

Stabilization of neurotoxic Alzheimer amyloid- β oligomers by protein engineering

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Soluble oligomeric aggregates of the amyloid- β peptide (A β) have been implicated in the pathogenesis of Alzheimer's disease (AD). Although the conformation adopted by A β within these aggregates is not known, a β -hairpin conformation is known to be accessible to monomeric A β . Here we show that this β -hairpin is a building block of toxic A β oligomers by engineering a double-cysteine mutant (called A β cc) in which the β -hairpin is stabilized by an intramolecular disulfide bond. A β ₄₀cc and A β ₄₂cc both spontaneously form stable oligomeric species with distinct molecular weights and secondary-structure content, but both are unable to convert into amyloid fibrils. Biochemical and biophysical experiments and assays with conformation-specific antibodies used to detect A β aggregates in vivo indicate that the wild-type oligomer structure is preserved and stabilized in A β cc oligomers. Stable oligomers are expected to become highly toxic and, accordingly, we find that β -sheet-containing A β ₄₂cc oligomers or protofibrillar species formed by these oligomers are 50 times more potent inducers of neuronal apoptosis than amyloid fibrils or samples of monomeric wild-type A β ₄₂, in which toxic aggregates are only transiently formed. The possibility of obtaining completely stable and physiologically relevant neurotoxic A β oligomer preparations will facilitate studies of their structure and role in the pathogenesis of AD. For example, here we show how kinetic partitioning into different aggregation pathways can explain why A β ₄₂ is more toxic than the shorter A β ₄₀, and why certain inherited mutations are linked to protofibril formation and early-onset AD.

amyloid- β peptide | protein aggregation | protein structure | protofibril | β -hairpin conformation

Alzheimer's disease (AD) is linked to the formation of neurotoxic oligomeric aggregates of the amyloid- β peptide (A β) in the brain and several such aggregates have been described (1, 2). These aggregates have, for instance, been identified in vivo in brain tissue of humans as 70 kDa or larger aggregates containing A β dimers (3), or from transgenic AD mice as smaller aggregates called A β *56 (4). Toxic A β oligomers and protofibrils have also been made in vitro, such as oligomeric ADDLs prepared by dilution from organic solvents (5, 6), smaller globulomers formed in SDS-containing solvents (7), oligomers formed in water at low pH (8, 9), and larger nonfibrillar aggregates known as protofibrils (10, 11). However, it is not clear if and how these different aggregates are related to the pathogenesis of AD. Indeed, although there is a large body of data on the conformation and "cross- β " packing of A β in amyloid fibrils (12, 13), which are the end-products of aggregation, little is known about the basic building blocks of the oligomers and protofibrils that precede them. Here we use protein engineering to address these issues and to provide a method to stabilize toxic A β oligomers for structural and functional studies.

Monomeric A β does not adopt a unique conformation in water solution. Nevertheless, NMR experiments (14) and molecular modeling (15) suggest that the central and C-terminal hydrophobic regions of A β have a propensity to form extended β -strand

conformations with a connecting turn between them. Such a "hairpin" conformation (Fig. 1A) is in fact also induced when A β forms a complex with a phage-display selected Affibody-binding protein (16, 17). The hairpin is topologically similar to the conformation of A β in fibrils. However, there is a distinct difference in that the hydrogen bonds are intramolecular, resulting in antiparallel β -strands, whereas they are intermolecular in fibrils, resulting instead in parallel β -sheets.

We have previously proposed that intermediate oligomeric species consist of A β subunits in the hairpin conformation and that a conformational change results in the formation of A β subunits held together by intermolecular hydrogen bonds that are seeds for polymerization into amyloid fibrils (17). There is also experimental evidence for the hairpin in oligomers of the globulomer kind (18), as well as for antiparallel β -sheet secondary structure in A β oligomers formed in TBS buffer or in cell culture medium (19). We therefore set out to test if stabilizing A β in the hairpin conformation observed in the Affibody complex would promote the formation of oligomeric aggregates but not fibrils, and whether such stabilized oligomers would possess antigenic and neurotoxic characteristics similar to those of wild-type A β oligomers found in AD.

Results

Protein Engineering. The structure of A β ₄₀ in complex with the ZA β 3 Affibody (PDB accession No. 2OTK) was examined for sites suitable for disulfide engineering that would constrain it in its hairpin conformation. Ala21 and Ala30 are ideally suited for this purpose, as their β -carbons are located on opposite β -strands at a distance of 4.2 Å from each other (Fig. 1A). Molecular modeling showed that a Cys21/Cys30 disulfide can be accommodated with favorable conformational energy and without perturbing the hairpin structure in which 12 backbone hydrogen bonds are predicted to form in A β ₄₀cc.

We produced A β cc in *Escherichia coli* bacteria by coexpression with the ZA β 3 Affibody (20). This Affibody binds A β in the hairpin conformation, which allows for the formation and purification of monomeric A β cc with an intramolecular disulfide bond. Recombinant A β produced in this way contains an N-terminal methionine residue, but this does not affect its biophysical properties (20).

