X-ray diffraction and far-UV CD studies of filaments formed by a leucine-rich repeat peptide: structural similarity to the amyloid fibrils of prions and Alzheimer's disease β-protein

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Abstract The development of neuro-degenerative diseases often involves amyloidosis, that is the formation of polymeric fibrillar structures from normal cellular proteins or peptides. For example, in Alzheimer's disease, a 42 amino acid peptide processed from the amyloid precursor protein forms filaments with a β-sheet structure. Because of this, the structure and dynamics of polymeric peptide filaments is of considerable interest. We showed previously that a 23 amino acid peptide constituting a single leucine-rich repeat (LRRN) polymerises spontaneously in solution to form long filaments of a β-sheet structure, a property similar to that of Alzheimer's β-amyloid and prion peptides. Here we report that a variant of LRRN in which a highly conserved asparagine residue is replaced by aspartic acid does not form either filaments or β structure. By contrast, a variant which replaces this asparagine residue with glutamine forms filaments ultrastructurally indistinguishable from those of LRRN. Electron micrographs of LRRN filaments show that many consist of two interleaved strands which appear to have a ribbon-like morphology. X-ray diffraction patterns from oriented LRRN fibres reveal that they are composed of long β-sheet arrays, with the interstrand hydrogen bonding parallel to the filament axis. This 'cross-β' structure is similar to that adopted by β-amyloid and prion derived fibres. Taken together, these results indicate that the LRR filaments are stabilised by inter- or intra-strand hydrogen bonded interactions comparable to the asparagine ladders of β-helix proteins or the 'glutamine zippers' of poly-glutamine peptides. We propose that similar stabilising interactions may underlie a number of characterised predispositions to neuro-degenerative diseases that are caused by mutations to amide residues. Our finding that amyloid-like filaments can form from a peptide motif not at present correlated with degenerative disease suggests that a propensity for β-filament formation is a common feature of protein sub-domains.

Key words: Leucine-rich repeat filament; X-ray diffraction; Alzheimer's disease; Prion amyloid; Stabilization

1. Introduction

The inappropriate formation of polymeric filaments by peptides and small proteins is thought to underlie the pathology of several neuro-degenerative conditions, for example Alzheimer's disease and prion induced dementia. It is thus of some interest to understand what structural features confer the ability to form such polymers and what the overall 3-dimensional structure of the filaments is. In this regard, fibre diffraction studies of fibres and fibrils derived from both the Alzheimer's β-peptide and prion protein show them to form arrays of β-sheet [1,2].

In these fibrils, the polypeptide chain direction of the subunits is oriented perpendicular to the axis of the fibre, which contrasts with the filamentous β-protein keratin in which the amino acid chain is parallel to the fibre axis.

The leucine-rich repeat (LRR) is a repetitive motif consisting of about 24 amino acids and conforms to the following consensus:

pXXX**F**X**X**L**X**X**L**X**X**L**X**L**X**X**N**X**I**X**X**L

About seventy proteins containing LRRs have been characterised (see [3] for a review) and the motif is present in these sequences in between 1 and 38 tandemly repeated copies. The crystal structure of a single LRR protein, ribonuclease angiogenin inhibitor (RI) has been determined [4]. Each LRR unit in RI folds into an unusual α/β structure and the molecule adopts a horse-shoe shape. The inner surface of the horseshoe consists of short parallel β-strands which, unusually, are exposed to solvent. The β-strands are connected to the helical regions, which form the outer circumference of the horseshoe, by a turn which includes highly conserved asparagine and cysteine residues (equivalent to residue 19 in the above consensus), residues which make a complex network of stabilising interactions. The RI repeats are unusual among LRR proteins, being 28 amino acids long and only the regions forming the β-strand and turn are conserved in the shorter repeats. Furthermore, standard and RI repeats are never found in the same protein, suggesting that they are structurally incompatible with each other. In view of this, it has been suggested that the shorter LRRs may form a more extended β structure such as a β-helix [5]. A striking feature of the short LRR family is the almost invariable conservation of asparagine 19. In the structure of the β-helical protein pectate lyase, asparagine residues form a ladder of hydrogen bonded interactions along the helical axis which play a critical role in stabilising the structure [6].

