

The Structure of Amyloid

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ABSTRACT

The local or systemic deposition of insoluble amyloid fibrils is characteristic of the pathogenesis of the heterogeneous group of diseases known as the amyloidoses. Normally soluble, innocuous proteins undergo a change in conformation and self assemble into insoluble, potentially toxic, amyloid fibrils. Electron microscopy shows amyloid fibrils to be straight, unbranching structures, 70 to 120 Å in diameter and of indeterminate length. The potential for amyloidogenesis may be a near universal property of protein. Knowledge of the structure of these fibrils is a crucial element in the development of an understanding of their stability and assembly. With this information, the rational design of drugs to prevent amyloidogenesis and promote disassembly might be enabled. Furthermore, it may grant some insight into the generality of protein folding. Single crystal X-ray crystallography and solution NMR are not possible due to the fibrillar inability to crystallise and to intrinsic insolubility. X-ray and recently electron fibre diffraction have proved to be of great value in the elucidation of the structure of amyloid. This review discusses the advances made and how fibre diffraction is used in conjunction with other structural techniques.

INTRODUCTION

Alzheimer's disease is the leading cause of dementia; it affects nearly half the population over eighty-five and costs the US economy some 100 billion dollars per annum (Alzheimer's Association, 2002). Abnormal amyloid-like aggregates are involved in the pathogenesis of this and an increasing list of other diseases, including the transmissible spongiform encephalopathies (e.g. Mad Cow disease) and type 2 diabetes (Rochet & Lansbury, 2000; Kelly, 1996). In these diseases, known collectively as the Amyloidoses, insoluble amyloid fibrils are self-assembled from normally soluble protein and deposited extracellularly. There is no apparent commonality between precursor proteins in amino acid sequence, molecular weight or morphology. Furthermore, many proteins not implicated in conformational disease have also been shown to form amyloid *in vitro*, leading to the hypothesis that the potential for amyloidogenesis may be a near universal feature of protein (Dobson, 2001).

Amyloidosis cases have been described for well over 300 years, whilst Rudolph Virchow first classified Amyloid in 1854 (Cohen, 1986; Sipe & Cohen, 2000). Yet it is only within the past 20 years that the specific makeup and structure of amyloid has begun to be understood. Amyloid is now characterised by specific staining, the X-ray diffraction pattern and its appearance under the electron microscope. An apple green birefringence pattern is observed after staining with Congo red dye and viewing between crossed polarisers under a light microscope (Khurana *et al.*, 2001). The X-ray diffraction pattern is described as cross- β , consisting of two major reflections, a sharp meridional at 4.7Å and a more diffuse equatorial at between 10 and 11 Å (Figure 1b). Electron micrographs reveal fibres with a similar ultrastructural morphology - being straight, unbranching, 70 to 120 Å in diameter and of indeterminate length (see Figure 3a).

Knowledge of the structure of the mature amyloid fibril is a necessary step in the rational development of drugs to prevent the formation and promote the disassembly of the fibrils. To this end, a great variety of structural techniques have been employed. Unfortunately, structural exegesis by means of X-ray crystallography is not possible since amyloid does not generally crystallise. Similarly, solution nuclear magnetic resonance (NMR) is thwarted due to the insolubility of amyloid. Successful techniques include electron microscopy, light scattering, solid state NMR, Fourier transform infrared spectroscopy (FTIR), circular dichroism (CD), selective mutation experiments, atomic force microscopy (AFM), small angle neutron scattering (SANS) and X-ray fibre diffraction. The utility of fibre diffraction might be established by means of comparison with the other techniques.

Following fibrillogenesis

The process of self-assembly is examined in kinetic studies using CD, AFM and light scattering. CD provides insight into the changes in the secondary structure and the environment and orientation of aromatic side chains, which accompany amyloidogenesis (Ganesh & Jayakumar, 2003). High-resolution data gathered using AFM has illuminated the mechanism and kinetics of the assembly (Goldsbury *et al.*, 1999), whilst light scattering allows one to establish the accompanying changes in particle size (Murphy, 1997). These studies have demonstrated that amyloidogenesis is a process of nucleation and growth, with nucleation being the rate determining step. In FTIR, certain vibrational bands have been assigned to particular conformations of β -sheets by empirical studies of model peptides and normal mode computations (Janek *et al.*, 1999). Fibre diffraction is used to study the mature amyloid fibril. It is therefore complementary to electron microscopy, FTIR and solid state NMR.