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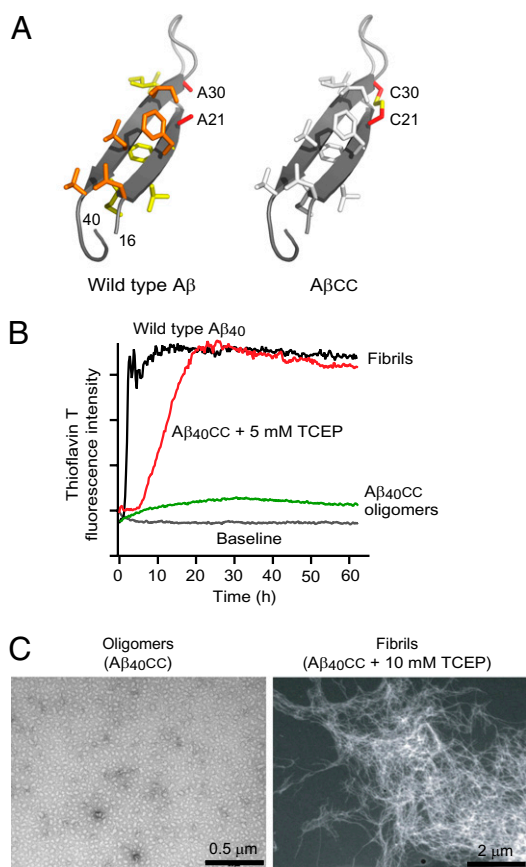


Fig. 1. Protein engineering. (A) (Left) The β -hairpin conformation of $A\beta_{40}$ observed in complex with an Affibody binding protein (17). Nonpolar side chains at the two hydrophobic faces are shown as sticks and colored yellow and orange, respectively. The Ala21 and Ala30 methyls are located in close proximity on opposite β -strands. (Right) Model of the $A\beta_{21C/A30C}$ double mutant ($A\beta_{CC}$) in which the β -hairpin conformation is locked by a disulfide bond. (B) ThT fluorescence assays of $A\beta_{40CC}$ aggregation in the absence or presence of TCEP reducing agent compared with wild-type $A\beta_{40}$ aggregation. (C) TEM micrographs of β -sheet oligomers of $A\beta_{40CC}$ (Left) and of fibrils formed in presence of TCEP (Right).

$A\beta_{CC}$ with an Intact Disulfide Bond Cannot Form Amyloid Fibrils. Our hairpin hypothesis postulates that $A\beta_{CC}$ should not form amyloid fibrils as long as the intramolecular disulfide is intact, and experiments show that this is indeed the case. Thioflavin T (ThT) fluorescence assays of fibril formation demonstrate that oxidized $A\beta_{40CC}$ remains soluble in a weakly ThT-binding state, whereas the addition of tris-2-carboxyethyl-phosphine (TCEP) reducing agent to break the disulfide bond results in accelerating ThT binding to levels equal to those observed with wild-type $A\beta_{40}$ fibrils (Fig. 1B). Transmission electron microscopy (TEM) confirms that oxidized $A\beta_{40CC}$ forms oligomeric aggregates, whereas reduced $A\beta_{40CC}$ forms fibrils (Fig. 1C). In addition, the longer $A\beta_{42CC}$ cannot form amyloid fibrils in its oxidized state (Fig. S1) but, as described below, instead forms β -sheet containing oligomers and protofibrils.

Formation of Oligomers with Different Secondary-Structure Content. Blocking of fibril formation allows for the enrichment of oligomeric aggregates that are otherwise only transiently formed by wild-type $A\beta$ (1, 2). Purified $A\beta_{40CC}$ and $A\beta_{42CC}$ were denatured into monomeric form in 7 M guanidinium chloride and subjected to size exclusion chromatography (SEC) with native (non-denaturing) phosphate buffer at pH 7.2 as running buffer. Different oligomers then form spontaneously during SEC. The SEC chromatograms of $A\beta_{40CC}$ and $A\beta_{42CC}$ reveal the separation of distinct oligomeric species with apparent molecular weights of ~ 16 ,

~ 30 , ~ 50 , and ~ 100 kDa, in addition to monomer (at 8–10 kDa) and high molecular weight (HMW) aggregates (larger than $\sim 1,000$ kDa) eluting at the void volume (Fig. 2A and B). $A\beta_{42CC}$ and $A\beta_{40CC}$ form oligomers with similar molecular weights and higher concentration favors the formation of larger oligomers of both species, as expected (Fig. S2). Sometimes, ~ 185 -kDa oligomers are also observed; these are not well separated in SEC, but can be distinguished in the SEC profiles in Fig. 2B and Fig. S1A.

Circular dichroism (CD) spectroscopy (Fig. 2C) reveals that $A\beta_{40CC}$ and $A\beta_{42CC}$ oligomers with an apparent molecular weight of ~ 100 kDa contain $\sim 40\%$ β -sheet secondary structure (β -sheet oligomers). Low molecular-weight (LMW) oligomers display a “random coil” CD spectrum similar to that of a disordered peptide and therefore lack regular secondary structure. LMW oligomers may, however, convert into either β -sheet oligomers or HMW aggregates and β -sheet oligomers may assemble further into protofibrils (Fig. 2D), as described below. The presence of antiparallel β -sheet in the larger oligomers was confirmed by Fourier transform infrared (FTIR) spectroscopy (Fig. S3).

