Stabilization of a β-hairpin in monomeric Alzheimer’s amyloid-β peptide inhibits amyloid formation

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According to the amyloid hypothesis, the pathogenesis of Alzheimer’s disease is triggered by the oligomerization and aggregation of the amyloid-β (Aβ) peptide into protein plaques. Formation of the potentially toxic oligomeric and fibrillar Aβ assemblies is accompanied by a conformational change toward a high content of β-structure. Here, we report the solution structure of Aβ(1–40) in complex with the phage-display selected affibody protein ZAβ3, a binding protein of nanomolar affinity. Bound Aβ(1–40) features a β-hairpin comprising residues 17–36, providing the first high-resolution structure of Aβ in β conformation. The positions of the secondary structure elements strongly resemble those observed for fibrillar Aβ. ZAβ3 stabilizes the β-sheet by extending it inter-molecularly and by burying both of the mostly nonpolar faces of the Aβ hairpin within a large hydrophobic tunnel-like cavity. Consequently, ZAβ3 acts as a stoichiometric inhibitor of Aβ fibrillation. The selected Aβ conformation allows us to suggest a structural mechanism for amyloid formation based on soluble oligomeric hairpin intermediates.

Results and Discussion

Disulfide-Linked ZAβ3 Dimer Binds Aβ(1–40) with Nanomolar Affinity.

The 16 Affibody variants, for which binding to both Aβ(1–40) and Aβ(1–42) was tested and confirmed (20) (Fig. 1A), all have a cysteine residue at position 28, suggesting that disulfide-linked dimers were selected. Dimeric ZAβ3 binds monomeric Aβ(1–40) with 1:1 stoichiometry and an affinity of KD = 17 nM as determined by isothermal titration calorimetry (ITC) (Fig. 2A). In contrast, a monomeric mutant, obtained by replacing Cys-28 with serine (ZAβ3C28S), binds Aβ(1–40) with much lower affinity and in agreement with a cooperative association of two ZAβ3C28S molecules with one Aβ(1–40) monomer (Fig. 2B). Cooperative binding of two affibody units to distinct sites on Aβ(1–40) is supported by 15N heteronuclear single quantum correlation (HSQC) NMR spectroscopy, as shown for the glycine-rich region: The two glycines Gly-13 an Gly-14 give rise to one peak each for free ZAβ3C28S (Fig. 2C). Upon titration with unlabeled Aβ(1–40), four new peaks appear with identical intensities, corresponding to Gly-13 and Gly-14 of ZAβ3C28S bound to two distinct Aβ(1–40) sites (Fig. 2D). The chemical shifts of these peaks are practically identical to those observed for saturated ZAβ3 (Fig. 2E), suggesting a common binding mode for the monomeric and dimeric constructs. Binding is coupled to folding of sufficiently high affinity could potentially dissolve plaques by shifting the dynamic equilibrium between central nervous system Aβ and plasma Aβ toward the latter (14, 19). On this account, affibody ligands to monomeric Aβ(1–40) have recently been selected (20). Affibody ligands represent one class of engineered affinity proteins with applications in biotechnology, biochemical assays, disease diagnosis, and therapy (21–23). They are based on the Z domain derived from staphylococcal protein A and selected by phage display from a combinatorial protein library in which 13 of the 58 amino acid residues are randomized. The randomized positions, distributed over helix 1 and 2 of the three-helix bundle scaffold, have been chosen because of their location to the binding interface in the complex of the Z domain with its target, the Fc fragment from IgG (22).

Here, we investigate the interaction of Aβ(1–40) with the affibody protein ZAβ3 and report the solution structure of the complex. ZAβ3 binds to the central/C-terminal part of Aβ(1–40), which adopts a β-hairpin conformation reminiscent of the Aβ fibril structure.

The α-hairpin model is a specific feature of antibodies to its hydrophobic central part (14). Recognition of the hydrophobic central part is also a strategy pursued in the search for peptide or peptidomimetic inhibitors of Aβ fibrillation (15–17). Recently, the structures of the antigen binding fragments of two antibodies to the N-terminal Aβ(1–8) peptide were reported (18). However, the structural basis of interactions with the central and C-terminal parts of Aβ remains elusive.

The peripheral sink mechanism implies that not only antibodies, but any peripherally administered Aβ binding molecule


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Data deposition: Atomic coordinates and experimental constraints have been deposited in the Protein Data Bank, www.pdb.org [accession no. 2OTK (ZAβ3/Aβ(1–40) complex)].

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of both Aβ(1–40) and the affibody ligand, as indicated by the greatly improved resonance dispersion in HSQC spectra upon complex formation (Fig. 2 F and G). Particularly, several amide proton resonances are shifted downfield to values typical for β-sheet conformation upon binding [supporting information (SI) Fig. S1]. Concomitantly, the thermostability of ZAβ3 increases from a melting temperature of 47°C for free ZAβ3 to 64°C for the complex (Fig. 2F).

