7.4. Amyloid formation was quantified by using the thioflavin T (ThT) fluorescence method. ThT binds specifically to amyloid and this binding produces a shift in its emission spectrum and a fluorescent signal proportional to the mass of amyloid formed [30]. After incubation, Aβ peptides were added to 50 mM glycine, pH 9.2, containing 2 μM ThT in a final volume of 2 ml. Immediately thereafter, fluorescence was monitored at excitation 435 nm and emission 485 nm in a Hitachi F-2000 fluorescence spectrometer. A time scan of fluorescence was performed and three values after the decay reached the plateau (280, 290 and 300 s) were averaged after substracting the background fluorescence of 2 μM ThT.

Sedimentation assays

Aβ peptides were incubated at the indicated concentrations in 0.1 M Tris/HCl, pH 7.4, for different times at room temperature. The soluble and aggregated peptide were separated by centrifugation at 11400 ***g*** for 15 min in an Eppendorf microfuge at 25 °C. The amount of peptide remaining soluble was analysed on reverse-phase HPLC. Absorbance was monitored at 220 nm. The percentage of the non-sedimentable peptide was estimated by comparing the area of the peak corresponding to the soluble peptide in each incubated sample with an identical control of non-incubated sample.

Electron microscopy

For fibril formation, Aβ peptides at 1 mg/ml were incubated for 6 d at room temperature. Samples were placed on carbon Formvar-coated 300-mesh nickel grids for 1 min, blotted and stained for 1 min with 2 % (w/v) uranyl acetate. Grids were examined on a Zeiss EM 10 electron microscope at 80 kV.

Protease-resistance assay

Aliquots of Aβ peptides at a concentration of 50 μg/ml in 20 mM Tris/HCl, pH 7.4, were incubated for 30 min or 4 h with 0.5 μg/ml of trypsin (Promega) at 37 °C. After the indicated times, samples were subjected to reverse-phase HPLC on a Vydac C_4 column (0.46 cm × 25.0 cm) and a linear gradient of 5–70 % acetonitrile in 0.1 % trifluoroacetic acid in 25 min. Absorbance was monitored at 220 nm. The HPLC profile was compared with that obtained with peptides incubated alone at the same concentration. The relative amount of peptide resistant to proteolysis by trypsin was estimated by comparing the area of the peak corresponding to the intact peptide.

Circular dichroism studies

CD spectra were recorded on a Jasco spectropolarimeter Model J-720 at room temperature in a 0.1 cm pathlength cell. Aliquots of peptides at a concentration of 0.1–0.2 mg/ml in 20 mM Tris/HCl, pH 7.4, were first centrifuged to remove aggregated material; the spectra were then recorded at 1 nm intervals over the wavelength range 190–260 nm. Results were expressed as mean residue ellipticity in units of $deg \cdot cm^2 \cdot dmol^{-1}$. CD data were analysed by the Lincomb [31] algorithm to obtain the percentages of the different secondary structure motifs. To calculate the percentages of two Aβ conformers in different Aβ variants by CD, the experimental spectra of these variants were fitted with the obtained spectra of purified Aβnac and Aβac, by using:

$$\text{CD spectrum of A}\beta\text{ variant} = a \times \text{CD spectrum of A}\beta\text{nac} + b \times \text{CD spectrum of A}\beta\text{ac}$$

In the above equation a and b represent the fractions of Aβnac and Aβac present on the Aβ variant studied.

RESULTS

Comparison of the fibrillogenic properties and resistance to partial proteolysis of Aβnac and Aβac

Aliquots of both Aβ populations isolated as described in the Materials and methods section were used to study their ability to form amyloid by the fluorimetric assay. Figure 1, top, shows the ThT fluorescence values obtained with Aβnac and Aβac after different incubation times at a peptide concentration of 1 mg/ml. Aβac showed a ThT fluorescence signal that was approx. 10–15-fold higher than that obtained with Aβnac throughout the incubation period. A similar difference was obtained when Aβ conformers were incubated at different concentrations for 24 h at room temperature (Figure 1, bottom). Electron microscopic examination showed that Aβac, incubated for 6 d at 1 mg/ml, formed typical non-branching amyloid fibrils 10 nm wide, whereas Aβnac under the same conditions was completely unable to form amyloid-like fibrils (Figure 2). Aβnac required higher peptide concentrations or longer incubation times to show small amounts of amyloid-like fibres.

