High-Resolution NMR Spectroscopy of the β-Amyloid(1–28) Fibril Typical for Alzheimer's Disease

Emmanuel Mikros,* Dimitra Benaki, Eberhard Humpfer, Manfred Spraul, Spyros Loukas, Chariklia I. Stassinopoulou, and Maria Pelecanou*

One of the major markers of Alzheimer's disease (AD) is the extracellular deposition of fibrous protein aggregates, called senile or amyloid plaques, in brain tissue.[1] Even though a causal link between plaque formation and AD has not been firmly established, increasing evidence suggests that amyloid deposition may play a critical role in the neurodegenerative process.[2] The major component of amyloid plaques is the 39- to 42-aminoacid β-amyloid peptide (β-AP) which is deposited in the form of protease-resistant amyloid fibrils.[3]

Knowledge of the β-AP fibril structure is essential for understanding the abnormal assembly and deposition of this peptide in AD and could lead to the rational design of therapeutic agents for the prevention or even disaggregation of the fibrils. Furthermore, elucidation of the structure of β-AP fibrils would be an important step towards understanding the propagating structure of other fibrillar proteins involved in systemic amyloidoses, all of which form fibrils with very similar morphology and histochemical properties.[4]

Fortunately, the highly ordered structure of β-AP fibrils can be replicated in vitro by using synthetic β-APs of various lengths,[5] a fact that has allowed systematic structural studies. Electron microscopy has revealed that amyloid fibrils are rigid, nonbranching, with lengths exceeding 1 μm and diameters in the order of 10 nm.[6] X-ray diffraction measurements from amyloid fibrils showed the "cross-β" pattern characteristic for an extended β-sheet structure with polypeptide chains running perpendicular to the long axis of the fibril and interchain hydrogen bonds roughly parallel to this axis.[7]

Since amyloid fibrils are noncrystalline, insoluble, high molecular weight aggregates, they are intractable to study by the usual high-resolution structural methods, like X-ray crystallography and solution NMR spectroscopy, and, therefore, atomic details of their structure remained unknown until recently. Interestingly enough, most of the structural models proposed for the amyloid fibril are based on an antiparallel β-sheet organization.[6b, 7d, 8] These structural models, however, are largely derived from molecular modeling and are unconstrained by experimental data. Experimental support for the antiparallel arrangement of the strands is limited to IR spectroscopy and specifically to the observation of a weak amide I vibrational band near 1690 cm^{-1}[9] which has been assigned to antiparallel β-sheets by empirical studies of model peptides and by theoretical studies.[10]

The application of solid-state NMR techniques to the elucidation of the structure of amyloid fibrils is of critical importance because solid-state NMR spectroscopy is uniquely capable of providing high-resolution, site-specific structural constraints at the level of specific interatomic distances and torsion angles.[11] The applications reported so far have utilized ^{13}C-labeled peptides and have determined ^{13}C-^{13}C dipolar couplings and hence ^{13}C-^{13}C distances. The first set of measurements on β-AP fibrils was carried out on a doubly ^{13}C-labeled nine-residue β-AP(34–42) fibril and, in combination with molecular modeling, the antiparallel structure was proposed.[12] Recently, additional experiments on seven-residue β-AP(16–22) indicated an antiparallel β-sheet structure as well.[13] However, experiments carried out with longer peptide fibrils provide solid experimental evidence for a parallel β-sheet structure. Specifically, ^{13}C-^{13}C dipolar couplings were determined in singly ^{13}C-labeled β-AP(10–35) fibrils[14] as well as in full-length β-AP(1–40) fibrils.[15] In both studies, the measured distance between labeled carbons was approximately 5 Å, a fact which is in agreement with a parallel β-sheet with residues in exact register. The different results in the case of β-AP(34–42)[12] and β-AP(16–22)[13] are attributed to the limited length of the peptide chain.[11]

We now report high-resolution magic angle spinning (HR-MAS) ^1H NMR studies on the β-AP(1–28) fibrils. β-AP(1–28) readily forms fibrils in vitro that are similar in ultrastructure to the in vivo amyloid and aggregate into large bundles that resemble those of senile plaque cores.[5d,e, 6b, 16] The spectral resolution achieved with the HR-MAS method, which is applied to systems that maintain some degree of motional freedom,[17] enabled the application of two-dimensional NMR spectroscopy and the assignment of the spectra on the basis of total correlation (TOCSY) and nuclear Overhauser effect (NOESY) experiments. To our knowledge, this is the first time that high-resolution ^1H NMR data are reported for amyloid fibrils. The data are in agreement with an in-register, parallel organization of the fibrils.

