Alzheimer β-Amyloid Homodimers Facilitate Aβ Fibrillation and the Generation of Conformational Antibodies*

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We reported previously that stabilized β-amyloid peptide dimers were derived from mutant amyloid precursor protein with a single cysteine in the ectodomain juxtamembrane position. In vitro studies revealed that two forms of SDS-stable Aβ homodimers exist, species ending at Aβ40 and Aβ42. The phenomenon of the transformation of the initially “unaggregated” 42-residue β-amyloid peptide into the amyloid fibrils of Alzheimer’s disease plagues remains to be explained in physical terms, i.e. energetically and structurally. We therefore performed spectroscopic analyses revealing that engineered dimeric peptides ending at residue 42 displayed a much more pronounced β-structural transition than corresponding monomers. Specifically, the single chemically induced dimerization of Aβ peptides significantly increased the β-sheet content by a factor of 2. The C-terminal residues Ile-41 and Ala-42 of dimeric forms further increased the β-sheet content by roughly one-third. In contrast to Aβ42, the β-sheet content of the α- and γ-secretase-generated p3 fragments did not necessarily correlate with the tendency to form fibrils, although p3/17–42 had a pronounced thread forming character with fibril lengths of up to 2.5 μm. Electron microscopic images show that forms of p3/17–42 generated smaller granular particles than forms ending at residue 40. We discuss these findings in terms of Aβ1–42 dimers representing paranuclei, which self-aggregate into ribbon-like ordered fibrils by elongation. Based on Aβ42 dimer-specific titers of a polyclonal antiserum we propose that the Aβ homodimer represents a nidus for plaque formation and a well defined novel therapeutic target.

In the amyloidogenic processing of APP,1 the Aβ peptide is produced, circulates extracellularly, and usually does not de-
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The high content of β-sheet structure in the Aβ42 dimer has been confirmed by Fourier-transformed infrared (FTIR) mass spectrometry, and a conformation-dependent epitope of Aβ dimers has been detected by surface plasmon resonance (SPR).

EXPERIMENTAL PROCEDURES

CD Spectroscopy—Alterations in the secondary structure content of the Aβ-derived peptides were monitored by CD spectroscopy. Spectra were taken using a Jasco J-710 instrument with a PFD-350S temperature control device set at a sensitivity of 10 mdeg, a time constant of 4 s, and a scan speed of 5 nm/min. Typically, peptides were diluted to 100 μg/ml in 1 mM Tris-HCl buffer, pH 7.5, with or without 1 mM dithiothreitol and measured in a 1-mm pathlength cuvette, scanning from 240 to 190 nm. Protein concentrations for the calculation of mean residue ellipticity (θam), were determined by amino acid analysis of the sample after measurement. Curves are presented as the signal average of four transients with a similarly signal-averaged base line subtracted. A Fourier transform operation was carried out to remove high frequency noise from the signal.

Secondary structure content of the peptides was estimated from the far UV CD spectra as mean residue ellipticity using the PEPFIT program (19).

EM—Each peptide was dissolved to 1 mg/ml in deionized water (Milli Q, Millipore) supplemented with 5 μL of 25% ammonium solution (measured pH 9.8) and incubated for 24 h at 37 °C. Aliquots (15 μL) of the aged peptide solutions (1 mg/ml) were applied to freshly glow-discharged carbon-coated copper grids (300 mesh size) and negatively stained with 2% aqueous uranyl acetate. The negative staining preparations for all peptide samples were done in parallel within a time interval of about 10 min after the incubation period. All staining preparations were performed in duplicate. Grids were examined in a Zeiss EM 10A electron microscope (Oberkochen, Germany) at an acceleration rate of 80 kV. The magnification indicator of the microscope was routinely controlled by using a grating replica.

FTIR Measurements—The peptide was dissolved in 10 mM phosphate buffer D2O. The pH was adjusted to 8.7 with DCl and NaOH, dissolved in D2O, and subsequently incubated at 37 °C for 4 h to reach a proton-deuterium exchange. To remove residual protons, the sample was lyophilized twice and dissolved in D2O again. For measurements, a peptide solution of 30 μg μl−1 in a temperature-controlled CaF2 cell with a path length of 15 μm was used. Spectra were recorded using a Phillips PU 9600 FTIR spectrometer whereby instrument optics and sample compartment were flushed with dry nitrogen gas. Buffer spectra recorded under the same conditions were subtracted from the sample spectra. The position of individual IR transitions in the amide I region between 1,700 and 1,600 cm−1 were resolved by second derivative spectra and by self-deconvolution routines. For secondary structure analysis the spectra were fitted using a set of six Lorentzian line shaped transitions.

