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In Pursuit of the Molecular Structure of Amyloid Plaque: New Technology Provides Unexpected and Critical Information†

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The deposition of a fibrous protein aggregate, or amyloid plaque, is characteristic of many diseases (Cohen & Skinner, 1990; Glenner, 1980a,b), including several forms of systemic amyloidosis (Castano & Frangione, 1988) and localized conditions such as Alzheimer's disease (AD)[1] (Katzman & Saitoh, 1991; Selkoe, 1991; Yanker & Mesulam, 1991) and type II diabetes (Nishi et al., 1990). A causal link between plaque formation and AD has not been firmly established. However, mounting evidence suggests that amyloid deposition may be a critical step in the neurodegenerative process (Golde et al., 1992; Selkoe, 1991; Yanker & Mesulam, 1991). Interest in the detailed molecular structure of the AD amyloid plaque and its constituent amyloid fibril has increased with its identification as a potential therapeutic target (Selkoe, 1991; Yanker & Mesulam, 1991).

Since amyloid is, by definition, insoluble and noncrystalline, the popular methods for the determination of protein structure (i.e., multidimensional ^{1}H NMR[1] and X-ray crystallography) cannot be used to elucidate the molecular details of the plaque structure. Amyloid plaque is characterized by extreme insolubility under physiological conditions and is commonly defined by three physical properties: birefringent staining, fibrous morphology, and a distinct X-ray fiber diffraction pattern (Glenner, 1980). None of these properties provide information which can be directly translated into a high-resolution structural model of the plaque. X-ray fiber diffraction analysis of amyloids that contain repeating primary structure, such as polyalanine and silk has been used to generate low-resolution models of the amyloid fibril (Fraser & MacRae, 1973). The first such model was Pauling's cross-β fibril[2] (see Figure 1) (Marsh et al., 1955), which has been widely accepted as the general amyloid fibril structure. An equally plausible model, also based on diffraction data, has been proposed (Arnott et al., 1967). The cross-β fibril,[2] like the protein sequences which inspired it, is extremely regular and may represent only one of a spectrum of possible structures. Since amyloid protein sequences are quite diverse, it follows that the structures of naturally-occurring amyloid plaques will also vary. Structural studies of two amyloidogenic peptides conducted in our laboratory have demonstrated this point (vide infra). These studies were made possible by new technology which allows elucidation of the plaque at the molecular level. We have developed a new application of Fourier transform infrared spectroscopy (FTIR)[1] (Ashburn et al., 1992; Halverson et al., 1991) and have utilized a solid-state NMR (ssNMR)[1] technique developed by Robert Griffin to analyze amyloid structure (Spencer et al., 1991). Two peptides which share all the traditional characteristics of amyloid have been shown to differ at the level of secondary structure. One of these peptides contains unusual structural features which are inconsistent with the regular models which have been proposed for the amyloid fibril. The unexpected results of our studies, which will be summarized herein, precipitated a reexamination of the literature concerning amyloid structure, specifically, the experimental basis of the cross-β fibril model.

The purpose of this review is to summarize what is known about the physical properties of amyloid plaque and to emphasize that *the shared properties of amyloid proteins do not require that all amyloid proteins are identical, or even similar, at the level of secondary structure.* The applications

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[1] Abbreviations: AD, Alzheimer's disease; CR, Congo red; EM, electron microscopy; FTIR, Fourier transform infrared spectroscopy; IAPP, islet amyloid polypeptide; NMR, nuclear magnetic resonance spectroscopy; ssNMR, solid-state NMR.

[2] In this review, the term cross-β fibril will be used to refer to Pauling's structural model (Marsh et al., 1955) as opposed to an experimentally verified protein structure.

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of FTIR to the study of amyloid proteins will be reviewed, and the limitations of this technique will be enumerated. Finally, two new spectroscopic methods, which make it possible to elucidate the molecular structure of amyloid plaque, will be discussed. Our initial results suggest that unusual secondary structure may characterize certain amyloid proteins.