NMR samples were prepared in 90 % H_2O/10 % D_2O under conditions described for rapid fibril formation.[5f] Evidence for full fibrillization was obtained through optical polarizing microscopy of the fibrillized sample after staining with Congo red. Strong green-yellow birefringence, that is characteristic of amyloid fibrils,[18] was observed from more than 95 % of the sample indicating full fibrillization (microscope image provided as Supporting Information). In addition, no signals were observed in either a usual liquid-state 500 MHz ^1H NMR spectrum or a circular dichroism spectrum of the sample indicating that the HR-MAS signals really come from amyloid fibrils.

[*] Prof. Dr. E. Mikros, Dr. D. Benaki
Division of Pharmaceutical Chemistry
Department of Pharmacy
University of Athens
15771 Athens (Greece)
Fax: (+30) 1-7274747
E-mail: mikros@pharm.uoa.gr

Dr. M. Pelecanou, Dr. S. Loukas, Dr. C. I. Stassinopoulou
Institute of Biology
National Centre for Scientific Research "Demokritos"
15310 Athens (Greece)
E-mail: pelmar@mail.demokritos.gr

Dipl.-Chem. E. Humpfer, Dr. M. Spraul
Bruker Analytik GmbH
Silberstreifen, 76287 Rheinstetten (Germany)

Supporting information for this article is available on the WWW under http://www.angewandte.com or from the author.

  1433-7851/01/4019-3603 $ 17.50+.50/0

The identification of most of the spin systems was unambiguously achieved from amide proton TOCSY connectivities (Figure 1). The assignment of resonances to

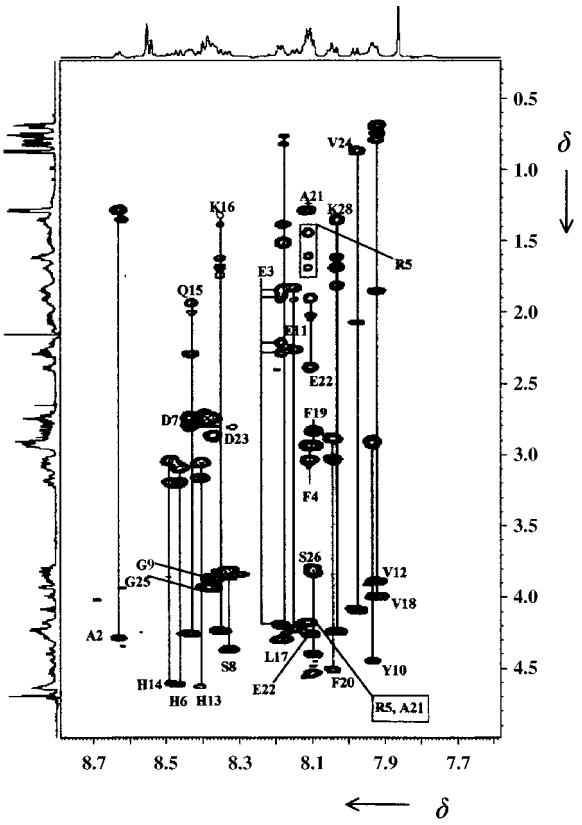

Figure 1. Region of a TOCSY spectrum of β-AP(1–28) fibrils (H_2O/D_2O 9:1, 300 K, 600 MHz , mixing time 80 ms) with assignments of intraresidue connectivities.

individual amino acids was accomplished by the combined analyses of the αH_i-NH_{i+1} connectivities in the fingerprint regions of the TOCSY and NOESY spectra and ascertained by the examination of the βH_i-NH_{i+1} connectivities. The complete assignments for the ^1H resonances of all amino acid residues are provided as Supporting Information.

All NOEs recorded in the NOESY spectra and their relative intensities are presented in Figure 2. αH_i-NH_i and αH_i-NH_{i+1} connectivities are present over the full length of

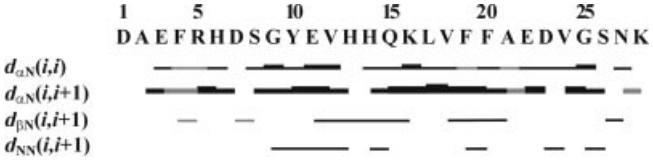

Figure 2. Visualization of NOE connectivities measured for β-AP(1–28) fibrils (H_2O/D_2O 9:1, 300 K). The intensities of NOE crosspeaks are indicated by the thickness of the bars, grouped into strong, medium, weak, and very weak. Where unambiguous assignment was not possible because of peak overlap, NOE connections are drawn with gray boxes.

the peptide. Weak NH_i-NH_{i+1} NOEs were observed for residues 9–13, 14/15, 19/20, 23/24, and 25/26, as shown in the contour plot of the NH-NH region (Figure 3). No long-range NOEs were observed.