Previously, we reported that a 23 amino acid peptide constituting a single unit of a leucine-rich repeat (LRRN) forms spontaneously into a gelatinous solution consisting of filaments with a β-sheet structure [7]. In this report we show

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that LRRN filaments have a 'cross-β' structure similar Alzheimer β-amyloid and prion protein fibrils. The LRRN filaments appear to be stabilised by asparagine ladder interactions.

2. Materials and methods

2.1. Materials

Peptides were synthesised and purified as previously described [7]:

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LRRN    PANLLTDMRNLSHLELRANIEEM
LRRQ                    Q
LRRD                    D
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2.2. Circular dichroism spectroscopy

LRRN, LRRQ and LRRD peptide was dissolved in 50 mM Tris-Cl, pH 8.2 at a concentration of 2.6 mg ml^{-1} and left at room temperature for 2 h. The samples were then diluted to 260 μg ml^{-1} in 10 mM sodium phosphate buffer (pH 7.8) and spectra measured with a Jovin-Yvon CD6 spectrograph. Spectra were recorded at 22°C, in a 0.1 mm path length cell, between 190 and 240 nm and subtracted from the spectrum of 50 mM Tris-HCl (pH 8.2) diluted in the same manner. Each spectrum is the average of five scans and is smoothed. The concentration of the peptide solutions was calculated by reference to amino acid analysis of the purified product. The CD is expressed as mean residue delta-epsilon (Δε). For far-UV CD measurements, UV radiation from station 3.1 SRS with a SEYA monochromator at Daresbury Laboratory, CLRC, Daresbury, Warrington, Cheshire, UK was used. In these experiments the peptide was dissolved at 1 mg/ml directly in 10 mM sodium phosphate buffer (pH 7.8) and allowed to polymerise over time in a 0.1 mm path length cuvette.

2.3. X-ray diffraction

LRRN peptide was dissolved in water at 10 mg/ml and polymerisation initiated by addition of phosphate buffer (10 mM final concentration) to give a pH in the range 7.0 to 8.2. Ten-μl aliquots were immediately suspended vertically between glass-rods aligned with the magnetic field of a 9 T superconducting magnet. The solutions dried into fibres over 30 h at 7°C and 92% R.H. When dry they were removed and stored at 75% R.H. Fibres formed at pH 7.4 were transparent and showed blue birefringence (see Fig. 3A) and these were chosen for fibre diffraction studies. The pH 7.0 fibres were transparent but had red birefringence and gave diffraction patterns suggesting greater disorientation. Fibres from pH 8.2 were not transparent and gave much weaker birefringence. Diffraction patterns were obtained from a pH 7.4 fibre mounted vertically in the Keele fibre camera (0.2 mm collimator, 75% R.H.) on Station 7.2 SRS Daresbury ($\lambda = 1.488$ Å). The pattern was recorded on a MAR research 120 mm image plate (20 min exposure). A calibration ring at 3.136 Å was obtained by dusting the fibre with silicon powder. The pattern was converted to CCP13 image format using the program CONV [8] displayed and calibrated using the program FIX. This allowed the fibre-to-detector distance, centre, rotation, and tilt to be estimated. It was also used to measure the position of intensity maxima in reciprocal space [8].

3. Results

3.1. LRRN filaments are stabilised by a conserved asparagine residue

To determine whether the conserved asparagine residue is important for filament formation by LRRN, we synthesised variants in which this residue is replaced by either aspartic acid (LRRD) or glutamine (LRRQ). In contrast with LRRN, we found that a solution of LRRD did not form into a gel and had a circular dichroism spectrum indicating that it was in a random coil conformation. By contrast, solutions of LRRQ did form into a gel and had a CD spectrum very similar to that of LRRN (Fig. 1A). Our data also show that both LRRN and LRRQ form filaments with pseudo-first-order kinetics but that LRRQ filaments form more slowly (Fig. 1B and C). This suggests that the fibres are being generated by end dependent polymerization at a constant number of nuclei, a property held in common with amyloids of Alzheimer's β-peptides [9]. Taken together, these findings indicate that asparagine 19 plays a critical role in the formation of LRRN filaments and that this residue can be substituted by glutamine but not by aspartic acid.