Chemical Synthesis, Purification, and Analysis of Synthetic Peptides—For solid phase synthesis of Aβ-derived peptides the Fmoc (N-(9-fluorenylethoxycarbonyl) strategy (20) was used. Peptide chain assembly was performed using in situ activation of amino acid building blocks by 2-(1H-benzotriazole-1-y1)-1,1,3,3-tetramethyldiisylammonium hexafluorophosphate (HBTU). The purified material was analyzed by HPLC and laser desorption mass spectrometry (Vision 2000, Finnigan MAT). Purified peptides were lyophilized and stored at −20 °C until use.

The peptides to be cross-linked were allowed to form S-S bonds in 20% dimethyl sulfoxide. After a 12-h incubation, the sample was freeze-dried, dissolved in diluted ammonia, and injected onto a Jupiter C4 300 Å reversed phase column (Phenomenex, Torrance, CA). The dimeric fraction was collected, and identity was verified with mass spectrometry again.

Concentrations of dissolved peptides were determined by amino acid analysis according to the manufacturer’s protocol after hydrolysis with 6 N HCl for 24 h at 110 °C (420A Amino Acid Analysis System, Applied Biosystems).

Antibodies and Western Blot—The monoclonal antibody W0-2 recognizing the N-terminal region of Aβ has been used as a control (22). Polyclonal Aβ17–40 K28C dimer antibodies were raised against the synthetic peptide. Unconjugated peptide was used for rabbit immunization. Prior to immunization peptide solutions were prepared as described for EM, and complete Freund’s adjuvant was added to a final concentration of 500 μg/ml. After 4 weeks, subsequent immunizations were given with incomplete Freund’s adjuvant three times at 2-week intervals. Blood samples were collected 10 days after each injection and stored at 4 °C until assayed by SPR with immobilized synthetic peptides.

Snap-frozen hemispheres of APP23 (23) transgenic mouse brains (15 months of age) were homogenized in lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 8.0, 120 mM NaCl) with complete protease inhibitor mixture (Roche Applied Science). Samples were diluted in 2% SDS sample buffer (8% SDS, 5% 2-mercaptoethanol, 0.1% Comassie Blue G, 3 mM Tris-HCl, pH 8.45), electrophoresed on 16% Tricine SDS-polyacrylamide gels (Novex, San Diego), and proteins were transferred to Hybond nitrocellulose membranes (Amersham Biosciences). Aβ was detected after heating the membrane in preboiled phosphate-buffered saline for 5 min to enhance the signal and blocking in 10% non-fat dry milk (W0-2) or 20% horse serum (MX-02) containing 0.05% Tween 20 for 1 h. Monoclonal antibodies were incubated overnight at 4 °C. Signals were detected by horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibodies followed by ECL detection (Amersham Biosciences) according to the manufacturer’s instructions.

RESULTS

We assessed uniform and standardized engineered Aβ1–42, Aβ1–40, Aβ17–42, and Aβ17–40 homodimers and compared their biochemical and biophysical characteristics with corresponding wild-type “monomers” (set into quotations because freshly dissolved synthetic Aβ always yields a certain ratio of monomers, dimers, and trimers (25)). The generation of homodimers was achieved by introducing a Cys at position 28. This approach is based on the following assumptions. First, dimerization of Aβ is not only the first but also an essential step for Aβ fibrillation. Second, although it is distinct from the Aβ dimer in vivo, the K28C substitution is nearly authentic Aβ because this mutation was selected for an enhanced production of Aβ dimers from APP695 K624C-transfected SH-SY5Y cells (18).

