Structural and Dynamic Features of Alzheimer’s Aβ Peptide in Amyloid Fibrils Studied by Site-directed Spin Labeling*

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Electron paramagnetic resonance spectroscopy analysis of 19 spin-labeled derivatives of the Alzheimer’s amyloid β (Aβ) peptide was used to reveal structural features of amyloid fibril formation. In the fibril, extensive regions of the peptide show an in-register, parallel arrangement. Based on the parallel arrangement and side chain mobility analysis we find the amyloid structure to be mostly ordered and specific, but we also identify more dynamic regions (N and C termini) and likely turn or bend regions (around residues 23–26). Despite their different aggregation properties and roles in disease, the two peptides, Aβ40 and Aβ42, homogeneously co-mix in amyloid fibrils suggesting that they possess the same structural architecture.

Protein deposits are commonly associated with a wide range of degenerative diseases, including Alzheimer’s disease, Parkinson’s disease, type-2 diabetes, macular degeneration, and several others. These deposits contain a number of proteins that often have fibrillar morphology. In Alzheimer’s disease, the extracellular deposits are largely made up of a short 39–42-amino-acid-long peptide referred to as Aβ.

Like all other amyloid fibrils, Aβ fibrils are thought to have a cross β-structure, in which individual β-strands are organized roughly perpendicular to the fiber axis. According to fiber x-ray diffraction studies of Aβ fibrils (1, 2) the unit spacing between these individual β-strands along the fibril axis is 4.7 Å, while the sheets are spaced at about 10 Å. However, our understanding of the exact structure of Aβ or that of other amyloid fibrils is still very incomplete. For example, it is not known which regions of the peptide are directly involved in the formation of the cross β-structure. Simple geometric considerations suggest that not all amino acids in Aβ40 or Aβ42 can be part of the cross β-structure in a linear, fully extended β-strand. Assuming an average distance between residues in an extended β-strand of 3.5 Å per residue, a completely extended structure model predicts a minimum extended length of 140 Å for Aβ40. This length is considerably larger than the diameter of the putative building blocks of the fibrils, protofibrils with an individual diameter of 40–55 Å (3–6). In fact, it even exceeds the 60–100-Å diameter of the entire fibril. Thus, turn or bend regions are needed to account for the fiber dimensions. Indeed, hydrogen-deuterium exchange measurements of fibrillar Aβ (7) suggest that only about half of the peptide is involved in a protective hydrogen-bonded structure. In addition, Fourier transform infrared spectroscopic data on fibrils indicate the presence of at least one turn (8, 9). However, the locations of these turns or the location of the β-strands are still unclear.

Furthermore, there has also been some controversy regarding the arrangement of individual peptides with respect to each other. Fourier transform infrared spectroscopic data indicate an antiparallel organization of the β-sheets within the Aβ fibril (8, 10). An antiparallel organization is also supported by other studies on the molecular structures of amyloid fibers formed from shorter Aβ peptide analogs, Aβ(11–25) (11), Aβ(16–22) (12), and Aβ(34–42) (13). In contrast, solid state NMR analysis of Aβ(10–35) fibrils (14–16) suggests that the fibrils are organized with the peptides in parallel β-strands where the amino acids are in exact register. The distances measured between single 13C backbone atoms at 12 separate locations in the amyloid fibril averaged 5.3 ± 0.2 Å. In addition, multiple quantum solid state NMR measurements on 13C-labeled Aβ40 by Tycok and co-workers are consistent with the parallel, exact register organization of the Aβ fibril (17). As an independent means of clarifying the structural organization of Aβ40 and Aβ42 within the amyloid fibril, we have used site-directed spin labeling (SDSL).1

The basic strategy of SDSL requires the introduction of a spin label into the protein sequence at a specific site. This is typically accomplished by labeling specific cysteine residues resulting in the formation of side chain R1 (Fig. 1), which is remarkably well tolerated in proteins (18). Important structural information can be extracted from the mobility information contained in the EPR spectra of spin-labeled proteins. SDSL studies on proteins with known crystallographic structures have established that mobility information is sufficient to identify a residue as a loop site, exposed, in tertiary contact, or buried (18–20). Together with the ability to measure distances between multiple spin labels in a range of 5–25 Å, these features have been proven to be sufficient to model the structure of proteins (21) with the resolution of the backbone fold.