**ZAβ3 Inhibits Aβ(1–40) Fibrillation at Stoichiometric Concentrations.**

Thioflavin T fluorescence was used to monitor Aβ(1–40) fibrillation in the absence and presence of ZAβ3 (Fig. 2J). ZAβ3 acts as a potent fibrillation inhibitor. Stoichiometric concentrations of ZAβ3 dimer are required for complete inhibition, revealing that the binding of monomeric Aβ(1–40) is responsible for the inhibitory function.

**Structure of the ZAβ3:Aβ(1–40) Complex.** NMR was used to determine the structure of the complex between Aβ(1–40) and the disulfide-linked dimer of ZAβ3 (Fig. 3). The complex consists of...
a four-stranded antiparallel β-sheet and four α-helices. The selected conformation of Aβ(1–40) is a β-hairpin in which the Aβ17–Aβ23 and Aβ30–Aβ36 fragments make intramolecular backbone hydrogen bonds to form the two central strands of the β-sheet (Fig. 3E). Both faces of the Aβ hairpin are predominantly nonpolar, and both are buried within a large hydrophobic tunnel-like cavity in the ZAβ3 dimer (Fig. 3F). Hence, 1,400 Å² of surface area, of which 71% is nonpolar, is inaccessible to water in the complex. In the cavity, the Aβ hairpin is flanked on each side by β-strands formed by residues 15–18 of the two ZAβ3 subunits, respectively. This fragment is part of helix 1 in the originating Z domain (24) and affibody complexes reported in refs. 25 and 26. However, it is unfolded in ZAβ3 and presumably in all ZAβ binders, because they contain helix-destabilizing glycine and proline replacements at positions 9–11 and 13–14 (Fig. 1 A and B). Selected residues at positions 17 and 18 in the (former) helix do, however, show β-sheet propensity in agreement with the observed structure (Fig. 1C). A further consequence of helix 1 unfolding is that it opens a large hydrophobic cleft in which the core of the ZAβ3 dimer becomes exposed. The “interior” face of the Aβ hairpin containing the AβLeu-17, AβPhe-19, AβIle-32, AβLeu-34, and AβVal-36 side chains docks into the cleft to form a large intermolecular hydrophobic core. The ZAβ3 side of the core is held tightly by the selected Cys-28–Cys-28 disulfide linking helices 2 of the two subunits, and it includes the conservatively selected Leu-27, entailing a comparatively strong hydrophobicity in this sequence region (Fig. 3D), and the two Ile-31 side chains. Interestingly, although Ile-31 is not varied in phage display selection, it is still found at the hydrophobic interface of all Z domain and affibody complexes studied so far (25). The “exterior” face of the Aβ hairpin is embraced from both sides by the Ile-16 and (selected) Tyr-18 side chains. In this position, Tyr-18 of one ZAβ3 subunit (Z18 in Fig. 3E) also forms a hydrogen bond to the AβGlu-22 carboxyl. The N-terminal ZAβ3 β-strands are further anchored against helix 3 in both subunits by nonpolar interactions involving (selected) Val-17 and a salt bridge between Glu-15 and Lys-49. This salt bridge is in fact also present in other Z domain and affibody structures when Glu-15 is in a helical conformation. The selection of alanine or proline at position 24 can be rationalized...
by the tight packing of the disulfide-linked helices, which requires a small nonpolar residue at this position. Residues 25, 32, and 35 are solvent-exposed and not involved in binding, resulting in a less-conserved selection of polar side chains. The terminal fragments Aβ1-15 of Aβ(1–40) and 1–13 and 57–58 of both ZAβ3 subunits are not well defined by NMR data; NMR chemical shifts close to random coil values and lack of observable NOEs indicate that they are disordered. The C-terminal AβVal-39 and AβVal-40 of Aβ(1–40) are ordered as judged from several medium- and long-range NOEs, but their conformation is nevertheless not uniquely defined following structure determination by simulated annealing. NOEs supporting the formation of a salt bridge between the side chains of AβVal-23 and Aβlys-28, which is populated in Aβ fibrils formed under certain conditions (27, 28), could not be detected.

Short peptides, which are homologous to Aβ but contain proline residues as β-sheet blockers, have been developed as fibrillogenesis inhibitors (16). The aim of this and related peptide- and protein-based approaches that target Aβ aggregation is to bind the hydrophobic part of Aβ by exploiting the same intermolecular interactions formed in Aβ self-assembly, e.g., β-sheet backbone hydrogen bonds, and to consequently disrupt the potential for further β-sheet extension (15–17, 29). Although no structure of a β-sheet breaker peptide in complex with Aβ has been reported, certain aspects of the concept appear to be reflected in the ZAβ3-Aβ(1–40) interaction: The ZAβ3 β-strands cap the edges of the Aβ(1–40) β-sheet; the strands are short and terminated on their C-terminal side by a proline and on the N-terminal side by a sequence region selected to have little propensity for either α- or β-structure, resulting in an inability to serve as a template for further β-sheet extension.

