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Acetylcholinesterase Accelerates Assembly of Amyloid-β-Peptides into Alzheimer's Fibrils: Possible Role of the Peripheral Site of the Enzyme

Nibaldo C. Inestrosa, Alejandra Alvarez, Cristián A. Pérez, Ricardo D. Moreno, Matias Vicente, Claudia Linker, Olivia I. Casanueva, Claudio Soto,* and Jorge Garrido

Departamento de Biología Celular y Molecular
Facultad de Ciencias Biológicas
Pontificia Universidad
Católica de Chile Santiago
Chile

Summary

Acetylcholinesterase (AChE), an important component of cholinergic synapses, colocalizes with amyloid-β peptide (Aβ) deposits of Alzheimer's brain. We report here that bovine brain AChE, as well as the human and mouse recombinant enzyme, accelerates amyloid formation from wild-type Aβ and a mutant Aβ peptide, which alone produces few amyloid-like fibrils. The action of AChE was independent of the subunit array of the enzyme, was not affected by edrophonium, an active site inhibitor, but it was affected by propidium, a peripheral anionic binding site ligand. Butyrylcholinesterase, an enzyme that lacks the peripheral site, did not affect amyloid formation. Furthermore, AChE is a potent amyloid-promoting factor when compared with other Aβ-associated proteins. Thus, in addition to its role in cholinergic synapses, AChE may function by accelerating Aβ formation and could play a role during amyloid deposition in Alzheimer's brain.

Introduction

Acetylcholinesterase (AChE) is the enzyme believed to be involved in the hydrolysis of the neurotransmitter acetylcholine in the central nervous system of mammals (Inestrosa and Perelman, 1990; Taylor and Radic, 1994; Silman et al., 1994). Its catalytic properties and role in synaptic transmission are well-documented (Hall and Kelly, 1971; Sussman and Silman, 1992). Alzheimer's disease (AD), the most common form of dementia in adults, is characterized by extensive neuronal loss and the presence of amyloid plaques and neurofibrillary tangles (Selkoe, 1994; Soto et al., 1994). In 1984, Smith and Cuello suggested that a common feature shared by the different neuronal groups in which lesions occur in AD is the presence of AChE. This enzymatic activity decreases in the brain of AD individuals, with a major decline in the G_4 form (Atack et al., 1983; Fishman et al., 1986) and a parallel increase in G_1 and A_{12} forms (Younkin et al., 1986; Arendt et al., 1992). This latter form increases from nearly undetectable levels to almost 4% of total activity in some areas (Younkin et al., 1986). Most of the cortical AChE activity present in AD brain was found associated with neuritic plaques (Mesulam, 1986; Geula and Mesulam, 1989), in which it colocalized with amyloid-β peptide (Aβ) deposits, including both the preamyloid diffuse deposits and the mature senile plaques (Carson et al., 1991; Morán et al., 1993). The diffuse deposition of AChE together with Aβ, is particularly interesting because it represents an early step in the development of the senile plaques (Tagliavini et al., 1988), suggesting that AChE, an enzyme that is localized on the presynaptic nerve terminal and also secreted as a soluble form (Massoulié et al., 1993) may be present together with the soluble Aβ at a very early stage of amyloid plaque formation. Progressive deterioration of memory and learning is a characteristic manifestation of AD (Bondi et al., 1994). The molecular mechanisms that underlie this phenomenon are still obscure, particularly in the sporadic cases, which correspond to the great majority of AD patients (Katzman and Kawas, 1994). Therefore, there is a need to search for potential reasons to explain the progressive cognitive decline in AD. Recent support for a role of neocortical acetylcholine in spatial memory has been obtained (Winkler et al., 1995); moreover, transgenic mice that overexpress human AChE in brain neurons showed a progressive cognitive deterioration in the Morris water maze paradigm (Beeri et al., 1995). A number of studies with synthetic Aβ in vitro have shown that this peptide aggregates and forms amyloid fibrils similar to the filaments found in the brains of patients with AD (Castano et al., 1986; Kirschner et al., 1987). A successful approach in the study of the structure and solubility of Aβ has been the alteration of the primary structure of the Aβ fragments and the study of their properties (Soto et al., 1994). The substitution of Glu-22→Gln, found in hereditary cerebral hemorrhage with amyloidosis of the Dutch type (Levy et al., 1990), yields a peptide with increased ability to form amyloid fibrils (Wisniewski et al., 1991). On the other hand, the single mutation of Val-18→Ala induces a significant increment of the α-helical content of Aβ, and dramatically diminishes fibrillogenesis (Soto et al., 1995). It was our aim to investigate whether the in vivo colocalization of AChE and Aβ could be reproduced under in vitro conditions. If such interactions do occur, they might influence the process leading to amyloid deposition and the appearance of AD. We report here that AChE induces amyloid fibril formation. Such effect is independent of the source and structural polymorphism of the enzyme, is not affected by an active site inhibitor, is diminished by a nonselective bisquaternary inhibitor and by a peripheral site blocker, and is not shared by butyrylcholinesterase (BuChE).

Result

jAChE Promotes Aggregation of Aβ Peptides and Amyloid Formation

The effect of AChE on the aggregation of Aβ synthetic peptides was studied through the turbidimetric measurement of the aggregation process (Jarret et al., 1993).

*Present address: Department of Neurology, New York University Medical Center, New York, New York 10016.

During the 24 hr incubation period, the Aβ peptide alone became aggregated to a lesser extent (2-fold less) than the Aβ peptide plus purified brain G_4 AChE (240 μM Aβ with AChE at 2.4 μM) (Figure 1A). To quantitate the level of peptide aggregation further, we measured the amount of remaining soluble peptide after incubation by a sedimentation assay. Aβ peptide aggregated only partially in the absence of AChE (26%); however, addition of AChE at 100:1 molar ratio increased the aggregate material up to 80%. To quantitate partially the amount of amyloid formed under each condition, we used a novel method based on the fluorescence emission by thioflavine-T bound to amyloid (Naiki et al., 1991; LeVine, 1993). Thioflavine-T binds specifically to amyloid, and this binding produces a shift in the emission spectra and a fluorescence enhancement that is directly proportional to the amount of amyloid formed (LeVine, 1993). Freshly suspended $Aβ_{1–40}$ either alone or coincubated with AChE in the fluorescence buffer showed no specific thioflavine-T emission at 485 nm. During the incubation period, there was a gradual increase of the fluorescence in both 240 μM $Aβ_{1–40}$ alone, and in Aβ coincubated with 2.4 μM AChE up to 24 hr (Figure 1B). However, the rate of increase was greater for Aβ coincubated with AChE. In fact, $Aβ_{1–40}$ coincubated with AChE displayed fluorescence values that were 2.8-fold higher than for Aβ alone. AChE alone (2–10 μM) showed no increase in fluorescence over this time period. $Aβ_{1–40}$ incubated with bovine serum albumin, ubiquitin, collagen type IV, or fibronectin gave similar fluorescence values to Aβ alone (data not shown). The next step was to study the effect of AChE concentration upon amyloid formation. After 24 hr, an increase in the fluorescence emission was observed with increasing AChE concentrations up to 2.4 μM. At 1.2 μM (1:200 molar ratio, AChE:$Aβ_{1–40}$ peptide), 60% of the maximal increase was seen (Figure 1C). These results are consistent with a faster aggregation of the $Aβ_{1–40}$ in the presence of AChE.