THE RELATIONSHIP BETWEEN THE PHYSICAL PROPERTIES AND THE MOLECULAR STRUCTURE OF AMYLOID IS NOT UNDERSTOOD

Insolubility May Depend on β-Sheet Structure. The physical basis for protein insolubility is unclear. Empirically-derived hydropathy scales (Kyte & Doolittle, 1982) are used to estimate the hydrophobicity of proteins; however, this value does not correlate well with solubility. No primary sequence homology has been shown to characterize all amyloid proteins, although subtle similarities may exist between certain sequences. We proposed that sequences rich in amino acids which are rarely found in helical or "random-coil" secondary structure and often found in "extended" or β-sheet structure (Chou & Fasman, 1974) such as valine and isoleucine (but not leucine) may be particularly insoluble (Halverson et al., 1990). In addition, the periodic occurrence of glycine residues may serve to allow access to unusual and potentially amyloidogenic structures (Richardson & Richardson, 1989; Spencer et al., 1991). Many amyloidogenic proteins have a large amount of β-sheet structure in the soluble form. However, the preexistence of β-sheet structure is not a requirement for amyloid formation, since precipitation of peptides which have little or no β-sheet structure in the soluble state often affords aggregates which seem to contain primarily antiparallel β-sheet structure [e.g., Halverson et al. (1990)]. Studies of amyloid proteins in soluble form, while providing critical information concerning the conditions (pH, [salt], etc.) which favor aggregation, do not neccessarily provide information relevant to the structure of amyloid plaque (Barrow & Zagorski, 1991; Hilbich et al., 1991). Analysis of a protein that was precipitated by "salting-out" also demonstrated a correlation between increased β-sheet structure and decreased solubility (Przybycien & Bailey, 1989). Finally, studies of in vivo protein folding have suggested that aggregation, precipitation, and the formation of β-sheet structure are coupled (Haase-Pettingell & King, 1988).

The classification of an insoluble protein aggregate as amyloid depends on three criteria (Castano & Frangione, 1988; Crowther, 1991): (1) birefringent staining with Congo red (CR),[1] (2) an unbranched, fibrillar morphology as determined by electron microscopy (EM),[1] and (3) the observation of an X-ray fiber diffraction pattern which is similar to those of *Bombyx mori* silk and polyalanine (Fraser & MacRae, 1973). The structural basis for each of these three properties will be discussed below.

Birefringent Staining Suggests an Ordered Dye–Amyloid Complex. The staining properties of amyloid proteins are responsible for their misnomer, as they were originally thought to be composed of carbohydrate. Birefringent staining with CR is observed using a light microscope with a polarizing light stage (Cooper, 1974). The stained amyloid appears red when observed under nonpolarized light and green when the incident light is polarized. In addition, a circular dichroism signal is induced upon binding of CR to amyloid fibrils (Benditt et al., 1970; Taylor et al., 1974). These effects suggest that the bound CR molecules are ordered with respect to each other (Cooper, 1974); however, the structural basis for the CR–amyloid fibril interaction remains a mystery due to the technological problems associated with analyzing insoluble, noncrystalline amyloid proteins. Two models of CR binding, both based on the cross-β fibril,[2] have been proposed (Cooper, 1974; Klunk et al., 1989a,b). We believe that amyloid proteins share a feature, not necessarily at the level of secondary structure, which is recognized by CR. It should be emphasized that birefringent staining with CR is not *specific* to cross-β fibrillar structure.[2] The failure to observe birefringent staining of fibrillar peptides which would otherwise be classified as amyloid has been noted (Caputo et al., 1992). In addition, our recent work has demonstrated that the exhibition of all the physical properties of amyloid, including birefringent staining, does not prove cross-β structure (Halverson et al., 1990; Spencer et al., 1991).

