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α_1-Antichymotrypsin regulates Alzheimer β-amyloid peptide fibril formation

(β-amyloid 1–40 peptide/Alzheimer disease)

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ABSTRACT **The major component of the cerebral plaques in Alzheimer disease is the β-amyloid peptide, but serine proteinase inhibitors like α_1-antichymotrypsin (ACT) are also present. Their role in the pathogenesis of amyloid formation is unsettled. In addition to their function as proteinase inhibitors, serine proteinase inhibitors can interact with various hydrophobic compounds, a reaction accompanied by a transition from the stressed to the relaxed conformation. We report here on the ability of ACT to regulate the formation of β-amyloid fibrils *in vitro*. In a molar ratio of 1:10 (ACT to β-amyloid) ACT inhibits β-amyloid fibril formation. Furthermore, ACT promotes rapid disaggregation of β-amyloid fibrils when added in the same molar ratio to preformed β-amyloid fibrils. These processes are accompanied by increased thermostability of ACT and loss of its biological activity, consistent with a conformational transition of ACT from the stressed to the relaxed state. The influence of ACT on β-amyloid fibril formation may be an example of a hydrophobic interaction between the β-amyloid peptide and the hydrophobic domain C terminal to the reactive center of ACT.**

Alzheimer disease (AD) is a neurodegenerative disorder characterized by abnormal deposition of extracellular congophilic plaques in the brain (1). The main constituent of senile plaques is a 39- to 43-amino acid peptide, called β-amyloid, which is a proteolytic fragment of the amyloid precursor protein (2). The metabolism of amyloid precursor protein is only partially understood. The proteases responsible for amyloid precursor protein processing and the sites in the brain where these proteolytic events occur are unknown (3). Recently, it was demonstrated that β-amyloid is a product of normal cellular metabolism (4–6). Different lengths of the peptide exist, but the major form is 40–42 amino acids, which in solution exist in a monomeric α-helical conformation. *In vitro* studies have shown that changes in pH, concentration, and temperature result in conformational rearrangements from an α-helix to an oligomeric β-sheet, which precipitates from solution and produces amyloid-like deposits (7). β-Amyloid is thought to be important early in the pathogenesis of AD (L.L., unpublished data).

Serine proteases and their inhibitors like α_1-antichymotrypsin (ACT) and α_1-proteinase inhibitor have been identified in different types of amyloid fibril isolates (8). The presence of these proteins in the extracellular amyloid deposits suggests a number of potential roles they may play in the pathogenesis of AD. Recently, it has been shown that a specific sequence within the N-terminal 10 amino acid residues of the β-amyloid peptide is responsible for the formation of an ACT–amyloid complex (9). These authors also noted that ACT induced fibril disaggregation *in vitro*. The increased expression of ACT by astrocytes in areas of Alzheimer lesions in the brain known to have many neuritic plaques support such a role (9).

Understanding how a normally soluble protein is transformed into insoluble fibrils is a critical part of understanding the mechanism of amyloid diseases. Structure-activity studies of ACT, α_1-proteinase inhibitor, and other related members of the serine proteinase inhibitor (serpin) super-family show that serpins have evolved mobile reactive cores and that in complex physiological environments their conformation may vary. During formation of a complex between a serpin and its target proteinase, a specific peptide bond of the serpin active site is cleaved. Cleavage results in a structural transition of the protein from a stressed to a relaxed form. During this transformation the inhibitor loses its antiprotease activity and becomes thermostable (10). We have previously shown that hydrophobic surroundings result in similar conformational alterations of the serpins; the serpins lose biological activity and become thermostable (S.J. and S.E., unpublished data). Hydrophobic compounds that have been used to induce such conformational transitions include cholesterol and the secondary bile acid lithocholic acid. Indirect evidence has suggested that interaction with α_1-proteinase inhibitor takes place through binding with the hydrophobic domain C terminal to the reactive center of the serpin. This 36-amino acid peptide has recently been isolated from human tissues, such as bile, blood, and spleen (11). The closely related serpin ACT possesses a homologous, hydrophobic C-terminal domain (12), which may be expected to interact in a similar fashion. The presence of ACT in AD disease plaques, the expression of ACT in brain tissue, and the ability of ACT to interact with hydrophobic compounds suggested to us that ACT may have a regulatory role in the formation of β-amyloid fibrils. The present report documents such a role for ACT.