Characterization of the Amyloid Fibrils Induced by AChE

The morphology of the amyloid fibrils produced in the presence of affinity purified G_4 AChE form was examined by electron microscopy after 5 days of incubation at room temperature. Negatively stained $Aβ_{1–40}$ assembled at (pH 7.4) revealed that the fibrous aggregates are composed of fibrils, both in the presence and absence of tetrameric AChE (Figures 2A and 2B). No morphological differences were detected between the fibrils formed by Aβ alone and by Aβ incubated with AChE. In both cases, the amyloid fibrils showed the typical features described, 7 nm–10 nm thick unbranched fibrils, up to 3 mm in length (Castano et al., 1986; Kirschner et al., 1987). Although the morphology of the amyloid fibers was similar, the amount of these structures deposited on the electron microscope grids was higher in the presence of AChE than in its absence, strengthening the results described above. No fibril formation was noted with G_4 AChE alone (Figure 2C). The above results suggest that brain AChE constitutes an important cofactor in $Aβ_{1–40}$ fibrillogenesis.

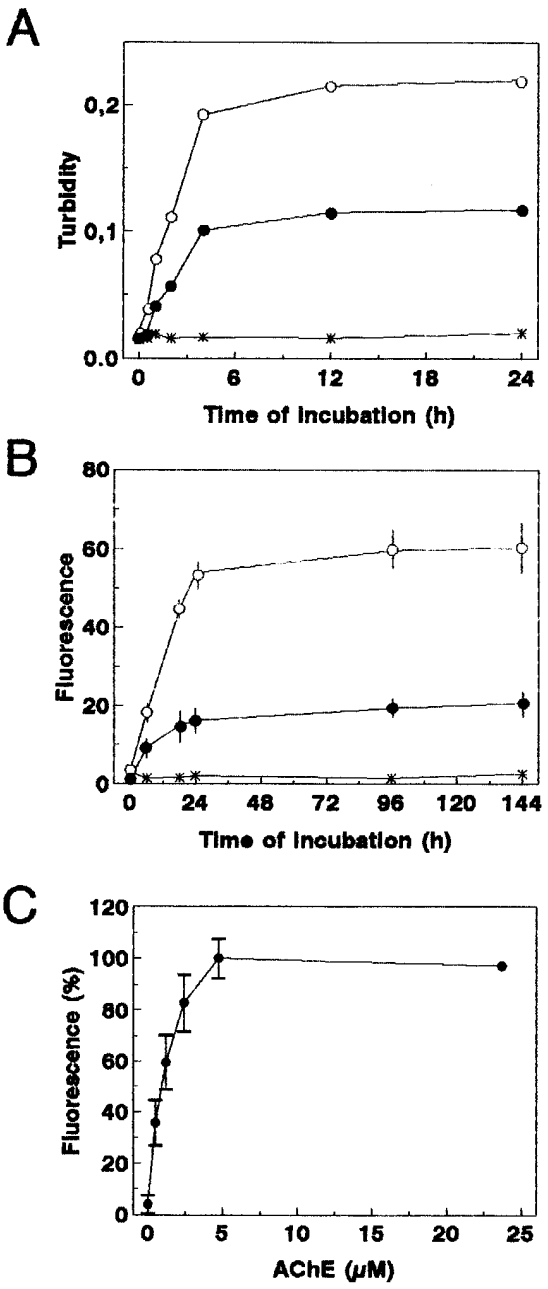

Figure 1. AChE Induces Aggregation of the $Aβ_{1–40}$ Peptide and Amyloid Formation

(A) The aggregation was measured by the turbidity at 405 nm. The $Aβ_{1–40}$ peptide was incubated alone (240 μM) and with AChE (2.4 μM) at different times at room temperature in 0.1 M sodium acetate buffer, (pH 5.0). The values shown represent the mean of two different experiments.

(B) Thioflavine-T fluorescence of $Aβ_{1–40}$ alone and with AChE at different times of incubation. Fluorometric data from three identical samples in separate experiments, represent the mean ± SD.

(C) AChE concentration dependence of $Aβ_{1–40}$ enhanced aggregation. Values represent the mean ± SD of three separate experiments, with the exception of the molar ratio 10:1 (Aβ:AChE) in which only one experiment was carried out because 200 μg of enzyme was required for each assay.

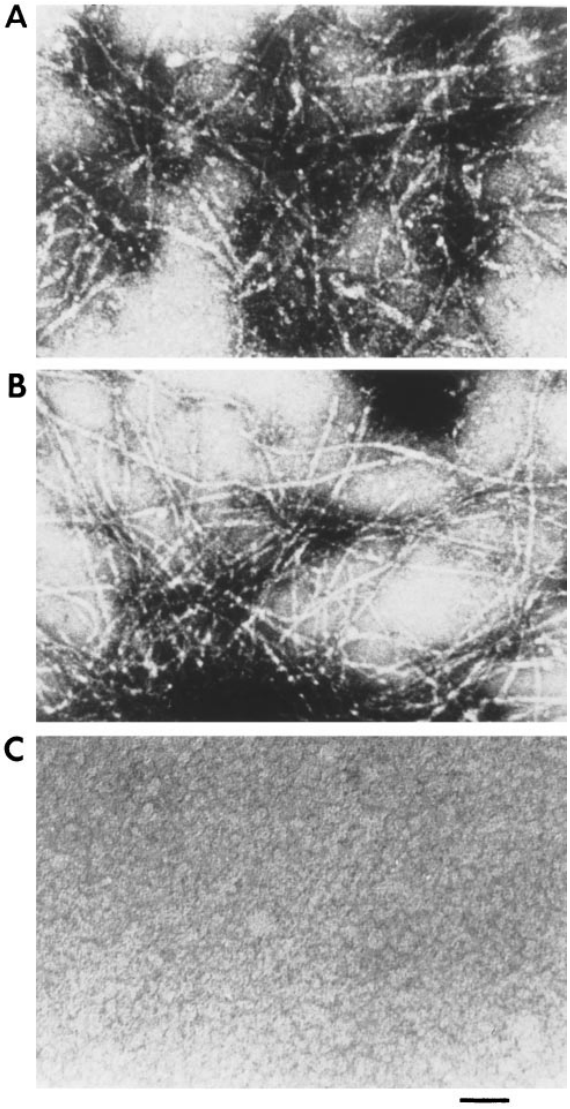

Figure 2. Electron Micrographs of Negatively Stained Preparations of $A\beta_{1-40}$ Fibrils with and without AChE

Aliquots of both preparations, $A\beta_{1-40}$ (A), $A\beta_{1-40}$ plus AChE (B) and AChE alone (C) were adsorbed onto 300-mesh Formvar-coated grids and negative-stained with 2% uranyl acetate. The specimens were viewed for fibrils with a Philips electron microscope. Original magnification 80,000×.