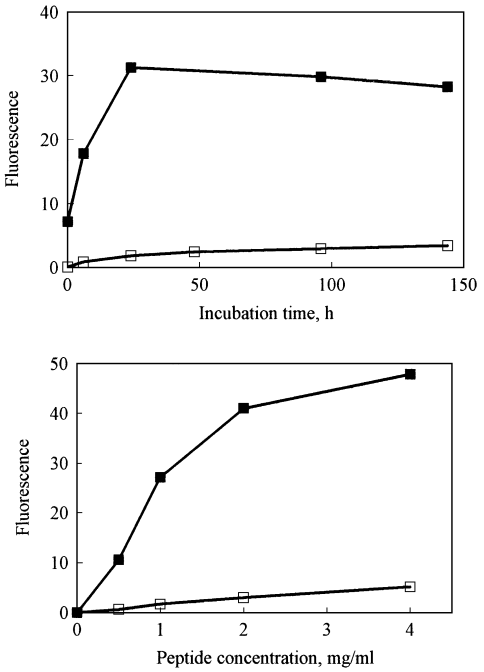

Figure 1 Fibrillogenic properties of Aβac (■) and Aβnac (□)

Time dependence (top) and concentration dependence (bottom) of amyloid formation by using isolated Aβnac and Aβac was studied by ThT fluorescence as described in the Materials and methods section. Top: aliquots of each species at 1 mg/ml (0.25 mM) were incubated for different times in 0.1 M Tris/HCl, pH 7.4, in 15 μl at room temperature. Bottom: peptides were incubated at different concentrations in 15 μl of 0.1 M Tris/HCl, pH 7.4, for 24 h at room temperature. Note that the total mass of peptide is different at each concentration point and is different from that shown in Table 2.

To investigate the nature of the amyloidogenic differences between Aβnac and Aβac, the chemical structure and the initial assembly state were studied by laser desorption mass spectroscopy, reverse-phase HPLC and denaturating or non-denaturating gel electrophoresis. Both populations have the same molecular mass (4329.6 and 4329.8 Da), indicating that there were no detectable chemical modifications. Furthermore no significant differences in the initial assembly state were evident, because Aβnac and Aβac were eluted in the same position in reverse-phase HPLC, and showed the same pattern in SDS/PAGE and PAGE without SDS (results not shown). Furthermore, both Aβnac and Aβac were > 90 % monomeric or dimeric, shown by gel filtration FPLC using a Superose 12 column (C. Soto, unpublished work). These results indicate that both Aβ populations correspond to single non-aggregated molecules.

To characterize further the properties associated with the two conformational states of Aβ, the resistance to proteolysis was evaluated. After 30 min of incubation at 37 °C with 1 % (w/w) trypsin, Aβnac was almost completely digested, whereas approximately 85 % of Aβac remained intact (Figure 3). At incubation times longer than 4 h, both Aβnac and Aβac were completely digested. When an aliquot of the stock solution of Aβ(1–40) was subjected to the protease-resistance assay for 30 min, approx. 30 % of the peptide remained undigested under the conditions used, suggesting that Aβac was already present in the Aβ stock solution. It is important to note that in these experiments only soluble Aβnac and Aβac are detected, because the aggregated peptide cannot enter into the HPLC column. Therefore our

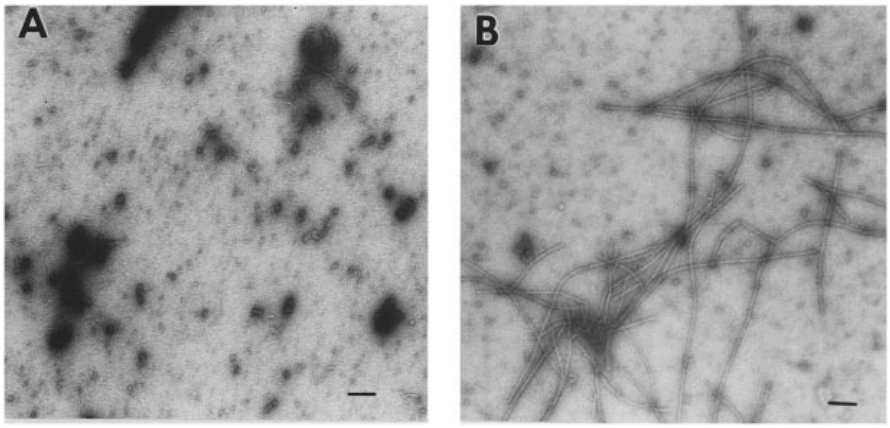

Figure 2 Electron micrographs of negative-stained preparations of Aβnac (A) and Aβac (B)

Aliquots of both conformational states of Aβ incubated at 1 mg/ml for 6 d were absorbed onto 300-mesh Formvar-coated grids and negative-stained with 2% (w/v) uranyl acetate. Scale bar = 100 nm.

