

## Arrest of $\beta$ -Amyloid Fibril Formation by a Pentapeptide Ligand\*

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**Polymerization of amyloid  $\beta$ -peptide ( $A\beta$ ) into amyloid fibrils is a critical step in the pathogenesis of Alzheimer's disease. Here, we show that peptides incorporating a short  $A\beta$  fragment (KLVFF;  $A\beta^{16-20}$ ) can bind full-length  $A\beta$  and prevent its assembly into amyloid fibrils. Through alanine substitution, it was demonstrated that amino acids Lys<sup>16</sup>, Leu<sup>17</sup>, and Phe<sup>20</sup> are critical for binding to  $A\beta$  and inhibition of  $A\beta$  fibril formation. A mutant  $A\beta$  molecule, in which these residues had been substituted, had a markedly reduced capability of forming amyloid fibrils. The present data suggest that residues  $A\beta^{16-20}$  serve as a binding sequence during  $A\beta$  polymerization and fibril formation. Moreover, the present KLVFF peptide may serve as a lead compound for the development of peptide and non-peptide agents aimed at inhibiting  $A\beta$  amyloidogenesis *in vivo*.**

The preeminent neuropathological feature of Alzheimer's disease is the deposition of amyloid in the brain parenchyma and cerebrovasculature (1, 2). The basic components of the amyloid are thin fibrils of a peptide termed  $A\beta$  (3, 4). This peptide is a 40- to 42-amino acid-long proteolytic fragment of the Alzheimer amyloid precursor protein (APP),<sup>1</sup> a protein

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<sup>1</sup> The abbreviations used are: APP, Alzheimer amyloid precursor protein;  $A\beta$ , Alzheimer amyloid  $\beta$ -peptide; RPLC, reverse phase liquid

expressed in most tissues (5). Genetic and neuropathological studies provide strong evidence for a central role of  $A\beta$  amyloid in the pathogenesis of Alzheimer's disease (6), but the pathophysiological consequences of the amyloid deposition are unclear. However, it has been suggested that  $A\beta$  polymers and amyloid are toxic to neurons, either directly or via induction of radicals, and hence cause neurodegeneration (7–9).

Previous studies indicate that  $A\beta$  polymerization *in vivo* and *in vitro* is a specific process that probably involves interactions between binding sequences in the  $A\beta$  peptide (10–12). A rational pharmacological approach for prevention of amyloid formation would therefore be to use drugs that specifically interfere with  $A\beta$ - $A\beta$  interaction and polymerization. We hypothesized that ligands capable of binding to and blocking such sequences might inhibit amyloid fibril formation as outlined schematically in Fig. 1. Our strategy in searching for an  $A\beta$  ligand was to identify binding sequences in  $A\beta$  and then, based on their primary structures, synthesize a peptide ligand. Binding sequences were identified by systematically synthesizing short peptides corresponding to sequences of the  $A\beta$  molecule. The minimum length of an identified binding sequence was determined by truncating the peptide. Residues critical for binding were identified by alanine scanning. These critical residues were then substituted in an  $A\beta$  fragment ( $A\beta^{1-28}$ ) that normally is capable of forming amyloid fibrils (13, 14) in order to determine if they indeed are important for  $A\beta$  amyloid fibril formation. Finally, it was determined if the identified ligand, in addition to binding to the  $A\beta$  molecule, was capable of inhibiting fibril formation of  $A\beta^{1-40}$ .

### EXPERIMENTAL PROCEDURES

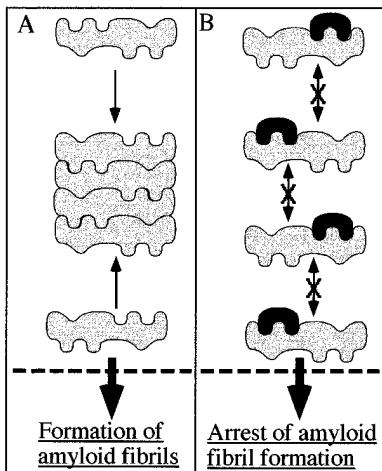
**Materials**—Synthetic  $A\beta^{1-40}$  and all other soluble peptides were synthesized by Fmoc chemistry. Unless otherwise indicated, all reagents were from Sigma.  $^{125}\text{I}-A\beta^{1-40}$  was iodinated using the Bolton-Hunter technique. Following the reaction, the iodinated peptide was purified on a Vydac C-4 RPLC column (0.21 × 15 cm) using a solvent system containing 0.1% trifluoroacetic acid in water (buffer A) and 0.1% trifluoroacetic acid, 100% acetonitrile (buffer B) (15).