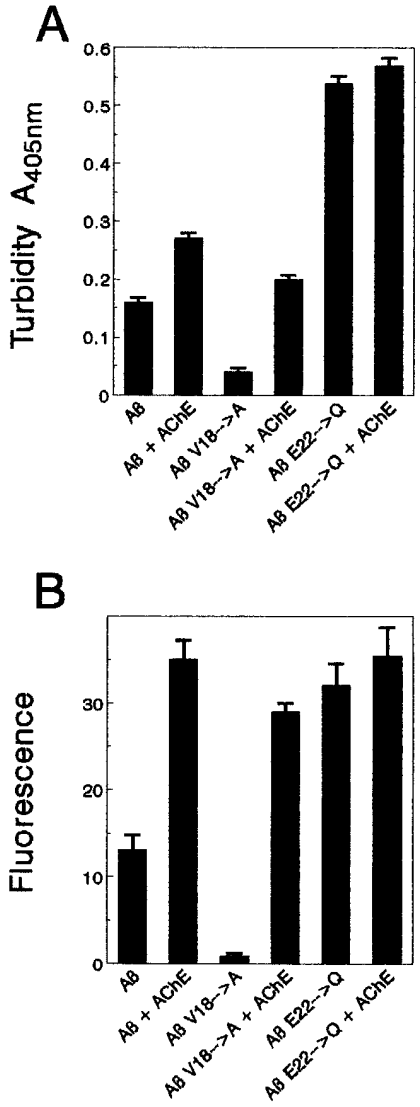

Figure 3. Effect of AChE on Aggregation and Amyloid Formation by Aβ Analogs Containing Different Substitutions

Experimental conditions were those described in Figure 1. In both the turbidity measurement (A) and in the fluorescence studies (B), the time of incubation was 24 hr. Different peptides ($A\beta_{1-40}$, $A\beta_{Val-18\rightarrow Ala}$ and $A\beta G_{\alpha lu-22\rightarrow Gln}$) alone (1 mg/ml in 0.1 M Tris–HCl, [pH 7.4]) and with 2.4 μM AChE were incubated at room temperature. The mean ± SD of three different experiments made in triplicate is shown. The graph shows the fluorescence emission, in arbitrary units, of thioflavine-T bound to the amyloid formed in the presence of each peptide with and without AChE.

AChE Induces the Mutant $A\beta_{Val-18\rightarrow Ala}$, but Not the Dutch Variant $A\beta_{Glu-22\rightarrow Gln}$, to Form Amyloid

To better understand the effect of AChE on amyloid formation, Aβ analogs containing single amino acid substitutions were used. First, we studied the single mutation of Val-18→Ala that induces a significant increment of α-helical structure and dramatically diminishes fibrillogenesis (Soto et al., 1995). When the $A\beta_{Val-18\rightarrow Ala}$ was incubated at a concentration of 240 μM, only a small increase in turbidity was observed after 24 hr of incubation. However, coincubation with purified brain AChE (2.4 μM) resulted in a 5-fold increase in turbidity (Figure 3A). When the amyloid formation was measured by thioflavine-T fluorescence, a clear increase in fluorescence (9-fold) was observed after 24 hr of incubation (Figure 3B). These results indicate that AChE produces a greater effect on those peptides that have less β-sheet structure.

The Dutch variant $A\beta_{Glu-22\rightarrow Gln}$ is found in hereditary cerebral hemorrhage with amyloidosis, Dutch type (Levy et al., 1990). This variant is highly amyloidogenic in vitro, forming more stable fibrils more rapidly than wild-type $A\beta_{1-40}$ (Wisniewski et al., 1991). To test the effect of AChE on the aggregation of the Dutch variant, the peptide was incubated at 240 μM with AChE at 2.4 μM. Both turbidity

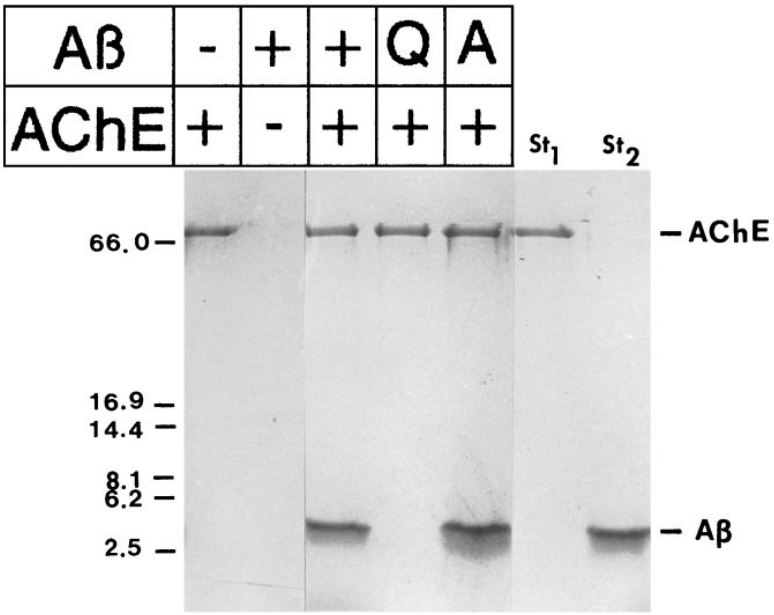

Figure 4. Binding of Aβ Analogs to AChE

Binding of different peptides with AChE was studied by size exclusion chromatography (Barrow et al., 1992). 10 μg of each peptide (wild-type $A\beta_{1-40}$ and the variants $A\beta_{Val\ 18\rightarrow Ala}$ [A] and $A\beta_{Glu\text{-}22\rightarrow Gln}$ [Q]) with AChE (molar ratio of Aβ:AChE, 15:1) for one hr at room temperature in a final volume of 40 μl. After incubation, the samples were loaded in Sephadex G-25 columns preequilibrated in 0.1 M Tris–HCl, (pH 7.4), and eluted by a brief spin. Eluted samples were analyzed by SDS–polyacrylamide gels, using the Tris–Tricine system (Schagger and van Jagow, 1987). The samples in the gel from left to right correspond to AChE alone (lane 1), $A\beta_{1-40}$ peptide alone (lane 2), $A\beta_{1-40}$ incubated with AChE (lane 3), $A\beta_{Glu\text{-}22\rightarrow Gln}$ (Q) with AChE (lane 4) and $A\beta_{Val\text{-}18\rightarrow Ala}$ (A) with AChE (lane 5). St1 AChE was directly loaded in the gel (lane 6), as was St2 $A\beta_{1-40}$ peptide (lane 7). The molecular weight markers are as follows: myoglobin (1–153) 16,950 Da; myoglobin I+II (1–131) 14,440 Da; myoglobin I (56–131) 8,160 Da; myoglobin II (1–55) 6,210 Da, and myoglobin III (132–153) 2,510 Da.

and fluorimetry showed no significant differences after 24 hr, respectively (Figures 3A and 3B). These results indicate that tetrameric AChE had little effect on the self-aggregation of the highly amyloidogenic Dutch variant. No differences were detected in the morphology (electron microscopy) of the amyloid fibrils formed by the mutant $A\beta_{Val\text{-}18\rightarrow Ala}$, or the Dutch variant plus AChE compared with the wild-type $A\beta_{1-40}$ (data not shown). Previously we have shown that Aβ peptide binds AChE (Inestrosa et al., 1994, LatinoAmerican Congress of Pharmacology, abstract; Alvarez et al., 1995). Therefore, we tested the ability of the two Aβ analog ($A\beta_{Val\text{-}18\rightarrow Ala}$ and the Dutch variant) to bind G_4 AChE using size exclusion chromatography (Barrow et al., 1992). The esterase was preincubated for one hr with $A\beta_{1-40}$ and the Aβ analog containing single amino acid substitutions. As shown in Figure 4, SDS–polycrylamide gels of the eluates of Sephadex G-25 filtration experiments indicated that both the wild-type $A\beta_{1-40}$, as well as the mutant $A\beta_{Val\text{-}18\rightarrow Ala}$(A) were able to bind AChE, while the Dutch variant $A\beta_{Glu\text{-}22\rightarrow Gln}$ (Q) was not. These results are consistent with those presented above, showing that enhancement of amyloid formation by AChE is relatively higher on less amyloidogenic peptides ($A\beta_{1-40}$ and $A\beta_{Val\text{-}18\rightarrow Ala}$) than on highly amyloidogenic variants such as $A\beta_{Glu\text{-}22\rightarrow Gln}$.

Different Molecular Forms of AChE Bind Aβ and Induce Amyloid Formation

AChE exhibits considerable structural polymorphism (Inestrosa and Perelman, 1989; Massoulié et al., 1993); therefore, we studied the ability of several molecular forms of AChE to promote the assembly of $A\beta_{1-40}$ into amyloid fibrils and to bind the Aβ peptide. As shown in Table 1, hydrophobic and hydrophilic tetrameric (G_4) AChE, as well as hydrophilic monomeric (G_1) AChE forms, were able to bind $A\beta_{1-40}$ and to induce amyloid fibril formation. These results suggest that the capacity of AChE to promote assembly of Aβ into amyloid filaments resides in the molecular structure of its monomer.

Human and Mouse Recombinant AChE Promotes Amyloid Formation

To make sure that the effect of AChE on amyloid formation was not the result of an Aβ aggregation-promoting contaminant that copurified with the bovine brain G_4 form, amyloid formation was studied in the presence of both human and mouse recombinant AChE. The recombinant enzymes were purified by affinity chromatography from the medium of transfected HEK-293 cells (Velan et al., 1991; Bourne et al., 1995). Mouse AChE

Table 1. Aβ Binding and Formation of Aβ Fibrils with Different AChE Forms

Molecular Form of AChE	Aβ Binding	Aβ Fibrils
Hydrophobic G_4 AChE	35[a]	100[b]
Hydrophilic G_4 AChE	25	90
Hydrophilic G_1 AChE	20	85

[a] Binding of Aβ peptide to the different forms of AChE was performed using gel filtration analysis on Sephadex G-25 followed by SDS–PAGE as described in Experimental Procedures. Binding is expressed as the percentage of total Aβ peptide that bound to the AChE. The data are mean values of three separate experiments that did not differ by more than 10%.

[b] The formation of Aβ fibrils was established by electron microscopic examination of the fibrils formed under the conditions described in Figure 4. Data are expressed as the percentage of the total aggregation of Aβ in separate grids, containing either the Aβ peptide alone (30% aggregation) or the corresponding AChE form. The hydrophobic G_4 AChE form containing the 20 kDa noncatalytic anchor domain was affinity purified from bovine caudate nucleus on an acridine–Sepharose column, as described previously (Inestrosa et al., 1987). The hydrophilic G_4 AChE was prepared from the hydrophobic G_4 after proteolytic digestion using proteinase K, and the monomeric hydrophilic AChE form was prepared from the hydrophobic G_4 form by mild proteolytic digestion with trypsin (Fuentes et al., 1988). In both cases, the hydrophilic G_4 and the monomeric forms of AChE were separated from the reaction products of each proteolytic digestion plus the corresponding protease by subsequent chromatography on acridine–Sepharose as described for the native G_4 AChE (Inestrosa et al., 1987). In addition, for each enzyme form, homogeneity was established by SDS–PAGE.

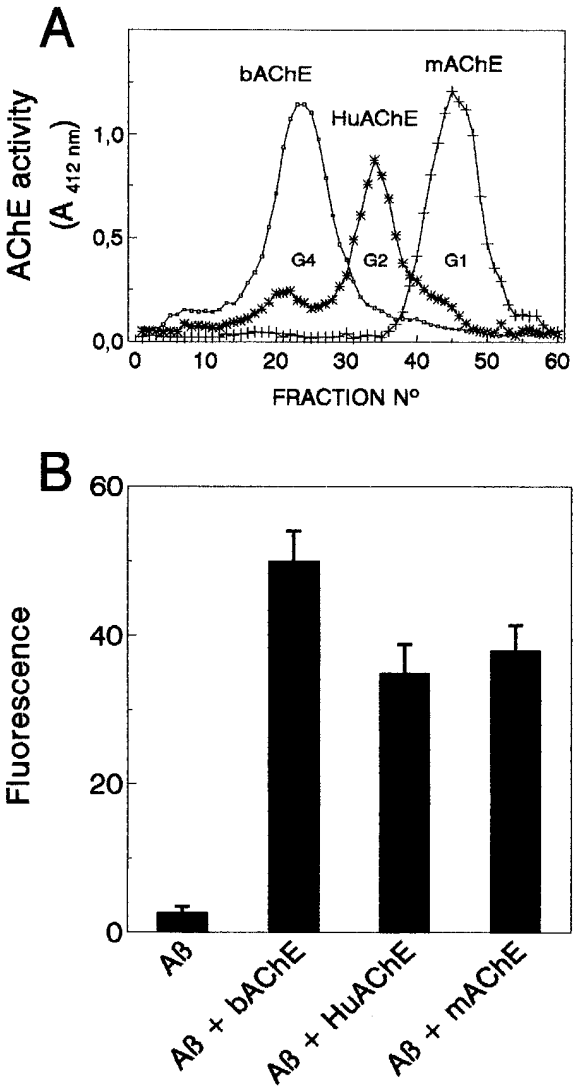

Figure 5. HuAChE and mAChE Recombinants Promote the Assembly of $A\beta_{1-40}$ Peptide on Amyloid Fibrils

(A) Hydrodynamic analysis of purified brain bAChE, HuAChE, and mAChE. A sample of each purified enzyme (50 μl), supplemented with sedimentation standards, was centrifuged in a 5%–20% sucrose gradient in the presence of Triton X-100, and then fractionated, and the AChE activity was determined by the Ellman method (Ellman et al., 1961).

(B) Amyloid formation by AChE from several sources. Purified G_4 bovine AChE, and HuAChE recombinant, containing 90% of G_2 form and 10% of G_1 form, and mAChE recombinant monomeric G_1 AChE form were incubated with 240 μM $A\beta_{Val\text{-}18\rightarrow Ala}$ peptide (1 mg/ml) with or without bAChE (2.4 μM), HuAChE (3.6 μM) and mAChE (9.6 μM) for 48 hr at room temperature. Then, amyloid formation was quantified by thioflavine-T fluorescence. Data from three different samples run in parallel, results are mean ± SD.

(mAChE) sediments as a 4.8 S monomeric G_1 form and human AChE (HuAChE) sediments as two molecular forms of 6.5 S and 10.7 S, which correspond to G_2 and G_4 species (Figure 5A). Both mAChE and HuAChE were assayed for their capacity to accelerate amyloid formation. As Figure 5B indicates, coincubation of Aβ with either mAChE or HuAChE resulted in a clear increase in thioflavine-T fluorescence. In both cases, the effect is appproximately 75% of that observed with the affinity-purified bovine brain G_4 tetramer. These results indicate that AChEs from different sources are able to accelerate the aggregation of $A\beta_{1-40}$ into amyloid fibrils, making unlikely that an aggregation-promoting factor other than AChE could be responsible for the results presented in this paper.

Propidium, a Peripheral Anionic Site Ligand, Affects the Formation of Amyloid Induced by AChE

Besides active site inhibitors, AChE activity is also inhibited by peripheral anionic site ligands (Radic et al., 1991; Eichler et al., 1994). When the effect of AChE on amyloid formation was studied in the presence of propidium, an exclusive peripheral anionic site ligand (Taylor and Lappi, 1975), this compound inhibited almost 75% of the enhancement of amyloid formation triggered by AChE (Figure 6A). Bisquaternary ligands are well-known inhibitors of AChE (Bergmann et al., 1950; Bergmann and Segal, 1954) and their inhibitory capacity has been ascribed to their ability to span the distance between the active center and the peripheral anionic site (Main, 1976). When the effect of AChE on amyloid formation was studied in the presence of the nonselective bisquaternary inhibitor decamethonium, this compound inhibited nearly 80% of the enhancement of amyloid fibril formation (Figure 6A). However, BW 284C51, a selective bisquaternary inhibitor, did not inhibit amyloid formation at 2 μM. On the other hand, when the cationic active center ligand edrophonium (Wilson and Quan, 1958; Silman et al., 1994) was included, no effect on amyloid formation was observed (Figure 6A).

These results suggest that the catalytic active center of AChE does not participate in the interaction of the enzyme with the Aβ peptide, whereas it is possible that the peripheral anionic binding site of AChE may be involved in amyloid formation.

BuChE Does Not Promote Amyloid Formation

BuChE shares many structural and physicochemical properties with AChE (Chatonnet and Lockridge, 1989), and it has been detected in senile plaques and also in neurofibrillary tangles (Mesulam et al., 1987; Gómez-Ramos et al., 1992) in which it colocalized with the Aβ peptide (Morán et al., 1993). Therefore, we evaluated whether BuChE can affect the known spontaneous in vitro amyloid formation. Thioflavine-T fluorimetry of $A\beta_{1-40}$ incubated with BuChE, at 240 μM and 2.4 μM, respectively, showed no significant differences after 24 hr (Figure 6B). The same results were obtained when the relative concentration of BuChE in relation to Aβ was increased in the incubation sample (2.4 μM BuChE with 120 and 60 μM $A\beta_{1-40}$). Because BuChE lacks Tyr-72, Tyr-124 and Trp-286, residues that form the peripheral anionic binding site of AChE (Harel et al., 1992; Radic et al., 1993; Barak et al., 1994), it is possible that the absence of such amino acids may be involved in the lack of effect of BuChE on the formation of amyloid fibrils.

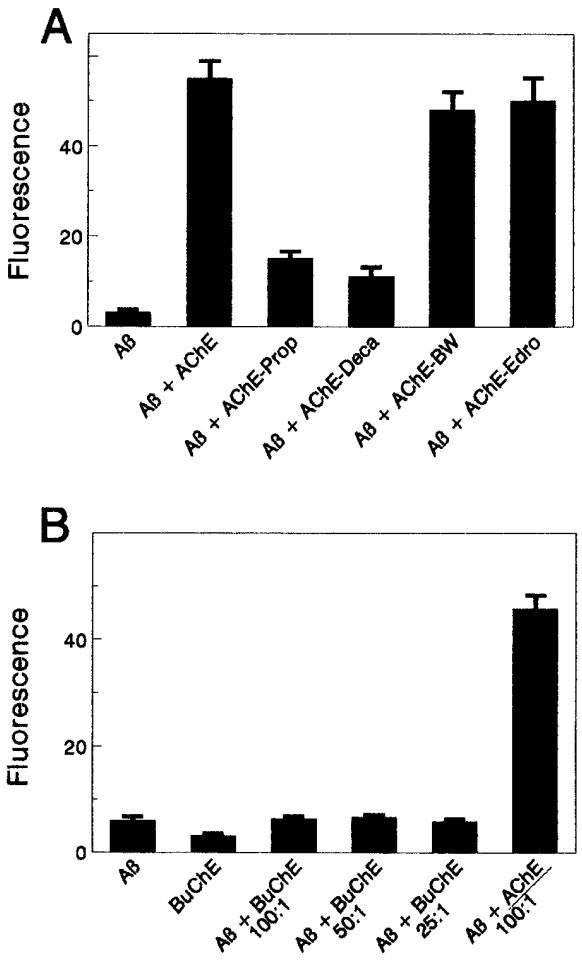

Figure 6. Propidium, but Not Active Site Inhibitors, Inhibits Amyloid Formation Induced by AChE