Fibrillar Morphology Does Not Require β-Sheet Structure. The formation of rigid unbranched fibrils with width and depth of approximately 100 Å and length of 0.01–3 μm is typical of amyloidogenic peptides and proteins; however, the detailed molecular architecture of these fibrils is not understood (Cooper, 1974; Crowther, 1991). In fact, fibrils can be constructed from secondary structures other than the antiparallel β-sheet, which is the building block of the cross-β fibril[2] (Fraser & MacRae, 1973). For example, the nine amino acid peptide β34–42 forms amyloid fibrils which are extremely resistant to denaturation and contain atypical secondary structure (Spencer et al., 1991). Thus fibrillar morphology, while important for the histochemical identification of amyloid, cannot be interpreted at the molecular level.

A Distinctive X-ray Fiber Diffraction Pattern Indicates Shared Repeating Structure. X-ray fiber diffraction reveals the presence of related repeating structure in all amyloid proteins which have been analyzed (Crowther, 1991). However, the similarity may be at the level of intermolecular packing, rather than secondary structure. Although every diffraction pattern theoretically specifies a single structure, it is very difficult to directly translate a diffraction pattern into a molecular structure. Therefore, most patterns are classified by analogy to that of a simple repeating-sequence polypeptide such as polyalanine. A protein aggregate is classified as amyloid if the spacing and orientation of the major reflections (4.7 Å on the meridian and 5–10 Å on the equator) are similar to those observed for polyalanine and for the silk protein of *B. mori* (Arnott et al., 1967; Fraser & MacRae, 1973; Marsh et al., 1955). The polyalanine X-ray diffraction pattern has been used to construct models of the fibril and its subunit antiparallel β-sheet (see Figure 1). The iterative model-building process involves fitting of the observed diffraction pattern to one or more molecular models, assessing the energy of each consistent structure, and matching other available data, such as the infrared absorption spectrum, in order to arrive at a model (Marsh et al., 1955). Two consistent models have been proposed for the simple case of polyalanine (Arnott et al., 1967; Marsh et al., 1955). Both models are based on the β-strand secondary structure; however, they differ with respect to the interchain orientation and, therefore, the hydrogen bond geometry (Figure 1 shows Pauling's cross-β fibril[2]). These models were based on an amide bond geometry which was constrained to be planar and trans ($\omega = 180°$). It has recently been pointed out that this constraint does not reflect the amide geometry which is observed in crystalline

