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An Unusual Peptide Conformation May Precipitate Amyloid Formation in Alzheimer's Disease: Application of Solid-State NMR to the Determination of Protein Secondary Structure†

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ABSTRACT: The formation of insoluble proteinaceous deposits is characteristic of many diseases which are collectively known as amyloidosis. There is very little molecular-level structural information available regarding the amyloid deposits due to the fact that the constituent proteins are insoluble and noncrystalline. Therefore, traditional protein structure determination methods such as solution NMR and X-ray crystallography are not applicable. We report herein the application of the solid-state NMR technique rotational resonance (R^2) to the accurate measurement of carbon-to-carbon distances in the amyloid formed from a synthetic fragment (H_2N-LeuMetValGlyGlyValValIleAla-CO_2H) of the amyloid-forming protein of Alzheimer's disease (AD). This sequence has been implicated in the initiation of amyloid formation. Two distances measured by R^2 indicate that an unusual structure, probably involving a cis amide bond, is present in the aggregated peptide amyloid. This structure is incompatible with the accepted models of fibril structure. A relationship between this structure and the stability of the amyloid is proposed.

Amyloid deposits are characteristic of many diseases (Castaño & Frangione, 1988; Glenner, 1980a,b; Stone, 1990; Goate et al., 1991) including Alzheimer's disease (Selkoe, 1990; Glenner, 1988) and type II diabetes (Nishi et al., 1990). The deposits, or plaques, comprise protein fibrils which share affinity for certain dyes (Cooper, 1974) and a regular, repeating structure (Crowther, 1991). The naturally derived amyloid plaque from AD[1] brain has been analyzed by X-ray fiber diffraction and produces a distinctive pattern of reflections (Kirschner et al., 1986). This diffraction pattern was observed for *Bombyx mori* silk (Marsh et al., 1955) and for polyalanine (Pauling & Corey, 1953b) and gave rise to Pauling's widely accepted model of a cross-β fibril (see Figure 1) (Marsh et al., 1955). The cross-β fibril model consists of antiparallel peptide chains which are arranged perpendicular to the direction of fibril growth, to form a β-pleated sheet. The strands in each β-sheet interact via a network of interstrand hydrogen bonds (Marsh et al., 1955; Arnott et al., 1967). Sheets are stacked in a parallel fashion, the intersheet distance being governed by the identity of the amino acid side chains (Marsh et al., 1955; Geddes et al., 1968). Although lamellar silk crystals have been grown (Lotz et al., 1982), single crystals suitable for crystallographic studies have not been produced; hence, the details of the cross-β fibrillar structure and its constituent antiparallel β-sheet have not been elucidated. This information is required in order to fully understand the factors which direct amyloid formation and, more generally, the sequence dependence for β-sheet formation (von Heijne & Blomberg, 1977; Lifson & Sander, 1980).

The synthetic nine amino acid peptide H_2N-LMVGGVVIA-CO_2H (β34–42) (Halverson et al., 1990) represents the carboxy-terminal portion (residues 34–42) of the 42 amino acid β/A4 protein. The β/A4 protein is the major constituent of the extracellular amyloid plaque that characterizes the brains of victims of AD and advanced Down's syndrome (Masters et al., 1985). The 34–42 portion of the β/A4 sequence is thought to be part of the transmembrane sequence in the amyloid precursor protein (Selkoe, 1990; Glenner, 1988). Proteolytic excision of the β/A4 sequence from the precursor protein may be characteristic of the disease state (Sisodia et al., 1990). The β34–42 peptide is extremely insoluble and readily forms cross-β fibrils which can be observed by electron microscopy (Halverson et al., 1990). This

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[1] Abbreviations: R^2, rotational resonance; AD, Alzheimer's disease; FABMS, fast atom bombardment mass spectrometry; MM, molecular mechanics; ssNMR, solid-state NMR; CPMAS, cross-polarization magic-angle spinning.