(A) Amyloid formation was quantified through the thioflavine-T assay (Naiki et al., 1989; LeVine, 1993). Aliquots of $A\beta_{Val\text{-}18 \rightarrow Ala}$ in a concentration of 1 mg/ml (0.25 μM) were incubated, with or without 2.4 μM AChE in the presence of 50 μM propidium, 100 μM decamethonium, 2 μM BW284C51, and 100 μM edrophonium for 24 hr in 0.1 M Tris–HCl (pH 7.5), at room temperature. Then, amyloid formation was quantified by thioflavine-T fluorescence.

(B) BuChE does not promote amyloid formation. Aliquots of $A\beta_{1\text{–}40}$ (1 mg/ml) were incubated, with or without 2.4 μM BuChE or AChE for 24 hr in 0.1 M Tris–HCl, (pH 7.5), at room temperature. Then, the amyloid was quantified by the thioflavine-T method. As in (A), the values shown correspond to the mean ± SD of three different experiments made in duplicate.

AChE Is a Potent Amyloid-Promoting Factor

We examined the relative ability of purified brain G_4 AChE, recombinant apolipoprotein E3 (apoE3), and α_1-antichymotrypsin (α_1-ACT) to promote amyloid formation. As shown in Table 2, a small effect was observed with apoE3. This result is consistent with recent studies showing that recombinant apoE is not an efficient promoter of Aβ fibrillogenesis (Castano et al., 1995; Evans et al., 1995). Unexpectedly, α_1-ACT showed only a relatively weak capacity to form amyloid (Ma et al., 1994). We conclude that AChE is an effective promoting factor of amyloid formation.

Table 2. AChE Is an Efficient Promoter of Amyloid Formation

Protein	Amyloid Formation (Thioflavine-T Fluorescence)	Relative Enhancement
Aβ alone	14.1 ± 2.7	1.00
Aβ plus apoE3	17.6 ± 3.9	1.25
Aβ plus α_1-ACT	19.2 ± 4.1	1.36
Aβ plus AChE	58.2 ± 3.7[a]	4.12

Aliquots of the $A\beta_{1\text{–}40}$ peptide in a concentration of 1 mg/ml (240 μM) were incubated for 24 hr in 0.1 M Tris–HCl (pH 7.5) at room temperature in the presence of 2.4 μM of AChE, apolipoprotein E3 (apoE3), and α_1-antichymotrypsin (α_1-ACT) (1:100 molar ratio). ApoE3 and α_1-ACT alone did not produce fluorescence above the background. In the case of AChE alone, the value of fluorescence was 2. The values shown correspond to the mean fluorescence ± SD of three different experiments made in duplicate.

[a] Student's t test, $p \leq 0.001$.

Discussion

AChE Is an Amyloid-Promoting Factor

In the present study, the ability of purified AChE to promote the assembly of $A\beta_{1\text{–}40}$ peptide into amyloid filaments was demonstrated. AChE induced a higher increase in the amount of amyloid formed on a poorly amyloidogenic Aβ analog created by substituting alanine for valine at position 18. Conversely, AChE was not able to increase the formation of amyloid by the Dutch variant of Aβ, in which a Glu-22→Gln substitution generates a higher amyloidogenic variant (Wisniewski et al., 1991; Soto et al., 1995). These results are in agreement with our recent findings that AChE modulates amyloid formation by inducing a conformational change in Aβ (Alvarez et al., 1995). Alternatively, AChE could act as a heterogeneous nucleator, increasing the rate of fibrillogenesis, especially in the less amyloidogenic peptides. An additional possibility is that AChE could bind to and stabilize the growing amyloid fibrils.

AChE from Different Sources and Quaternary Structure Promotes Amyloid Formation

Human, bovine, and mouse AChE were able to accelerate the aggregation of $A\beta_{1\text{–}40}$ into amyloid fibrils. These results indicate that an AChE-specific domain, not related to different species, is involved in such a function. The fact that recombinant HuAChE promotes amyloid formation is interesting because AD affects human beings, and therefore, if one desires to make any suggestion about the effect of AChE on AD pathology, it is mandatory to demonstrate that HuAChE is able to promote amyloid formation. Also, recent studies have shown that the transgenic expression of HuAChE induces progressive memory deterioration in mice (Beeri et al., 1995), a fact that may be related to the effect described in this paper, particularly considering that G_1 or A_{12} AChE forms may be involved. From a biological point of view, it is interesting that two different recombinant enzymes (HuAChE and mAChE) were able to accelerate amyloid formation, because it would be relatively easy to employ site-directed mutagenesis and molecular modeling techniques to examine different mutants

of AChE for their capacity to bind Aβ and to promote amyloid formation.

The quaternary structure adopted by AChE did not affect the capacity of the enzyme to accelerate the amyloid formation; in fact, tetrameric (both hydrophobic and hydrophilic), as well as dimers and monomers of the enzyme, were able to accomplish such a function. This is important because changes in the distribution of the AChE molecular forms occur during AD (Younkin et al., 1986; Nakamura et al., 1990; Arendt et al., 1992), including the appearance of an anomalous molecular form of this enzyme (Navaratnam et al., 1991).

The Catalytic Active Center of AChE Is Not Required for Amyloid Formation

Some features of AChE suggest that the enzyme may have a potential noncatalytic function(s) other than acetylcholine hydrolysis (Layer et al., 1993; Massoulié et al., 1993). Our results, showing that the ability of AChE to promote amyloid fibril formation was not affected by a specific active center inhibitor, indicate that the enzyme was able to perform such function by using a structural domain other than the active center. This result suggests that the aggregation of the Aβ peptide promoted by AChE is independent of its well-known catalytic activity.