Differences Between $A\beta_{40CC}$ and $A\beta_{42CC}$ Oligomerization. There is a clear difference between the aggregation patterns of $A\beta_{40CC}$ and $A\beta_{42CC}$, as the latter more readily forms β -sheet oligomers (Fig. 2A and Fig. S2). However, $A\beta_{40CC}$ LMW oligomers form β -sheet oligomers when they are concentrated to ~ 0.6 mM and subjected to a 10-min heat treatment at 60°C (Fig. 2C and Fig. S4). Hence, a kinetic barrier for formation of β -sheet oligomers is more easily overcome by $A\beta_{42CC}$ than by $A\beta_{40CC}$. This finding explains the different size distributions observed in SEC of $A\beta_{42CC}$ and $A\beta_{40CC}$ (Fig. 2A) and it may also explain differences in toxicity, as described in the Discussion.

β -Sheet Oligomers Contain SDS-Stable Dimers and Trimers. Aggregated $A\beta$ isolated from the brains of AD patients contains neurotoxic dimeric and trimeric species that are either formed in SDS-containing solutions or are resistant to denaturation in SDS/PAGE electrophoresis experiments. It has been suggested that such species may constitute building blocks of toxic $A\beta$ aggregates (3, 18). If this suggestion is true and if the aggregates formed by $A\beta_{CC}$ are structurally identical to wild-type aggregates, then one would expect $A\beta_{CC}$ to form SDS-stable dimers. We find that dimeric and trimeric bands of purified but unfractionated $A\beta_{42CC}$ are more prominent in SDS/PAGE than those of wild-type $A\beta_{42}$. But more importantly, ~ 100 kDa β -sheet oligomer fractions of $A\beta_{42CC}$ are only observed as dimer and trimer units in SDS/PAGE with Coomassie staining (Fig. 2E). The absence of a monomer band implies that dimers and trimers form in the oligomers and remain stable in SDS-containing buffers.

β -Sheet Oligomers Can Form Protofibrils. Pooling and concentrating SEC fractions containing $A\beta_{42CC}$ or $A\beta_{40CC}$ β -sheet oligomers results in the formation of higher-order aggregates (Fig. 2D), which are distinct from the HMW aggregates observed to elute in the void of the SEC profiles in Fig. 2A and B (also shown by antibody assays below). TEM images of such samples (Fig. 2F) show spherical oligomers with an average diameter of ~ 6 nm, as well as longer and curved aggregates with the same diameter. The morphology of the larger aggregates is such that they appear to be assemblies of the 6-nm oligomers. Their size and appearance are also very similar, if not indistinguishable, to those of protofibrils formed by wild-type $A\beta$; one may, for instance, compare Fig. 2F to figure 1C in the work of Goldsbury et al. (figure 1C in ref. 21). A globular protein, which in SEC elutes at a volume corresponding to that of the β -sheet oligomers (~ 100 kDa) will have a diameter of ~ 6 nm, in agreement with the size of the small spherical oligomers. A molecular weight of ~ 100 kDa per 6-nm oligomer subunit in $A\beta_{CC}$ protofibrils is also close to the observed mass per length of wild-type $A\beta$ protofibrils: 114 ± 12 kDa per 6 nm (21). Hence, it appears that the smaller particles in Fig. 2F are β -sheet oligomers and that these associate to form protofibrils. $A\beta_{40CC}$ β -sheet oligomers also form protofibrils upon con-