3.2. Ultrastructure of LRRN filaments

We have investigated the ultrastructure of LRRN and LRRQ filaments using electron microscopy. Samples were stained using the technique of rotary shadowing or with uranyl acetate and were viewed with a Philips EM300 electron microscope. The fibres formed by LRRN and LRRQ look very similar, having a thickness of about 10 nm, as reported previously for LRRN filaments (Fig. 2). Rotary shadowed images show that many of the filaments are constituted of two strands interleaved together (Fig. 2C). The pattern of staining suggests that the strands of each filament are ribbon-like, a structure consistent with the proposal that the peptides form into long arrays of β-sheet.

3.3. LRRN filaments have a 'cross-β' structure

To further investigate the structure of LRRN we have used the technique of fibre diffraction. Oriented preparations of LRRN fibres were prepared by slowly drying a polymerizing solution of peptide to room humidity (75% R.H.) between two supporting glass rods in the room temperature bore of a 9T superconducting magnet. A birefringent fibre was obtained suggesting alignment of the LRRN filaments (Fig. 3A). The

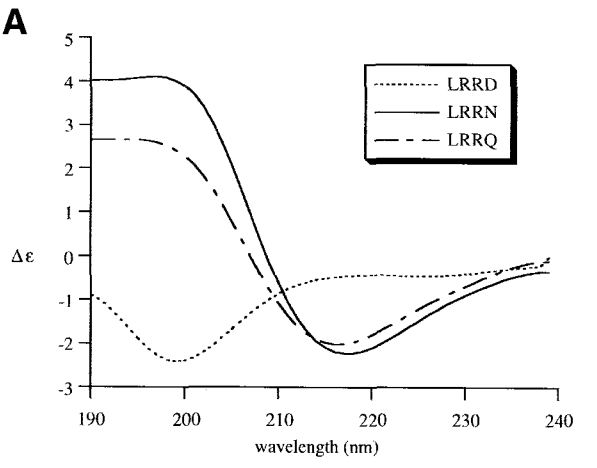
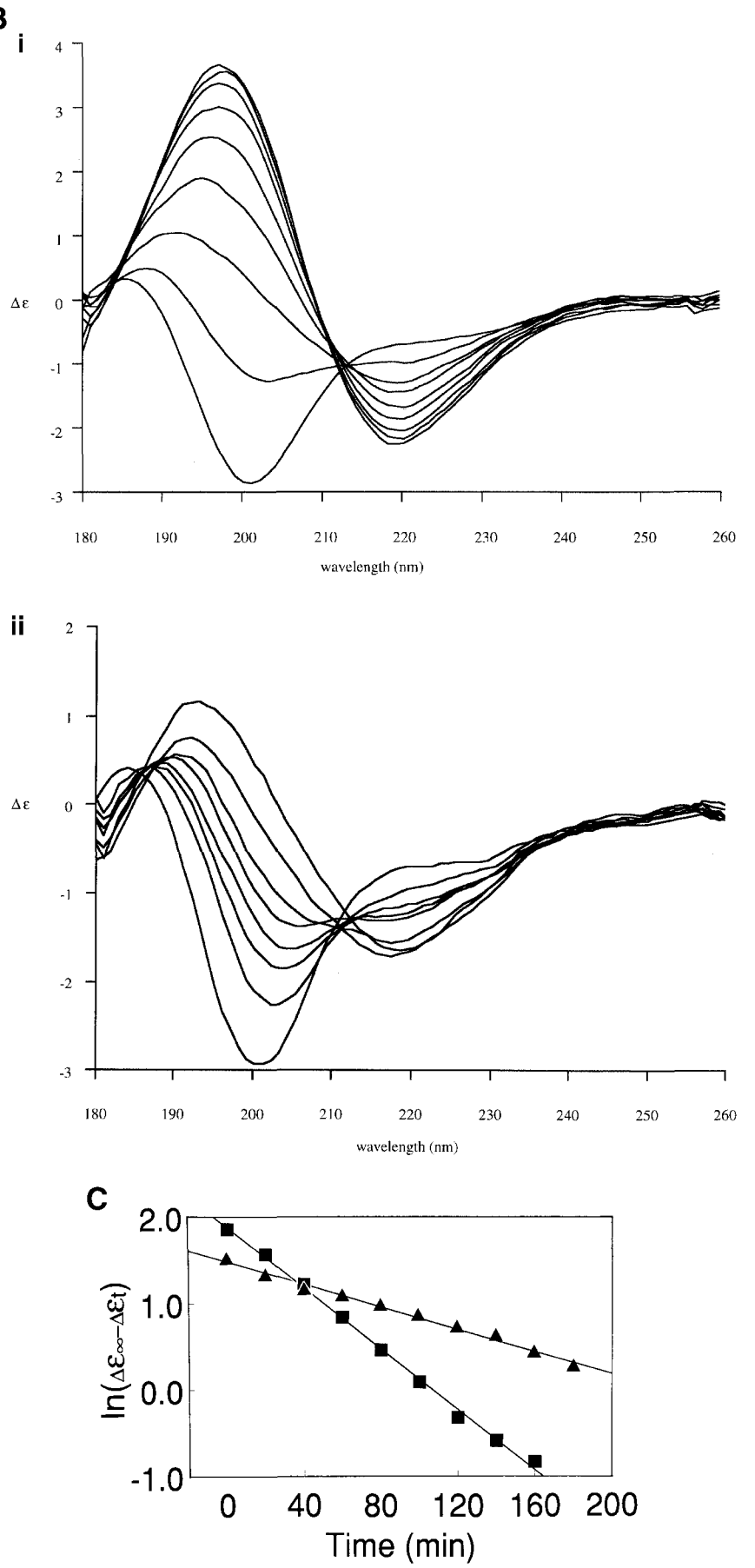