Relation of the Aβ β-Hairpin to the Conformation Within Amyloid Fibrils. The structure of Aβ(1–40) bound to ZAβ3 shares important characteristics with fibrillar Aβ. Within amyloid fibrils, Aβ peptides form β-strand-turn-β-strand motifs, with the precise extent of β-strands and turn varying somewhat depending on Aβ variant and preparation conditions (28, 30–32). Overall, the positions of the secondary structure elements are in good agreement with those determined in this study for bound Aβ(1–40). The hydrophobic cluster identified in Aβ(1–40) fibrils (28), including AβLeu-17, AβPhe-19, Aβlle-32, AβLeu-34, and AβVal-36, is also present in the Aβ(1–40):ZAβ3 complex (Figs. 3F and 4A). Aβ amyloid fibrils are stabilized by backbone hydrogen bonding within β-sheets and packing of hydrophobic side-chains. Similarly, ZAβ3 forms an intramolecular β-sheet with Aβ(1–40) and provides a hydrophobic interface for both faces of the Aβ(1–40) β-sheet. Thus, ZAβ3 captures Aβ(1–40) in an amyloid-like, but monomeric, conformation and consequently inhibits fibrillation.

Although the two Aβ(1–40) β-strands form in-register, intermolecular, parallel β-sheets in amyloid fibrils, they hydrogen bond to each other in the Aβ(1–40):ZAβ3 complex. The two conformations are related by a 90° rotation of both β-strands around their axes (Fig. 4B). Applying a 90° rotation with either direction of rotation to both β-strands in the ZAβ3 bound conformation yields four possible molecular conformations for fibrillar Aβ(1–40) (28, 32). Although the conformation allowing for formation of the hydrophobic cluster AβLeu-17, AβPhe-19, Aβlle-32, AβLeu-34, and AβVal-36 within one molecular layer appears to be preferred, other conformations might also be populated depending on the conditions of fibril formation, reflecting the structural plasticity of amyloid fibrils (28, 32). In this context, it can be speculated that the β-hairpin presented here constitutes an intermediate conformation on the pathway to amyloid fibrils, e.g., in the form of a transiently populated conformation sampled by the disordered monomer (33) or as a constituent of oligomeric Aβ (Fig. 4B). In small oligomers, which presumably account for most of the toxicity of amyloidogenic proteins (3, 4, 34), intramolecular β-sheets might be preferred to intermolecular ones because the fibril core structure with its characteristic long-range order is not fully established. Thus, oligomers might form by hydrophobic stacking of β-hairpins and remain soluble as a consequence of the hydrogen bonding capacity of exposed peptide backbones. Fibril seeds could subsequently be generated by a concerted conformational transition toward intermolecular in-register β-sheets (Fig. 4B). The presence of the β-hairpin conformation in oligomers would be in agreement with the observation of a clearly resolvable peak at 1,693 cm−1, indicating an antiparallel β-sheet structure, in the attenuated total reflectance Fourier-transform infrared (ATR-FTIR) spectrum of Aβ oligomers but not fibrils (35).

Amyloid pores, oligomeric assemblies that have been suggested to insert into membranes and confer neurotoxicity by disrupting cell homeostasis (36), could also be composed of Aβ(1–40) hairpins (data not shown). It is unclear, however, whether their dimensions would suffice to span neuronal membranes and whether their mainly nonpolar inner surface would be compatible with a membrane-permeabilizing activity.

Conclusion

This study establishes the β-hairpin as an accessible conformational state of Aβ peptides. Its relation to the conformation of Aβ within amyloid fibrils suggests a role of the β-hairpin in oligomerization and fibrillation. The successful selection of an affibody binding protein that adapts to the conformational
preferences of its target by tolerating limited but essential
modifications of the scaffold structure highlights the potential of
the combinatorial engineering approach. The availability of a
binder to monomeric A\beta and its detailed structural and bio-
physical description will potentiate further investigation of A\beta
oligomerization, aggregation, and disaggregation and will help to
elucidate to what extent binding and stabilization of monomeric
A\beta can interfere with early pathogenic events in AD.