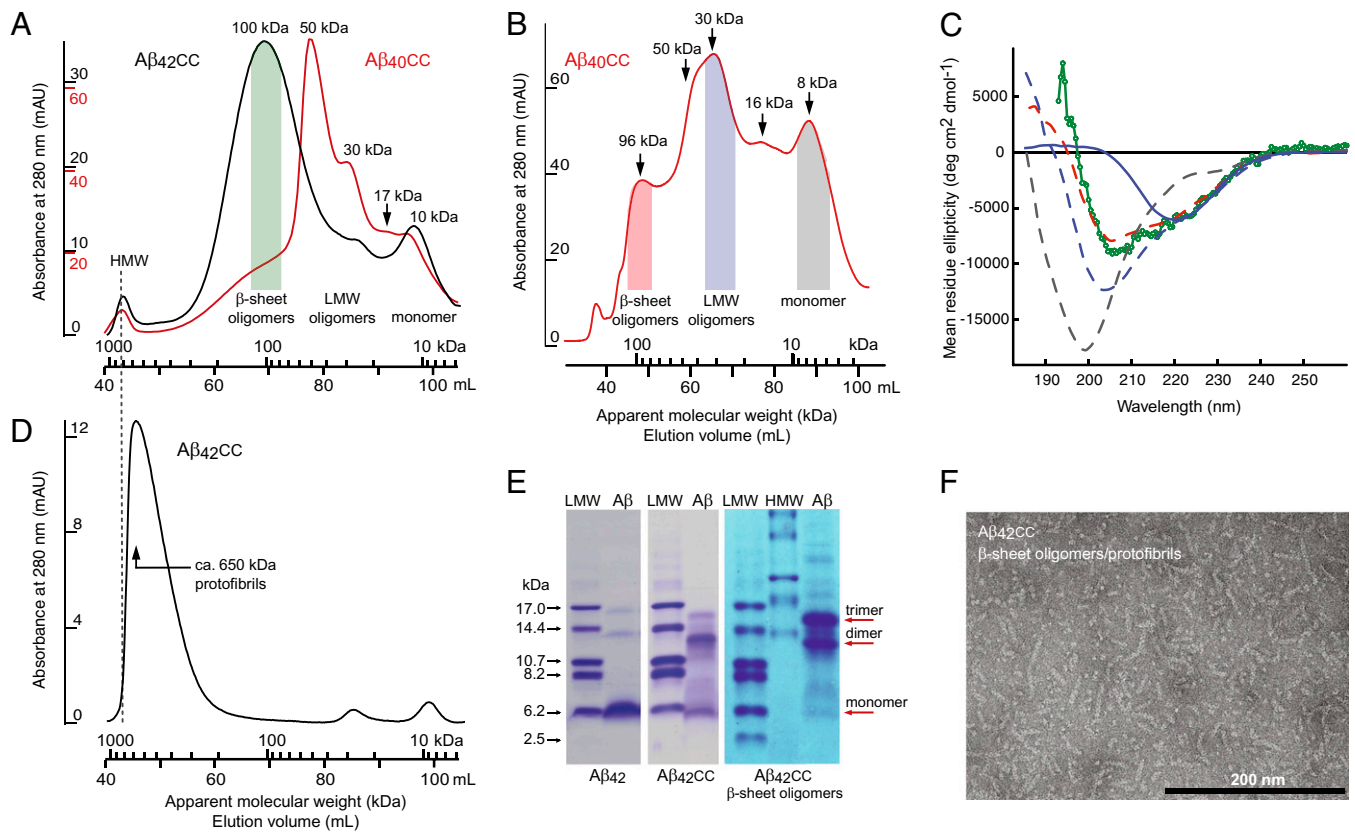


Fig. 2. Biophysical and biochemical characterization of A β cc oligomers and protofibrils. (A) Formation and separation of A β_{42} CC (black) and A β_{40} CC (red) oligomers during SEC on a Superdex 200 PG 16/600 column. Monomer peptide samples were loaded in denaturing buffer and eluted with native phosphate buffer at pH 7.2. Apparent molecular weights and classification of eluted oligomers have been indicated. HMW aggregates elute with the void volume. Sample amounts: 2.7 mg A β_{40} CC and 1.7 mg A β_{42} CC; see also Fig. S2. (B) SEC as in A showing separation of A β_{40} CC oligomers on a Superdex 75 PG 16/60 column on which smaller aggregates become better separated. Sample amount: ~3 mg. (C) CD (mean residue ellipticity) of SEC fractions pooled as indicated by shaded areas in A and B. Dashed lines: 8-kDa monomer (gray, 12 μ M), 30-kDa LMW oligomers (blue, 16 μ M), and 96-kDa β -sheet oligomers (red, 8 μ M) of A β_{40} CC. Green: ~100-kDa β -sheet oligomers of A β_{42} CC (13 μ M). Solid blue line: the 30-kDa LMW oligomer fraction of A β_{40} CC after concentration and heat treatment showing formation of β -sheet oligomers. (D) SEC of concentrated A β_{42} CC β -sheet oligomers (1 mL, 145 μ M), which form protofibrils with an average apparent molecular weight of ~650 kDa. The dotted line shows that these are smaller than HMW aggregates in A. (E) SDS/PAGE of wild-type A β_{42} (Left), purified but unfractionated A β_{42} CC (Center), and A β_{42} CC β -sheet oligomers formed during SEC as in A (Right). The right lanes in all panels contain A β samples, and other lanes contain high and low molecular weight standards (HMW and LMW). Weights corresponding to monomer and SDS-resistant dimers and trimers have been indicated. The loading buffer contained 2.5 mM TCEP (heat-stable reducing agent) to completely break all disulfide bonds. (F) TEM micrograph of a concentrated sample of A β_{42} CC β -sheet oligomers (190- μ M monomer concentration) showing assembly of β -sheet oligomers into protofibrils.

centration. Dilution of protofibril samples followed by SEC suggest that they are stable (Fig. 2D). Such diluted samples, which we will call β -sheet oligomers/protofibrils, were used for antibody and neurotoxicity experiments described in the following section.

Recognition of A β cc by a Physiologically Relevant Conformation-Specific Antibody. We used two different conformation-specific antibodies that bind wild-type A β aggregates to examine if their binding epitopes are preserved in A β cc and to shed light on how the many A β cc aggregates are related. The mAb158 monoclonal antibody (22) was selected based on its affinity for protofibrils of A β_{42} carrying the Arctic mutation (11). The mAb158 antibody also recognizes protofibrils of wild-type A β_{42} , a protofibrillar form that is present in the medium of APP-expressing cells, and A β aggregates in brains of transgenic mice (22). ELISA experiments show that β -sheet oligomers/protofibrils of A β_{42} CC bind mAb158 with the same affinity, or better, than wild-type protofibrils (Fig. 3A). This assay is conformation-specific because monomeric A β_{42} CC or SDS-treated β -sheet oligomers are not recognized by mAb158 (Fig. 3B). β -Sheet oligomers of A β_{40} CC that have been converted from LMW oligomers by concentration and heat treatment are also recognized by mAb158 (Fig. S4). Hence, β -sheet

A β cc oligomers expose a mAb158 conformational epitope that is also present in wild-type A β protofibrils in vitro and in vivo.