Fig. 1. Circular dichroism spectra of LRRN and variants with either aspartic acid (LRRD) or glutamine (LRRQ) at position 19. (A) CD spectra of LRRN, Q and D peptide solutions. (B) (i) Far-UV CD spectra of LRRN. Data were collected every 20 min over 160 min and the spectra were superimposed to show the change in structure. (ii) Far-UV CD spectra of LRRQ. Data collected as in (i). The spectra are derived from single scans subtracted from a buffer baseline and are slightly smoothed. (C) Time course of development of positive delta-epsilon at 197 nm was plotted semilogarithmically to show pseudo-first-order kinetics. The data were from LRRN spectra (B(i)) (squares) and LRRQ spectra (B(ii)) (triangles). Final delta-epsilon was assumed to be 4.1 and 2.2 for plots of LRRN and LRRQ kinetics, respectively. Non-linear least squares fitting gave pseudo-first-order rate constants of 1.6×10^{-2} min^{-1} and 0.7×10^{-2} min^{-1} for LRRN and LRRQ, respectively.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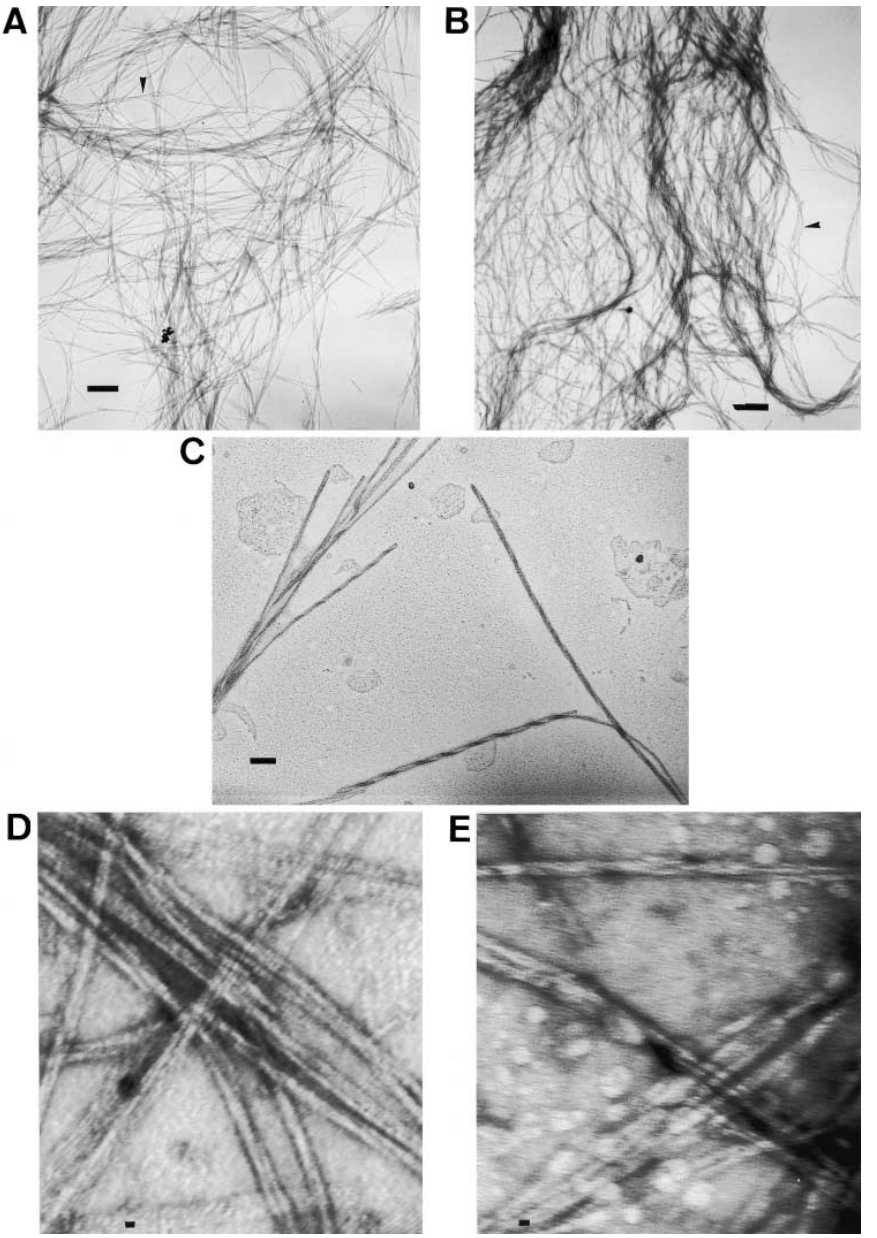