A Hydrophobic Environment Close to the Peripheral Anionic Binding Site(s) of AChE Is Probably Involved in the Formation of Amyloid

In 1950, on the basis of binding studies of bisquaternary compounds to AChE, Nachmansohn and coworkers proposed the existence of a second anionic site on the enzyme, which became known as the peripheral anionic site (Bergmann et al., 1950). Later, Changeux (1966) showed that the action of reversible inhibitors of AChE could not be explained simply on the basis of a single site, but rather that certain ligands affect catalysis through binding to an allosteric site. The ligand propidium binds to AChE and is selective for the peripheral site (Taylor and Lappi, 1975). The peripheral site of AChE appears to be located at or close to the enzyme surface, near the entrance of the active site gorge (Weise et al., 1990; Amitai and Taylor, 1991; Sussman et al., 1991; Sussman and Silman, 1992; Silman et al., 1994), and peripheral site ligands might act by partially obstructing the entrance to the gorge (Taylor and Radic, 1994). Within this context, it is possible that the presence of a peripheral site blocker will not permit the Aβ peptide to interact with AChE, determining a lack of effect of the esterase over the amyloid fibril formation process. Consistent with this possibility is the finding that BuChE, an enzyme that lacks most of the amino acids of the peripheral anionic site (Harel et al., 1992; Vellom et al., 1993; Radic et al., 1993), was not able to stimulate the formation of amyloid fibrils. Our results, which show that the ability of AChE to promote amyloid formation was inhibited by propidium and decamethonium, point to the involvement of the peripheral anionic binding site in the interaction of the enzyme with the Aβ peptide; however, the result with BW284c51 is not consistent with this possibility. Barak et al. (1994) proposed that the peripheral anionic site of HuAChE consists of a number of binding sites close to the entrance of the active site gorge. Their data suggest that propidium, decamethonium, and BW284C51 bind to the peripheral site in a distinctive manner and that the nature and extent of participation of the individual residues is ligand dependent. This functional degeneracy is in part the result of the ability of the Trp-286 indole moiety to interact either via stacking (with propidium), via p-cation attractions (with decamethonium), or aromatic–aromatic interactions (with BW284c51). Another view is provided by the recent analysis of the crystal complex of AChE and fasciculin, a 61 amino acid peptide isolated from mamba venoms (Bourne et al., 1995; Harel et al., 1995). This toxin binds to the residues belonging to the peripheral anionic site of AChE, although other residues like Pro-78 and Gly-342 in mAChE also contribute to the fasciculin binding site. Whether they should also be considered as part of the peripheral anionic site is not certain (Bourne et al., 1995; Harel et al., 1995). In this context, we recently found that 1 nM fasciculin inhibits only 30% of the enhancement of amyloid formation triggered by the bovine brain G_4 AChE (C. A. P., F. Dajas., N. C. I., unpublished data). Therefore, it is possible that the interaction of the Aβ peptide with the peripheral anionic binding site(s) of AChE may be more complex than we would expect, by simply following the well-established specificity of the enzyme. Of course, further studies are necessary to identify the structural AChE domain involved in the interaction with the Aβ peptide. For the time being, we would like to propose the following as a working hypothesis: the domain of AChE involved in the acceleration of amyloid formation is related to a hydrophobic environment close to the peripheral anionic binding site(s) of the catalytic subunit of the enzyme.

Role of Other Senile Plaque-Associated Proteins in Amyloid Formation

Amyloid plaque is an ordered, fibrous protein aggregate characteristic of AD brain (Lansbury, 1992; Soto et al., 1994). It has been suggested that endogenous factors that accelerate amyloid deposition could play a significant role in the pathogenesis of AD (Jarrett and Lansbury, 1993). Although the present investigation has determined that AChE is an Aβ-associated component that promotes amyloid fibril formation, it is possible that other Aβ-associated proteins may be involved. These include amyloid-P component (Kalaria et al., 1991), apo E (Wisniewski and Frangione, 1992), apo J (Ghiso et al., 1993), α_1-ACT (Ma et al., 1994), extracellular matrix components (Brandan and Inestrosa, 1993), and proteoglycans (Snow et al., 1988). Recent studies indicate that perlecan, a heparan sulfate proteoglycan, infused together with $A\beta_{1-40}$ into rat brain, induces the formation of amyloid deposits adjacent to the infusion site (Snow et al., 1994). Therefore, it is possible that AChE and a heparan sulfate proteoglycan might play a role in promoting the assembly of Alzheimer Aβ peptide into filaments. In this context, it is interesting to mention that

asymmetric AChE, an enzyme form that increases severalfold in AD brain (Younkin et al., 1986) and is apparently bound to senile plaques (Nakamura et al., 1990), binds heparan sulfate proteoglycans and heparin (Brandan et al., 1985; Deprez and Inestrosa, 1995). Further studies of amyloid formation in vitro, using additional amyloid-associated proteins, should be carried out in order to determine the relative importance of their eventual contributions.

Has AChE Any Role in Triggering Amyloid Deposition in AD?

The cholinergic system is affected in AD (Perry et al., 1978; Whitehouse et al., 1981; Coyle et al., 1983; Geula and Mesulam, 1994), and despite a decrease in the level of AChE in the AD brain, the enzyme accumulates within amyloid plaques (Geula and Mesulam, 1989; Gómez-Ramos et al., 1992; Wright et al., 1993). Moreover, primate studies have shown that AChE may be deposited in plaques during the early stages of amyloid formation (Struble et al., 1982). In mature plaques, AChE is predominantly associated with the amyloid, rather than with the neuritic component (Carson et al., 1991). Within this context, the fact that AChE behaves as an amyloid-promoting factor suggests that the enzyme may play a role in amyloid deposition. Regarding the neuronal damage and the deposition of amyloid fibrils that exhibit a restricted localization in Alzheimer's brain (Terry et al., 1994), it is unclear how the low (nanomolar) levels of soluble Aβ present in the brain (Selkoe, 1994) give rise to aggregated Aβ in the form of stable amyloid plaques. Therefore, the availability of the Aβ peptide itself is not a rate-limiting step in its deposition (Selkoe, 1994) and other local promoting factors like AChE may play an active role in the formation of mature toxic plaques (Iversen et al., 1995). Loss of cellular AChE and deposition of the secreted enzyme within preamyloid diffuse plaques and senile plaques (Struble et al., 1982; Morán et al., 1993), may be due to the degeneration of AChE-containing neurons (Geula and Mesulam, 1994) some of which may not be cholinergic (Mesulam and Geula, 1988). The importance of AChE in AD was emphasized by Smith and Cuello (1984), who indicate that different cell groups in which lesions occur in AD share as a common feature their high content of AChE. In this context, it was recently proposed that the alteration of the AChE systems, especially those more vulnerable in AD such as the lightly stained neurons located in the entorhinal cortex, the CA1/subiculum of the hippocampus, and the amygdala, are the first to be affected in the pathological process of AD (Shen, 1994). Consistent with the possibility that AChE would play a relevant role in AD is a recent work of Soreq and coworkers (Beeri et al., 1995), showing that transgenic expression of Hu-AChE induces progressive cognitive deterioration in mice. Therefore, it is intriguing to speculate that AChE may have a more fundamental role in amyloid formation, by providing a potential molecular link between the degeneration of AChE-containing presynaptic nerve terminals and the formation of senile plaques in Alzheimer's brain.