A β cc Can Access Two Distinct Aggregation Pathways. In addition to oligomers and protofibrils, A β cc also forms HMW aggregates (Fig. 2A and Fig. S2). These aggregates are less homogeneous and contain both large amorphous aggregates and long protofibrils (Fig. S5), the latter of which may account for mAb158 binding observed for this fraction (Fig. 3B). Interestingly, the HMW SEC fractions also bind the A11 antibody (Fig. S6), which also is conformation-specific (23). However, β -sheet oligomers/protofibrils of A β cc that bind mAb158 do not bind A11, and these are therefore structurally distinct from the HMW aggregates. To identify which oligomeric species represent the precursors of the A11 binding HMW aggregates of A β cc, we aged both LMW and β -sheet oligomer fractions of A β_{42} CC at 37 $^{\circ}$ C. (LMW oligomers were kept in diluted form to avoid the transformation into β -sheet oligomers). Within a few days, LMW oligomer samples of A β_{42} CC assemble into large, stable A11-positive aggregates with a diameter of 19 to 25 nm, whereas β -sheet oligomers do not form A11-binding species even after 8 wk of aging (Fig. 3C and Fig. S6). These experiments show that the conformational specificity of A11 is different from that of mAb158: mAb158 binds an epitope pre-

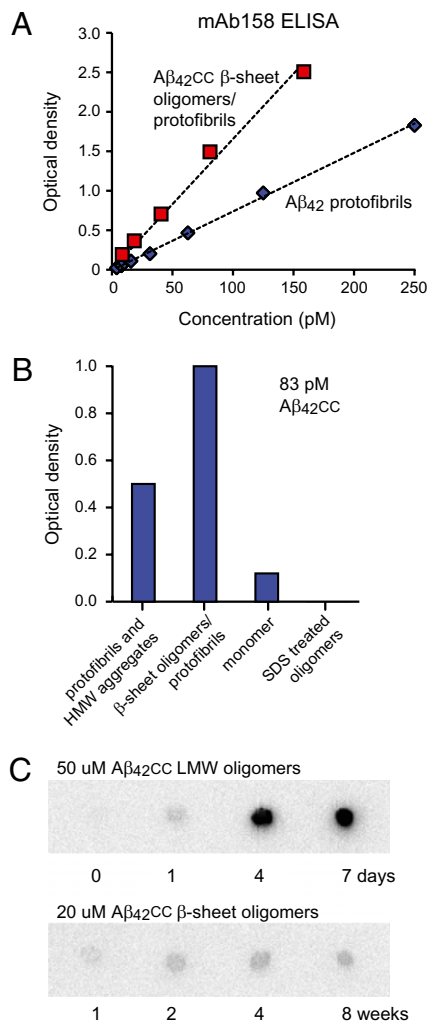


Fig. 3. Recognition of Aβ_{cc} oligomers by antibodies that are conformation specific for wild-type Aβ aggregates. (A) mAb158 antibody sandwich ELISA detection (22) of Aβ₄₂CC β-sheet oligomers/protofibrils compared with detection of wild-type Aβ₄₂ protofibrils. (B) mAb158 ELISA analysis of different Aβ₄₂CC species at 83 pM total peptide concentrations. (C) Aging of Aβ₄₂CC LMW and β-sheet oligomers at 37 °C followed by A11 antibody dot blot analysis showing that A11 binding aggregates form from LMW oligomers but not from β-sheet oligomers. Concentrations were equalized before blotting.

sent in aggregates containing Aβ₄₂CC or Aβ₄₀CC β-sheet oligomers, whereas A11 binds aggregates that are formed by LMW Aβ₄₀CC or Aβ₄₂CC oligomers or monomeric samples. There must therefore be two aggregation pathways for Aβ_{cc} with different end products: the β-sheet pathway, involving aggregation of β-sheet oligomers into protofibrils (which are detected in the mAb158 ELISA), and the coil pathway, involving aggregation of monomer or disordered LMW oligomers into HMW A11-binding aggregates (which are also detectable *in vivo*) (23). (The naming of the aggregation pathways reflects the secondary-structure content of the originating oligomers.)