Fig. 2. Electron micrographs of LRRN and LRRQ filaments. (A) LRRN fibres (×6400). The arrowhead indicates an example of an interleaved filament. (B) LRRQ fibres (×6400). The arrowhead indicates an example of an interleaved filament. (C) LRRN fibres (×54 700). (D) LRRN fibres (×272 000). (E) LRRQ fibres (×272 000). Scale bars indicate 1 μm (A, B); 100 nm (C); 10 nm (D, E). Samples of peptide were polymerised as described in Fig. 1 and then diluted 1/50 in 10 mM sodium phosphate pH 7.8. Samples (A)–(C) were stained by the rotary shadowing method, (D) and (E) with 0.5% uranyl acetate. Samples were examined with a Philips EM300 instrument.

X-ray diffraction from the fibre was obtained on Station 7.2 SRS Daresbury. This confirms the alignment of the fibrils although disorientation is still considerable (Fig. 3B). The pattern shows no evidence for a large helical repeat. The main feature is strong meridional intensity corresponding to a spacing of 4.76 Å. This is consistent with a 'cross-β' structure in which interstrand hydrogen bonds of a β-sheet are aligned along the fibril and hence fibre axis [1,10,11]. No intensity is observable corresponding to the 9.4 Å repeat of antiparallel β-strands [12]. Equatorial intensity corresponding to the intersheet distance is observed at 11 Å: this spacing results from intersheet side-chain packing and is therefore rather variable among cross-β structures. There is additional less distinct equatorial intensity centred on 22 Å. There is no observable intensity due to the disposition of peptide units at 3.3 Å across the filament sheets [12] but this is most likely due to the high disorientation and water content of the fibres obtained (see, for example, [13]). There are no observable maxima in the low angle region comparable to the prominent 71 Å feature of Alzheimer fibres. This probably relates to differences in the packing of filaments across the fibre [11]. Fibres prepared from the LRRQ peptide have a very similar fibre diffraction pattern and display no features additional to those seen for LRRN (Fig. 3B; result not shown).