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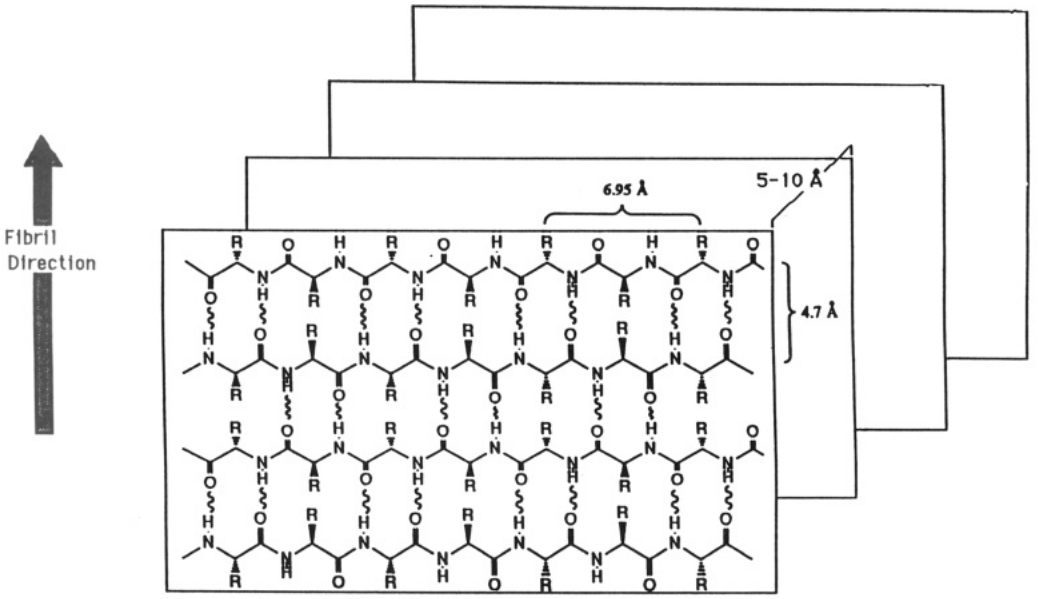

FIGURE 1: Schematic depiction of the cross-β fibril as proposed by Pauling et al. (Marsh et al., 1955). The alignment of the strands relative to each other is uncertain (Arnott et al., 1967).

structural similarity and the fact that amino acid deletions at the C-terminus have a profound effect on the solubility of cerebrovascular amyloid (Joachim et al., 1988; Prelli et al., 1988), led to our postulate that the β34–42 fibrils may represent a "core" structure which is responsible for initiating fibril formation in vivo (Halverson et al., 1990). The β34–42 fibrils have been analyzed using Fourier transform infrared spectroscopy (FTIR) in combination with isotope labeling, revealing irregularities in the β-sheet structure which cannot be observed by other methods (Halverson et al., 1991).

Solid-state NMR studies were initiated with the goal of describing the aggregating structure of β34–42 at the molecular level. The rotational resonance (R^2) determination of the distance between members of a homonuclear spin pair is based on rotation-enhanced transfer of Zeeman magnetization. This transfer occurs when the MAS frequency ω_r is adjusted to satisfy the R^2 condition $\delta = n\omega_r$, where δ is the isotropic chemical shift difference between the two resonances of the spin pair (Andrew et al., 1963; Raleigh et al., 1988; Levitt et al., 1990; Colombo et al., 1988), n is a small integer, and ω_r is the spinning speed. For carbon–carbon distances, appropriate samples may be engineered by selective pairwise ^{13}C labeling.

The R^2 technique has been successfully applied to distinguish between two possible structures on the basis of a single distance measurement (McDermott et al., 1990; Creuzet et al., 1991). In order to utilize this technique for the systematic elucidation of protein structure, each dihedral angle (ω, ϕ, ψ) in the peptide backbone must be defined. A simple method for the systematic translation of R^2 distances into protein structure is outlined in this paper. The approach is illustrated by the detection of an unusual local conformation in the amyloid-forming peptide β34–42.