**Synthesis of Peptides on Cellulose Membranes**—The technique used is essentially identical with the SPOT technique described by Frank (16). Briefly, cellulose membranes (Whatman 1Chr) were derivatized with *N,N'*-diisopropylcarbodiimide-activated  $\beta$ -alanine. A spacer, consisting of a  $\beta$ -alanine dipeptide, was coupled to derivatized cellulose membranes. The indicated peptides were then synthesized using Fmoc-protected and pentafluorophenyl-activated amino acids dissolved in N-methylpyrrolidone. Coupling efficiency was monitored using bromophenol blue.

**Radioligand Binding Studies**—Following blocking of the cellulose membranes with 0.05% Tween 20 in Tris-buffered saline (TBS), they were incubated in the presence of 20  $\mu\text{M}$   $^{125}\text{I}$ -labeled  $A\beta^{1-40}$  at 20 °C for 12 h in TBS, pH 7.4, supplemented with 1% bovine serum albumin. Subsequently, the cellulose membranes were washed repeatedly in the same buffer containing 0.5 M NaCl and dried. Radioactivity bound to the cellulose membrane was visualized by autoradiography and quantitated using densitometry. In experiments aimed at investigating the strength of the binding between soluble  $^{125}\text{I}-A\beta^{1-40}$  and immobilized peptides, the cellulose membranes were washed sequentially in 0.5 M NaCl, pH 7.4 (overnight at 20 °C), 6 M urea, pH 7.4 (3 h at 20 °C), 9 M urea, pH 11 (6 h at 20 °C). The efficiency of each washing step was monitored by autoradiography.

**Surface Plasmon Resonance Spectroscopy**—BIAcore 2000 (Pharmacia Biosensor AB, Sweden) was used for real-time studies based on

chromatography; TBS, Tris-buffered saline; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl.



**FIG. 1.** *In vitro* and *in vivo* studies of  $\text{A}\beta$  amyloid have shown that the  $\text{A}\beta$  molecules (shown in gray) interact during polymer growth and fibril formation. This probably involves interaction between one or several binding sequences in the  $\text{A}\beta$  molecules (A). It is therefore reasonable to assume that ligands (shown in black) that can bind to these sequences are capable of arresting further  $\text{A}\beta$  polymerization (B) and, assuming that  $\text{A}\beta$  polymerization is a dynamic and reversible process, possibly also dissolve  $\text{A}\beta$  polymers *in situ*.

surface plasmon resonance spectroscopy. The peptide was immobilized using thiol coupling. The running buffer consisted of 10 mM HEPES, 0.15 M NaCl, 3.4 mM EDTA, and 0.05% surfactant P20 as described by the manufacturer.

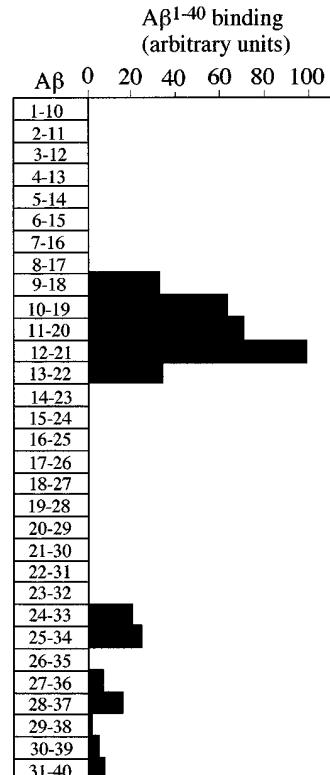
**Polymerization Studies**—Peptides at the indicated concentrations were incubated in TBS, pH 7.4, containing 0.02%  $\text{NaN}_3$  for 24–48 h. The incubations were terminated by separation of aggregated and soluble peptide by centrifugation at  $20,000 \times g$ . Soluble peptide was quantitated by RPLC as described above, whereas aggregated peptide was analyzed by electron microscopy.