Neurotoxicity of Aβ_{cc} Oligomers. The neurotoxicity of different Aβ_{cc} aggregates was assayed by measuring their ability to induce apoptosis, indicated by the level of caspase-3/7 activity in a human neuroblastoma cell line, SH-SY5Y. Aβ₄₂CC aggregates pooled from SEC all induce apoptosis 24 h after addition to the cells, but β-sheet oligomer fractions are considerably more toxic than LMW or HMW fractions of Aβ₄₂CC. In fact, the toxicity is comparable or

higher than that of wild-type Aβ₄₂ oligomers, as one would expect for a stabilized toxic aggregate (Fig. 4A). Nerve cell apoptosis is dose-dependent and measurable after 24 h of treatment at 1-μM peptide concentrations and the β-sheet Aβ₄₂CC oligomers are 50 times more potent than wild-type Aβ₄₂ monomer or amyloid fibril samples (Fig. 4B). In contrast, the equivalent monomer and LMW oligomer fractions of Aβ₄₀CC do not induce apoptosis. However, when Aβ₄₀CC LMW oligomers are transformed to β-sheet oligomers/protofibrils, as described above, these start to induce caspase activity, but at lower levels compared with Aβ₄₂CC (Fig. 4C). The neurotoxicity experiments therefore suggest that the actively toxic species are β-sheet oligomers of Aβ₄₂CC and Aβ₄₀CC, and larger protofibrillar aggregates that form when these are concentrated, as they have to be before the apoptosis assays. The results are also consistent with the lower propensity of Aβ₄₀CC monomers to form β-sheet oligomers and these are, once formed, intrinsically less toxic than those of Aβ₄₂CC. The fact that LMW oligomers and HMW aggregates of Aβ₄₂CC also induce apoptosis (as shown in Fig. 4A) does not contradict that β-sheet oligomers/protofibrils are the primary toxic species, because LMW and β-sheet oligomers are not completely separated in SEC (Fig. 2A and B) and large protofibrils are present in HMW fractions (Fig. S5). Overall, the neurotoxicity of different Aβ₄₂CC and Aβ₄₀CC aggregates (Fig. 4) matches specific recognition in the mAb158 ELISA (Fig. 3A and B, and Fig. S4), indicating that toxic aggregates formed by Aβ_{cc} are to be found along the β-sheet aggregation pathway.

Discussion

Do Aβ_{cc} Oligomers Mimic Wild-Type Aβ Oligomers? Aβ_{cc} contains alanine to cysteine replacements, at positions 21 and 30, designed to stabilize a β-hairpin conformation by forming an intramolecular disulfide cross-link. The hairpin is predicted to comprise residues 17 to 23 and 30 to 36 as antiparallel β-strands connected by a turn involving residues 25 to 29. There is considerable evidence that such a conformation is accessible in monomeric Aβ: (i) it forms in complex with binding proteins (16, 17), (ii) its secondary structure elements persistently appear in computer simulations of different Aβ fragments (15, 24, 25), and (iii) NMR data suggest that turn formation of residues 24 to 28 nucleates monomer folding (26). We previously postulated that metastable Aβ oligomers contain hairpin subunits and that conversion into a related cross-β conformation transforms oligomers into fibril seeds and primes these for the runaway aggregation that is typical of amyloid fibril formation.

Here we test if soluble Aβ oligomers indeed consist of hairpins as constituent monomer building blocks and, if this is the case, if hairpin stabilization then provides a method for stabilization of physiologically relevant oligomers. We find that a Cys21-Cys30 disulfide prevents the formation of amyloid fibrils, resulting instead in the formation of stable oligomeric aggregates and protofibrils. These aggregates are indistinguishable from wild-type Aβ aggregates in TEM and they contain the dimeric and trimeric SDS-resistant units that are regarded as fingerprints of neurotoxic Aβ *in vivo*. Conformation-specific antibodies that recognize wild-type Aβ in the brains of AD patients (23) and transgenic mice (22) also recognize Aβ_{cc} oligomers. Finally, β-sheet oligomers and protofibrillar species formed by Aβ₄₂CC or Aβ₄₀CC are powerful inducers of apoptosis in neuronal cells in culture. Aβ_{cc} oligomers are therefore biologically functional, and we propose that they constitute an appropriate model for structural and functional studies of oligomers relevant to the pathogenesis of AD. Aβ_{cc} may potentially also be used for therapy development based on immunization or for small-molecule drug discovery.

Architecture of Neurotoxic Aβ Oligomers and Protofibrils. The conformation of Aβ_{cc} in toxic β-sheet oligomers is most likely a β-hairpin, as shown in Fig. 1A, but β-hairpin conformations in which the turn is shifted from residues 25 to 29 toward, for instance, 24 to 28 or 23 to 27 may in fact also be compatible with a Cys21/Cys30 disulfide. The proposition that toxic Aβ oligomers are built of hairpin monomer subunits arranged to form larger