Experimental Procedures

Materials, Synthetic Peptides and Proteins

Aβ peptide corresponding to residues 1–40 of the human wild-type sequence, and peptides with amino acid substitutions, were synthesized by Chiron Corporation Incorporated, Emeryville, CA. The variant peptides were a mutant peptide $A\beta_{Val\text{-}18\rightarrow Ala}$, containing a valine to alanine substitution (Soto et al., 1995), and the Dutch variant of Aβ, containing a single amino acid substitution, Glu-22→Gln (Levy et al., 1990). Results for the $A\beta_{1\text{-}40}$ peptide were replicated with peptides from Sigma. Human serum BuChE was a gift of Dr. Oksana Lockridge, University of Nebraska, Omaha, NE. Recombinant apoE3 isoform was obtained from PanVera (Madison, WI). α_1-ACT was purchased from Calbiochem (La Jolla, CA). Other reagents, where not otherwise specified, were obtained from commercial sources.

Substrates and Inhibitors

AChE was assayed with acetylthiocholine iodide (Sigma) as substrate. The inhibitors 3,8-diamino-5-(3′-trimethylammonium)propyl-6-phenyl-phenanthridinium diiodide (propidium), 1,10-bis (trimethylammonium)decane (decamethonium), di(p-allyl-N-dimethylaminophenyl)-pentane-3-one (BW284C51), and ethyl(m-hydroxyphenyl)-dimethylammonium chloride (edrophonium) were all purchased from Sigma.

Purification of Bovine Brain AChE

The tetrameric G_4 AChE form (sedimentation coefficient 10.7 S) was purified from bovine caudate nucleus, using acridine-affinity chromatography (Inestrosa et al., 1987). Both specific activities (6,000 U/mg protein), and staining intensities following polyacrylamide gel electrophoresis (a single band of 68 kDa) were used to verify purity.

Recombinant AChEs

Recombinant human AChE was purchased from Sigma, and consists of 90% of dimers (G_2, 6.5 S) and 10% of tetramers (G_4, 10.7 S) of catalytic subunits (Velan et al., 1991), and a G_1 (4.8 S) mouse recombinant. mAChE was a gift of Dr. Palmer Taylor, University of California, San Diego, La Jolla, CA.

Aggregation Studies of Aβ Peptides: Turbidity

Lyophilized aliquots of the peptides were resuspended in 0.1M sodium acetate, (pH 5.0). At various times, the aggregation was detected via turbidity measurements at 400 nm, as described previously (Jarret et al., 1993; Soto et al., 1995). Specifically, $A\beta_{1\text{-}40}$ synthetic peptide (240 μM) was incubated alone or in the presence of affinity purified AChE (2.4 μM) at a molar ratio Aβ:AChE of 100:1. The incubation was carried out at room temperature in phosphate-buffered saline, (pH 7.4). Sedimentation assay $A\beta_{1\text{-}40}$ peptide was incubated for 5 days in 0.1 M Tris–HCl, (pH 7.4), at room temperature. The soluble peptide was separated from the aggregated one, by centrifugation at 14,000 rpm for 15 min. Aliquots of the supernatant were analyzed by HPLC using a Waters apparatus. Absorbance was monitored at 220 nm. The percentage of the nonsedimentable peptide was measured by comparing the area of the peak corresponding to the soluble peptide at 5 days of incubation with a nonincubated sample.

Amyloid Detection in Suspension: Thioflavine-T Based Fluorometric Assay

Aliquots of peptides at the indicated concentrations were incubated for different times at room temperature in 0.1M Tris–HCl, (pH 7.4). For coincubation experiments, aliquots of AChE to a final molar ratio of 100:1 were added. To quantitate amyloid formation we used a thioflavine-T fluorescence method as described (LeVine, 1993; Soto et al., 1995). Thioflavine-T binds specifically to amyloid and this binding produces a shift in its emission spectrum and a fluorescent signal proportional to the amount of amyloid formed (Naiki et al., 1991). After incubation, Aβ peptides were added to 50 μM glycine, (pH 9.0), 1.5 μM thioflavine-T in a final volume of 2 ml. Fluorescence was monitored at excitation 435 nm and emission 485 nm using a Hitachi F-2000 spectrofluorometer. A time scan of fluorescence was performed, and three values after the decay reached the plateau

(around 300 s) were averaged after subtracting the background fluorescence of 1.5 μM thioflavine-T. For coincubation experiments, fluorescence of AChE alone preincubated at 10 μM was determined. The esterase or the Aβ peptide with or without 0.1% Triton X-100 gave fluorescence values of 2 or less. To study the effect of the AChE concentration on the Aβ aggregation, $A\beta_{1-40}$ at 240 μM in 0.1 M Tris–HCl, (pH 7.4) was incubated for 24 hr in the presence of increasing concentrations of AChE and fluorometry performed as described above. All of the experiments were performed in triplicate.

Electron Microscopy of Amyloid Fibrils

For fibril formation, peptides (1 mg/ml) were incubated in 0.1 M Tris–HCl, (pH 7.4) for 5 days at room temperature. Aβ peptides alone or incubated with AChE at room temperature, were placed on Formvar-carbon coated 300-mesh nickel grids (Ladd) for 1 min in under 2% glutaraldehyde vapors, blotted, and negatively stained with 2% uranyl acetate (Ladd) for 1 min. Grids were examined on a Philips EM-300 electron microscope at 80 kV.

Binding of Aβ to AChE and Size Exclusion Chromatography

The binding of the $A\beta_{1-40}$, $A\beta_{Val\text{-}18\rightarrow Ala}$ and the Dutch peptides to AChE was determined by size exclusion chromatography (Barrow et al., 1992). Sephadex G-25 columns equilibrated in phosphate saline buffer (pH 7.4) were packed to a final volume of 150 μl. The columns were loaded with different samples of Aβ peptides with AChE, previously incubated for 1 hr in a final volume of 40 μl at room temperature. A molar ratio of 1:20 (AChE:Aβ peptide) was used. Each column was eluted by a spin in an Eppendorf centrifuge, and each sample was then submitted to SDS–PAGE.

SDS–PAGE Analysis of AChE and the Aβ Peptides

Proteins were resolved using Tris–Tricine 16% SDS–PAGE (Schagger and von Jagow, 1987).

AChE Activity Measurements

AChE activity was determined by the method of Ellman et al. (1961).

Sedimentation Velocity Analysis

Affinity-purified brain AChE, as well as recombinant HuAChE and mAChE were submitted to sedimentation velocity analysis as described previously (Inestrosa et al., 1983, 1988).

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