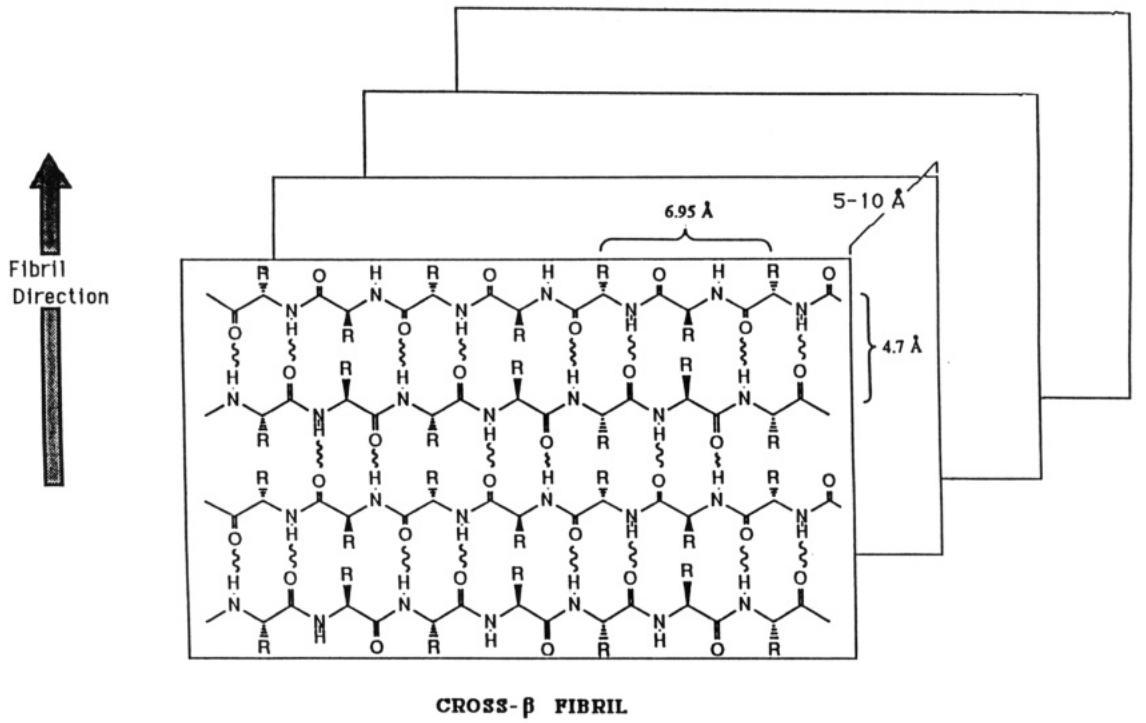

FIGURE 1: Schematic depiction of Pauling's cross-β fibril model (Marsh et al., 1955). This model is generally accepted to represent all amyloid. The point of this review is to emphasize that this structure may represent one member of a structural class. The antiparallel arrangement of the peptide chains and the resultant linear hydrogen-bonding network are shown for the front sheet. Another model has been proposed in which the interstrand orientation (Δz = 0.6 Å, as opposed to 0 Å in the Pauling cross-β fibril), and hence the hydrogen bond geometry, differs (Arnott et al., 1967). The intersheet distance will vary, depending on the side chains involved. The major reflections in the X-ray fiber diffraction pattern which are considered to be diagnostic of this structure are the interstrand distance of 4.7 Å along the meridian and the variable intersheet distance (5–10 Å) along the equator.

proteins[3] (Stewart et al., 1990). The prominent 4.7-Å reflection which is perpendicular to the fiber axis is thought to arise from the interstrand spacing, according to the cross-β fibril[2] (Figure 1). The 9.4-Å meridional reflection that is expected for the antiparallel sheet (distance between parallel strands) is not always seen, possibly due to intersheet packing disorder. Thus, the distinction between two structures which are quite different at the molecular level, that is, the antiparallel and parallel β-sheets, cannot necessarily be made by observation of the diffraction pattern (Fraser & MacRae, 1973). A reflection of 5–10 Å along the equator is thought to arise from the spacing between antiparallel β-sheets, which is side-chain dependent. It should be emphasized that unusual structures may not perturb the prominent reflections usually assigned to the antiparallel β-sheet and that secondary structures other than the antiparallel β-sheet may give rise to an indistinguishable diffraction pattern [e.g., Spencer et al. (1991)]. It is possible that the reflection at 4.7 Å may be commonly observed due to the preferred geometry of any interchain hydrogen bond network, rather than the existence of β-sheet structure.

TRADITIONAL INFRARED SPECTROSCOPY CAN BE MODIFIED TO PROVIDE LOW-RESOLUTION INFORMATION ABOUT LOCAL STRUCTURE

Because amyloid is by definition insoluble in water, most spectroscopic methods are not applicable. However, FTIR has provided useful information regarding amyloid structure (Ashburn et al., 1992; Halverson et al., 1991; Caughey et al., 1991). Given a simple structure (a small molecule or the unit cell of a simple repeating structure such as the cross-β fibril), a theoretical infrared absorption spectrum can be calculated using normal mode analysis and compared to the experimentally determined spectrum (Krimm & Bandekar, 1986). Unfortunately, this process does not work in reverse; that is, a protein or peptide structure cannot be determined by analysis of the infrared absorption spectrum. This is due to the fact that different structures can produce indistinguishable spectra.

