

# $\beta$ -Amyloid Fibril Structures, In Vitro and In Vivo

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**Abstract** Since 1998, a great deal of progress has been made towards determining and understanding the molecular structures of amyloid fibrils, including fibrils formed by the  $\beta$ -amyloid peptide that is associated with Alzheimer's disease. Much of this progress has resulted from solid state nuclear magnetic resonance (NMR) measurements, which provide experimental constraints on molecular conformations and interatomic distances without requiring solubility or crystallinity. In general, amyloid fibrils are polymorphic, meaning that fibrils formed by a given peptide or protein can have multiple, distinct molecular structures, depending on the precise conditions under which the fibrils grow. From solid state NMR, electron microscopy, and other measurements, we have developed two detailed molecular structural models for fibrils formed by the 40-residue wild-type  $\beta$ -amyloid ( $A\beta_{1-40}$ ) peptide. These two  $A\beta_{1-40}$  fibril polymorphs share a common, parallel  $\beta$ -sheet organization and contain similar peptide conformations but differ in overall symmetry and in other structural aspects. We have also identified and characterized a surprising antiparallel  $\beta$ -sheet structure in metastable fibrils formed by a disease-associated mutant, D23N- $A\beta_{1-40}$ , which reveals how similar sets of interactions can stabilize both parallel and antiparallel  $\beta$ -sheets within amyloid fibrils. We are currently extending our structural studies to  $\beta$ -amyloid fibrils that develop in human brain tissue, with the goal of testing whether variations in fibril structure correlate with variations in severity, progression rate, or other characteristics of Alzheimer's disease.

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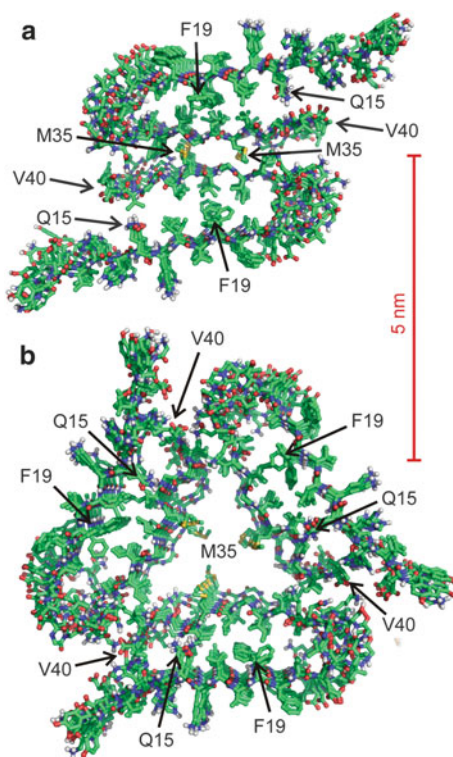
## Introduction

Knowledge about the structures of amyloid fibrils is important for several reasons: (1) detailed structural information at the molecular and atomic level is necessary for rational design of compounds that inhibit amyloid formation (Gordon et al. 2001; Sato et al. 2006; Sievers et al. 2011) or bind specifically to amyloid (Klunk et al. 2004; Schutz et al. 2011; Wong et al. 2010). Such compounds have therapeutic and diagnostic applications; (2) substantial evidence exists that amyloid fibrils are polymorphic at the molecular structural level, i.e., that the amino acid sequence alone does not determine the molecular structure uniquely (Goldsbury et al. 2000; Kodali et al. 2010; Luca et al. 2007; Paravastu et al. 2008; Petkova et al. 2005). The possibility exists that fibrils with distinct structures may have distinct biological effects (Meyer-Luehmann et al. 2006; Paravastu et al. 2009; Petkova et al. 2005; Tycko et al. 2009). Details of the molecular structures may therefore have biomedical consequences; and (3) detailed structural information is a requirement for any understanding of the intermolecular forces that drive amyloid formation and for any understanding of the mechanisms and pathways by which amyloid fibrils form from monomeric peptides and proteins (Fawzi et al. 2007; Klimov and Thirumalai 2003).