Materials and Methods

Synthesis, Purification, and Characterization of β34–42 ^{13}C-Labeled Analogues. The peptides used in this study (abbreviated according to ^{13}C label positions as α37–38, 37–α38, and 37–α39) were synthesized, purified, and characterized according to a published method (Halverson et al., 1990). ^{13}C-Labeled amino acids were purchased from Cambridge Isotope Laboratories and protected as the *tert*-butyl carbamates (Itoh et al., 1975). Label positions were verified by FABMS–collision MS sequence analysis. We thank Dr. Ioannis Papayannopoulos of the MIT mass spectrometry facility, supported by NIH Division of Research Resources, Grant RR00317 to Professor Klaus Biemann.

Solid-State ^{13}C NMR. Magic-angle spinning (MAS) spectra were collected on a home-built spectrometer operating at a proton frequency of 398 MHz and a ^{13}C frequency of 100 MHz. A home-built probe was used, with a stator and rotors from Doty Scientific, Inc. (Columbia, SC). Typical ^1H and ^{13}C 90° pulse lengths were 3.5 and 4.5 μs, respectively. Recycle delays were 3 s and the cross-polarization time used was 2.0 ms. Spectra were recorded at room temperature. Principal values of the shift tensors for each labeled carbon were extracted by computer simulation of the rotational side-band spectrum (Herzfeld & Berger, 1980). The values obtained were virtually identical to those observed for rigid lattices.

Rotation-enhanced magnetization transfer is observed as follows (Raleigh et al., 1988), with proton decoupling applied throughout. After initial enhancement of ^{13}C magnetization by cross-polarization and storage along the z direction of the rotating frame, one of the two resonances is selectively inverted. The resulting nonequilibrium state evolves for a specified delay time, after which an observation pulse is applied to detect remaining longitudinal magnetization. The entire experiment is repeated for a number of delay times (see Figure 2). The difference between the longitudinal magnetizations of the two sites as a function of delay time is then compared with simulations, which depend on several parameters, including the dipolar coupling (Levitt et al., 1990). In general, a larger dipolar coupling, that is, a shorter internuclear distance, leads to a more rapid monotonic decay in the difference magnetization. In all of the experiments described here, the $n = 1$ R^2 was used; that is, the spinning speed was equal to the difference in isotropic shifts between the labeled sites. On the 398-MHz (proton frequency) spectrometer employed, this difference was in the range of 11–13 kHz.

In order to determine the peptide backbone structure, a number of intercarbon distances must be determined to within a fraction of an angstrom, necessitating particularly close attention to potential sources of error. Systematic errors may result from the following factors. If the R^2 condition is not exactly satisfied due to spinning speed fluctuations or inaccuracies in shift determinations, magnetization exchange will