**Electron Microscopy**—Synthetic polypeptides were prepared for electron microscopy by placing 5  $\mu\text{l}$  of the polymerized and nonpolymerized peptide solutions on grids covered by a carbon-stabilized formvar film. After 2–3 min, excess fluid was removed with a filter paper, and the grids were then negatively stained with 2% uranyl acetate in water. The specimens were finally examined and photographed in a JEOL EM 100CX at 60 kV.

## RESULTS

**Identification and Characterization of Sequences in the  $\text{A}\beta$  Molecule Mediating  $\text{A}\beta$ - $\text{A}\beta$  Interactions**—We first synthesized the 31 possible 10-mers corresponding to amino acids 1–10 up to 31–40 of the  $\text{A}\beta^{1-40}$  molecule on a cellulose membrane matrix (16, 17). The  $\text{A}\beta$  fragments capable of binding full-length  $\text{A}\beta$  were identified by radioligand binding. Cellulose membrane-bound peptides were incubated with  $^{125}\text{I}-\text{A}\beta^{1-40}$  overnight. Following washing of the cellulose membrane in high-salt buffer, bound radioactivity was quantitated by autoradiography and densitometry (Fig. 2). The measured binding should be interpreted as semiquantitative, since the coupling efficiency during synthesis, and therefore the amount of peptide per spot, may vary. A region located in the central part of  $\text{A}\beta$  ( $\text{A}\beta^{9-18}$  to  $\text{A}\beta^{13-22}$ ) displayed prominent binding of radioactive  $\text{A}\beta^{1-40}$ . Another binding region was the hydrophobic C terminus of the molecule (18), but binding here was considerably less prominent. The binding between  $\text{A}\beta^{1-40}$  and the  $\text{A}\beta$  fragments on the cellulose membrane matrix was strong. In order to obtain dissociation, it was necessary to incubate the cellulose membranes in 9 M urea, pH 11. Incubation in 6 M urea at pH 7.4 did not induce any measurable dissociation (data not shown, see “Experimental Procedures” for details).

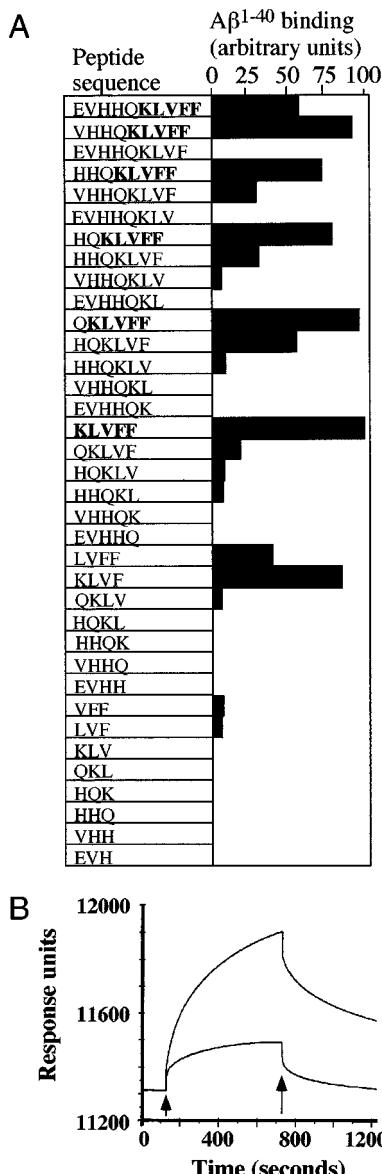
Being located in the center of the binding region,  $\text{A}\beta^{11-20}$  was selected for further studies of the structural requirements for binding. This peptide, as well as N- and C-terminally truncated