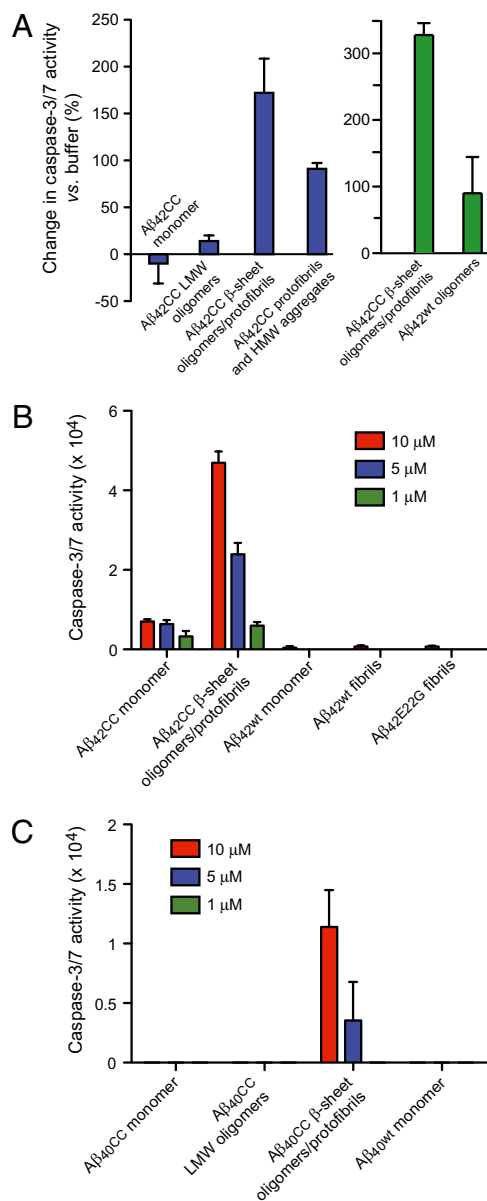


Fig. 4. Neurotoxicity of Aβ_{cc} to SH-SY5Y human neuroblastoma cells. Aβ_{cc} samples, except β-sheet oligomers/protofibrils in C, were prepared and isolated by SEC, as in Fig. 2A, concentrated to ~75 to 250 μM in phosphate buffer at pH 7.2, and added to cell cultures at 1-, 5-, or 10-μM concentrations. Caspase-3/7 activity reporting on apoptosis was measured after 24 h of treatment. (A) Aβ₄₂CC induced apoptosis following treatment with 10 μM of different species. Blue: comparison of different Aβ₄₂CC species. Green: Aβ₄₂CC oligomers compared with wild-type Aβ₄₂ oligomers in another experiment. (B) Dose-dependence of apoptosis induced by Aβ₄₂CC species compared with that of wild-type Aβ₄₂ monomer and fibrils and Aβ₄₂E22G. (C) Apoptosis induced by different Aβ₄₀CC species and wild-type Aβ₄₀. The sample marked "Aβ₄₀CC LMW oligomers" is a 75-kDa SEC oligomer fraction. The Aβ₄₀CC β-sheet oligomers/protofibrils were formed by pooling and concentrating monomer and LMW oligomer SEC fractions and heating the concentrated sample as described in the text and in Fig. S4.

units of antiparallel β-sheet secondary structure is in agreement with other observations. Infrared spectroscopy shows that antiparallel (and not parallel) β-sheet is present in oligomeric aggregates (19, 27). A β-hairpin similar to what is described here is also observed in synthetic Aβ globulomers (18). Aβ species with a mass equivalent to that of a dimer has been directly linked to disease (3). It is not certain that these are chemically identical to

dimers observed in vitro, but the present data linking neurotoxicity, β-sheet oligomers, and SDS-resistant dimers and trimers are consistent with such a view.

Our experiments are not conclusive with regard to the precise molecular weight of a minimal β-sheet oligomer from which protofibrils assemble. SEC elution volumes suggest ~100 kDa, but SEC columns are calibrated with globular protein standards and any disorder will result in a larger Stokes radius and an overestimate of the molecular weight. For example, the disordered nature of monomeric Aβ makes it appear larger in SEC than a globular protein of the same molecular weight. It is therefore not clear if oligomers of ~16, ~30, and ~50 kDa correspond to dimers, tetramers, and hexamers of Aβ_{CC}, respectively, or if some other stoichiometry applies. However, when also considering that disordered regions may remain in the ~100-kDa β-sheet oligomers, these should contain at least 12 monomer subunits. Hence, a dodecamer stoichiometry of toxic Aβ oligomers that has been favored in other research (4, 28–30) is consistent with our data.

Electron microscopy images indicate that β-sheet oligomers of ~6 nm in diameter assemble into protofibrils of variable lengths, thereby implying that the protofibrils are composed of β-sheet oligomers. Aβ_{CC} protofibrils are morphologically very similar to wild-type protofibrils. These protofibrils are also recognized in the mAb158 monoclonal antibody ELISA (22), which is conformation-specific for protofibrils of wild-type Aβ₄₂.

With regard to the structural differences between β-sheet and LMW oligomers, it is possible that the latter either do not form β-hairpins with intramolecular hydrogen bonds (despite the disulfide) or that hydrogen-bonded β-hairpins do not assemble into larger β-sheet structures in LMW oligomers.

Differences Between Aβ₄₀ and Aβ₄₂ Aggregation and Toxicity. We find that Aβ₄₂CC more readily forms β-sheet oligomers than Aβ₄₀CC. This occurs as a result of the presence of a kinetic barrier to oligomer formation that can only be overcome by Aβ₄₀CC with the aid of heating and concentration. Wild-type Aβ₄₂ is considered to be more toxic than Aβ₄₀. It is possible that this difference in toxicity reflects different barriers to β-sheet oligomer formation that we observe here with Aβ_{cc}.