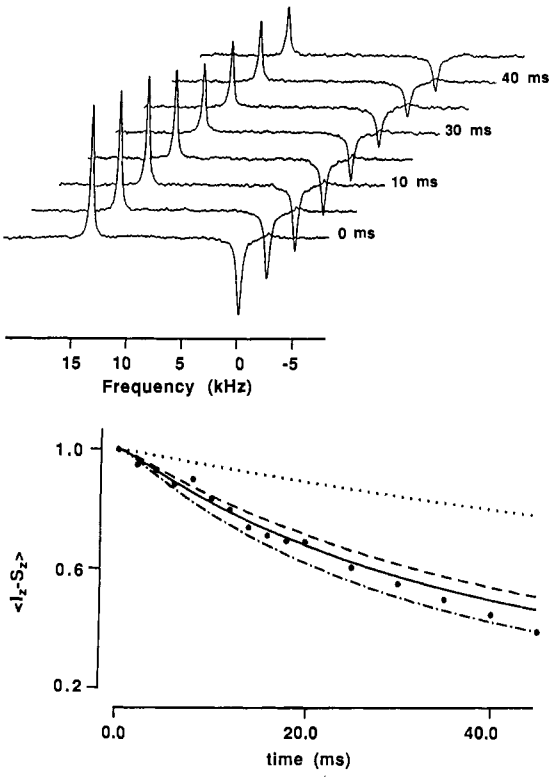

FIGURE 2: (Top) Series of ^{13}C MAS spectra of β34–42 labeled at the α carbon of Gly37 and the carbonyl carbon of Gly38 (α37–38 distance). The spectra were obtained at a ^{13}C frequency of 100 MHz and with a MAS frequency of 12.487 kHz corresponding to the n = 1 rotational resonance. The times shown denote the interval between inversion of the labeled methylene resonance, which occurs immediately after cross polarization, and application of the observation pulse. Thus, 0 ms corresponds to the initial nonequilibrium state established by the selective inversion. The rate of this decrease may be modeled to determine the distance between the labeled sites. (Bottom) Comparison of magnetization exchange data (filled circles) for α37–38 along with the calculated curves for four distances: 4.74 Å (dotted line), 4.0 Å (dashed line), 3.9 Å (solid line), and 3.8 Å (dot-dashed line). The 4.74-Å distance would be expected for an idealized antiparallel β-strand (Marsh et al., 1955). The best fit to the data is 3.9 Å. This data is for the undiluted α37–38 sample; final distance determination (α37–38 = 4.0 Å) was taken from the 1:5 dilution.

occur more slowly than predicted, leading to a distance overestimate. Since departures from exact R^2 were much less than the line widths, this was a small effect (F. Creuzet, D. P. Raleigh, M. H. Levitt, and R. G. Griffin, unpublished results), leading to an error of less than 0.05 Å. Distance underestimates may result from drifts off of the optimum cross-polarization condition, which is very narrow at these spinning speeds (Stejskal et al., 1977; Wind et al., 1988). The resulting decrease in ^{13}C signal would mimic greater magnetization exchange. This was controlled for by normalizing each exchange data point to an appropriate zero reference. To eliminate possible effects due to intermolecular interactions, measurements were performed on isotopically pure samples and on samples in which the doubly labeled peptide was diluted 1:5 or 1:10 in unlabeled peptide. A particularly strong dilution effect was seen in the 37–α39 exchange curve, which, for the undiluted sample, was fit by a distance of 3.8 Å. However, at 1:5 and 1:10 dilutions a distance of 4.8 Å was obtained. Finally, difficulty in exactly determining the time constant of the decay of zero-quantum coherence between the two spins, the T_2^{ZQ}, can lead to a distance underestimate (Creuzet et al., unpublished results). However, the success of control distance determinations (Raleigh et al., 1988; Creuzet et al., unpublished results; and see text) provides strong support for the model of uncorrelated random fields (Kubo & McDowell, 1988) used to estimate T_2^{ZQ}. A systematic error which may lead to an over- or underestimate of distance in a particular case is the dependence of the magnetization exchange on chemical shift orientation. Rigid lattice tensor elements, obtained from slow spinning spectra and chemical shift tensors taken from glycine (Haberkorn et al., 1980), were employed as input for the distance calculations. However, when the side band intensities are weak, as is the case for the n = 1 R^2 data presented here, these angular dependencies are small and typically lead to errors of less than 0.05 Å in our experiments. Random errors may result from the following factors. Finite spectral signal-to-noise appears as scatter of experimental data about simulated magnetization exchange curves. Data collection was designed so that multiple measurements were made at each delay time, allowing the magnitude of this effect to be estimated. In all cases, a distance can be defined to within 0.15 Å or less. Signal-to-noise considerations also lead to random error in the estimate of T_2^{ZQ}; appropriate signal averaging essentially eliminated this error.