Secondary structures of Aβ peptides generated by substituting Lys-28 to Cys-28, oxidation, and subsequent purification by HPLC were analyzed together with wild-type peptides by CD spectroscopy and confirmed by FTIR spectroscopy for the Aβ1–42 K28C mutant (Fig. 1). Most strikingly, wild-type and mutant peptides ending at residue 42 displayed an even more pronounced β-structural transition than forms ending at resi-
Fig. 1. CD and FTIR spectrum of full-length Aβ. CD spectra of Aβ1–42 K28C (red curve in A), wild-type Aβ1–42 (blue curve in A), Aβ1–40 K28C (red curve in B), and wild-type Aβ1–40 (blue curve in B) were recorded at room temperature and in aqueous solutions immediately after preparing the solutions; blue lines represent “monomeric peptides,” and red lines correspond to the disulfide linked dimeric K28C isoforms. A time course study was performed with Aβ1–42 K28C dimers under reducing conditions; the red line shows measurement at time 0, orange line after 10 min, and the black line after 24 h (C). FTIR spectra of Aβ1–42 K28C dimers are shown in D. The fit curve (●—●) overlays the experimental spectrum (——). The individual Lorentzian curves are shown underneath (——). CD spectra of Aβ17–42 K28C (red curve in E), wild-type Aβ17–42 (blue curve in E), Aβ17–40 K28C (red curve in F), and wild-type Aβ17–40 (blue curve in F) were processed as described for full-length forms of Aβ.
due 40 (Fig. 1, A versus B). Compared with Aβ1–42 it is apparent that the absence of the C-terminal two amino acids in Aβ1–40 leads to a marked increase in the negative maximum at −198 nm (Fig. 1, A and B, blue curves). Deconvolution of CD spectra indicates a β-sheet content of 48%, a content of unstructured coil of 22%, and negligible amounts of α-helix for the Aβ1–42 K28C dimer (Fig. 1A, red curve), for the Aβ1–42 “monomer” (Fig. 1A, blue curve) 23% β-sheet, and 52% random and 18% β-sheet and 70% random for the Aβ1–40 “monomer” (Fig. 1B, blue curve). Dimeric Aβ1–40 K28C (35% β-sheet, 55% unstructured coil) was even superior to “monomeric” Aβ1–42 (20% β-sheet, 52% unstructured coil; Fig. 1B, red curve, versus A, blue curve). To summarize, Aβ K28C dimerization significantly increased the β-sheet content by a factor of 2 for both the 40 and 42 series.

To determine the role of Cys-28 in maintaining the conformation, time course CD studies performed under reducing conditions with the Aβ1–42 K28C dimer revealed that its predominant β-sheet structure was unchanged even in the presence of a 50-fold molar excess of dithiobiotin. CD spectra collected within 30 s of mixing were superimposable on spectra at 24 h and the control (Fig. 1C). The reduction of disulfide bonds was analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (data not shown). This indicates that treatment of Aβ K28C dimers with dithiobiotin per se was not sufficient for changing the conformation adopted after initial dimerization.

To analyze more accurately the high content of β-sheets in the Aβ1–42 K28C dimer, FTIR spectroscopy was applied (Fig. 1D). The overall spectrum of the Aβ1–42 K28C dimer with an absorption maximum for the amide I band at 1,625 cm−1 observed. First, the estimated diameter and the length of the Aβ1–42 K28C was three times enlarged compared with the wild-type. Second, the fibrils did not appear beaded but straight. Third, the proportion of protofibrils was much lower, suggesting that the assembly/maturation was much faster or was an irreversible process. In each case, fibrillogenesis of Aβ1–42 K28C must have started with dimers as initial building blocks and further oligomerized to tetramers and higher oligomers as described for the nucleation and growth of wild-type amyloid β-protein fibrils (32).

Dimeric and also the “monomeric” forms of Aβ17–40 and Aβ17–42 polymerized into arrays of filaments (Fig. 3). Aβ17–42 K28C dimers (Fig. 3A), Aβ17–40 K28C dimers (Fig. 3B), and “monomeric” Aβ17–40 (Fig. 3C) formed granular particles with apparent diameters ranging from 10 nm for the Aβ17–42 K28C dimers, 13 nm for Aβ17–40 K28C dimers, and up to 21 nm for the Aβ17–40 “monomers.” Protofibril lengths of 60–100 nm and mature fibrils of 300 nm for the Aβ17–42 K28C dimer, uniform fibrils of 130–180 nm for the Aβ17–40 “monomer,” and of 200–400 nm for the Aβ17–40 K28C dimer were observed. “Monomeric” Aβ17–42 exhibited the smallest apparent diameter (5–10 nm) but the most pronounced thread forming character with fibril lengths of up to 2.5 μm (Fig. 3D), suggesting that Aβ17–42 fibrils grow by virtually irreversible binding of “monomers” to fibril ends. Strikingly and in contrast to full-length peptides the K28C mutation had a growth-arresting effect on particles, which was more pronounced for Aβ17–42 than for Aβ17–40. This can be best explained by the Cys-28 mutation, which might rather promote a parallel aggregation than an anti-parallel aggregation of Aβ17–40/42.

Next, we asked whether conformational changes of dimerization at the N- and C-terminal ends of the K28C peptides could be monitored by immunoreactivity. To produce antisera specific for conformational epitopes of the Aβ K28C dimer, rabbits were immunized with unconjugated freshly dissolved Aβ17–40 K28C dimers. The target antigen choice of the N-terminally truncated peptide was led by the following considerations. First, the majority of antibodies in mice immunized with Aβ fibrils are directed against the N-terminal 12 residues of Aβ and are capable of cross-reacting with the “monomeric” peptide (33). Second, the monoclonal antibody 4G8 recognizing Aβ residues 17–24, which was described to label naturally occurring SDS-stable Aβ dimers on the blot preferentially (17) indicated the minimum epitope required.