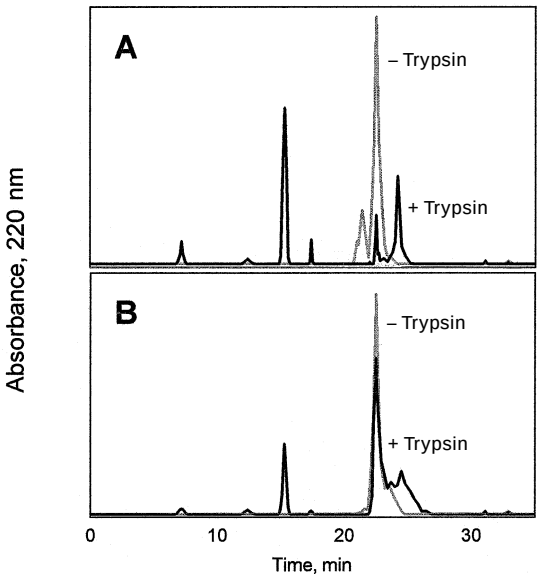

Figure 3 Trypsin resistance of Aβnac and Aβac

Aliquots of 5 μg of each Aβ conformational species were incubated with or without 50 ng of trypsin in 100 μl of 20 mM Tris/HCl, pH 7.4, for 30 min at 37 °C. The samples were then separated by HPLC on a C4 reverse-phase column and a 5–70% acetonitrile gradient. The peptide was detected by measuring absorbance at 220 nm. Panels (**A**) and (**B**) show the HPLC profiles of Aβnac and Aβac respectively, with (black line) or without (grey line) trypsin.

findings indicate that the differences in the protease sensitive are probably due to the different conformations adopted by the peptide in solution.

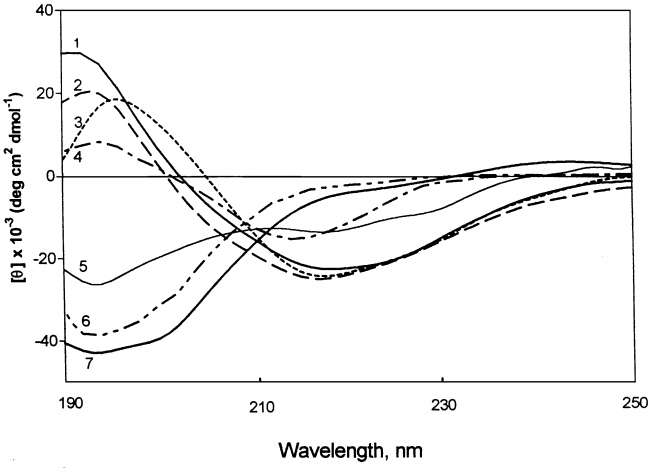

Figure 4 Circular dichroism studies of Aβ(1–40), Aβ(1–42) and Aβ analogues

Aliquots (60 μg) of each peptide were dissolved in 300 μl of 20 mM Tris buffer, pH 7.4. CD spectra were recorded, after centrifugation 11 400 ***g*** for 5 min at 25 °C to remove aggregated material, in a Jasco spectropolarimeter model J-720 at room temperature in a 0.1 cm pathlength cell. Results are expressed as mean residue ellipticity in units of deg · cm^2 · dmol^{-1} after substraction of buffer baseline spectra and smoothing with the algorithm provided by Jasco. The spectra of Aβnac (spectrum 7) and Aβac (spectrum 1) derived from Aβ(1–40) are shown, as are the spectra of Aβ(1–42) (spectrum 2), Aβ(1–40Q) (spectrum 3), Aβ(1–40G) (spectrum 4), Aβ(1–40) (spectrum 5) and Aβ(1–40A) (spectrum 6).

Table 1 Relative amounts of Aβnac and Aβac in different Aβ analogues

The values correspond to the percentages of Aβ conformers as determined by sedimentation assay and by the fitting of CD spectra.