Research on amyloid fibril structures began in my laboratory in 1998. At that time, relatively little was known about the molecular structures of amyloid fibrils, primarily because amyloid fibrils are inherently noncrystalline and insoluble, making direct structure determination by X-ray crystallography and liquid state nuclear magnetic resonance (NMR) impossible. The only aspect of amyloid fibril structures that was firmly established by experimental data was the fact that amyloid fibrils contain ribbon-like  $\beta$ -sheets, running the length of the fibrils and arranged in a “cross- $\beta$ ” motif, i.e., a motif in which  $\beta$ -strand segments are oriented approximately perpendicular to the long axis of the fibril and are connected by backbone hydrogen bonds that are oriented approximately parallel to the long axis (Sunde et al. 1997). Other structural aspects, including the nature of the  $\beta$ -sheets within the cross- $\beta$  motif (parallel or antiparallel), the identities of  $\beta$ -strand segments, and even the extent to which amyloid fibrils contain well-defined molecular structures (rather than being highly disordered at the molecular level) were uncertain.

Our understanding of amyloid fibril structures has advanced quite substantially over the past 14 years, to the point where many of the generic features of these structures have been elucidated and detailed structural models, based on large sets of experimental data, have been developed for fibrils formed by specific peptides and proteins (Jaroniec et al. 2004; Paravastu et al. 2008; Petkova et al. 2006; Qiang et al. 2012; Van Melckebeke et al. 2010). The remainder of this article reviews contributions from my laboratory, especially our results for fibrils formed by the  $\beta$ -amyloid (A $\beta$ ) peptide that is associated with Alzheimer’s disease (AD). Structural models for two distinct polymorphs of A $\beta$  fibrils (Paravastu et al. 2008; Petkova et al. 2006), discussed in detail below, are shown in Fig. 1. Our work relies heavily on solid state NMR methods, which are specialized NMR techniques that are applicable to noncrystalline, insoluble biomolecular systems such as amyloid fibrils. Much of the

**Fig. 1** Molecular structural models for  $A\beta_{1-40}$  fibrils that were prepared in vitro from synthetic peptide. Cross-sections of the models are shown, with the fibril growth axis perpendicular to the page and with six repeat units. The models have approximate twofold (a) or threefold (b) symmetry about the growth axis, and represent regularized averages of atomic coordinates in Protein Data Bank files 2LMN (a) and 2LMP (b). Residues 9–40 are shown, and sidechains of certain residues are indicated. These models were developed from a combination of solid state NMR and electron microscopy data (Paravastu et al. 2008; Petkova et al. 2006)



recent progress in our understanding of amyloid fibril structures can be attributed directly to information from solid state NMR measurements (Benzinger et al. 1998; Cheng et al. 2011; Comellas et al. 2011; Debelouchina et al. 2010; Heise et al. 2005; Helmus et al. 2008; Jaroniec et al. 2004; Kammerer et al. 2004; Lansbury et al. 1995; Nielsen et al. 2009; Van Melckebeke et al. 2010). The principles behind these measurements have been reviewed elsewhere (Tycko 2006, 2011). The following discussion focuses on the results we have obtained with solid state NMR methods, supplemented by electron microscopy and other physical measurements.

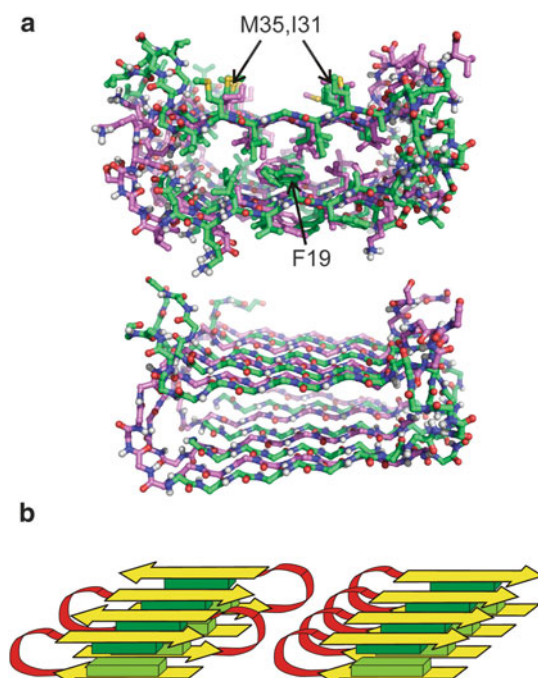
## Organization of $\beta$ -Sheets in Amyloid Fibrils

The naturally occurring  $A\beta$  peptide is primarily 40 or 42 residues in length ( $A\beta_{1-40}$  or  $A\beta_{1-42}$ ), with  $A\beta_{1-40}$  accounting for roughly 80 % of the  $A\beta$  molecules in humans. Early models for fibrils formed by full-length  $A\beta$  assumed that the cross- $\beta$  motif was constructed from antiparallel  $\beta$ -sheets (Chaney et al. 1998; George and Howlett 1999; Lazo and Downing 1998; Li et al. 1999; Tjernberg et al. 1999). Solid state NMR experiments on fibrils formed by a nine-residue peptide, representing residues 34–42

of A $\beta$ <sub>1–42</sub>, provided support for an antiparallel  $\beta$ -sheet structure (Lansbury et al. 1995). However, subsequent solid state NMR experiments on fibrils formed by a 26-residue peptide, representing residues 10–35, provided the first evidence that amyloid fibrils could contain parallel  $\beta$ -sheets, in which neighboring peptide chains are aligned precisely “in-register” (Benzinger et al. 1998). Our own measurements on A $\beta$ <sub>1–40</sub> (Antzutkin et al. 2000; Balbach et al. 2002) and A $\beta$ <sub>1–42</sub> (Antzutkin et al. 2002) fibrils showed that these fibrils contain the same in-register, parallel  $\beta$ -sheet organization. Parallel  $\beta$ -sheets in full-length A $\beta$  fibrils were also found by electron paramagnetic resonance measurements (Torok et al. 2002). Moreover, in-register parallel  $\beta$ -sheets have also been found in amyloid fibrils formed by the islet amyloid polypeptide (Luca et al. 2007), tau (Margittai and Langen 2004),  $\beta_2$ -microglobulin (Debelouchina et al. 2010),  $\alpha$ -synuclein (Der-Sarkissian et al. 2003), and both mammalian and yeast prion proteins (Baxa et al. 2007; Chan et al. 2005; Cobb et al. 2007; Kryndushkin et al. 2011; Shewmaker et al. 2006; Tycko et al. 2010; Wickner et al. 2008). Fibrils formed by the fungal prion protein HET-s have been shown to have a “pseudo-in-register” structure, in which homologous segments align with one another in parallel  $\beta$ -sheets (Van Melckebeke et al. 2010). Thus, the in-register, parallel  $\beta$ -sheet organization predominates in biologically relevant fibrils.

In addition to A $\beta$ <sub>34–42</sub>, several other short A $\beta$  fragments have been shown by solid state NMR to form fibrils containing antiparallel  $\beta$ -sheets (Balbach et al. 2000; Bu et al. 2007; Kammerer et al. 2004; Petkova et al. 2004). These peptides contain only one  $\beta$ -strand segment, whereas full-length A $\beta$  and other full-length amyloid-forming peptides and proteins contain two or more  $\beta$ -strand segments. In addition, both parallel and antiparallel  $\beta$ -sheets have been observed in crystal structures of short amyloid-forming peptides (Sawaya et al. 2007). These observations gave rise to the idea that antiparallel  $\beta$ -sheet structures are limited to fibrils that are formed by short peptides with one  $\beta$ -strand segment and that optimal interactions within and between  $\beta$ -sheets in a cross- $\beta$  motif generally require a parallel  $\beta$ -sheet organization if more than one  $\beta$ -strand segment is involved.

It was therefore surprising to us when we recently found that fibrils formed by the Asp23-to-Asp mutant of A $\beta$ <sub>1–40</sub> (D23N-A $\beta$ <sub>1–40</sub>), which produces familial early-onset neurodegeneration (Grabowski et al. 2001), could contain antiparallel  $\beta$ -sheets (Tycko et al. 2009). A molecular model for antiparallel D23N-A $\beta$ <sub>1–40</sub> fibrils, developed from a substantial set of solid state NMR and electron microscopy measurements (Qiang et al. 2012), is shown in Fig. 2a. Figure 2b shows schematically how parallel and antiparallel  $\beta$ -sheet structures can have similar combinations of favorable hydrophobic interactions, as suggested by our results for D23N-A $\beta$ <sub>1–40</sub> fibrils. On the other hand, experimental data show conclusively that antiparallel D23N-A $\beta$ <sub>1–40</sub> structures are only metastable, gradually converting to parallel structures when the two types of structures are mixed (Qiang et al. 2011, 2012).



**Fig. 2** (a) Molecular structural model for D23N-A $\beta$ <sub>1-40</sub> fibrils that contain antiparallel  $\beta$ -sheets, showing all atoms except non-polar hydrogens (*top*) or only backbone atoms (*bottom*). This model is the first set of atomic coordinates in Protein Data Bank file 2LNQ (Qiang et al. 2012). Residues 15–40 are shown, and the model is viewed in cross-section, with the fibril growth axis nearly perpendicular to the page. Carbon atoms of successive D23N-A $\beta$ <sub>1-40</sub> molecules are alternately colored *green* or *magenta* to emphasize the antiparallel organization. (b) Schematic representation of the antiparallel (*left*) and parallel (*right*) cross- $\beta$  structures in D23N-A $\beta$ <sub>1-40</sub> and wild-type A $\beta$ <sub>1-40</sub> fibrils, showing how both structures produce favorable contacts among sidechains of hydrophobic segments (*green bars*)

## Polymorphism of Amyloid Fibrils

One of the central principles of biology and biochemistry is that protein structures are fully and uniquely determined by amino acid sequences. This principle does not apply to amyloid fibrils. We have shown that subtle variations in growth conditions lead to significant, reproducible, and self-propagating variations in the molecular structures of A $\beta$ <sub>1-40</sub> fibrils (Petkova et al. 2005; Qiang et al. 2011). Structural variations at the molecular level lead to obvious variations in solid state NMR spectra, in addition to variations in the appearance of the fibrils in electron microscope images (i.e., variations in fibril morphology). The same ideas apply to fibrils formed by most other amyloid-forming peptides and proteins, including mammalian and yeast prion proteins, where self-propagating structural variations are almost certainly the source of distinct prion strains or variants (Bessen and Marsh 1992; Telling et al. 1996; Toyama et al. 2007).

Given that amyloid fibrils are highly polymorphic, one must be very careful when comparing structural measurements performed by different research groups on amyloid fibrils formed by the same peptide or protein. Unless the fibrils are known to have been prepared identically and to exhibit precisely the same morphologies (and ideally to exhibit the same solid state NMR spectra), there is no reason to expect measurements by different research groups to be in quantitative, or even qualitative, agreement.

Some of the sources of amyloid polymorphism are revealed by the structural models in Figs. 1 and 2. In these models, the  $\beta$ -sheet-forming segments are nearly the same and the overall peptide conformations are similar. The largest differences are in (1) overall symmetry, with the models for wild-type  $A\beta_{1-40}$  fibrils having either twofold or threefold rotational symmetry about the fibril growth direction and the model for D23N- $A\beta_{1-40}$  fibrils having no rotational symmetry and (2) the nature of the  $\beta$ -sheets, as discussed above. The details of the conformations of the non- $\beta$ -strand segments, the details of contacts between  $\beta$ -sheets, and the fraction of the peptide sequence that becomes structurally ordered in the fibrils (Bertini et al. 2011) also vary. We find that  $\beta$ -sheets formed by the N-terminal and C-terminal  $\beta$ -strands of  $A\beta_{1-40}$  are “staggered,” meaning that the two  $\beta$ -strands from a given peptide molecule do not make contact with one another (Paravastu et al. 2008; Petkova et al. 2006). The direction of stagger is difficult to determine from experimental measurements and could also be a source of polymorphism.

It is worth noting that fibrils formed by HET-s are not polymorphic, except under extreme pH conditions (Mizuno et al. 2011; Wasmer et al. 2008), that distinct HET-s prion strains have not been observed, that HET-s fibrils are proposed to have an evolved biological function (Coustou et al. 1997), and that solid state NMR spectra of HET-s fibrils are exceptionally well-resolved and reproducible (Tycko and Hu 2010; Van Melckebeke et al. 2010). As mentioned above, the molecular structure of HET-s fibrils is also unusual (Van Melckebeke et al. 2010), suggesting that HET-s fibrils are a rather unique system.

Measurements in cultures of primary embryonic rat hippocampal neurons indicated statistically significant, although not very large, differences in toxicity for  $A\beta_{1-40}$  fibrils with different morphologies (Petkova et al. 2005). In these experiments, the fibrils were prepared in vitro from synthetic  $A\beta_{1-40}$ . These results do not necessarily imply differences in neurotoxicity in the human brain, but such differences certainly seem possible. In the case of transmissible spongiform encephalopathies caused by mammalian PrP, there is strong evidence that distinct molecular structures of PrP aggregates produce distinct strains, characterized by distinct patterns of PrP deposition and incubation periods between infection and neurodegeneration (Bessen and Marsh 1992; Telling et al. 1996). Analogous phenomena may exist in AD. Conflicting observations regarding correlations between cognitive impairment in AD and  $A\beta$  amyloid deposition (Aizenstein et al. 2008; Cummings et al. 1996) can be understood if the neurotoxicity of the amyloid deposits varies with the molecular structure of the  $A\beta$  fibrils. Studies on transgenic animals have shown that the histopathology of amyloid deposition induced by inoculation with amyloid-containing tissue extracts depends on the source of the inoculums (Meyer-Luehmann

et al. 2006). These studies also show that amyloid deposits within brain tissue cannot be induced by synthetic A $\beta$  material, suggesting the possibility of structural differences between purely synthetic fibrils and the fibrils that develop within brain tissue. Thus, the phenomenon of amyloid polymorphism may have consequences for AD and other amyloid diseases.

## Structures of Nonfibrillar Aggregates

In electron microscopy or atomic force microscopy studies of amyloid formation in vitro, one commonly observes nonfibrillar aggregated structures in addition to the bona fide, mature fibrils (Goldsbury et al. 2005). These structures, described by various terms that include “oligomer” and “protofibril,” generally appear early in the amyloid formation process and eventually disappear as the amyloid-forming peptide or protein converts fully to the fibrillar state. Such nonfibrillar structures have attracted great interest as toxic agents in AD and other amyloid diseases. Due to the transient nature of oligomers and protofibrils, as well as the difficulty of preparing structurally homogeneous samples, the molecular structures within nonfibrillar aggregates are less well known than the structures within certain amyloid fibrils. However, rather surprisingly in light of the large morphological differences between nonfibrillar and fibrillar aggregates, structural information that has been obtained to date suggests that the molecular structures within oligomers and protofibrils closely resemble the structures within mature fibrils. For wild-type A $\beta$ , studies by electron microscopy and by solid state NMR indicate that elements of both the molecular conformation and the  $\beta$ -sheet organization found in mature fibrils are retained in oligomers and protofibrils (Ahmed et al. 2010; Chimon et al. 2007; Goldsbury et al. 2005; Kheterpal et al. 2006). Results for the D23N-A $\beta_{1-40}$  fibrils described above constitute a case in which the molecular conformation is largely retained but the  $\beta$ -sheet organization is quite different in a metastable fibrillar state that resembles protofibrils (Qiang et al. 2012).

## Structural Studies of A $\beta$ Fibrils from Human Brain Tissue

To clarify the connections between amyloid structures and amyloid diseases, we obviously need to identify and characterize structures that actually develop in human tissue. Solid state NMR methods that have been used successfully to characterize the molecular structures of amyloid fibrils require milligrams of isotopically ( $^{13}\text{C}$  and  $^{15}\text{N}$ ) labeled fibrils. Thus, direct structural measurements on amyloid extracted from tissue are not possible. However, we have shown that amyloid fibrils extracted from brain tissue of AD patients, obtained at autopsy, can be used as a “seed”







to grow fibrils from synthetic or recombinant  $A\beta_{1-40}$ , thereby allowing both amplification from the microgram to the milligram scale and isotopic labeling (Paravastu et al. 2009). Studies of seeded fibril growth in vitro have shown that, when fibrils are grown from seeds (i.e., short fibril fragments, generally produced by sonication of longer fibrils), the resulting fibrils retain the molecular structure of the seeds (Petkova et al. 2005), although the relative abundances of different polymorphs within a heterogeneous mixture can evolve as seeded growth is performed through multiple generations (Paravastu et al. 2008; Qiang et al. 2011). Initial experiments with brain tissue used a rigorous extraction procedure to isolate nearly pure  $A\beta$  fibrils from the tissue (Paravastu et al. 2009). Three generations of seeded growth were used to create 5- to 10-mg samples, starting with 10  $\mu$ g of amyloid extract. Solid state NMR spectra indicated a mixture of structures but with the same predominant structure being derived from brain tissue from two different AD patients. Moreover, the NMR chemical shifts in spectra of brain-derived fibrils were different from the chemical shifts in spectra of the synthetic fibrils for which structural models are shown in Fig. 1.

More recently, we have developed a simplified extraction and seeding protocol that requires only 1–2 g of brain tissue, is unlikely to alter fibril structures or structural distributions in the brain tissue, and produces sufficient quantities of isotopically labeled fibrils in a single generation of seeded growth. Figure 3a, b shows electron microscopy data for  $A\beta_{1-40}$  fibrils that were grown from brain tissue of an 72-year-old female AD patient. Figure 3c, d shows solid state  $^{13}\text{C}$  NMR spectra of these fibrils. A single set of NMR signals appears in these spectra, indicating the predominance of a single fibril structure. Moreover, tissue samples from two brain regions of the same patient (occipital lobe and temporal/parietal lobe) produce the same set of strong NMR signals, indicating the same predominant fibril structure in both regions. These data, plus many additional solid state NMR measurements on fibrils derived from the same tissue samples, imply a molecular structure that is again different from structural models in Fig. 1. Development of a full molecular structural model for the fibrils that developed in the brain of this particular AD patient is currently in progress.

We are now applying the same approach to brain tissue samples from AD patients with diverse clinical histories. The results may reveal whether variations in  $A\beta$  fibril structure correlate with variations in AD progression rate, severity, or other characteristics. A similar approach can be applied to other amyloid diseases. If we find that the development of amyloid diseases depends on the details of amyloid fibril structures, it then becomes important to use structural information from solid state NMR, electron microscopy, and other sources to guide the development of diagnostic imaging agents and fibril inhibitors that have precise structural specificity. Thus, we

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**Fig. 3** (continued) residues F19 (dark blue), V24 (light blue), A30 (purple), I31 (yellow), L34 (green), and M35 (red). The same main signals are observed in both spectra. (d) 2D  $^{15}\text{N}$ – $^{13}\text{C}$  solid state NMR spectrum of fibrils derived from occipital lobe tissue, with assignments of the NMR signals to specific residues. Data were acquired by Dr. Junxia Lu

have reached the stage where advanced physical measurement techniques, and the understanding of amyloid structure that has resulted from these techniques, can be applied directly to problems with important consequences for human health.

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