The presence of conformation-specific antibodies was detected by a direct binding assay with the polyclonal serum
Fig. 2. EMs of full-length Aβ. Average widths and diameters were measured from different views of an individual grid (i-iii). Considerable variance was observed in the appearance of the fiber structure reaching from straight, very long smooth fibers of Aβ1–42 K28C dimers (A) to regularly twisted fibers of Aβ1–42 monomers (B). Aβ1–40 K28C dimers showed longer fibers (C), whereas Aβ1–40 “monomers” exhibited protofibrils (D). Scale bar, 100 nm.

Fig. 3. EMs of N-terminally truncated Aβ. After a 24-h incubation protofibrils with differing sizes were observed for Aβ17–42 K28C dimers (A) and Aβ17–40 K28C dimers (B), wild-type Aβ17–40 “monomers” (C), and protofibrils of Aβ17–42 “monomers” (D). A mature fibril formation was restricted to Aβ17–42 monomer preparations (D).
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Fig. 4. Sensorgrams of the complete antibody/Aβ binding cycle. Polyclonal serum MX-02 raised against the Aβ1–40 K28C dimer (diluted 1:50) and monoclonal W0-2 (5 μg/ml) was injected onto an Aβ1–42 K28C dimer surface (immobilized to 300 RU on the chip surface) (A), an Aβ1–40 K28C dimer surface (immobilized to 1,500 RU) (B), and an Aβ1–42 monomer surface (immobilized to 3,000 RU) (C). The injections were started at 100 s at a flow rate of 5 μl/min. Fast association phases (100–270 s) and varying dissociation rates (270–420 s) were observed for W0-2 and the polyclonal serum depending on the specific antigen coated to the chip surface. The rapid drop in signal at 270 s of the diluted MX-02 serum is the result of changes in the refractive index caused by contaminating proteins within the crude serum. A detailed kinetic analysis of the monoclonal W0-2 will be published elsewhere. Immunoblots of APP23 mice brain homogenate (100 μg of protein/lane), synthetic peptides Aβ1–40 (100 ng/lane), and Aβ1–42 (100 ng/lane) probed with monoclonal W0-2 (1 μg/ml) and polyclonal MX-02 (diluted 1:500) are shown in D. The proteins were resolved under reducing conditions on a 16% Tricine gel. Cortical immunohistochemical staining of 18-month-old APP23 transgenic mice E–G. E, staining of vacular amyloid Aβ using anti-Aβ1–40 K28C. F, staining of plaque amyloid Aβ using anti-Aβ1–40 K28C. G, staining of plaque amyloid Aβ40 using G2-10. Counterstaining was with hematoxylin.
face and varied between 180 RU (Fig. 4A) and 1,700 RU for the monoclonal W0-2 (Fig. 4C). For each individual chip surface, the overlay of sensorgrams allows a rough estimate of the avidity of the polyclonal MX-02 serum raised against Aβ17–40 K28C by comparing the relative resonance signal in the dissociation phase of Aβ-specific W0-2 and MX-02 curves. The reactivity of MX-02 was significantly higher than for W0-2 when Aβ1–42 K28C was immobilized (Fig. 4A) and similarly strong with the Aβ1–40 K28C surface (Fig. 4B) but was significantly below the W0-2 signal with “monomeric” Aβ1–42 on the chip surface (Fig. 4C). Taking into consideration that the conformation recognized by MX-02 is partially present in the “monomeric” Aβ, a quantitative assessment of the cross-reactivity is impossible. Nevertheless, this suggests that a major portion of the polyclonal antibodies are directed against conformational epitopes that only exist in the K28C dimerized peptides.

To characterize further the polyclonal MX-02 serum raised against Aβ17–40 K28C, synthetic peptides Aβ1–40, Aβ1–42, and naturally occurring SDS-stable Aβ dimers were analyzed by Western blot. Crude homogenates of APP23 mouse brain using Aβ K28C homodimers to scale down the assembly start to fibrillize (18), stabilized Aβ homodimers have important inter- or intramolecular interactions.

Thus, it can be assumed that in the Aβ K28C peptides the dimeric state was frozen by covalent cross-linking of the side chains of Cys-28 similarly to the interlayer hydrogen bonds observed for Lys-28 in wild-type Aβ and without altering any important inter- or intramolecular interactions.