MATERIALS AND METHODS

The β-amyloid peptide (1–40) was synthesized on solid phase (13) by David B. Teplow. The purity has been checked by HPLC and found to be approximately 80%.

Electrophoretically pure ACT was prepared according to Christensson and coworkers (14) and was a gift from Hans Lilja (Department of Clinical Chemistry, Lund University). The k_{ass} for ACT with human leucocyte cathepsin G was 1.05×10^6 as determined according to Travis and Morii (15). A monospecific antiserum against ACT was obtained from Dakopatts (Glostrup, Denmark). Standard incubation conditions were chosen so that samples were prepared in 0.01 M Tris·HCl, pH 7.4/0.15 M NaCl. In the text and legends, this buffer is referred to as Tris buffer. The amyloid peptide (0.2 mg) was solubilized at room temperature in the buffer containing 0.2 mg of ACT per ml. The molar ratio of β-amyloid to ACT was

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Abbreviations: ACT, α_1-antichymotrypsin; serpin, serine proteinase inhibitor; AD, Alzheimer disease.

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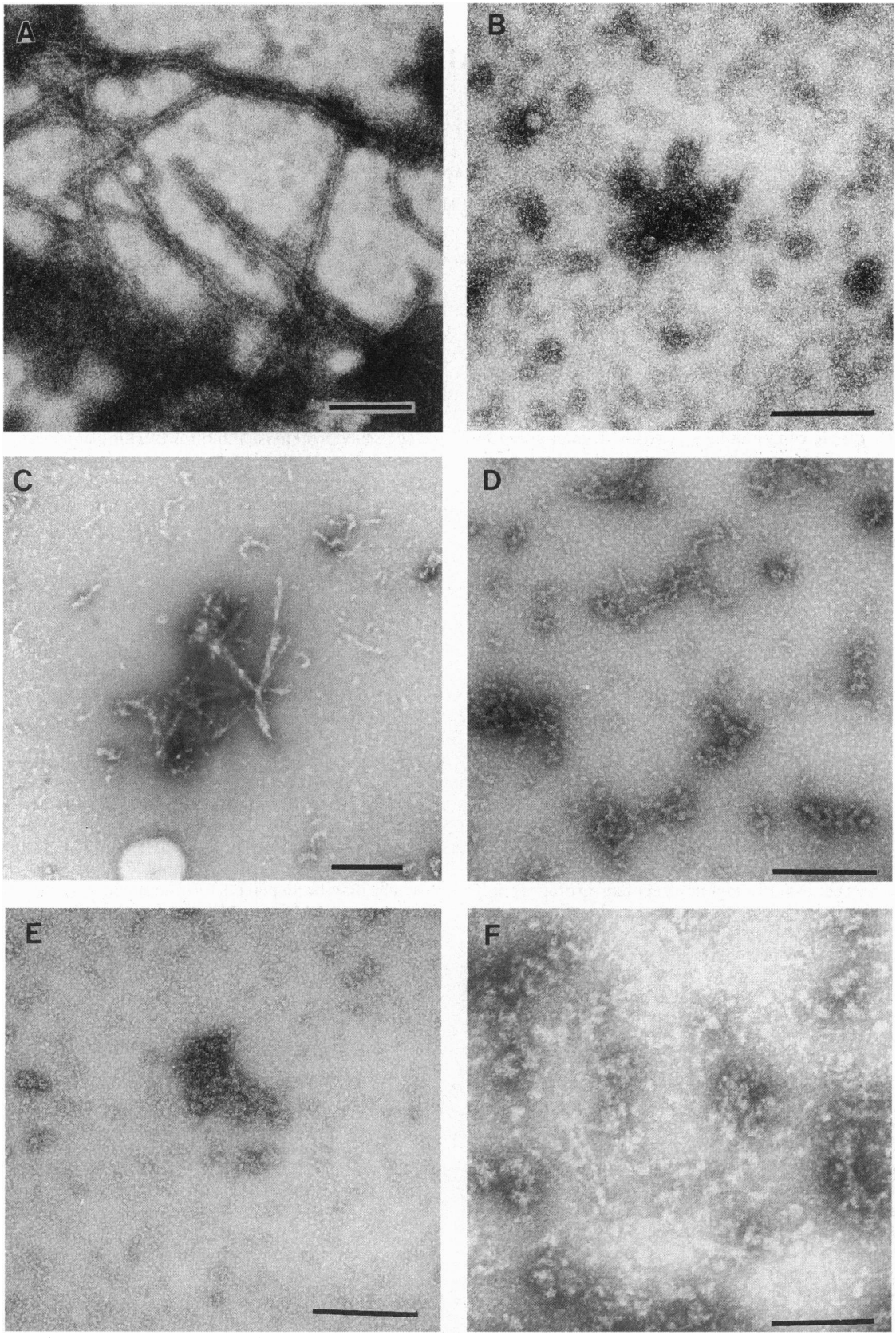