1 The abbreviations used are: SDSL, site-directed spin labeling; EPR, electron paramagnetic resonance spectroscopy; Aβ, amyloid β; SNARE, soluble NSF attachment protein receptors.
EXPERIMENTAL PROCEDURES

R1-labeled Aβ Peptides—All peptides were synthesized by fluoren-9-yl-methoxycarbonyl chemistry using a continuous flow, semiautomatic instrument, purified by reverse phase-high pressure liquid chromatography, and characterized by sequencing and electrospray mass spectrometry as described previously (22). Only samples exhibiting 90% or greater purity with less than 5% of a single contaminant were used. Several single cysteine substitution mutants were synthesized simultaneously by a similar approach, except that at locations where Cys was substituted, a portion of the resin was coupled separately with Cys (23). Crude peptides were reduced by addition of excess dithiothreitol prior to purification by reverse phase-high pressure liquid chromatography, and the purified peptides were lyophilized. Lyophilized peptides were dissolved in water (or Me2SO) at a concentration of 2.25 mg/ml to make stock solutions immediately before use. For R1 labeling, the stock solution was diluted into 10 mM Tris-HCl, pH 7.6, to a final concentration of 0.5–1 mg/ml, and immediately after dilution a 4-fold molar excess of MTSL spin-labeled (1-oxyl-2,2,5,5-tetramethyl-pyrroline-3-methyl methanesulphonate, kindly provided by Dr. Hideg) was added. After ~30 min of incubation at room temperature, excess spin label was removed using PD10 gel filtration column. The eluted peptide was collected and lyophilized. The absence of free R1 was checked by thin layer chromatography in chloroform:methanol (2:1). All samples contained less than 1% free label. The labeling efficiency, purity, and correct structure were determined by matrix-assisted laser desorption ionization mass spectroscopy. A molecular ion at the expected mass of the R1-labeled peptide was obtained, and the parent, unlabeled peptide was not observed at a limit of detection of ~2% of the labeled peptide.

Fibril Formation by R1-labeled Aβ Peptides—The lyophilized peptides were first dissolved in Me2SO to a concentration of 20 μg/ml. Subsequently, this solution was diluted into 20 mM HEPES, 100 mM NaCl, 0.2% NaN3, pH 7.4, to a final concentration of 50–80 μM. All concentrations were determined using a Micro BCA Protein Assay Reagent Kit (Pierce). Fibrils were grown at room temperature under constant stirring. Fibril formation occurred after as little as 1 day. However, to maximize our yields and minimize contributions from smaller aggregates or intermediates, fibrils were harvested after 10–20 days by centrifugation and washed repeatedly with the same buffer. The fibril quality was tested for each derivative by atomic force microscopy and/or by negative staining electron microscopy, and thioflavin T binding according to previously published protocols (24). Spin dilution experiments were performed for each of the spin-labeled derivatives. In these experiments the total concentration of Aβ was kept constant and the ratio of R1-labeled to wild type peptide was varied as indicated in the text.