	Sedimentation*		CD†	
Peptide	Aβnac	Aβac	Aβnac	Aβac
Aβ(1–40)	65.4	34.6	68	32
Aβ(1–42)	11.5	88.5	9	91
Aβ(1–40Q)	24.3	75.7	12	88
Aβ(1–40G)	27.8	72.2	33	67
Aβ(1–40A)	90.6	9.4	94	6

* The relative amounts of both Aβ species were determined by the amount of peptide in the pellet and supernatant after 10 d of incubation.

† The relative amounts of the Aβ conformers were obtained by the best fit of the experimental CD spectra of the Aβ variants with the CD spectra of Aβnac and Aβac, as described in the Materials and methods section.

Aβnac and Aβac derived from Aβ peptides of different lengths and bearing amino acid substitutions

It has been shown that Aβ peptides of different lengths or containing amino acid substitutions have different abilities to form amyloid fibrils *in vitro* [8,9,13,15,16,19–21,23]. To study whether the differences in the fibrillogenic properties of Aβ variants were due to the presence of distinct relative levels of amyloidogenic and non-amyloidogenic conformations, the analysis of the secondary structure of several Aβ synthetic peptides was performed in aqueous solution. The circular dichroism (CD) spectra of Aβ(1–42) and Aβ(1–40Q) showed mainly β-sheet structure and were similar to the spectrum of the Aβac species derived from Aβ(1–40) (Figure 4, Table 1). In contrast, the spectrum of Aβ(1–40A) showed predominantly a minimum between 190 and 200 nm, characteristic of random coil structures and is very similar to the spectrum of Aβnac derived from Aβ(1–40). Aβ(1–40) and Aβ(1–40G) adopted a mixture of β-sheet and random coil structures, with different relative proportions of each secondary structure (Figure 4). When CD spectra of Aβ peptides incubated in 20% TFE were recorded, random coil, but not β-sheet, was stabilized as α-helix, as previously reported [21,25].

The relative level of both conformational species in Aβ(1–42) and Aβ analogues was calculated by sedimentation assays and by fitting the experimental CD spectra, as described in the Materials and methods section (Table 1). Under these experimental conditions Aβ(1–42) and Aβ(1–40Q) had the highest levels of Aβac, and Aβ(1–40A) had the lowest level. Amyloid formation by the Aβ peptides, before separation of both populations, measured by ThT fluorescence, is directly proportional ($r = 0.97$) to the content of β-sheet conformation as determined by CD spectra (Figure 5, top). In addition the protease resistance of several Aβ variants was also found to be directly dependent ($r = 0.96$) on the level of β-sheet secondary structure, i.e. the relative amount of amyloidogenic conformer (Figure 5, bottom).

Amyloid formation by the amyloidogenic conformer was high and fast as determined by fluorimetric assay. ThT fluorescence values of Aβac, incubated for 24 h at 1 mg/ml in 30 μl of 0.1 M Tris/HCl, was approx. 55–65 fluorescence units and aggregated almost completely after 4 d of incubation (Table 2). These values were independent of the Aβ peptide used to isolate Aβac. Aβnac showed 3–8 fluorescence units regardless of its parent Aβ variant (Table 2). Moreover, Aβnac isolated from Aβ(1–40), Aβ(1–42)

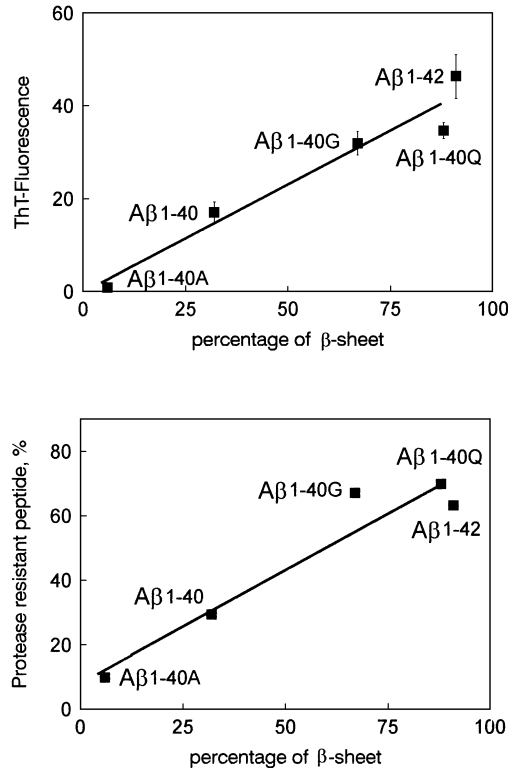

Figure 5 Dependence of fibrillogenesis and protease resistance on the percentage of β-sheet secondary structure in different Aβ peptides