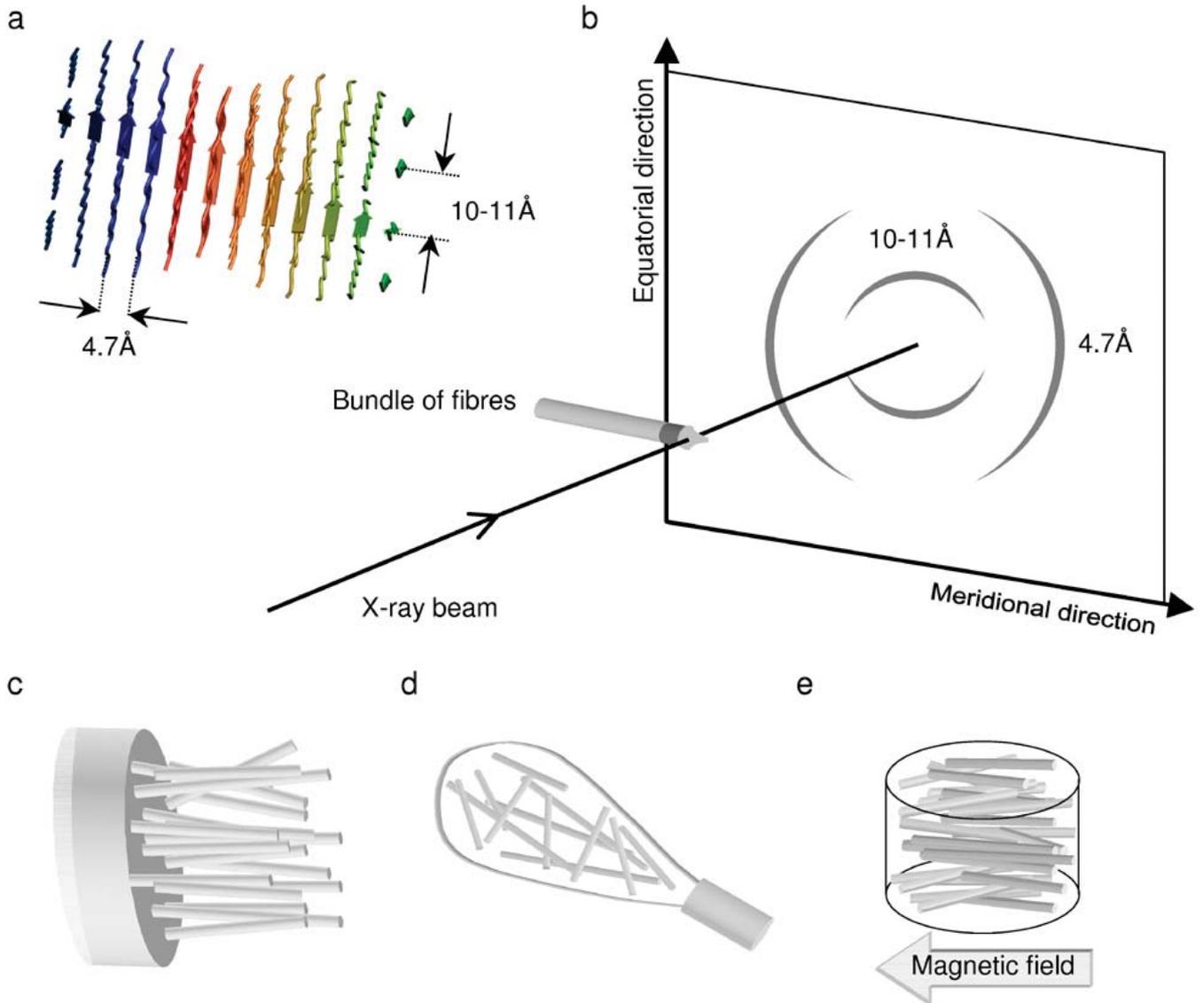


Figure 1. Upper panel: illustration of amyloid fibre diffraction showing the position and orientation of the β -strands and the resulting diffractogram. Lower panel: three different possible fibre orientations are shown, from left to right, stretch frame, mat and magnetically aligned.