EPR Spectroscopy—For EPR analysis, 1–5 μl of a fibril suspension or soluble Aβ were loaded into glass capillaries. EPR spectroscopy was performed on a Bruker EMX spectrometer fitted with a loop gap resonator. X-band EPR spectra were obtained at ambient temperature using optimized modulation amplitude of 1.5–3 G and an incident microwave power of 2 mW. The scan range for each spectrum is 150 G. Spectra represent the average of 10–25 scans (40 s/scan). All first derivative EPR spectra are normalized to the same number of spins using double integration and are shown normalized. The use of magnetic dipolar interaction has been proven to be a reliable tool for distance determination and the validity of the approach used in the current study has already been demonstrated (25, 26). To determine the intermolecular distances between same R1-labels on Aβ40 we employed simulation software that has kindly been provided by Drs. Hubbell and Altenbach (25, 26). The underlying approach is based upon calculation of the magnetic dipolar interaction that occurs in the slow motion regime, which is applicable for the large fibrillar amyloid structures. Distances were obtained by comparing the spectra of fully labeled fibrils and spin-diluted fibrils. Most simulations were performed with the 50% spin-diluted fibrils. In these cases, the spin-diluted spectra were corrected for the presence of residual spin coupling (26). The validity of this approach was tested for a few test cases by comparing the resulting distance distribution to that obtained for more highly diluted samples, which resulted in similar results.

RESULTS

Fibril Formation of R1-labeled Aβ Results in Significant EPR Spectral Changes—To probe the structural organization of Aβ40 and Aβ42 in amyloid fibrils we grew fibrils from 19 different R1-labeled derivatives. Spin-labeled sites were chosen to evenly cover most regions of the peptide to provide local structural information from all segments of the peptide. 18 of these derivatives were based on the Aβ40 peptide, and one derivative, 42R1, was based on the longer Aβ42 peptide. All fibrils grown from the R1-labeled Aβ peptides display morphologies similar to those previously reported for wild type Aβ (27–30). According to atomic force microscopy, electron microscopy of negatively stained samples, and thioflavin T fluorescence measurements, the introduction of R1 at each of the 19 positions did not significantly affect the ability of these modified peptides to form fibrils (data not shown). In addition, the ability of the R1-labeled Aβ peptides to co-assemble interchangeably with wild type Aβ in the same amyloid fiber lattice provides strong evidence that the structures of the labeled and wild type peptides are very similar (see also next section).

As illustrated for the Aβ40 25R1 derivative, fibril formation has a profound effect on the EPR spectral lines (Fig. 2A). The EPR spectrum of soluble 25R1, like those of all other derivatives, is dominated by sharp and narrowly spaced lines (blue trace, Fig. 2A) that indicate a high degree of motion on the sub-nanosecond time scale. This motion can arise from very rapid tumbling of the short Aβ peptide as well as its very dynamic structure (31–33). A more detailed study of soluble Aβ will be forthcoming. The main purpose of showing this spectrum is for a qualitative comparison to the EPR spectra of the
fibrils, which as in the case of a prion peptide (34) are very different from those of the soluble form. As illustrated with the example of 25R1, the EPR spectral lines become very broad and of very low amplitude in the fibril (black trace, Fig. 2A).

In fact, the spectrum was redrawn at 20-fold magnification to visualize the actual line shape (dashed trace, Fig. 2A). Similar effects were observed at all other sites as illustrated by the EPR spectra of the fibrillar state in Fig. 3. Such dramatic spectral changes could be due to immobilization and/or spin-spin interaction, which requires close proximity of spin labels.

Labeled and Unlabeled Aβ Co-mix and Reveal Regions of Parallel Arrangement—The EPR spectra at most sites (except for 40R1, 42R1, and some N-terminal sites) are broadened beyond 100 G, a characteristic feature of strong spin-spin interaction (note the EPR spectral scan width of 150 G, Fig. 3). To characterize the effects of spin-spin coupling, we performed dilution experiments in which fibrils were grown from a mixture of R1-labeled and wild type Aβ. Assuming random co-mixing of R1-labeled and wild type peptide, such dilutions will increase the distance between spin-labeled derivatives and therefore reduce the line broadening and amplitude loss caused by spin-spin interaction.