Top: amount of amyloid formed by Aβ(1–40), Aβ(1–42), Aβ(1–40Q), Aβ(1–40G) and Aβ(1–40A), as estimated by ThT fluorescence, in relation to its content of β-sheet structure. The fluorescence values were measured in triplicate after 24 h of incubation at 1 mg/ml in a final volume of 30 μl of 0.1 M Tris/HCl, pH 7.4. Bottom: percentage of peptide resistance to trypsin digestion in the Aβ variants studied in relation to the proportion of β-sheet structure. The protease resistance was evaluated under the conditions described in Figure 3. The percentages of β-sheet were calculated by using the Lincomb algorithm.

Table 2 Aggregation and amyloid formation of Aβnac and Aβac obtained from Aβ(1–40), Aβ(1–42) and Aβ(1–40Q)

Aβnac and Aβac were obtained from the Aβ variants as described in the Materials and methods section. Aliquots of both Aβ conformers at a concentration of 1 mg/ml were incubated for 24 or 96 h and the ThT fluorescence or the percentage of peptide remaining soluble was measured by the fluorimetric and sedimentation assays respectively.

	ThT fluorescence		Soluble peptide (%)	
Peptide	Aβnac	Aβac	Aβnac	Aβac
Aβ(1–40)	3.9	54.8	93.7	3.2
Aβ(1–42)	8.6	66.4	81.6	1.1
Aβ(1–40Q)	6.6	59.2	85.8	1.7

or Aβ(1–40Q), incubated at 1 mg/ml, remained more than 80% soluble after 96 h of incubation (Table 2).

DISCUSSION

In this study we have shown that synthetic peptides containing the sequence 1–40 and 1–42 of Aβ and several Aβ analogues are composed of a mixture of two major species: one is highly amyloidogenic, partly resistant to proteolysis and contains a β-sheet structure; the other is poorly amyloidogenic, sensitive to proteolysis and adopts mainly a random coil conformation. In the absence of changes in external factors, the relative propensity of the peptides to adopt both conformational states is largely conditioned by the primary structure of the peptide (amino acid sequence and length). Aβ(1–42) and Aβ(1–40Q) consisted primarily of the Aβ amyloidogenic species (89.7% and 81.9% of Aβac respectively) (Table 1). In contrast, Aβ(1–40) and Aβ(1–40A) contained mainly the Aβ non-amyloidogenic species (66.7% and 92.3% of Aβnac respectively). There was a direct correlation between the percentage of β-sheet structure and both the amyloidogenicity and the resistance to proteolysis of the peptide. These results suggest that the differences in the fibrillogenesis and in the protease sensitivity among the Aβ peptides studied is due to a variable relative content of both Aβ species. This concept is consistent with the higher rate of amyloid formation obtained with Aβ progressively longer at their carboxyl terminus [13,16] and with Aβ(1–40Q) and Aβ(1–40G) analogues [15,19–22]. Although recent findings indicate a variability between lots in the relative levels of Aβnac and Aβac found with synthetic peptides of Aβ(1-40), it was always clear that there was a direct correlation between the secondary structure of the peptide and its fibrillogenic and protease resistance properties. These findings suggest that, besides the amino acid sequences, slightly different environmental conditions can determine important changes in the propensity of the peptide to adopt each conformational state. These results are in agreement with previous reports showing that an apparent source of variability in experiments involving synthetic Aβ peptides is the source of peptide [33–35].

It has been proposed than the length of the C-terminus of Aβ is crucial for amyloidogenesis in AD [13,16]. Our findings confirm the importance of the length of Aβ on the fibrillogenesis rate, indicating that the addition of hydrophobic residues at the C-terminal region of Aβ increases its propensity to adopt the amyloidogenic state. However, the presence of amino acid substitutions (Aβ1–40Q, Aβ1–40G) can produce a similar enhancement in the amyloidogenic potential of Aβ(1–40). These results suggest that the main determinant of amyloid formation is the conformation adopted by the peptide in the stage before aggregation. This conclusion is strengthened by the finding that the population of the non-amyloidogenic conformer present in Aβ(1–42) and Aβ(1–40Q) was completely random coil and was unable to form amyloid-like fibrils as determined by fluorimetric and sedimentation assays (Table 2). Although there are many reports in the literature indicating a relationship between the secondary structure of Aβ analogues and their fibrillogenicity, we are now showing evidence indicating that a solution of Aβ consists of multiple conformational states (at least two) with differential abilities to form amyloid. These findings may contribute to an explanation of why Aβ is found *in vivo* in both a soluble and a fibrillar form. In addition, the conformer that has the highest amyloidogenic capability is also more resistant to proteolytic degradation. It has been described in other systems that the protease sensitivity is modulated by the conformation of the protein [36], but this is the first time that it has been shown that the conformation adopted by Aβ determines its resistance to digestion by proteases.