**FIG. 2.** Identification of binding sequences in the  $\text{A}\beta$  molecule. Ten-mers corresponding to consecutive sequences of  $\text{A}\beta^{1-40}$  were synthesized on a cellulose membrane matrix using the SPOT technique. Following blocking with 0.05% Tween 20 in TBS, the cellulose membrane was incubated in the presence of 20  $\mu\text{M}$   $^{125}\text{I}-\text{A}\beta^{1-40}$  at 20 °C for 12 h in TBS, pH 7.4, supplemented with 1% bovine serum albumin. The cellulose membrane was then washed repeatedly in the same buffer containing 0.5 M NaCl and dried. Radioactivity bound to the cellulose membrane was visualized by autoradiography and quantitated using densitometry.

fragments, were synthesized using the same technique as described previously (Fig. 3A). The shortest peptide still displaying consistent high  $\text{A}\beta$  binding capacity had the sequence KLVFF (corresponding to  $\text{A}\beta^{16-20}$ ). In order to confirm binding between  $\text{A}\beta^{1-40}$  and the KLVFF peptide, it was decided to study this interaction in an additional test system. Surface plasmon resonance spectroscopy is a technique suitable for real-time studies of molecular interactions (19). By adding a cysteine residue via a linker of two  $\beta$ -alanine residues to the C terminus of AcKLVFF, the peptide could be attached through a disulfide bond to the sensor chip of the surface plasmon resonance spectrometer. As control for nonspecific binding, cysteine alone was coupled to another channel of the sensor chip. A solution of  $\text{A}\beta^{1-40}$  was injected onto the sensor chip.  $\text{A}\beta^{1-40}$  was found to bind to the AcKLVFF peptide and not nonspecifically to the sensor chip (Fig. 3B).

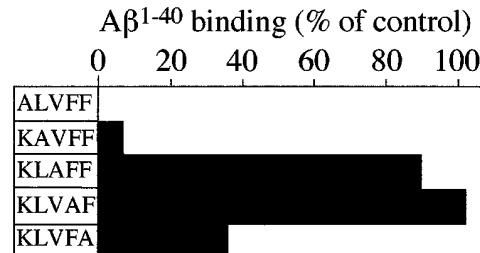
**Identification of Amino Acid Residues Mediating Binding**—By systematically substituting the amino acid residues in the KLVFF sequence with alanine, we found that the first, second, and fifth residues (*i.e.* KLXXF) were critical for binding (Fig. 4). To investigate if the KLXXF motif was required for  $\text{A}\beta$  polymerization, we synthesized  $\text{A}\beta^{1-28}$ , a well-studied  $\text{A}\beta$  fragment that readily forms amyloid fibrils (13), and mutated  $\text{A}\beta^{1-28}$  in which the KLVFF sequence was substituted with AAVFA ( $\text{A}\beta^{1-28(A16,17,20)}$ ). After incubation of  $\text{A}\beta^{1-28}$  at a concentration of 200  $\mu\text{M}$  for 24 h at 37 °C, only a small fraction was present as soluble peptide in the supernatant, whereas large fibril bundles were observed in the pellet (Fig. 5, A and C). The substituted peptide,  $\text{A}\beta^{1-28(A16,17,20)}$ , showed different proper-