The amide I absorption band, which is primarily a CO stretch (1700–1600 cm^{-1}), is known to be sensitive to secondary structure (Krimm & Bandekar, 1986). The antiparallel β-sheet is unique among commonly-observed secondary structures in that extensive interstrand dipole coupling leads to a splitting of the amide I absorption into low- and high-frequency bands. For example, the polyalanine solid-state IR spectrum is characterized by a strong low-frequency amide I absorption (ca. 1625 cm^{-1}) and a weak high-frequency band (ca. 1695 cm^{-1}) (Krimm & Bandekar, 1986). This spectrum is nearly identical to that calculated for the Pauling cross-β fibril (Krimm & Bandekar, 1986). Other common secondary structures such as the α-helix (ca. 1655 cm^{-1}) can be eliminated as a possible polyalanine structure by the comparison of the calculated IR spectra with the experimentally determined spectrum of polyalanine (Krimm & Bandekar, 1986). It has become commonplace to curve-fit IR spectra and to use this information to determine the percentages of standard secondary structures present in a sample protein. This process relies on empirical correlations derived from studies of amino acid homopolymers and crystalline globular proteins of known structure. However, it has recently been experimentally demonstrated that a strict equation of band position and

[3] Pauling built other models of β-sheet-like structure containing *cis*-amides (Pauling & Corey, 1953); however, these models could not explain the observed diffraction pattern and have been largely forgotten. These models should be reexamined, in light of recent findings (Spencer et al., 1991).

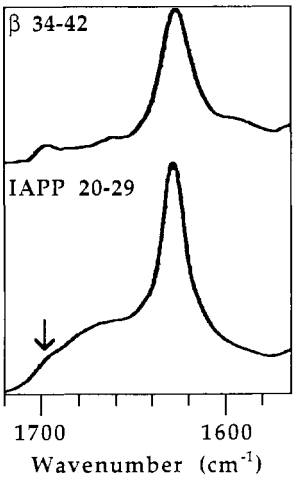

FIGURE 2: Solid-state FTIR spectra of peptides β34–42 (top) and IAPP20–29 (Ashburn et al., 1992; Halverson et al., 1991). Only the amide I absorption is shown. The "split" amide I absorption band is considered to be diagnostic for antiparallel β-sheet structure (Krimm & Bandekar, 1986). Note the characteristic low-insensity band at ca. 1695 cm^{-1}, which appears as a shoulder in the IAPP20–29 spectrum,[4] and the high-intensity band at ca. 1628 cm^{-1}. The broad absorption band centered at ca. 1655 cm^{-1} in the IAPP20–29 spectrum is due to the asparagine side chains.[4] The peptide β34–42 has been shown *not* to have antiparallel β-sheet secondary structure by isotope-edited FTIR and by rotational resonance ssNMR[5] (Halverson et al., 1991; Spencer et al., 1991).

secondary structure may not be legitimate (Wilder et al., 1992). Consequently, overinterpretation of IR spectra must be avoided, especially in the case of the amyloid proteins, which are a distinct structural class. The low-frequency amide I band is a necessary, but not a sufficient, spectral characteristic for the assignment of antiparallel β-sheet secondary structure.

A striking example of this fact has surfaced in our work; the two amyloid peptides which produce the FTIR spectra shown in Figure 2 seem to have very similar secondary structures.[4] However, structural differences between the two peptides were demonstrated through the application of isotope labeling to FTIR. Replacement of specific amide carbonyl carbons with ^{13}C results in a localized perturbation in the dipole coupling interactions. These interactions are characteristic of, but not unique to, antiparallel β-sheet structure (Ashburn et al., 1992; Halverson et al., 1991). The magnitude of the isotope-induced perturbation depends on the local environment of the labeled amide. We call this FTIR method isotope-edited dipole coupling analysis. The structural basis for the different sensitivities of the two peptides to isotopic substitution cannot be determined by IR alone. However, certain structures that are consistent with the unlabeled FTIR spectrum, but inconsistent with the isotope-edited dipole coupling analysis, can be ruled out. In the case of the peptide β34–42, the cross-β fibril can be ruled out as a possible structure for the aggregate.