Molecular Modeling. Molecular modeling was performed on a CAChe benchtop work system. Energy calculations utilized the CAChe MM2 program with augmented force field. Geometric parameters were taken from crystallographic data (Ramachandran & Sasisekharan, 1968). Due to difficulties in modeling intermolecular interactions, no energy minimization was attempted. Amide planarity was assumed, although small deviations from planarity were shown not to lead to significant variations in the derived structure.

RESULTS

Peptide β34–42 Exists as a Single Conformer in the Amyloid. Solid-state ^{13}C MAS NMR (Griffin et al., 1986; Mehring, 1983) spectra of the β34–42 fibrillar aggregate revealed the presence of two resolved amide carbonyl resonances; the minor line was upfield by ca. 3 ppm. This phenomenon was independent of sample preparation. Several β34–42 analogues, each ^{13}C-labeled at a single amide, were studied, resulting in the assignment of the minor upfield resonance to both the Gly37 and the Gly38 amide carbonyl carbons. However, the possibility exists that the carbonyl of Leu34, Met35, and/or Ile41 may have the same chemical shift as these glycines. This observation and the relatively narrow line width of the Gly38 amide resonance (ca. 200 Hz) suggested that the peptide exists as a single conformer in the aggregate. The possibility that motional averaging is affecting these measurements is remote, due to the similarity of the determined shift tensor elements to rigid lattice values (see Materials and Methods). The portion of the peptide backbone surrounding Gly38 became the focus of the studies reported herein.

A Known Distance in Glycylglycine and a Conformation-Independent Distance in β34–42 Were Correctly Measured. Although the accuracy of the R^2 method has been validated by measurement of several known distances (Raleigh et al., 1988; Creuzet et al., unpublished results), two additional controls were performed. The distance between the α carbon of the N-terminal glycine and the carboxyl carbon of the C-terminal glycine of the dipeptide glycylglycine hydrochloride, known from crystal structure data to be 4.56 Å (Koetzle et al., 1972), was measured. The R^2 experiment yielded a distance within ±0.1 Å of the crystallographic data. A distance determination was also made within the β34–42 peptide itself, between the carbonyl carbon of Gly37 and the α carbon of Gly38 (see Figure 3). A distance of 2.4 ± 0.2 Å was measured (see Figure 3), in accord with typical peptide-bond geometry (2.45 Å for a trans amide and 2.49 Å for a cis amide;

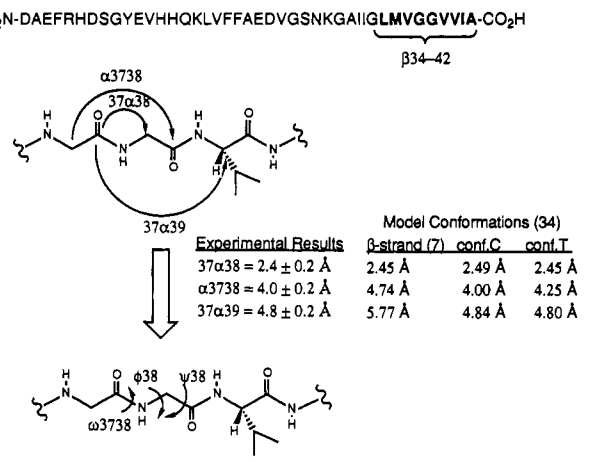

FIGURE 3: Schematic representation of the Gly37Gly38Val39 region of β34–42, showing the distances measured by R^2 and those dihedral angles which have been determined. The experimentally determined distances are listed in tabular form along with the distances derived from models of three conformations; the idealized β-strand (Marsh et al., 1955), conformation C, and conformation T. The sequence of the β/A4 protein is shown at the top, with the β34–42 sequence in boldface.

Ramachandran & Sasisekharan, 1968).