Conformational studies of different Aβ fragments in the presence of distinct solvents [25] and of Aβ containing single amino acid substitutions [21] suggest that the main variation of the conformers involves a change in an α-helix or random coil to a β-strand structure in the N-terminal domain of Aβ. Until some years ago, short peptides were believed to exhibit very little structure in aqueous solution. However, considerable evidence

has accumulated showing that many peptide fragments of proteins are able to adopt folded conformations in water [37,38]. Frequently, short peptides adopt multiple structures in equilibrium [37–40] and it has been proposed that this conformational flexibility, typical of small peptides, can play a fundamental role in the initiation of protein folding [37,38]. The existence of multiple conformational states in solution has been previously indicated for short fragments of Aβ [7]. A fragment identical with Aβ(1–28) solubilized in a membrane-mimicking solvent was found as a monomeric α-helical structure that became partly random coil with an increase in temperature at pH 1–4 or greater than 7. However, this fragment adopted an oligomeric β-sheet structure and aggregated rapidly at pH 4–7 [12,17,25,26,41]. Furthermore it has been shown that a peptide containing the region 25–35 of Aβ exists in a dynamic equilibrium between random coil and β-sheet structure in aqueous solution, modulated by pH and by small alterations in the chemical structure [42]. Moreover, only the fragments with β-sheet structure were found to form fibrils, whereas those adopting a random coil conformation remained as monomers [42].

A relevant property of amyloid deposits is that they can apparently escape the proteolytic clearance mechanisms *in vivo* [6]. It has been shown that Aβ develops protease resistance in association with its polymerization into fibrils [43] and that the interaction of serum amyloid P component, a universal constituent of amyloid deposits *in vivo*, including Aβ fibrils, makes the amyloid even more resistant to proteolytic degradation [44]. Our findings that Aβac was more resistant to proteolysis than Aβnac may determine a differential degradation in body fluids and raises the possibility that small amounts of Aβac can accumulate gradually *in vivo* to a concentration sufficient to give rise to amyloid deposition. In support of our results, Norstedt et al. have shown [43] that Aβ peptide becomes resistant to proteases as a result of structural changes associated with its polymerization into amyloid fibrils. Moreover, they demonstrated that fibril formation *per se* does not lead to protease resistance, and they proposed that conformational changes closely associated with Aβ polymerization determine whether or not the peptide acquires protease resistance [43]. Considering these results and our findings, we conclude that the conversion of Aβnac into Aβac not only produces a peptide much more amyloidogenic, but also a peptide more resistant to proteolysis. It has been shown that in prion diseases the prion protein can also adopt two conformational states, one (PrP^{sc}) with a higher content of β-sheet structure, resistant to proteolysis and associated with the disease process and a normal prion protein (PrP^{c}) conformer that contains a higher level of α-helix and is protease sensitive [45]. A relevant feature of prion proteins is that PrP^{sc} is able to induce the transformation of PrP^{c} into PrP^{sc}. It remains to be determined whether Aβac under some conditions can convert Aβnac into the amyloidogenic conformation.

Because no chemical modifications were detected in Aβnac or Aβac by mass spectrometry, the two Aβ species with different amyloidogenic properties are thought to arise from two different conformational states of Aβ. Our results suggest that the interconversion between both conformers is slow. We hypothesize that the primary structure of Aβ permits the existence of two minima of conformational energy with a barrier of activation energy between them, which slows down the spontaneous interconversion of the conformers. Thus the transformation of the non-amyloidogenic into the amyloidogenic conformation can be viewed as a problem of protein folding. It seems likely that this transition can be enhanced by several factors, including environmental changes (pH or free radicals) [12], metal ions (Zn^{2+} or Al^{3+}) [46,47], pathological chaperone proteins (apoE, α_1-antichymotrypsin, amyloid P component) [44,48–50], peptide concentration and post-translational modifications (oxidation and racemization) [51,52]. Whether the two conformational states observed in Aβ synthetic peptides are also present in sAβ obtained from human cerebrospinal fluid or from cell culture medium is unknown. Work is in progress to answer this question.

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