Several studies also indicate differences in the aggregation pathways of wild-type Aβ₄₂ and Aβ₄₀ (29) and multiple pathways for amyloid formation (1). We propose an aggregation scheme (Fig. 5) in which monomeric Aβ can form disordered LMW oligomers or larger β-sheet oligomers, and that LMW oligomers can convert into β-sheet oligomers. β-Sheet oligomers can associate into protofibrils recognized in the mAb158 ELISA. LMW oligomers, on the other hand, can also aggregate into A11-binding species, which

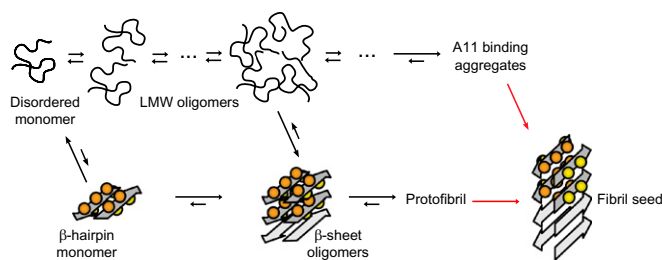


Fig. 5. Aβ aggregation via two pathways. One pathway involves LMW oligomers without regular secondary structure and eventually large non-fibrillar aggregates binding the A11 antibody (coil pathway; Upper) and the other involves assembly into β-sheet oligomers, or coil oligomers that are converted into β-sheet oligomers, which are building blocks of mAb158 binding protofibrils (β-sheet pathway; Lower). Neurotoxic Aβ aggregates are formed along the β-sheet pathway. The scheme is consistent with the present studies of Aβ_{cc} and overall features can be reconciled with a large body of work on wild-type and naturally occurring Aβ mutants as discussed in the text. Red arrows reflect the interconversion of Aβ subunits from β-hairpin conformation in soluble aggregates to cross-β structure in fibril seeds and mature amyloid fibrils.

cannot be formed by β -sheet oligomers or protofibrils. β -Sheet oligomer formation by $A\beta_{40CC}$ is slower than by $A\beta_{42CC}$, and this results in different populations of the two peptides (at a given concentration) aggregating along the LMW oligomer \rightarrow A11-binding HMW-aggregate pathway and the mAb158-binding β -sheet oligomer \rightarrow protofibril pathway.

It is not clear from which aggregate fibril seeds are formed in $A\beta_{CC}$ after the addition of reducing agent. However, protofibrils have been suggested to constitute the penultimate intermediate in amyloid fibril formation (1, 10), and fibril formation can also occur in solutions containing A11-binding aggregates (31). In the reaction scheme in Fig. 5, we therefore allow for seed formation by both these aggregates. Still, the aggregation equilibria also allow for other possibilities, such as seeding directly from small β -sheet oligomers as suggested previously (17), or by secondary nucleation events (32).

Possible Link Between β -Sheet Oligomer Formation and Early-Onset AD. Arguments relating toxicity to aggregation pathway may be extended to include inherited mutations in $A\beta$ that are linked to higher toxicity and early-onset AD. For example, $A\beta$ with the Arctic E22G mutation forms protofibrils more rapidly than wild-type $A\beta$ (11), which in our aggregation scheme is equivalent to a higher rate of β -sheet oligomer formation (aggregation along the β -sheet pathway). In accordance with such a mechanism, the $A\beta$ E22G peptide more easily forms SDS-resistant higher-order aggregates (20), which we also associate to β -sheet oligomers. An experimental linkage between kinetic partitioning of mutant $A\beta$ peptides into different aggregation pathways and their toxicity would, if it can be further substantiated, constitute an attractive mechanism to rationalize the relationship between inherited mutations and early-onset AD.

Conclusions

We have engineered an $A\beta$ peptide variant ($A\beta_{CC}$) in which a β -hairpin is stabilized by a disulfide bond. $A\beta_{CC}$ forms oligomeric

aggregates in which conformational and biological properties of wild-type $A\beta$ oligomers are preserved, and amyloid formation is prevented. $A\beta_{42CC}$ and $A\beta_{40CC}$ both aggregate via two distinct pathways that can be distinguished using conformation-specific antibodies. The coil pathway involves formation of less structured LMW oligomers with apparent molecular weights of 50 kDa or less, which can aggregate further into nonfibrillar HMW species. The β -sheet aggregation pathway involves initial formation of structured β -sheet oligomers with an apparent molecular weight of 100 kDa or more, which also contain SDS-stable dimeric and trimeric units. β -Sheet oligomers can associate into protofibrils that are morphologically identical to wild-type protofibrils and detected in a mAb158 antibody ELISA, which is specific for wild-type protofibrils. Aggregates on the β -sheet aggregation pathway are neurotoxic and induce neuronal apoptosis. A kinetic barrier for formation of β -sheet oligomers makes $A\beta_{40CC}$ more prone to aggregate along the coil pathway, but once formed, β -sheet oligomers of $A\beta_{40CC}$ induce neuronal apoptosis as well. Kinetic partitioning into two aggregation pathways in which one contains the neurotoxic aggregates may explain why $A\beta_{40}$ is less toxic than $A\beta_{42}$ and why certain $A\beta$ mutations lead to early onset AD.

Materials and Methods

Complete discussions of molecular modeling, peptide production and purification, SDS/PAGE, fibril formation assays, CD, infrared spectroscopy, TEM, atomic force microscopy, mAb158 ELISA, A11 dot blot, and neurotoxicity assays are in *SI Materials and Methods*.

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