Two Measured Conformation-Dependent Distances Indicate the Presence of an Unusual Local Structure. The R^2 method can be used in combination with molecular modeling to define the backbone structure of a peptide or protein, given a set of experimentally determined distances. Two conformation-dependent intercarbon distances were measured in the β34–42 aggregate. The distance between the α carbon of Gly37 and the carbonyl carbon of Gly38 (abbreviated α37–38; see Figure 3) was used to determine the dihedral angle ϕ38 and the configuration of the 37–38 amide bond (ω37–38). The α37–38 distance was measured to be 4.0 ± 0.2 Å, indicating that the Gly37–Gly38 amide bond has the unusual cis configuration (if ϕ38 = ±121°, then α37–38 = 4.00 Å) or that a strained conformation containing a trans amide is present (if ϕ38 = 0°, then α37–38 = 4.25 Å). The distance between the carbonyl carbon of Gly37 and the α carbon of Val39 (abbreviated 37–α39, see Figure 3) depends on ϕ38, ψ38, and ω38–39. Given the two alternative conformations dictated by the α37–38 measurement, the experimental 37–α39 distance of 4.8 ± 0.2 Å constrains the dihedral angle ψ38 such that two representative conformations must be considered (see Figure 4). It is important to note that for both of these, conformation C (ω37–38 = 0°, ϕ38 = 121°, ψ38 = −25° and ω37–38 = 0°, ϕ38 = −121°, ψ38 = 25°) and conformation T (ω37–38 = 180°; ϕ38 = 0°, ψ38 = ±134°), two mirror image conformations are possible.

Conformation C May Be More Stable Than Conformation T. Simple molecular mechanics calculations were used to determine the energies of a tripeptide GlyGlyVal in the conformations analogous to conformation C and conformation T and in the idealized antiparallel β-strand conformation. The conformation C model was calculated to be ca. 3 kcal/mol higher in energy than the β-strand, consistent with previous determinations of the cis–trans energy difference (Stewart et al., 1990). The conformation T model was calculated to be ca. 17 kcal/mol higher in energy than the β-strand. The energy of the conformation T model was very sensitive to the angle ψ38, rotation of which can relieve the unfavorable electrostatic interactions between the carbonyl oxygens of Gly37 and Gly38. However, these rotations result in conformations which deviate from the experimental distances to a greater degree than does conformation T. It is important to note that these MM2 calculations ignore solvation effects, resulting in an overestimate of the oxygen–oxygen interaction which exists in conformation T. It is also possible that conformation T could be stabilized by metal binding.

Discussion

A detailed understanding of the structure of amyloid is a prerequisite for the design of a compound which could bind to the amyloid plaque or, possibly, prevent its formation. This structure is difficult to study because the amyloid is extremely insoluble and fibrillar proteins do not form single crystals of the type required for crystallographic studies. The solid-state NMR technique of rotational resonance can provide structural information on proteins of this type, at a level of accuracy typical of most X-ray crystallographic studies. This method has been demonstrated in several experiments (Raleigh et al., 1988; Creuzet et al., unpublished results; McDermott et al., 1990; Creuzet et al., 1991); however, the experiment reported herein is the first application of this technique to the determination of protein structure.

For the determination of peptide backbone structure, two types of carbon-to-carbon distances must be measured. The distance between the α carbon of one amino acid and the carbonyl carbon of the next amino acid (α1–2, e.g., α37–38, Figure 3) determines ω1–2. If α1–2 ≤ 4.25 Å, then ω = 0° (cis), and if α1–2 ≥ 4.4 Å, then ω = 180° (trans). Otherwise, both cis and trans amide conformations must be considered. In addition, the ϕ2 dihedral angle can be determined from the α1–2 distance. The 1–α3 distance (e.g., 37–α39, Figure 3) depends on the angles ϕ2 (e.g., ϕ38) and ψ2 (e.g., ψ38) such that, given ϕ2, the experimental 1–α3 distance is sufficient to determine ψ2. In the general case, given ω1–2 and ω2–3, a maximum of two pairs of mirror-image conformations will fit a given pair of experimental α1–2, 1–α3 distances. For non-glycine residues, those conformations characterized by positive values of ϕ can be eliminated on energetic grounds (Ramachandran & Sasisekharan, 1968). Additional types of distances (e.g., α1–3) may need to be measured in order to differentiate between the possible conformers along the remainder of the β34–42 backbone.