Aβ1–40 and Aβ1–42 K28C homodimers demonstrated why Aβ1–42 is more amyloidogenic than Aβ1–40. To the best of our knowledge unaggregated and homogeneous starting material indicated that Ile-41 and Ala-42 of Aβ1–42 forces the fibril length and that the K28C mutant displayed an even further pronounced β-sheet transition than forms lacking of these residues. This conclusion is supported by K28C mutants of Aβ17–40 and Aβ17–42, which also showed a significantly increased β-sheet compared with wild-type peptides. The ribbon-like uniform morphology of Aβ1–42 dimers is best explained by the tendency of preformed fibrils to aggregate laterally. This supports an in-register parallel β-sheet organization as reported previously (39, 40) for protofibrils. Recently, the first NMR data on Aβ1–42 fibrils (41) found an in-register parallel β-sheet organization together with ~15% of fibrillized Aβ1–42 occurring in an antiparallel β-sheet structure. Also, IR spectroscopy data suggested that Aβ may form an antiparallel β-sheet with a turn located around residues 26–29 (Ser-Asn-Lys-Gly) (42).

Most interestingly, when we investigated the influence of the N-terminal β-strand segment (Aβ residues 10–22), a growth arresting effect was found for Ile-41 and Ala-42 of Aβ17–42 in sharp contrast to Aβ full-length peptides (see above). These findings, based on a contemporaneous analysis of both forms, are inconsistent with previous studies where a superior amyloidogenicity for Aβ17–42 over Aβ17–40 was suggested from sedimentation data (10). In this study, only Aβ17–42 was analyzed and exhibited a fibrillar morphology by transmission electron microscopy (10). Aβ17–40 was analyzed independently by others (27). As shown by our EM data the replacement of Lys-28 with Cys enhanced the inhibitory effect of the N-terminal deletions of Aβ17–40 and Aβ17–42 on fibril formation further indicating the distinct inverse relationship between the β-sheet structure and fibril formation. From the observations that the K28C mutant of Aβ17–40/42 is rather inhibitory to fibril growth and dimerization further lowers the mean fibril diameter can be concluded that Aβ17–40 aggregation normally occurs in an antiparallel manner.

Taken together, our findings support the emerging model that two β-strand segments in each peptide molecule span residues 10–22 and 30–35 with a loop or hinge between the two β-sheets located at residues 23–29 including Cys-28 of the mutant peptide, which enabled the regulated dimerization of Aβ1–42 (Fig. 5). In this model, the C terminus of Aβ1–42 would be in close contact to the residues 25–29 containing the bend of the peptide backbone that links the two β-sheets in mature fibrils. This is supported by SPR data obtained with C-terminal specific monoclonal antibodies revealing that the C-terminal region of the peptide is sensitive to conformational...
induced epitope masking in fibrils and implying that Aβ1–42 protofibrils associate by an end-to-end coalescence. The physiological relevance of our findings is emphasized by a number of reports on Aβ dimers occurring in vivo. The presence of Aβ dimers in the cortex has been suggested to initiate the accumulation of Aβ in the human brain (17). Nonfibrillar SDS-stable dimers have been characterized as neurotoxic derivatives (43) and selectively blocked hippocampal long term potentiation in the absence of monomers, protofibrils, or fibrils (12). Rather than being initiated soon after the generation of Aβ in discrete intracellular vesicles as suggested by Walsh et al. (12), our findings demonstrate for the first time that a biochemically defined assembly of Aβ into Aβ dimers probably represents the initial step in amyloidogenesis. It does not only offer an explanation as to why fibrils are formed despite the apparent low concentrations of Aβ in the nervous system but also answers the question of the origin of a unusually high activation energy for the transition of a monoclinic α-helical intermediate into a β-sheet conformation. A prevention of fibril formation by reducing dimer concentrations could have significant relevance to the treatment of amyloidoses where oligomers have already been implicated in disease processes (44).

The peptides used in this study should be useful for screens of such compounds and a monoclonal antibody recognizing Aβ1–42 homodimers (which are potentially the earliest forms of synaptotoxic Aβ oligomers) might be useful for Aβ amyloid related therapeutic approaches by impeding its precipitation into existing plaques. The biological or pathological relevance of Aβ homodimers remains to be elucidated further, and a monoclonal antibody generated against Aβ K28C dimers should be most useful to achieve this goal.

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Fig. 5. Suggested schematic model of primary parallel (A) followed by antiparallel (B) Aβ peptides containing two β-strand segments spanning residues 10–22 and 30–35 (arrows) connected by the loop region including Cys-28 of the mutant peptide (C), which enables a lateral aggregation of Aβ dimers into ribbon-like structures as implied in D. Dotted lines represent the unstructured N-terminal part of Aβ.