4. Discussion

The results outlined above suggest that LRRN filaments

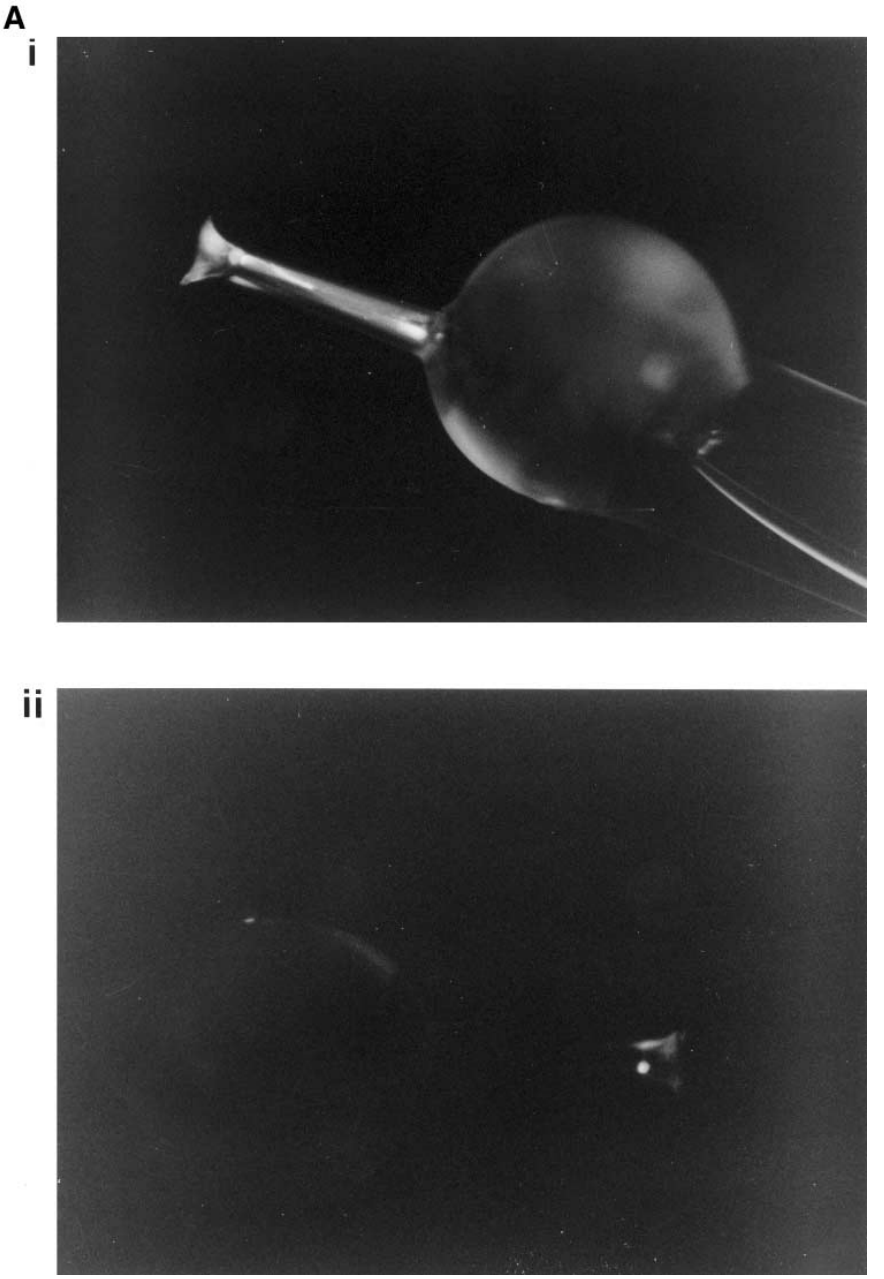
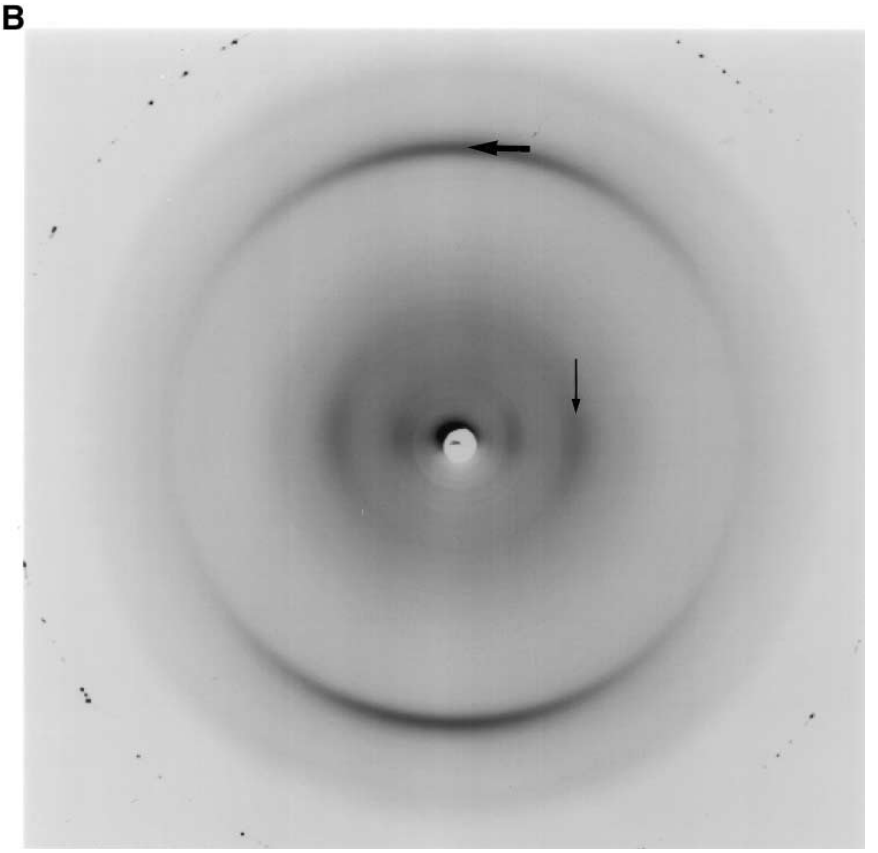