The results from a representative mixing experiment for the 25R1 derivative are shown in Fig. 2B. In this figure, all EPR spectra are normalized to the same number of spins (i.e., corrected for the fact that decreasing amounts of spin labels were used, see “Experimental Procedures”). The addition of unlabeled peptide leads to sharper lines and a reduced spectral breadth, two features which in turn result in an increased spectral intensity. Thus, these mixing experiments directly demonstrate the existence of intermolecular spin-spin interaction between R1 labels from neighboring subunits in the fibril. In addition, the co-assembly of R1-labeled and wild type Aβ strongly suggests that both peptides must take up similar structures in the fibril. This and the electron microscopy, atomic force microscopy, and thioflavin T results indicate that the introduction of R1 did not alter the inherent ability of Aβ to form fibrils.

Similar experiments were performed with all other derivatives. Because the higher dilutions had the tendency to give noisier spectra, Fig. 4 only shows the corresponding 50% dilutions of R1-labeled and wild type Aβ (red traces). The dilution with unlabeled Aβ results in a significant increase in the signal amplitude compared with the undiluted form (black traces) for nearly all cases. As expected, the strongest effects are seen at sites where the undiluted fibril spectra have relatively low signal amplitudes and exhibit broadening beyond 100 G. These sites include the derivatives at positions 14–38. To determine the distances between like spin labels in the fibrils we compared the diluted and undiluted fibril spectra using previously developed methods as described under “Experimental Procedures” (26). Using this approach the main distances for sites 14–38 are in the range of 9–11 Å, with small contributions of larger distances. Therefore, as in the solid state NMR studies (14–17), individual side chains in the peptide are arranged in a highly ordered, in-register, and parallel manner within the fibril. Although distance measurement by SDSL is quite precise, measured values reflect distances between the nitroxide moieties on different spin labels and not between the peptide backbone atom to which the spin labels are attached. Thus, the small differences between the distances obtained by NMR and in the present study are likely due to this effect (also see discussion below).

Outside of this central region, significantly less spin-spin coupling is observed. In the N-terminal region (4R1, 7R1, and 8R1) some spin-spin interaction can be detected, but the overall...
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At some sites, however, the immobilization is less pronounced—elevated mobility (Fig. 5). Although the values are generally low (also see below), we observe weak spin-spin interactions (distances beyond 20 Å) are observed in the C-terminal regions. Thus, the N- and C-terminal regions are not likely to be part of the overriding parallel, in-register structure.

Mobility Analysis Suggests the Domain Organization in Fibrillar Aβ—Mobility analysis is one of the most powerful features of SDS Gel. To obtain mobility information, one first needs to separate the line shape effects that arise from spin-spin interaction from those caused by motional averaging of the hyperfine anisotropy. To a close approximation, this is the case for the spin-diluted spectra shown in Fig. 4. Spin dilution attenuates the dipolar broadening enough so that the dominating line shape features arising from R1 dynamics at the various sites can be compared.

Although the values are generally low (also see below), we observe regions of very strongly suppressed mobility (regions outside the gray boxes in Fig. 5) and other regions of slightly elevated mobility (gray boxes in Fig. 5). It should be noted that a very similar behavior was also observed for higher spin dilutions (20% labeled peptide; data not shown). Regions of lowest mobility are generally found in the most highly ordered regions of a protein structure and, in the present case, are therefore likely to correspond to tightly packed β-strand regions. In contrast, the C- and N-terminal regions, as already suggested by the distance analysis, are less likely to be part of this amyloid core and could take up other structures. As discussed below, the region around residues 23 and 25 is still likely to be part of the parallel-arranged core, but could represent a turn or bend region.

Comparison of Motion in Aβ Fibrils and Globular Proteins—The R1 mobility in many soluble proteins has been analyzed in terms of the inverse of the central line width. For comparison to our amyloid data, we chose data from another highly stable protein complex, the SDS and heat stable neuronal SNARE complex (19). The dashed line in Fig. 5 represents the "mobility border" between buried residues and all other more surface-exposed residues. In the SNARE complex the majority of all buried sites have mobility values falling below the line, whereas most other sites are above the line. Interestingly, almost all of the line shapes observed for R1-labeled Aβ derivatives are below this cutoff line, and therefore the corresponding spectra correlate with those from buried sites in a folded globular protein. Again, very similar behavior was observed at the higher spin dilutions (20% labeled peptide; data not shown). It needs to be pointed out that this immobilization is not a direct consequence of the very slow tumbling of amyloid fibrils. For example, EPR analysis demonstrates that high mobility in dynamic or surface regions is even observed in crystals of R1-labeled proteins (35).