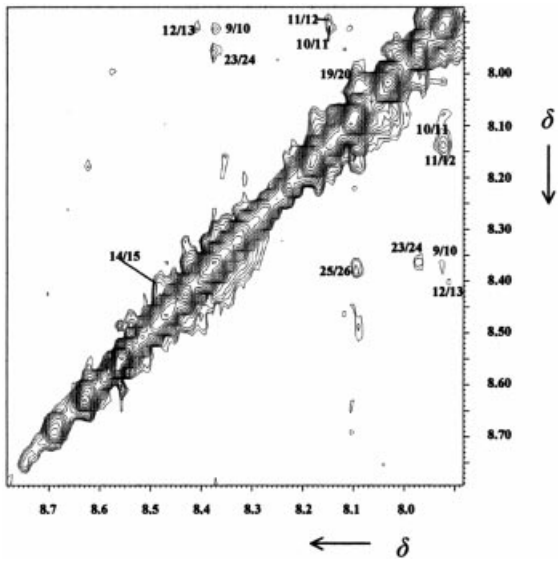

Figure 3. NH-NH region of a 150-ms NOESY spectrum of β-AP(1–28) fibrils (H_2O/D_2O 9:1, 300 K, 600 MHz) showing some weak sequential NH_i-NH_{i+1} connectivities.

The presence of strong αH_i-NH_{i+1} connectivities combined with the relatively weaker αH_i-NH_i crosspeaks and the absence of αH_i-NH_{i+3} and αH_i-βH_{i+3} connectivities is in agreement with a β-sheet structure.[19] Regarding the basic structural issue of a parallel or an antiparallel arranged β-sheet, the absence of NOEs between α-protons of adjacent strands is in agreement with a parallel arrangement since the distance $d_{\alpha\alpha}$ is 2.3 Å in the case of the antiparallel β-sheet and 4.8 Å in the case of a parallel β sheet.[19] Furthermore, the presence of weak consecutive NH_i-NH_{i+1} NOESY crosspeaks for residues 9 to 24 agrees with the in-register, parallel structure. As can be seen from Figure 4, this structure

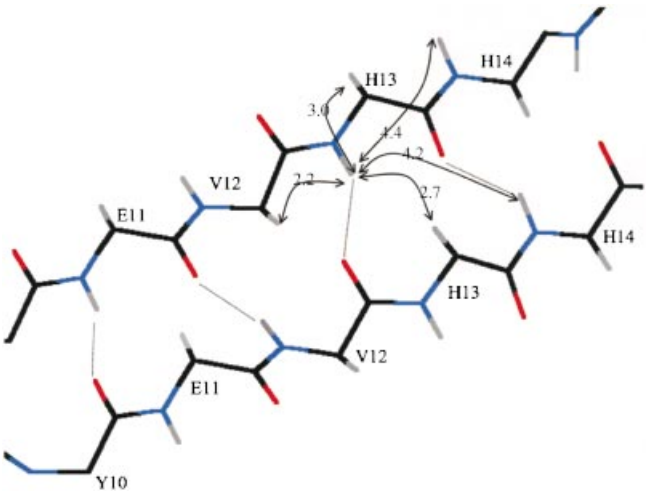

Figure 4. Two strands of β-AP(1–28) (only residues 10–14 are shown; C = black, O = red, N = blue, H = gray) in an in-register, parallel β-sheet arrangement. As shown with dotted lines, in this arrangement hydrogen bonds form between residue i of a given peptide strand and residues $i-1$ and $i+1$ of the neighboring strand. Distances corresponding to recorded NOEs are given in Å on curved arrows. The structure was calculated for β-AP(1–28) using Macromodel software, and the interatomic distances derived are in complete agreement with those in the literature.[19]

  1433-7851/01/4019-3604 $ 17.50+.50/0 *Angew. Chem. Int. Ed.* **2001**, *40*, No. 19

incorporates the observed NOE data well. If the strands were not in register, interstrand NOEs between amide protons at various positions in the peptide chain should exist, however, all the recorded NH-NH NOEs are between adjacent peptides.

The presence of consecutive NH_i-NH_{i+1} NOEs for residues 9 to 26 could also be explained by a continuous turn in the structure forming an open loop. This hypothesis cannot be excluded; however, it is not in agreement with the existing literature on the β-sheet structure of β-AP(1–28) fibrils.[5d,e, 6b, 16]

The lack of NH-NH connectivities for residues 1–8 may indicate structural disorder in the N-terminal part of the peptide. It has already been demonstrated that the first ten residues of the amyloid peptide are not required for fibril formation in vitro;[20] so it appears plausible that the N-terminal residues may extend outside the ordered β-sheet core.