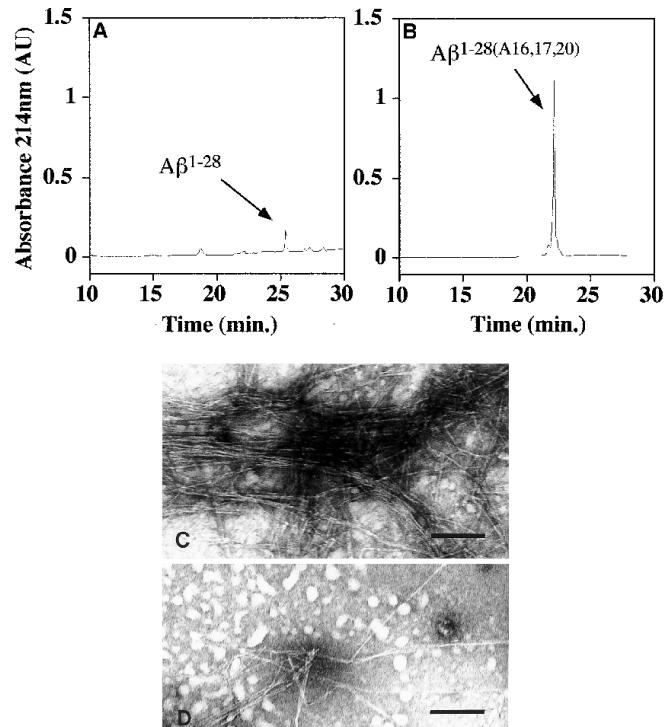
**FIG. 3. Identification of the shortest possible peptide capable of binding  $\text{A}\beta^{1-40}$ .** The  $\text{A}\beta^{11-20}$  molecule (EVHHQKLVFF) and indicated N- and C-terminal truncated fragments were synthesized using the same technique as described above and analyzed for affinity to  $^{125}\text{I}-\text{A}\beta^{1-40}$  (A). Sensorgram from surface plasmon resonance spectroscopy (BIAcore 2000). Solubilized  $\text{A}\beta^{1-40}$ , 100  $\mu\text{M}$ , was injected during 10 min over a sensorchip derivatized with the peptide AcKLVFFAAC (upper trace; AA and C served as spacer and linker to the chip, respectively) or cysteine alone (lower trace). Arrows indicate start and stop of injection (B).

ties. A large fraction was still present in soluble form after incubation, and only a few dispersed fibrils were found in the pellet (Fig. 5, B and D). The conclusions from these experiments were that the substitutions profoundly impair the ability of the peptide to aggregate and form amyloid-like fibrils.

**Arrest of  $\text{A}\beta^{1-40}$  Fibril Formation by an  $\text{A}\beta$  Ligand—Incubation of synthetic  $\text{A}\beta^{1-40}$  at 100  $\mu\text{M}$  for 48 h at 37 °C in TBS, led to polymerization of the peptide and formation of amyloid fibrils arranged in parallel in densely packed bundles (Fig. 6A), as shown previously (15). When  $\text{A}\beta^{1-40}$  was coincubated with AcQKLVFFNH<sub>2</sub>, at equimolar concentrations, this type of fibril bundles did not form. Instead, only a few occasional fibrils embedded in a diffuse background of small rod-like aggregates, similar to those formed by AcQKLVFFNH<sub>2</sub> itself (not shown), could be detected (Fig. 6B). In conclusion, the present  $\text{A}\beta$  ligand**



**FIG. 4. Identification of amino acid residues mediating binding.** Each amino acid residue in KLVFF was systematically replaced with alanine and analyzed for affinity to  $^{125}\text{I}-\text{A}\beta^{1-40}$  as described in the legend to Fig. 2. 100% represents  $^{125}\text{I}-\text{A}\beta^{1-40}$  binding to nonsubstituted KLVFF.



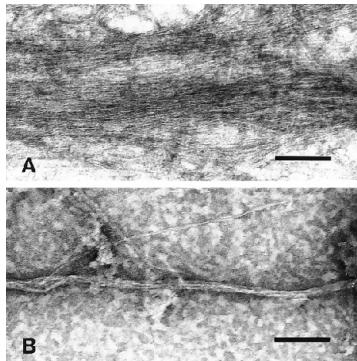
**FIG. 5. Aggregation and fibril formation of wild type and substituted  $\text{A}\beta^{1-28}$ .** Wild type  $\text{A}\beta^{1-28}$  (A and C) and  $\text{A}\beta^{1-28(A16,17,20)}$  (B and D) were incubated at 200  $\mu\text{M}$  in TBS for 24 h at 37 °C in a shaking water bath. After incubation, the tubes were centrifuged at 20,000  $\times g$  for 20 min. The content of nonaggregated peptide in the supernatants (A and B) was analyzed using an established C-4 RPLC system, whereas the aggregated peptides in the pellets were analyzed by electron microscopy after adsorption to formvar-coated grids and negative staining with 2% uranyl acetate in water (C and D; scale bars, 100 nm).

does not form amyloid-like fibrils *per se*, but it is capable of binding to, and inhibiting formation of, amyloid-like fibrils of the full-length form of the  $\text{A}\beta$  peptide.