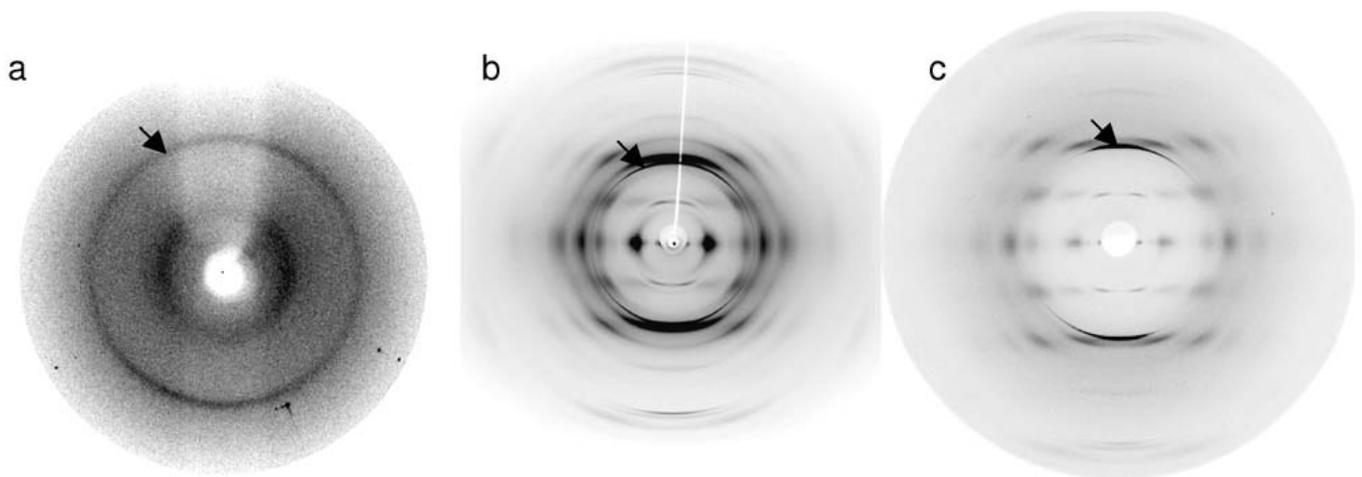


Figure 2. Comparison of diffraction patterns from amyloid fibrils formed from unaligned $A\beta(1-42)$ on the left, with stretch-frame aligned fibrils of $A\beta(11-25)$ in the centre and magnetically aligned $A\beta(11-25)$ in the right-hand panel (Sikorski *et al.*, 2003).

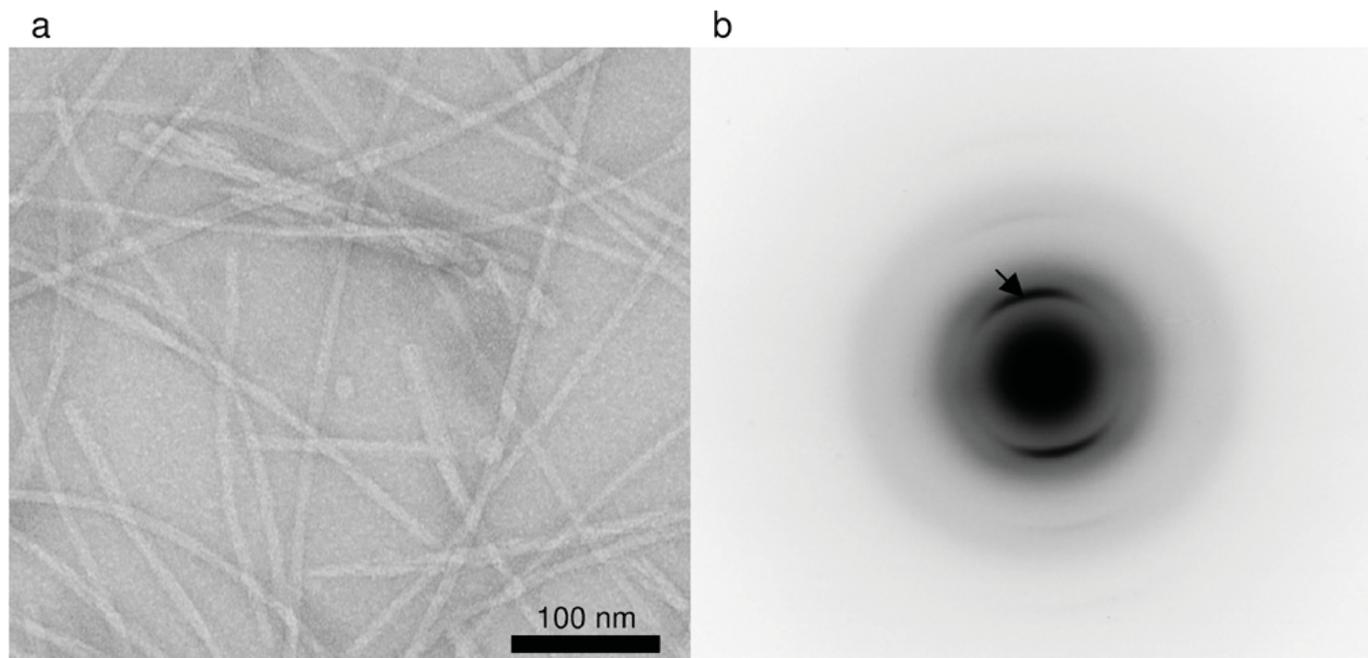


Figure 3. Electron microscopy and electron diffraction from amyloid fibrils formed from full-length islet amyloid polypeptide (IAPP), showing the long straight amyloid fibrils and the cross- β position of the 4.7Å meridional reflection from electron diffraction (Makin and Serpell, in press).

SANS and SAX

Small angle neutron scattering (Yong *et al.*, 2002; Yonezawa *et al.*, 2002; Lu *et al.*, 2003) has been used for the study of fibril assembly and the mature fibril. Its use is somewhat constrained by the requirement for a neutron source and much of the information can be gathered by alternative means. SANS of A β (10-35), used in conjunction with NMR and negative stain EM (Burkoth *et al.*, 2000), is an example of using various techniques to build up sufficient to construct a plausible model. Molecular dynamics simulation could then be employed to extend the structural assignment (Lakdawala *et al.* 2002). In the same manner, small angle X-ray equatorial diffraction has had the potential to determine the number and arrangement of protofilaments. Simple theoretical models were compared with equatorial traces from fibre diffraction patterns. On the meridian, small angle reflections indicate the long-range repeating unit along the hydrogen bonding direction, which may be consistent with helical twisting (Fraser *et al.*, 1991; 1992a; 1992b). Amyloidogenesis can be followed using time-resolved small angle X-ray diffraction to monitor the dimensions of the sample's constituents (Botelho *et al.*, 2003; Yonezawa *et al.*, 2002).

Solid state NMR and amyloid

Solid state NMR (ssNMR) is used to determine the distance between ^{13}C labels less than 6Å apart (Tycko, 2001). These measurements are then found to be consistent with either a parallel or antiparallel arrangement, since the peptide structure may be modelled given distance measurements between labelled residues. This is less information than might be expected from a well-aligned X-ray sample. Results may be misleading if the β -strands are simply out of register rather than antiparallel or a mixture of parallel and antiparallel. Distances of over 6 Å are unable to be measured. Consequently, there is a