In order to generate possible conformers, a simple computerized ball and stick model was used, although the conclusions presented herein can be reached using Dreiding models. Energy minimization was not applied due to the difficulties in modeling intermolecular interactions in the peptide aggregate. Commercial modeling programs must be carefully checked to assure that bond length and bond angle parameters are in agreement with crystallographic data (Ramachandran & Sasisekharan, 1968).

A conformation in which the Gly37Gly38 amide is cis and the Gly38Val39 amide is trans (conformation C, see Figure 4) is most consistent with the experimental data (modeled distances α37–38 = 4.00 Å and 37–α39 = 4.84 Å; see Figure 3). A trans, trans conformation (conformation T, Figure 4) should also be considered because it approaches the error limits of the R^2 measurements (α37–38 = 4.25 Å, 37–α39 = 4.80 Å). However, in conformation T, the ϕ38 dihedral angle (0°) which is required in order to minimize the α37–38 distance is very high in torsional strain energy (Ramachandran & Sasisekharan, 1968). In addition, conformation T suffers from unfavorable interactions between the oxygen lone pairs of the Gly37 and Gly38 amide carbonyls (this interaction is enforced by the experimental 37–α39 distance, which requires ψ38 to be ±134°). Molecular mechanics calculations indicate that conformation C is considerably more stable than conformation

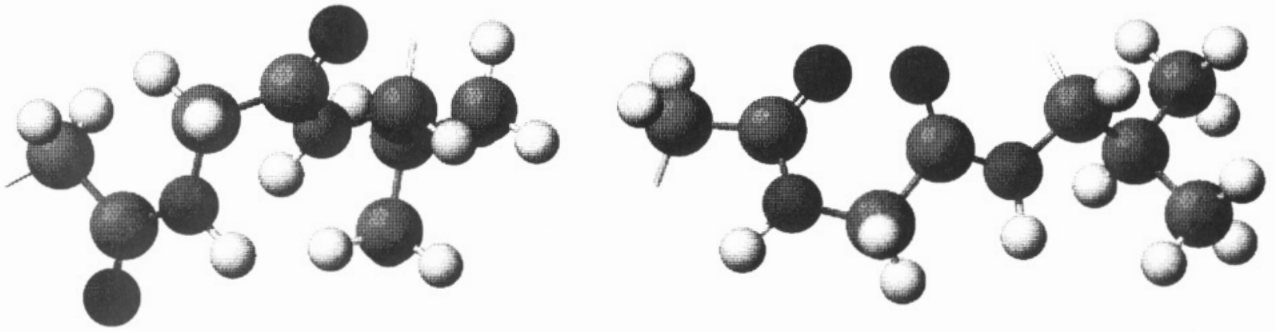

FIGURE 4: Two possible conformations for the region of the β34–42 peptide backbone extending from the α carbon of Gly37 to the α carbon of Val 39: conformation C (ω37–38 = 0°, φ38 = −121°, ψ38 = 25°, α37–38 = 4.00 Å, 37–α39 = 4.84 Å) at the left and conformation T (ω37–38 = 180°, φ38 = 0°, ψ38 = −134°, α37–38 = 4.25 Å, 37–α39 = 4.80 Å) at the right. For both conformations, φ39 = −41° and ω38–39 = 180°. No information is currently available concerning ψ37. Therefore, this dihedral angle and the angles of the Val side chain were modeled to minimize steric and torsional strain. The configuration of the Gly38–Val39 amide bond is unknown, but the trans amide is more likely on energetic grounds. This uncertainty does not affect the conclusions regarding the Gly37–Gly38 amide.