FIG. 1. Electron micrographs of the structures formed by synthetic β-amyloid (1–40) peptide in the absence or presence of ACT in a molar ratio of 10:1. (*A*) β-Amyloid fibrils formed after 48 h of incubation in Tris buffer. Structures formed by β-amyloid peptide in Tris buffer in the presence of ACT after 48 h (*B*) or after 1 week (*C* and *D*). Fibrils formed by β-amyloid peptide dissolved in ACT after 2 h (*E*) or 1 week (*F*). (Bars = 200 nm.)

10:1. Controls contained buffer without ACT. The antiproteinase activity of pure nondenatured ACT in combination with β-amyloid was assessed by using human leucocyte cathepsin G as the enzyme and *N*-Succinyl-Ala-Ala-Pro-Phe *p*-

nitroanilide as the substrate according to Barrett (16). Radiolabeling of β-amyloid (1–40) with ^{125}I was performed according to Thorell and Johansson (17).

Solubility Assays. The suspensions of β-amyloid (1–40) in Tris buffer in the presence or absence of ACT were incubated for 48 h at room temperature. After incubation, nonsolubilized peptide was removed by centrifugation at 10,000 × *g* for 10 min. Protein concentration in the supernatants was determined by measurement of the absorption at 280 nm. Results were corrected for the amount of pure ACT added. Mean values from three independent determinations were calculated.

Characterization of Soluble Monomers and Oligomers. Samples taken from supernatants after centrifugation were analyzed by electrophoresis through 1% agarose at pH 8.6 as previously described (18) and by SDS/PAGE under nonreducing conditions using a PhastGel 8–25% gradient according to the Phast System Users Manual (Pharmacia LKB). After electrophoresis, protein bands were stained with Coomassie blue R-250. Autoradiograms were produced by using Hyperfilm-MP1 (Amersham). Western blotting of electrophoretically separated proteins was performed as previously described (19).

Thermostability Analysis of ACT. Thermostability analysis of ACT mixed with synthetic β-amyloid during incubation was performed as described by Lomas *et al.* (20). Native ACT alone or in a mixture with β-amyloid was heated at constant temperatures of 40, 60, or 80°C for 2 h. After heating, the solutions were rapidly cooled on ice, centrifuged at 10,000 × *g* for 5 min, and filtered through a 0.20-μm pore diameter membrane (Micro Filtration System, Dublin, CA). The residual ACT concentration was determined by rocket electroimmunoassay (21).

EM. Synthetic β-amyloid filaments suspended in Tris buffer with or without ACT were applied to carbon-coated copper grids, negatively stained with 2% (wt/vol) uranyl acetate, and visualized in a JEOL 200cx electron microscope operating at 80 kV. Fibril formation was investigated after 2 h, 48 h, and 1 week of incubation.

RESULTS

Fibril Formation and Disaggregation. The fibril forming capacity of β-amyloid was monitored by EM, with the first examination occurring 48 h after incubation. At this time the β-amyloid peptide in deionized water formed no fibrils (not shown). In contrast, synthetic β-amyloid incubated in Tris buffer formed fibrillar structures of the same morphology as previously described (7). The majority of fibrils were long and irregularly twisted around each other (Fig. 1*A*). Some of the fibrils appeared single and not twisted. When the peptide was incubated for an identical time interval with ACT in a molar ratio of 10:1, fibril formation was inhibited and EM showed only amorphous, uncharacteristic protein aggregates (Fig. 1*B*). When samples were examined after 1 week of incubation, the fibrils in buffer alone appeared essentially unchanged (not shown). In the presence of ACT, some atypical, short, nontwisted fibril-like fragments were seen (Fig. 1*C*). Sometimes, more elongated structures surrounded by amorphous protein aggregates appeared (Fig. 1*D*).

When preformed fibrils (Fig. 1*A*) were incubated with pure ACT in a 1:10 molar ratio a rapid disaggregation of fibrils took place. This phenomenon was evident already 2 h after incubation (Fig. 1*E*) and also quite obvious after 48 h (Fig. 1*F*). After incubation for 1 week, atypical structures identical to those seen in Fig. 1 *C* and *D* appeared, but no typical fibrils formed by β-amyloid (1–40) peptide (Fig. 1*A*) could be seen.

The Interaction Between ACT and the β-Amyloid Peptide. The formation and dissolution, respectively, of fibrils was accompanied by an interaction between ACT and the β-amyloid peptide, as demonstrated by electrophoretic and solubility studies.

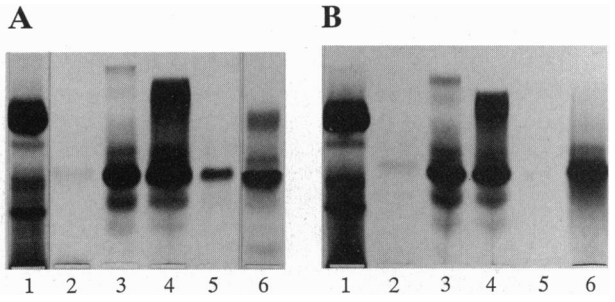

FIG. 2. Agarose electrophoresis of β-amyloid preparations after 48 h (*A*) and 1 week (*B*) of incubation in the indicated solutions. Standard incubation conditions were used. The anode is at the top. Lanes 1, human serum standard; lanes 2, pure ACT; lanes 3, β-amyloid dissolved in deionized water; lanes 4, β-amyloid in water and presence of ACT; lanes 5, β-amyloid in Tris buffer; and lanes 6, β-amyloid in Tris buffer after addition of ACT. The staining avidity of ACT alone is weak but increases after interaction with β-amyloid.

Fig. 2 compares the electrophoretic pattern produced by ACT alone and the effect on the solubility of the β-amyloid (1–40) peptide after 48 h of incubation with ACT. It is evident that the peptide is soluble in water but almost insoluble in Tris buffer. It is also seen that the addition of ACT to the buffer solution results in increased solubility of the peptide, as reflected in the appearance of multiple protein bands of varying charge in the supernatant, similar to that seen after the β-amyloid peptide has been dissolved in deionized water. During the 48 h of incubation, the solubility of β-amyloid peptide in the presence of ACT increased by a mean of 30% (range 20–37%) as reflected by measuring the absorbance of the supernatants at 280 nm. Prolonged incubation of β-amyloid peptide mixed with ACT for 1 or 2 weeks did not change the electrophoretic pattern or solubility to any large extent. As can be seen from SDS/8–25% gradient PAGE data (Fig. 3*A*) prolonged incubation of samples resulted in the interaction between β-amyloid peptide and ACT. Formation of a complex is evident from the autoradiogram shown in Fig. 3*B*. A new radiolabeled band appears in the ^{125}I-labeled peptide–ACT mixture compared to the ^{125}I-labeled peptide alone. In addi-

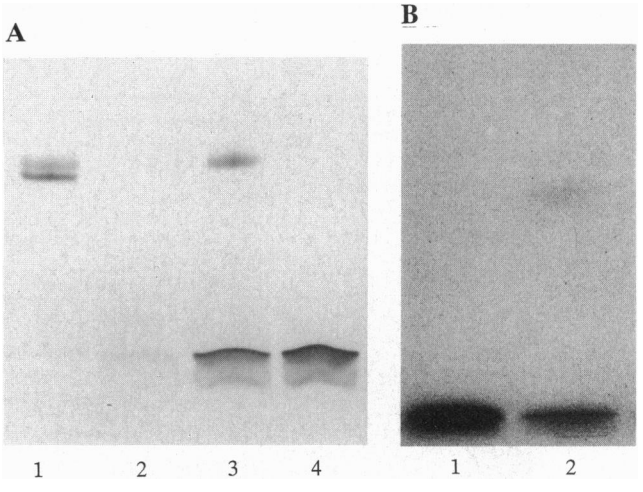

FIG. 3. SDS/8–25% gradient PAGE under nonreducing conditions of β-amyloid in a mixture with ACT at a molar ratio of 10:1. The anode is at the bottom. (*A*) Protein staining; lane 1, β-amyloid in mixture with ACT after 1 week of incubation; lane 2, β-amyloid alone after 1 week of incubation; lane 3, ACT and β-amyloid mixed immediately before electrophoresis; and lane 4, β-amyloid dissolved in Tris buffer. (*B*) Autoradiography; lane 1, β-amyloid; and lane 2, β-amyloid in a mixture with ACT.

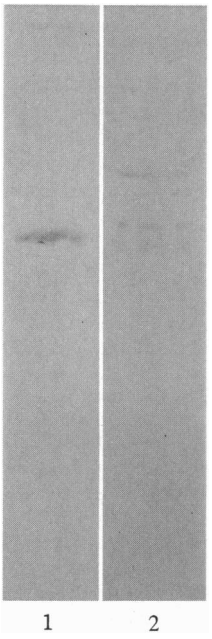

FIG. 4. Interaction between β-amyloid peptide and ACT studied by Western blot analysis. Non-reduced samples were applied to an SDS/8–25% gradient polyacrylamide gel and immunoblotted with monospecific antibodies against ACT. Lane 1, native ACT; and lane 2, ACT in a mixture with β-amyloid at a molar ratio 1:10.

tion, results from Western blot analysis using antibodies against ACT confirm the formation of a complex between ACT and β-amyloid peptide (Fig. 4). It should be noted that Western blot analysis of ACT–β-amyloid peptide mixtures shows one higher molecular mass immunoprecipitation band not visible in the autoradiogram (Fig. 3*B*), possibly representing ACT or ACT–β-amyloid peptide polymers. Fig. 5 demonstrates the effect of added ACT on preformed fibrils. After 48 h in Tris buffer the β-amyloid peptide alone is barely detectable after electrophoresis through 1% agarose. Addition of ACT results in the appearance of one strongly stained protein band with a somewhat retarded mobility compared to that of native ACT (Fig. 5, lane 2) and several weakly stained bands (Fig. 5, lane 4). Preformed fibrils dissolved in deionized water show a similar electrophoretic profile but with predominantly cathodal protein bands (Fig. 5, lane 5).