It has previously been reported that Aβ fibrils are polymorphic and display different diameters and morphologies, including smooth, twisted, and branched fibers and fiber bundles (27–30). These polymorphisms are dependent upon the time of incubation and conditions used. Physiological pH and ionic strengths are known to result in the formation of fibrillar structures that are made up of multiple, individual protofilaments (30). As in actual Alzheimer’s plaques, there is some variation in the length and number of these individual filaments per fibril. Furthermore, there is a tendency of these fibrils to form bundles of fibrils, a property that we also noted in the present study. Despite their polymorphic appearance, the structure of the individual filamentous building blocks is thought to be very similar because fibers of different morphologies give the same x-ray diffraction pattern (36). The strong immobilization we observe at most of the labeled positions does not appear to be significantly influenced by the fibril morphology as similarly strong immobilization is also observed for fibrils grown under conditions that promote the formation of a homogeneous population of smooth, straight fibers (pH 3) (37) (data not shown). We can therefore conclude that the apparent overall low mobility in this amyloid fibril is a common feature that is independent of fibril morphology and is much more suppressed than in a globular protein.

Co-mixing of Aβ40 and Aβ42 in Amyloid Fibrils Suggests Similar Structural Architecture—The longer Aβ42 peptide is significantly more prone to aggregation and fibril formation (22), and it is thought that elevated levels of Aβ42 could contribute to the pathogenesis of Alzheimer’s disease. Although it has been demonstrated that Aβ42 can act as a seed that promotes the fibril formation of the shorter derivative (38), it has not been clear whether the different peptides co-mix within fibrils or whether they form separate domains or protofilaments. The ability to monitor the loss of spin-spin coupling in the R1-labeled derivatives of Aβ40 provides a tool to investigate this question. If R1-labeled Aβ40 co-mixes with unlabeled Aβ42, one expects a loss of spin-spin interaction resulting in spectra similar or identical to those obtained when diluted with unlabeled Aβ40.
Such dilution experiments were performed analogously to those outlined above. The similarity of the spectra obtained by dilution with unlabeled Aβ40 (Fig. 4, red traces) and Aβ42 (Fig. 4, green traces) is remarkable, suggesting that Aβ40 and Aβ42 co-mix equally well and that the resulting structure must be very similar. The similarity of the structures formed is further illustrated in Fig. 5, which shows that the measured line widths of the respective spectra for the Aβ40 (circles) and Aβ42 (diamonds) dilution are very similar.

DISCUSSION

Motivated by the successful application of SDSL to globular and membrane proteins of any molecular weight (25), we employed SDSL to reveal structural and dynamic features of amyloid fibril formation. Together with a recent SDSL study on transthyretin amyloid formation (39), the current data show that SDSL can be successfully applied toward determining the structures of amyloid fibril assemblies.

One of the main findings of the present study is the demonstration that amyloid fibrils of Aβ have domains of highly ordered, densely packed, and in-register parallel aligned structures. Based on spin-spin interaction between same residues on different peptides and mobility analysis we can define three regions (Fig. 5B): a central region of pronounced parallelism from approximately residue 14 to 38, a more disordered N-terminal region with less specific parallelism, and a short C-terminal stretch where little parallelism could be detected.

A parallel, in-register orientation has been reported for a shorter Aβ10–35 peptide (14–16, 40) using solid state NMR. More recently, a similar in-register orientation has been demonstrated based on analysis of four sites in the central region of the longer Aβ40 peptide (17). Thus, our data are in good agreement with these findings and also provide a more comprehensive analysis of the entire peptide. Together these data can already exclude some of the more complicated models for amyloid fibril structure that lack this essential in-register parallelism.