T, although the magnitude of this difference is in question. Conformation C was calculated to be less stable than the β strand (Marsh et al., 1955) by 2–3 kcal/mol, consistent with experimental cis vs trans ΔG determinations for model amides (Stewart et al., 1990). It is important to note that the relative energy of conformation C is very sensitive to the identity of the side chains involved (the lowest energy case is GlyGly), whereas the energy of conformation T is relatively insensitive to sequence. Rotation around the Gly37Gly38 amide bond, which is required to produce conformation C from the β-strand, requires an activation energy of ca. 20 kcal/mol, which translates to a half-life of ca. 7 s (Stewart et al., 1990). Although this isomerization may be slower than the well-studied proline imide rotation (King et al., 1986; Harrison & Stein, 1990), it is still likely to be a physiologically relevant event. We conclude, therefore, that the structure of the Gly37–Gly38 region of the peptide β34–42 in the amyloid probably resembles that of conformation C.

Cis amide bonds have been identified in several crystalline proteins (Stewart et al., 1990); however, the apparent frequency of their occurrence is far less than one would expect on the basis of thermodynamic arguments (a free energy difference of ca. 3 kcal/mol translates to an equilibrium distribution of ca. 99:1 at 298 K) (Stewart et al., 1990). This discrepancy may be due to the misassignment of amide stereochemistry in structures derived from the relatively low-resolution electron density maps which are available for most proteins (Stewart et al., 1990). The cis amides present in globular proteins must enable the formation of a conformation that affords stabilizing tertiary interactions which offset the energetic cost (ΔG) of amide isomerization. One amide bond in the backbone of the heptadecapeptide bombolitin assumes the cis conformation when the peptide is in a micellar environment (Bairaktari et al., 1990). In that case, favorable peptide–micelle interactions must be sufficient to pay the energetic cost of amide isomerization. Similarly, the peptide β34–42 must derive a significant amount of intermolecular interaction energy from the aggregation of a conformer (C or T) which probably represents <1% of the equilibrium mixture. This aggregation energy, which is apparently unavailable to the idealized β-strand, may result from interstrand interactions (hydrogen bonding, side-chain packing) or sheet–sheet stacking interactions. We believe that the structure present at Gly38 facilitates structural changes which lead to the formation of unusually stable aggregates. It is interesting to note that Pauling presented two models for the cross-β fibril in which amide bonds were alternately cis and trans (Pauling & Corey, 1953a). Construction of a model for the β34–42 amyloid awaits the complete elucidation of the aggregating conformation.

Solution NMR techniques such as nuclear Overhauser effect spectroscopy (NOESY) are commonly used to determine the structure of soluble proteins. The determination is based on a number of relatively inaccurate distance measurements which are used as constraints. In contrast, the R^2 method allows the accurate measurement of distance and therefore requires fewer distances for local structure determination. The technique of rotational resonance allows the first detailed description of the structure of amyloid proteins. This approach may also be useful for the study of other proteins which are difficult to crystallize, including transmembrane proteins (analyzed in a lipid bilayer; Creuzet et al., 1991) and noncrystalline globular proteins.[2]

Conclusions

Amyloid comprising a peptide derived from a portion of the β/A4 protein which may initiate amyloid formation (Halverson et al., 1990) satisfies the traditional criteria of cross-β fibrillar structure (Marsh et al., 1955). However, we have identified an unusual structural feature, probably involving a cis amide bond, in the peptide amyloid. We expect that the aggregating conformation is sparsely populated at equilibrium, but due to the favorable intermolecular interactions which accompany aggregation, it is the exclusive structure present in the amyloid. This phenomenon may be unique to the β34–42 peptide or may characterize amyloid proteins in general. Further studies are in progress to completely define the backbone structure of the β34–42 peptide amyloid and to elucidate the intermolecular interactions which drive amyloid formation.

Acknowledgments

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