CRYSTALLINE β-SHEET STRUCTURE MAY BEAR LITTLE RESEMBLANCE TO AMYLOID SECONDARY STRUCTURE

The only available molecular-level information regarding antiparallel β-sheet structure is derived from X-ray crystallographic studies of soluble, crystalline peptides and proteins. Several reviews of the structural features of crystalline antiparallel β-sheets have appeared (Chothia, 1973; Richardson & Richardson, 1989; Salemme, 1983). One common characteristic of the crystalline sheets is a twist around the axis defined by the the polypeptide chain, which is caused by nonbonded interactions between side chains (Chothia, 1973; Chou et al., 1982; Chou & Scheraga, 1982; Raghavendra & Sasisekharan, 1979; Richardson & Richardson, 1989; Salemme, 1983). This twist is *not* a feature of the Pauling cross-β fibril model (Figure 1). The amyloid homopolymers which inspired the cross-β fibril[2] are not easily crystallized. Unfortunately, twisted lamellar crystals of *B. mori* silk protein are not of the quality required for single-crystal X-ray analysis (Lotz et al., 1982). However, if one assumes a cross-β fibrillar structure, then the magnitude of the observed twist in the lamellar silk crystal is negligible when compared to the twist of β-sheets in soluble, crystalline proteins (0.1° per strand vs 10–30° in soluble proteins) (Lotz et al., 1982). This finding suggests that β-structure in crystalline, soluble proteins and β-structure in noncrystalline, insoluble amyloid proteins are different. That proposal is supported by sequence analysis of amyloidogenic polypeptides. The Chou–Fasman empirical structure prediction method, which is based on the frequency of occurrence of individual amino acids in particular secondary structures within soluble, crystalline proteins (Chou & Fasman, 1974), incorrectly predicts that the *B. mori* silk sequence should have random-coil structure and should not contain significant amounts of β-sheet structure. The prevalence of glycine in the silk sequence as compared to soluble β-sheet structure suggests a stabilizing effect of glycine on amyloid structure. The failure of the Chou–Fasman empirical method in these cases suggests that there are significant differences between crystalline and fibrillar (amyloid) β-structure. Polyalanine and *B. mori* silk may contain regular, flat β-sheet structure, similar to that of the cross-β fibril. However, one amyloid peptide with a nonrepeating primary sequence (β34–42) has been shown to contain unusual secondary structure that is inconsistent with the cross-β fibril (Spencer et al., 1991).

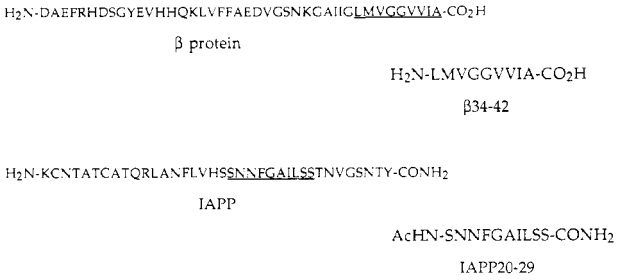

FIGURE 3: Primary sequences of the amyloid proteins of AD (β protein) and type II diabetes (IAPP). In each case, a peptide derived from the underlined sequence has been shown to be amyloidogenic (Glenner et al., 1988; Halverson et al., 1990). These amyloid peptides are shown.

UNUSUAL SECONDARY STRUCTURE CHARACTERIZES CERTAIN AMYLOIDS

We have studied two synthetic peptides, β34–42 (Halverson et al., 1990) and IAPP20-29 (Glenner et al., 1988), which form amyloid in vitro. Each is a fragment of an amyloidogenic protein (Figure 3). The synthetic peptide β34–42 is a C-terminal fragment of the β-protein of AD. We have proposed that the C-terminus of the β-protein of AD is critical in the initiation of amyloid deposition (Halverson et al., 1990). Recent studies of the β-protein support this contention but do not provide a molecular explanation (Burdick et al., 1992). The peptide IAPP20–29 is a fragment of the islet amyloid

[4] When corrected for the asparagine side-chain absorbance in the IAPP20–29 spectrum (Figure 2, lower spectrum), the spectrum is very similar to that of β34–42 (Figure 2, upper spectrum).

polypeptide of type II diabetes which has been shown to form amyloid fibrils (Glenner et al., 1988). Each of these peptide amyloids exhibits all of the traditional properties of amyloid and has an IR spectra which is consistent with the cross-β fibril model (figure 2) (Halverson et al., 1990). However, isotope-edited FTIR studies reveal differences between the two aggregates. These differences derive from variations in secondary structure and/or interchain interactions. The IAPP20–29 isotope-edited FTIR spectra are consistent with a cross-β fibril in the central portion of the sequence (Ashburn et al., 1992). In contrast, the β34–42 fibril does not appear to be a cross-β fibril. Furthermore, the central Gly-Gly region of peptide β34–42 behaves anomalously in the FTIR experiments (Halverson et al., 1991). The existence of an unusual structure at that location has been confirmed by rotational resonance ssNMR studies of the β34–42 amyloid, which reveal the presence of an unusual *cis*-amide bond between Gly37 and Gly38 (Spencer et al., 1991). This structural feature was invisible to the traditional biophysical approaches. Rotational resonance ssNMR is a novel technique, developed by Robert Griffin, which allows one to accurately (±0.1–0.2 Å) measure intercarbon distances of up to 6 Å in the solid state. By systematically measuring α-carbon to carbonyl carbon distances along the peptide backbone, one can define a family of possible secondary structures.[5] These structures can then be individually evaluated with respect to their agreement with IR spectra, their energetics, and their ability to form stable aggregates. The rotational resonance ssNMR technique does not require a crystalline sample and thus promises to revolutionize the study of amyloid protein structure.

CONCLUSIONS

Each of the three properties which led to the designation of amyloid proteins as a structural class is related to structure, although this relationship has not been elucidated at the molecular level. Two structural models have been proposed which are consistent with the X-ray diffraction pattern and the fibrillar morphology (Arnott et al., 1967; Marsh et al., 1955). These models represent extremes in terms of their structural regularity. Congo red binding can be rationalized in terms of these models; however, the molecular details of the dye–amyloid interaction have not been elucidated. We propose that the three defining properties of amyloid characterize a class of related structures, of which the cross-β fibril[2] is one member, rather than the single structure. The shared physical properties of the amyloid proteins may be due to similar intermolecular packing motifs, rather than shared secondary structure.

We have shown that amyloid fibrils which produce practically indistinguishable infrared absorption spectra do not necessarily have identical structures. Therefore, IR spectra must be interpreted with caution and should only be used to disprove the existence of a particular structure or to reveal structural differences between amyloids. FTIR can provide additional information about local structure via the incorporation of ^{13}C at specific amides along the peptide backbone. Most importantly, the recent emergence of rotational resonance ssNMR enables the determination of the detailed molecular architecture of this class of proteins. This technique has been used to prove that amyloid plaque does not necessarily contain pure antiparallel β-sheet structure[5] (Spencer et al., 1991). In fact, amyloid structures may contain secondary structures and/or hydrogen-bonding motifs which do not occur in crystalline proteins. Further studies will lead to elucidation of the molecular structures of the various members of the amyloid class and an understanding of the intermolecular packing interactions which determine their insolubility.

[5] A total of ten intercarbon distances have been measured in the β34–42 amyloid. These measurements indicate that much of the peptide backbone does *not* resemble typical antiparallel β-strand structure (Michèle Auger, Richard Spencer, Kurt Halverson, Ted Ashburn, Robert Griffin, and P. T. Lansbury, unpublished results).

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