Methods

Proteins. ZA\alpha\beta and ZA\alpha3C28S were expressed in Escherichia coli BL21 DE3 cells
from plasmid pYA442 containing a T7 promoter (20). In addition to the ZA\alpha3
affibody sequence displayed in Fig. 1A, the constructs contained an N-
terminal (His)6-tag (sequence MGSSHHHHHHQ) and two C-terminal residues
(VD). Cultures were grown at 37°C in LB medium or minimal medium
enriched with 1 g/liter 15NH4SO4 and/or 2 g/liter 13C-glucose supplemented with 30
\muM kanamycin. Protein expression was induced at OD 0.6–0.8 with IPTG
(final concentration 1 mM), followed by further incubation for 4 h. Cells were
harvested by centrifugation at 4,000 \times g and frozen at −20°C. For purification,
cells were resuspended in 50 mM Na-phosphate (pH 7.0), 0.2 mM NaCl, and 1
mM PMSF and lysed by sonication. Insoluble material was removed by cen-
trifugation at 16,000 \times g. The (His)6-tagged protein was isolated by TALON
metal affinity chromatography (BD Biosciences) according to the manufac-
turer’s instructions. Further purification was achieved by size exclusion chro-
matography employing an ÄKTA Explorer system equipped with a HiLoad
16/60 Superdex 75 prep grade column (GE Healthcare). The purified affibody
proteins were dialysed against 20 mM Na-phosphate (pH 7.2).

Circular Dichroism (CD) Spectroscopy. CD was performed on a JASCO J-810
spectropolarimeter. Melting curves were recorded at 220 nm, using a 0.1 cm
path-length cell containing proteins at concentrations of 17.5 \muM [A\beta(1–40),
ZA\alpha\beta dimer] or 35 \muM [ZA\alpha3C28S] in 20 mM Na-phosphate, pH 7.2.

Aggregation Assay. Thioflavin T fluorescence was recorded in 96-well plates
(Nunc), using a FLUOstar Optima reader (BMG Labtech) equipped with 440-nm
excitation and 480-nm emission filters. The samples contained 100 \muM of 115
\muM A\beta(1–40) in 50 mM Na-phosphate (pH 7.1), 0.1 M NaCl, 10 \muM thioflavin
T, and 0.1% Na-azide, supplemented with the indicated fraction of D2O.
Plates were sealed with polyolvinyl tape (Nunc) and incubated at 37°C. Data points
were recorded every 15 min with 5 min of orbital shaking (width 5 mm) before
the measurement.

NMR and Structure Determination. NMR data were collected at 25°C, using
Varian Inova 800 and 900-MHz spectrometers, the latter of which was
equipped with a cryogenic probe. NMR samples for structure determination
contained ~400 \muM 15N-labeled or 13C,15N-labeled A\beta(1–40) or disulfide-
linked ZA\alpha\beta dimer, and 15% molar excess of unlabeled ZA\alpha\beta dimer or A\beta(1–40),
respectively, in 20 mM Na-phosphate buffer at pH 7.2. Resonance assignments
were obtained from standard triple-resonance experiments. Interproton dis-
tance constraints were derived from 3D 15N-NOESY and 3D 13C-15N NOESY
spectra recorded with mixing times of 120 ms and calibrated by using known distances
in regular secondary structure elements. Intermolecular nuclear Overhauser
effects (NOEs) were also identified in 3D F1(13C)-filtered F2(15N)-filtered F3(13C or 15N)-
edited NOESY experiments (37). Backbone dihedral angle constraints were
derived from chemical shifts, using TALOS (38). Backbone hydrogen bond
donors were identified in amide hydrogen exchange experiments (see SI Text
and Fig. S2) and acceptor carbonyl oxygens were identified based on initial
structure calculations (see SI Text). The final constraint dataset (Table S1)
contained 3,438 NOE distances, of which 387 are unambiguous intermolecular
distances, 160 are backbone dihedral angle constraints, and 39 are hydrogen
bonds. Structures were calculated with Xplor-NIH 2.15.0 (39), using ab initio
simulated annealing with r6-averaging. A pseudopotential for the radius of
gyration (r6) was used on residues 16–40 of A\beta(1–40) and residues 14–56 of the two ZA\alpha\beta subunits to improve packing, and a conformational database
potential (41) was used to improve dihedral angle distributions. The full
Lennard–Jones potential, an electrostatic potential scaled to 25% of the
default value, and parameters for proper covalent disulfide bond geometry
were applied during the final slow-cooling refinement. An ensemble of 24 (of
100) structures was selected. Backbone and all-heavy atom rms deviations
between structures in the ensemble are 0.32 Å and 0.71 Å, respectively, and
93% of the residues are found in the most favored regions of the Ramachand-
ran diagram (see Table S2 for additional statistics). Hydrogen bonds shown
in Fig. 3E have donor–hydrogen–acceptor distances <2 Å and angles between
the donor bond vector and the vector connecting the two heavy atoms of<35°
in at least 50% of the structures in the ensemble. Molecular graphics figures
were created by using PyMOL (DeLano Scientific).

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