Thermal Stability and Inhibitory Activity of ACT. Fig. 6 illustrates the pronounced change in the thermal stability of ACT after 48 h of interaction with β-amyloid. It is evident that

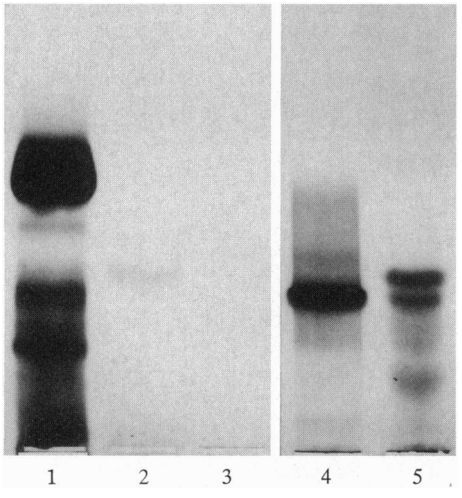

FIG. 5. Electrophoresis through 1% agarose of preformed β-amyloid fibrils. The anode is at the top. Lane 1, human serum standard; lane 2, pure ACT; lane 3, β-amyloid fibrils preformed in Tris buffer; lane 4, β-amyloid fibrils preformed in Tris buffer and dissolved by addition of ACT at a molar ratio of 10:1, and lane 5, β-amyloid fibrils preformed in Tris buffer and dissolved in deionized water.

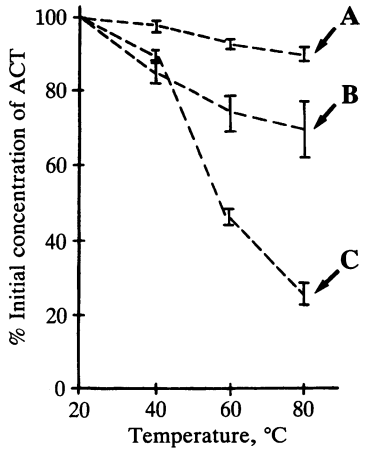

FIG. 6. Thermostability studies. (*A*) ACT in a complex with cathepsin G. (*B*) ACT in a mixture with β-amyloid at a molar ratio of 1:10. (*C*) Native ACT. The midpoint of each bar represents the mean of five determinations, and the endpoints of the bars represent the SD.

at 80°C only 25% of native ACT remains nondenaturated compared to 70% of the ACT in the presence of the β-amyloid and 90% after digestion with cathepsin G. In addition, there is a mean loss of antiproteinase activity of ACT of 70% (range, 60–83%) in presence of β-amyloid (data not shown).

DISCUSSION

We initiated the present studies hypothesizing that serpins, in particular ACT, could interact with the insoluble β-pleated structures that are characteristic of all forms of amyloid depositions to form complexes similar to those previously described for other strongly hydrophobic compounds, such as cholesterol and lithocholic acid (18, 19). We also had evidence (28) of the ability of α_1-antitrypsin to destabilize and solubilize amyloid fibrils extracted from patients with primary amyloidosis. Such interactions result in conformational changes in the serpins revealed by an increased thermal stability (S. J. and S. E., unpublished data) and a loss of proteinase-inhibitor activity (19). The rise in thermal stability reflects a transition of the serpin conformation from the stressed to a relaxed state and is a measure of the degree of protein polymerization (20). We chose ACT as a probe serpin in these experiments because ACT and β-amyloid always occur together in the AD plaques. Our approach to the enigmatic presence of a proteinase inhibitor in the Alzheimer lesions was different from that of other authors (9), who believed that the biological role of ACT in the Alzheimer lesions was mainly that of a proteinase inhibitor. We postulated that ACT is present in the lesions predominantly as a serpin capable of hydrophobic interaction.

In the present *in vitro* studies, we used a simple model based on electrophoretically pure ACT of high biological activity mixed with the synthetic β-amyloid (1–40) peptide that was accessible to us. We monitored the β-amyloid fibril formation process by E M and the concomitant conformational transitions of ACT by physicochemical methods.