Nevertheless, there is one significant difference between the EPR and NMR results. On average the EPR distances for the central region are roughly 4–6 Å longer than those obtained by NMR. This difference may be attributed to the fact that SDSL measures distances between side chains, whereas NMR measures distances between the backbone atoms. Thus, our data can also be interpreted in terms of a parallel arrangement of β-strands, although other models such as parallel β-hairpins, parallel arrangement of β-sheets (41), and some parallel, in-register β-helical structures (42) cannot be excluded based on the EPR data alone.

A structural model of Aβ in the amyloid fibril must consider the hydrogen-deuterium exchange data (7) as well as the overall fiber and filament dimensions. These dimensions restrict the overall width of a single protofibril subunit to about 60 Å or less. As outlined in the introduction, an extended structure of 40 or 42 amino acids would exceed this length. Thus turns, loops, or bends are expected somewhere in the sequence. The occurrence of such regions should be reflected by an increase in the local mobility as such structures would have less stabilizing and constraining factors.

Can we use the present data to identify the possible location of β-strand and turn regions? Despite the strong suppression of mobility throughout we observe three regions of relatively increased mobility (see shaded areas in mobility plot, Fig. 5A and Fig. 5B). One such region comprises the first 10 N-terminal residues, where higher mobility components are observed in the EPR spectra. In concert with this increased mobility we also observe a larger distance between same residues and a significantly increased range of distances. Together, these data suggest that the N terminus is too disordered to be part of the presumably tightly packed β-sheets regions. This notion is in good agreement with protease digestion experiments (43) suggesting that the N terminus is sufficiently exposed in the amyloid fibrils to account for rapid protease digestion.

Other regions of increased mobility include sites 23–29 and the C terminus. Interestingly, the former region is made up of residues that have a high propensity for turn formation (Fig. 5B) and evidence of loop formation has been shown for this region in soluble forms of Aβ (8, 31, 44). Furthermore, the formation of a turn or loop in this region would be in agreement with Fourier transform infrared spectroscopic studies (8) and limit the length of possible β-strands to ~10 amino acids, thereby fulfilling the geometric constraints given by the fibril dimensions. It should be noted that not each of the sites in the putative strand regions was spin-labeled. Therefore we cannot completely exclude the possibility that other (albeit short) turn regions (45) exist in these regions, and the estimate of 10 amino acids per β-strand should be viewed as an upper limit.

The increased mobility at the C terminus might be somewhat surprising considering that the addition of two amino acids in the case of Aβ42 strongly favors the formation of fibrils and that this region is often thought of as being at the core of the fibrils. However, there are several other reasons besides the mobility data that make it unlikely that the C-terminal region is at the core of the fibril. The co-mixing data presented here argue that the Aβ40 and Aβ42 can be mutually substituted by each other. Thus, the incorporation of two additional residues in Aβ42 does not appear to affect the overall structure or position of Aβ42 in the fibril. This result would not be expected if the additional amino acids had to reside in a tightly packed core. The notion of less ordered C-terminal (and N-terminal) regions excluded from the core structure is also in good agreement with a recent study on β-microglobulin light chain amyloid fibrils (46). The exclusion of the N and C termini from the cross β-core of the fibril may be a common feature of amyloids, especially those amyloids that form from the local misfolding of the intact protein where large regions of protein structure must be accommodated outside of the fiber lattice.

In summary, the results show that SDSL is a powerful new tool for studying structural features of amyloid fibrils. The EPR spectra presented here significantly constrain the number of possible structures in the amyloid fibrils. Systematic mapping of inter- and intramolecular distances by SDSL should be able to further resolve this issue and perhaps ultimately provide sufficient experimental constraints to generate a three-dimensional structure. Finally, the dynamic range of SDSL will also make it possible to compare the structures in fibrils to those of smaller intermediates.

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