Fig. 3. Fibre diffraction studies of LRRN filaments. (A) Birefringence of oriented LRRN fibres. The fibre was formed at pH 7.4 as described in (B) below. The birefringence was observed through a polarizing microscope with crossed polaroids. (i) and (ii) are photographs of the same sample turned through 45 degrees with respect to the polaroids. (B) Fibre diffraction pattern. Large arrow indicates the 4.76 Å meridional intensity. Small arrow indicates broad intensity in the 11 Å region. Other less intense maxima are observable at 22 Å on the equator and in the 4.2 Å region slightly off the meridian. No intense features were observed close to the central maximum, and no distinct feature is observable on the meridian corresponding to a spacing of 9.4 Å. Crystalline spots at outer corners are portions of calibration ring.

form into a similar structure to that of the class A peptides [1] deriving from Alzheimer's disease protein β-amyloid. In particular the hydrogen bonding pattern is parallel to the filament axis which rules out the possibility that the fibres are formed by stacking of the side chains of dimeric peptide units. Nevertheless, it is clear that the overall structure of the filament requires stabilisation by interactions mediated by amide side chains. For example, it seems possible that there are inter- or intra-filament hydrogen bonded interactions similar to those observed in the asparagine ladders of β-helix proteins and RI. In that regard the absence of a meridional intensity at 9.4 Å may indicate that the filaments are composed of parallel β-sheets which would be consistent with the arrangement seen in both pectate lyase and RI [5,6]. The glutamine zippers formed by poly-glutamine peptides may also be an appropriate model for LRRN and LRRQ filaments [14].

In a previous study [7], we modelled LRRN filaments as parallel β-sheets. We noted that such an arrangement would cause the fibres to have an amphipathic character with hydrophobic and charged amino acid residues segregating on opposite surfaces. The fibre diffraction data presented here support such a model and also rules out the presence of β-helical structures. The observation that many, if not all, filaments are interleaved together (Fig. 2C) suggests that the hydropho-



bic surfaces of two strands may pack together while the hydrophilic residues interact with solvent. The side chain of the conserved asparagine residue segregates on the hydrophilic surface and could make hydrogen bonded interactions between adjacent β strands similar to those seen in pectate lyase and RI [6,4]. In this regard, it is interesting that glutamine can substitute for asparagine in LRRN as this residue is nearly invariant in the short LRR sub-family. Presumably the additional methylene group in the glutamine side-chain can be accommodated in the LRRQ filaments but not in the native structures of most LRR proteins. This suggests that there is a structural constraint, as there is in β-helix proteins, which disallows glutamine at this position.

The properties of LRRN peptide described here suggest that stabilisation caused by intra-molecular interactions between side chains may have a role in the formation of amyloid deposits in neuro-degenerative diseases. Indeed, many mutations causing a pre-disposition to such diseases involve amino acid changes to glutamine or asparagine (see, for example, [15–19]).

Recently, Aggeli et al. [20] have described the physical properties of designed peptides which like LRRN and LRRQ form into gels and they suggest that such responsive gels may find a wide range of applications. It will be of interest to determine whether LRRN gels have similar viscoelastic properties.

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