Recently, it has been demonstrated that under certain conditions β-amyloid peptides and related fragments can exist in monomeric α-helical conformations (22). Changes in the surroundings may affect the peptide conformation. Rearrangement from an α-helix to an oligomeric β-pleated sheet promotes aggregation in aqueous solutions as shown in Fig. 1. Peptide concentration, pH, and time of incubation have been considered important parameters for β-pleated sheet formation *in vitro* (22). Our standard incubation procedure included a physiological pH of 7.4, a molar ratio of β-amyloid to ACT of 10:1, and incubation at room temperature. Except for time we have not systematically varied the conditions in this study. It is,

however, quite clear that the rate of both fibril formation and dissolution may be affected by the conditions chosen—i.e., fibril formation is faster at a lower pH (23). In addition, the length of the β-amyloid peptide may influence the rate of fibril formation. It has been shown that the soluble model β-amyloid (1–42) and (1–43) peptides, particularly when coincubated with other hydrophobic peptides, aggregate immediately (7). It has also been pointed out that amyloid formation, like crystallization, is a nucleation-dependent phenomenon (24). That means that soluble β-amyloid (1–40) peptide may exist in an α-helical conformation. Presence in the surroundings of even a very small amount of an insoluble variant may be critical for amyloid formation.

In the present *in vitro* study, we demonstrated profound and partly unexpected effects of ACT, both on the rate of β-amyloid fibril formation and, above all, on its ability to disaggregate preformed β-amyloid fibrils. Both processes were accompanied by changes in the physicochemical properties of ACT, most important, its change in thermal stability and loss of proteinase-inhibiting activity.

It is evident (Fig. 1) that after 48 h of incubation, the β-amyloid peptide in Tris buffer forms fibrils with a "twisted ribbon" morphology. Addition of ACT retards this fibril-forming process such that during this interval no fibrils were formed. The retardation, however, seems to some extent to be reversible; after prolonged (1 week) incubation some form of aggregation occurs and atypical short filaments appear. These atypical filaments may possibly act as the seed for fibril growth.

The addition of ACT to preformed fibrils has pronounced effects on fibril solubility. Our findings confirm those of Fraser *et al.* (9). After 2 h of incubation, all fibrils have "dissolved" and only small fragments are seen (Fig. 1*D*). If a similar process occurs *in vivo,* one may imagine that such small fragments can easily be removed by the circulation. The electrophoretic findings (Figs. 2 and 3) support the E M results. Supernatants from preparations of β-amyloid in buffer show no demonstrable protein bands. In contrast, addition of ACT restored the β-amyloid solubility to that observed in deionized water. The electrophoretic results are in agreement with the absorbance measurements at 280 nm.

We propose that the interaction between the β-amyloid peptide and ACT is an example of a hydrophobic interaction. By analogy with our previous results (18, 19), we suggest that the interactive part of the ACT molecule is the hydrophobic domain, C terminal to the enzymatic cleavage site. The interaction with β-amyloid changes the conformation of ACT corresponding to a transition from the stressed to the relaxed state.

Rearrangement of β-pleated sheet is believed to be central to changes that occur during serpin inactivation (25). The buried hydrophobic core becomes exposed, and as a result, the protein becomes thermostable (Fig. 6). Following interaction, the serpin loses its biological activity. Our data in this respect are in agreement with Potter and coworkers (26).

To conclude, this study clearly documents an inhibitory effect of ACT on the rate of fibril formation from the synthetic β-amyloid (1–40) peptide and the ability of the serpin to dissolve preformed fibrils. Preliminary experiments with synthetic β-amyloid (1–42) peptide, which is believed to be the main component of cerebral vascular amyloid (27) and forms fibrils more rapidly, have demonstrated a similar type of serpin interaction (S. J. and S. E., unpublished data). Although the exact mechanism remains to be explained, it is intimately associated with a conformational transition of ACT and not necessarily with its role as a proteinase inhibitor. The *in vivo* importance of these findings remains speculative but should stimulate interest in the role of serpins as regulatory factors in amyloid deposition in general. The involvement of ACT in the Alzheimer plaques may be part of a specific protective response mechanism. The understanding of this mechanism may have implications in the development of therapeutic strategies in AD.

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