## DISCUSSION

The aim of the present study was to identify regions in the  $\text{A}\beta$  molecule being important for binding during polymerization and, based on the structure of such a binding sequence, synthesize a small peptide ligand capable of binding to full-length  $\text{A}\beta$  and inhibiting its polymerization into amyloid fibrils.

The binding sequence identified in the present study is located in a region of the  $\text{A}\beta$  molecule that previously has been shown to be important during proteolytic processing of APP. During nonamyloidogenic processing of APP (*i.e.*  $\alpha$ -secretase cleavage), the molecule is cleaved between amino acid residues Lys<sup>16</sup> and Leu<sup>17</sup> (20). This leads, after further processing, to the formation of an  $\text{A}\beta$  fragment termed p3, corresponding to



**FIG. 6. Arrest of fibril formation by AcQKLVFFNH<sub>2</sub>.**  $\text{A}\beta^{1-40}$  was incubated at 100  $\mu\text{M}$  in TBS for 48 h at 37 °C in a shaking water bath, either alone (A) or together with 100  $\mu\text{M}$  AcQKLVFFNH<sub>2</sub> (B). The polymerized material was adsorbed to formvar-coated grids and negatively stained with 2% uranyl acetate in water. Scale bars, 100 nm.

$\text{A}\beta^{17-40}$  or  $\text{A}\beta^{17-42}$  (21). Through this metabolic pathway, the present binding sequence is disrupted. This may explain why p3 is not capable of forming amyloid *in vitro* or *in vivo* (11, 12). However, experimental studies show that p3 is capable of forming fibril-like structures *in vitro* (12). It is therefore highly probable that binding sequences other than the KLVFF sequence are involved in  $\text{A}\beta$  and p3 polymerization. The C terminus of the  $\text{A}\beta$  peptide may be of great importance in that respect (10, 18).

Previous studies of putative inhibitors of amyloid fibril formation showed that cyclodextrins (22) and Congo red (23) may have such properties. The usefulness of these molecules as lead substances in development of anti-Alzheimer amyloid drugs is, however, compromised by their lack of specificity. Cyclodextrins have primarily been used to increase the solubility of a wide range of drugs, and it is unlikely that they will display any specificity for  $\text{A}\beta$  *in vivo*. Congo red, which is used to detect amyloid histochemically, binds to a wide array of non- $\text{A}\beta$  amyloids as well as to other proteins with a high content of  $\beta$ -pleated sheet structures (24).

Due to the extreme insolubility of  $\text{A}\beta$  amyloid (strong chaotropic agents or potent organic solvents are required for its dissolution (4)), the concept of breaking up amyloid deposits *in situ* under physiological conditions may seem futile. However, the bulk of the individual molecules in amyloid are probably not joined by covalent bonds, and the deposition of  $\text{A}\beta$  into amyloid is, at least at some stages, a dynamic and reversible process (25). Hence, a molecule capable of binding to a site in the  $\text{A}\beta$  molecule being critical for fibril formation, and with an affinity higher than native  $\text{A}\beta$ , may inhibit amyloid growth and possibly also specifically dissolve amyloid fibrils *in situ*.

Previous studies suggest that amino acid residues within or close to  $\text{A}\beta^{16-20}$  are important for the adoption of the correct

$\beta$ -pleated sheet structure of  $\text{A}\beta$  (26, 27) and the proteolytic processing of its precursor (20). Here, it was shown that this region harbors a binding sequence required for the polymerization of  $\text{A}\beta$  into amyloid fibrils. It was also demonstrated that short peptides incorporating  $\text{A}\beta^{16-20}$  can function as ligands that bind to  $\text{A}\beta$  and inhibit the formation of amyloid fibrils. Since these peptide ligands are relatively small, they are amenable to investigation using organic chemistry. Non-peptide homologues of KLVFF may thus turn out to be useful as pharmacological tools for the treatment of Alzheimer's disease in the future.

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