range of reported results. Some have not been consistent with those from FTIR (Antzutkin *et al.*, 2000), explained by noting that the FTIR experiments used models significantly less complex than β -amyloid peptides. Balbach *et al.* (2000) analysed A β (16-22) using multiple quantum NMR and rotational echo double resonance, whilst Lansbury *et al.* (1995) examined A β (34-42) using rotational resonance, in both cases results were consistent with an antiparallel model. In contrast, A β (1-42) and A β (10-35) have been shown to have a parallel arrangement using multiple quantum NMR and dipolar recoupling in windowless sequence measurements (Antzutkin *et al.*, 2000; Benzinger *et al.* 2000), though it is unclear how the forty residue chain is folded such that the fibre diameter matches that observed in electron micrographs, since an extended strand of 40 residues would span ~140 Å (Sikorski *et al.*, 2003). Unfortunately, the fibre diffractogram (Figure 2a) (Sikorski *et al.*, 2003) is unable to provide further insight, but it is suggested that the data are consistent with intersheet β -hairpins.

The macromolecular structure of amyloid from electron microscopy and atomic force microscopy

AFM is clearly useful due to the measurements being taken in real time in near physiological conditions (Goldsbury *et al.*, 1999; Wang *et al.*, 2003; Stolz *et al.*, 2000). Resolutions down to 10 Å have been achieved, with the mechanical damage due to dragging a tip across the sample being reduced by the use of tapping modes. Unfortunately, this is insufficient to reveal the arrangement of β -strands even though a helical twist might be evident.

Negative stain, platinum shadowing and cryo electron microscopy (EM) have been employed to visualise the fibrils. Fibrils assembled from the same peptide may have different

morphologies (Goldsbury *et al.*, 1997), which may or may not be dependent on the conditions. It has been possible to directly visualise the β -strands making up the fibrils formed from A β (11-25) (Serpell & Smith, 2000). Thin cross-sections of fibrils have been analysed using single particle methods to determine the number and arrangement of protofilaments (Serpell *et al.* 2000), these results can be compared to those extracted from small angle X-ray and neutron scattering. Scanning tunnelling microscopy (Wang *et al.*, 2003; Shivji *et al.*, 1995; Baxa *et al.*, 2003) has had some use determining the mass per unit length of fibres to confirm or eliminate possible models. Finally, reconstruction using single particle methods of images of SH3 amyloid (Jiménez *et al.*, 1999) has allowed a three-dimensional model to be built within the electron density. Hence EM has provided information commensurate with the models developed from fibre diffraction. Whilst these techniques may be described as high resolution, fibre diffraction is more so and helps untangle the disagreements between methods based on the modelling of idealised peptides.

The cross- β diffraction pattern

X-ray diffraction from amyloid reveals the characteristic cross- β pattern, comprised of two spots (Figure 1b). A sharp reflection on the meridian is observed at a resolution of 4.7 Å. This corresponds to the distance between the β -strands that comprise a β -sheet. The other, more diffuse spot is found on the equator between 10 and 11 Å, which can be explained as the distance between stacked β -sheets (Figure 1a,b). There is variation in the distance between β -sheets according to the size of the side chains. Geddes *et al.* described the cross- β pattern in egg stalk of the green lacewing fly *Chrysopa flava* (Geddes *et al.* 1968) and the same pattern was noted for amyloid fibrils formed from Bence Jones protein (Eanes & Glenner, 1968). In many amyloid diffraction patterns a layer line at 9.4 Å is observed (Sikorski *et al.*, 2003) (see Figure 2b and c). The presence of a 9.4 Å (=2 x 4.7 Å) spacing suggests that the strands must be in an antiparallel arrangement. Unfortunately, the absence of a 9.4 Å spot (*eg.* Figure 2a) does not necessarily imply that the strands are parallel to one another since a systematic absence may suppress the reflection.

It is thought that individual peptides fold to form β -strands, which are orthogonal to the fibre. Long protofilaments are formed from β -sheets stacked face-to-face (Pauling & Corey, 1951), running parallel to the fibre axis. Diffraction from *ex vivo* transthyretin fibrils implies a model in which several β -sheets turn about a central axis (Blake & Serpell, 1996) (Figure 1). Finally the fibre itself is comprised of protofilaments, which may be twisted about one another in a helical manner.