Our results, suggestive of a parallel, in-register arrangement of the β-AP(1–28) fibrils, are in agreement with the solid-state ^{13}C NMR results recently reported on the structure of the β-AP(10–35)[14] and the full-length β-AP(1–40)[15] fibrils. HR-MAS ^1H NMR spectroscopy proves to be a powerful tool for probing amyloid fibril structure since it provides solid experimental evidence for the precise arrangement of the individual β-APs in the amyloid fibril, an issue that was until recently elusive. Hopefully, the elucidation of the structure of the β-AP fibrils will provide clues as to the mechanism of amyloid assembly in Alzheimer's disease and will identify potential targets for controlling the aggregation process.

Experimental Section

β-AP(1–28) (H-Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-OH) was purchased from AnaSpec Inc. and was >95% pure. 0.5 mg were dissolved in 20 µL of 90% H_2O/10% D_2O. At that concentration, fibril formation is very rapid.[5f]

HR-MAS ^1H NMR spectra were obtained with a Bruker AVANCE 600 (DRX) spectrometer. A 4-mm HR-MAS probe was used together with a rotor containing an inner spacer in order to improve $\boldsymbol{B}_o$ homogeneity and spinning stability as well as to improve the suppression of solvent signals. The spinning rate was 6 kHz, and the temperature was stabilized at 300 K. TOCSY spectra were obtained with a spectral width of 6613 Hz in both dimensions, a mixing time of 80 ms, 512 t_1 increments, 32 scans, and 2 K t_2 data points. The NOESY spectra were obtained with the same spectral parameters and mixing times of 80, 150, 200, and 250 ms. Suppression of the water peak was performed with presaturation.

For the Congo red staining procedure, the NMR sample was diluted 1:10 with H_2O for easier handling. Aliquots of the NMR sample were air-dried on glass slides, dehydrated for 5 min with 80% ethanol, and stained for 20 min with 5% Congo red prepared in 80% ethanol saturated with NaCl.[21] Excess Congo red was removed by washing with 90% ethanol. The stained samples were examined for green-yellow birefringence using an upright microscope (Zeiss Axioplan 2) equipped with polarizers. Images were acquired using a ×40 objective (Plan-Neofluar) and a Peltier-cooled CCD color camera (Hitachi KP-C571).

Received: April 24, 2001
Revised: July 20, 2001 [Z16993]

[1] a) C. L. Masters, G. Simms, N. A. Weinman, G. Multhaup, B. L. McDonald, K. Beyreuther, *Proc. Natl. Acad. Sci. USA* **1985**, *82*, 4245; b) J. Kang, H.-G. Lemaire, A. Unterbeck, J. M. Salbaum, C. L. Masters, K.-H. Grzeschik, G. Multhaup, K. Beyreuther, B. Müller-Hill, *Nature* **1987**, *325*, 733.
[2] D. J. Selkoe, *J. Biol. Chem.* **1996**, *271*, 18295.
[3] a) G. G. Glenner, C. W. Wong, *Biochem. Biophys. Res. Commun.* **1984**, *120*, 885; b) F. Prelli, E. Castaño, G. G. Glenner, B. Frangione, *J. Neurochem.* **1988**, *51*, 648.
[4] L. C. Serpell, M. Sunde, M. D. Benson, G. A. Tennent, M. B. Pepys, P. E. Fraser, *J. Mol. Biol.* **2000**, *300*, 1033, and references therein.
[5] a) E. M. Castaño, J. Ghiso, F. Prelli, P. D. Gorevic, A. Migheli, B. Frangione, *Biochem. Biophys. Res. Commun.* **1986**, *141*, 782; b) P. D. Gorevic, E. M. Castaño, R. Sarma, B. Frangione, *Biochem. Biophys. Res. Commun.* **1987**, *147*, 854; c) K. Halverson, P. E. Fraser, D. A. Kirschner, P. T. Lansbury, Jr., *Biochemistry* **1990**, *29*, 2639; d) P. E. Fraser, L. K. Duffy, M. B. O'Malley, J. Nguyen, H. Inouye, D. A. Kirschner, *J. Neurosci. Res.* **1991**, *28*, 474; e) P. E. Fraser, J. T. Nguyen, W. Surewicz, D. A. Kirschner, *Biophys. J.* **1991**, *60*, 1190; f) C. J. Barrow, M. G. Zagorski, *Science* **1991**, *253*, 179; g) C. J. Barrow, A. Yasuda, P. T. Kenny, M. G. Zagorski, *J. Mol. Biol.* **1992**, *225*, 1075; h) D. Burdick, B. Soreghan, M. Kwon, J. Kosmoski, M. Knauer, A. Henschen, J. Yates, C. Cotman, C. Glabe, *J. Biol. Chem.* **1992**, *267*, 546.
[6] a) T. Shirahama, A. S. Cohen, *J. Cell Biol.* **1967**, *33*, 679; b) D. A. Kirschner, H. Inouye, L. K. Duffy, A. