Arrest of β-Amyloid Fibril Formation by a Pentapeptide Ligand*

(Received for publication, January 16, 1996, and in revised form, February 19, 1996)
Lars O. Tjernberg,† Jan Näslund§§, Fredrik Lindqvist,§ Jan Johansson,**
Anders R. Karlström, ‡ Johan Thyberg***, Lars Terenius †‡, and Christer Nordstedt†§§

From the Laboratory of Biochemistry and Molecular Pharmacology, the Department of Clinical Neuroscience, the Karolinska Hospital, S-171 76 Stockholm, Sweden, the Department of Medical Biochemistry and Biophysics, the Karolinska Institute, S-171 77 Stockholm, Sweden, the Department of Biochemistry and Pharmacology, Pharmacia & Upjohn, S-112 87 Stockholm, Sweden, and the Department of Cell and Molecular Biology, the Medical Nobel Institute, the Karolinska Institute, S-171 77 Stockholm, Sweden.

Polymerization of amyloid β-peptide (Aβ) into amyloid fibrils is a critical step in the pathogenesis of Alzheimer’s disease. Here, we show that peptides incorporating a short Aβ fragment (KLVFF; Aβ(16–20)) can bind full-length Aβ and prevent its assembly into amyloid fibrils. Through alanine substitution, it was demonstrated that amino acids Lys16, Leu17, and Phe20 are critical for binding to Aβ and inhibition of Aβ fibril formation. A mutant Aβ molecule, in which these residues had been substituted, had a markedly reduced capability of forming amyloid fibrils. The present data suggest that residues Aβ(16–20) serve as a binding sequence during Aβ 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 Aβ 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 Aβ (3, 4). This peptide is a 40- to 42-amino acid-long proteolytic fragment of the Alzheimer amyloid precursor protein (APP), a protein expressed in most tissues (5). Genetic and neuropathological studies provide strong evidence for a central role of Aβ amyloid in the pathogenesis of Alzheimer’s disease (6), but the pathological consequences of the amyloid deposition are unclear. However, it has been suggested that Aβ polymers and amyloid are toxic to neurons, either directly or via induction of radicals, and hence cause neurodegeneration (7–9).

Previous studies indicate that Aβ polymerization in vivo and in vitro is a specific process that probably involves interactions between binding sequences in the Aβ peptide (10–12). A rational pharmacological approach for prevention of amyloid formation would therefore be to use drugs that specifically interfere with Aβ-Aβ 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 Aβ ligand was to identify binding sequences in Aβ 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 Aβ 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 Aβ fragment (Aβ(1–28)) that normally is capable of forming amyloid fibrils (13, 14) in order to determine if they indeed are important for Aβ amyloid fibril formation. Finally, it was determined if the identified ligand, in addition to binding to the Aβ molecule, was capable of inhibiting fibril formation of Aβ(1–40).

EXPERIMENTAL PROCEDURES

Materials—Synthetic Aβ1–40 and all other soluble peptides were synthesized by Fmoc chemistry. Unless otherwise indicated, all reagents were from Sigma. 125I-Aβ1–40 was iodinated using the Bolton-Hunter technique. Following the reaction, the iodinated peptide was purified on a Vydac C-4 RPLC column (0.21 x 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 β-alanine. A spacer, consisting of a β-alanine dipeptide, was coupled to derivatized cellulose membranes. The indicated peptides were then synthesized using Fmoc-protected and pentfluorophenyl-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 nM 125I-labeled Aβ1–40 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 125I-Aβ1–40 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 (Pharma- cia Biocensor AB, Sweden) was used for real-time studies based on chromatography; TBS, Tris-buffered saline; Fmoc, N-(9-fluorenyl)methoxycarbonyl.
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% NaN₃ for 24–48 h. The incubations were terminated by separation of aggregated and soluble peptide by centrifugation at 20,000 × 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 μl 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 J EOL EM 100CX at 60 kV.

**RESULTS**

Identification and Characterization of Sequences in the Aβ Molecule Mediating Aβ-Aβ Interactions—We first synthesized the 31 possible 10-mers corresponding to amino acids 1–10 up to 31–40 of the Aβ₁⁻⁴⁰ molecule on a cellulose membrane matrix (16, 17). The Aβ fragments capable of binding full-length Aβ were identified by radioligand binding. Cellulose membrane-bound peptides were incubated with 125I-Aβ₁⁻⁴⁰ 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 Aβ₁⁻¹⁸ (Aβ₁⁻¹⁸ to Aβ₁⁻²⁴) displayed prominent binding of radioactive Aβ₁⁻⁴⁰. Another binding region was the hydrophobic C terminus of the molecule (18), but binding here was considerably less prominent. The binding between Aβ₁⁻⁴⁰ and the Aβ 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, Aβ₁⁻¹⁰ was selected for further studies of the structural requirements for binding. This peptide, as well as N- and C-terminally truncated fragments, were synthesized using the same technique as described previously (Fig. 3A). The shortest peptide still displaying consistent high Aβ binding capacity had the sequence KLVFF (corresponding to Aβ₁⁻³⁰). In order to confirm binding between Aβ₁⁻⁴⁰ and the KLFF 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 β-alanine residues to the C terminus of AβKLFF, the peptide could be attached through a disulfide bond to the sensor chip of the surface plasmon resonance spectroscopy. As control for nonspecific binding, cysteine alone was coupled to another channel of the sensor chip. A solution of Aβ₁⁻⁴⁰ was injected onto the sensor chip. Aβ₁⁻⁴⁰ was found to bind to the AβKLFF 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 KLFF 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 Aβ polymerization, we synthesized Aβ₁⁻²⁸, a well-studied Aβ fragment that readily forms amyloid fibrils (13), and mutated Aβ₁⁻²⁸ in which the KLFF sequence was substituted with AAVFA (Aβ₁⁻²⁸[A₁₆,₁₇,₂₀]). After incubation of Aβ₁⁻²⁸ at a concentration of 200 μM 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, Aβ₁⁻²⁸[A₁₆,₁₇,₂₀], showed different proper-
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 Aβ1–40 Fibril Formation by an Aβ Ligand—Incubation of synthetic Aβ1–40 at 100 μM 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 Aβ1–40 was coincubated with AcKLVFFNH2 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 AcKLVFFNH2 itself (not shown), could be detected (Fig. 6B). In conclusion, the present Aβ ligand 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 Aβ peptide.

**DISCUSSION**

The aim of the present study was to identify regions in the Aβ 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 Aβ and inhibiting its polymerization into amyloid fibrils.

The binding sequence identified in the present study is located in a region of the Aβ molecule that previously has been shown to be important during proteolytic processing of APP. During nonamyloidogenic processing of APP (i.e. α-secretase cleavage), the molecule is cleaved between amino acid residues Lys16 and Leu17 (20). This leads, after further processing, to the formation of an Aβ fragment termed p3, corresponding to
Fig. 6. Arrest of fibril formation by AcQKLVPFFNH₂. Aβ₁-₄₀ was incubated at 100 μM in TBS for 48 h at 37 °C in a shaking water bath, either alone (A) or together with 100 μM AcQKLVPFFNH₂ (B). The polymerized material was adsorbed to formvar-coated grids and negatively stained with 2% uranyl acetate in water. Scale bars, 100 nm.

Aβ₁⁷⁻⁴₀ or Aβ₁⁷⁻⁴₂ (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 Aβ and p3 polymerization. The C terminus of the Aβ 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 inhibit amyloid growth and inhibit the formation of amyloid fibrils. Due to the extreme insolubility of Aβ 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 Aβ 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 Aβ molecule being critical for fibril formation, and with an affinity higher than native Aβ, may inhibit amyloid growth and possibly also specifically dissolve amyloid fibrils in situ.

Previous studies suggest that amino acid residues within or close to Aβ₁⁶⁻²⁰ are important for the adoption of the correct β-sheet structure of Aβ (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 Aβ into amyloid fibrils. It was also demonstrated that short peptides incorporating Aβ₁⁶⁻²⁰ can function as ligands that bind to Aβ 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.

Acknowledgments—We thank Drs. Magnus Edlund and Björn Obrink, Karolinska Institute, and Samuel E. Gandy, Cornell Medical College, NY, for stimulating discussions and reviewing the manuscript.

REFERENCES

1. Selkoe, D. J. (1991) Neuron 6, 487–498
2. Selkoe, D. J. (1994) Annu. Rev. Cell Biol. 10, 373–403
3. Glennie, G. D., and Wong, C. W. (1984) Biochim. Biophys. Res. Commun. 120, 895–890
4. Masters, C. L., Simms, G., Weinman, N. A., Multhaup, G., McDonald, B. L., and Beyreuther, K. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 4245–4249
5. Kang, J., Lemaire, H. G., Unterbeck, A., Salbaum, J. M., Masters, C. L., Graesich, K. H., Multhaup, G., Beyreuther, K., and Müller-Hill, B. (1987) Nature 325, 733–736
6. Selkoe, D. J. (1994) J. Neurophil. Exp. Neurol. 53, 438–447
7. Behl, C., Davis, J. B., Lesley, R., and Schubert, D. (1994) Cell 77, 817–827
8. Pike, C. J., Burdick, D., Walencewicz, A. J., Glabe, C. G., and Cotman, C. W. (1993) J. Neurosci. 13, 1676–1687
9. Loo, D. T., Capani, A., Pike, C. J., Whittmore, E. R., Walencewicz, A. J., and Cotman, C. W. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7951–7955
10. Jarrett, J. T., Berger, E. P., and Lansbury, P. T., Jr. (1993) Biochemistry 32, 4693–4697
11. Näslund, J., Schierhorn, A., Hellman, U., Lannfelt, L., Roses, A. D., Tjernberg, L. O., Silberring, J., Gandy, S. E., Winblad, B., Greenard, P., Nordstedt, C., and Terenius, L. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8378–8382
12. Näslund, J., Jensen, M., Tjernberg, L. O., Thyberg, J., Terenius, L., and Nordstedt, C. (1994) Biochem. Biophys. Res. Commun. 204, 780–787
13. Kirschner, D. A., Inouye, H., Dufy, L. K., Sinclair, A., Lind, M., and Selkoe, D. J. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 6953–6957
14. Barrow, C. J., and Zagoski, M. G. (1991) Science 253, 179–182
15. Nordstedt, C., Näslund, J., Tjernberg, L. O., Karlström, A. R., Thyberg, J., and Terenius, L. (1994) J. Biol. Chem. 269, 30773–30776
16. Frank, R. (1992) Tetrahedron 48, 9217–9232
17. Edlund, M., Blikstad, I., and Obrink, B. (1996) J. Biol. Chem. 271, 1393–1399
18. Burdick, D., Soreghan, B., Kwon, M., Kosmoski, J., Knauer, M., Henschig, A., Yates, J., Cotman, C., and Glabe, C. (1992) J. Biol. Chem. 267, 546–554
19. Jansson, U., and Malmqvist, M. (1992) J. Biol. Chem. 267, 546–554
20. Esch, F. S., Kilm, P. S., Beattie, E. C., Blacher, R. W., Culwell, A. R., Oliveros, T., McClure, D., and Ward, P. J. (1999) Science 284, 1122–1124
21. Haass, C., Seifert, J. A., Schlossmacher, M. G., Teplow, D. B., and Selkoe, D. J. (1993) J. Biol. Chem. 268, 3021–3024
22. Camilleri, P., Haskins, N. J., and Howlett, D. R. (1994) FEBS Lett. 341, 256–258
23. Lorenzo, A., and Yankner, B. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12243–12247
24. Turnell, W. G., and Finch, J. T. (1992) J. Mol. Biol. 227, 1205–1223
25. Maggio, J. E., Stimson, E. R., Lamberger, A., Allen, C. J., Dahl, C. E., Whitcomb, D. C., Vigna, S. R., Vinters, H. V., Labenski, M. E., and Mantyh, P. W. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5462–5466
26. Hilibich, C., Kisters-Walke, B., Reed, J., Masters, C. L., and Beyreuther, K. (1992) J. Mol. Biol. 228, 460–473
27. Wood, S. J., Wetzell, R., Martin, J. D., and Hurle, M. R. (1995) Biochemistry 34, 724–730