Synthetic peptides are generally used to form fibrils owing to their greater availability and purity, compared to those extracted from tissue. Truncated peptides have also been used to grow fibrils which are straighter and more ordered, whilst maintaining morphological and chemical similarity to fibrils grown from the full length peptide (Jarvis *et al.*, 1993; Nguyen *et al.*, 1995; Serpell, 2000).

Methods of alignment for preparing amyloid fibre diffraction specimens

Fibril alignment is important since a higher degree of orientation will result in more information being extractable from the resulting diffractogram (Figure 2a versus 2b and c). The main methods of alignment are a stretch frame, glass capillary magnetic alignment and diffraction from a film or mat (Figure 1c,d,e). The nature of the sample often determines the preferred method. If the sample contains many long fibrils, then the high viscosity will make it far more amenable to stretch frame alignment (Sunde *et al.*, 1997). In the case of small crystallites, magnetic alignment may be favoured (Inouye *et al.*, 1993; Malinchik *et al.*, 1998; Sikorski *et al.*, 2003). Here the sample is dried between the poles of a permanent 2 Tesla magnet. This large magnetic flux density orients the fibres so that they are parallel to the field lines. Alignment is due to anisotropy in diamagnetic susceptibility due to bond resonance in certain side groups (Glucksman *et al.*, 1986; Worcester, 1978; Pauling, 1979), which was first exploited by Kirschner (Inouye *et al.*, 1993) to examine fibrils formed from fragments of the Alzheimer's A β peptide and has since been used for other peptides (Inouye and Kirschner, 1998, Sikorski *et al.*, 2003). A mat of fibrils can be used as to provide an additional texture for extra information. The substrate can be glass, Parafilm or Teflon (Fandrich & Dobson, 2002; Perutz *et al.*, 1994). This method has been used for AA amyloid fibrils (Turnell *et al.*, 1986), and poly-Q (Perutz *et al.*, 1994; Perutz *et al.*, 2002). Alternatively, a cryo-loop normally used for freezing single crystals can be dipped into the fibrillar solution and allowed to dry, resulting in a thin, flat film (Serpell and Makin, unpublished data). This avoids the requirement for the film to be carefully lifted off the substrate, which may be difficult if the fibres are short and therefore non-viscous.

The contribution of electron diffraction to amyloid structure

Until recently, electron diffraction (ED) has been of limited benefit often only confirming the cross- β structure, with only the 4.7 Å reflection and little else being visible (Berriman *et al.*, 2003; Perutz *et al.*, 1994) (Figure 3b). High-resolution information, which is already faint due to the intensity being spread about reciprocal space, is further reduced by the k -squared term in the Mott formula for calculating electron structure factors. Furthermore, the beam saturates the film concealing low-resolution spots. In the case of nanocrystals (Diaz-Avalos *et al.*, 2003a; 2003b), electron diffraction has proved invaluable. Single crystals can be selected for examination owing to the electron beam having a far smaller diameter than is the case with X-rays. The diffraction spots can also be widened to produce an image of the sample area being diffracted; thus allowing direct confirmation of the relationship between spot location and fibre orientation. Radiation damage is a problem; one may observe the spots fading as the beam and sample interact. There is therefore a trade-off between long exposures, which ensure weak spots are observable and a higher signal to noise ratio is achieved and short exposures, which fulfil the necessity to keep the specimen intact. Unfortunately, the measurement of structure factors in electron diffractograms is unreliable, owing to electrons interacting with

matter in a far stronger manner than X-rays, leading to multiple scattering, which is difficult to account for. The micrograph film would also need to be well calibrated to ensure the expected relationship between exposure and image intensity. These lead to electron diffraction being a potentially useful technique whilst one is aware of the potential issues. ED has proved to be useful in the study of the structure of fibrils of α -synuclein (Serpell *et al.*, 2000a), nanocrystals (Makin *et al.*, unpublished), IAPP (Makin & Serpell, 2004) and other amyloid (Ferguson *et al.*, 2003). One expects the development of ED in relation to amyloid to be useful in future.

X-ray fibre diffraction and amyloid models

X-ray fibre diffraction has demonstrated that amyloid fibrils have a common core structure, which is a series of continuous beta sheets running parallel to the fibre axis (Sunde *et al.*, 1997; Blake & Serpell, 1996). In the case of transthyretin amyloid, indexing of meridional reflections revealed a helical repeat distance of 115.5Å, commensurate with twisted β -sheets known to be energetically favourable (Chothia, 1973). From this work, a model was presented for the core structure of the generic amyloid fibril (Blake & Serpell, 1996, Sunde *et al.*, 1997). X-ray diffraction patterns from amyloid fibrils formed from A β (1-40) were interpreted as arising from three to five tubular protofilaments, each composed of pairs of β -sheets (Malinchik *et al.*, 1998). A similar structure was concluded from amyloid fibrils formed from a range of shorter fragments of A β (Inouye *et al.*, 1993). Further detailed discussion of these models is included in Serpell, 2000.

More recently, Sikorski *et al.* (2003) presented a detailed model of A β (11-25) from data taken using a magnetically aligned sample. Three different diffractions patterns were obtained from orthogonal directions around the crystalline specimen suggesting a preferred orientation of the fibrils. In the fibrillar structure, A β (11-25) forms extended β -strands that stack into β -ribbons that then slip relative to each another by the length of two amino-acid units (0.70 nm). X-ray diffraction patterns simulated using Cerius2 (Accelrys, San Diego, CA, USA) were compared with empirical data and shown to be very similar (additional details may be found in Sikorski *et al.*, 2003). Very recently, the structure of A β (11-25) amyloid fibrils has been examined using ssNMR (Petkova *et al.*, 2004). Interestingly, it was shown that A β (11-25) was able to form two slightly different packing arrangements at different pH. This seems to suggest that A β (11-25) peptides have the ability to pack differently depending on environment and presumably are able to slide across one another. The fibre diffraction sample examined by us (Sikorski *et al.*, 2003) is a crystalline sample, so it may be that the environment favoured the particular packing arrangement described. The structure of A β (31-35) has also been obtained (Bond *et al.*, 2003), though in this case powder diffraction provided much of the required information.

There has been discussion about the amount of native structure remaining - how much of the original protein unfolds before forming amyloid. Inouye *et al.* (1998) suggest a transthyretin amyloid model in which the monomer structure is retained with the β -sheets simply lining up in an appropriate manner, thus contradicting the unfolding suggested in Blake & Serpell's earlier model. However, in order to satisfy the cross- β pattern, it is essential that the β -strands are perpendicular to the fibre

axis. In the case of the yeast prion, Ure2p it is disputed as to whether the fibres are amyloid at all (Baxa *et al.*, 2003; Bousset *et al.*, 2003), since it appears that the native structure of Ure2p (predominantly α -helical) may be retained within the fibres. The fibres have the characteristic birefringence pattern and appearance under the electron microscope. Other alternative structures for amyloid have been proposed. Lazo and Downing (1998) suggest an α -helical model in which protofilaments are formed from β -strands forming a tetrahedral shape. Perutz *et al.* (2002) have studied fibres formed from polyglutamine and concluded that amyloid fibrils are water filled nanotubes. These models are schematically similar in having β -strands wrapped around a core in antiparallel and parallel configurations respectively. Nevertheless, further evidence will be required if the received understanding, of the structure consisting of stacked β -sheets, is to be displaced, since the β -helical models do not appear to satisfy the cross- β diffraction pattern (Sikorski, unpublished).

CONCLUSION

X-ray fibre diffraction has always been an important part of the structural analysis of amyloid. Indeed, the cross- β pattern is an intrinsic characteristic of amyloid. It has provided information unavailable by other means, on both large and small length scales. Fibre diffraction using X-rays, neutrons and electrons is now an indispensable component of the armoury of structural techniques. The papers reviewed herein have demonstrated that increased efficacy is achieved by means of a cocktail of such techniques, particularly with regard to electron microscopy. Knowledge of amyloid structure and assembly are important in our understanding of the amyloidoses. Additionally, the implications of amyloid self-assembly to the production of fibres in nano-biotechnology have yet to be understood (Scheibel *et al.*, 2003). These ensure that the application of fibre diffraction to the elucidation of amyloid structure is likely to increase in importance.

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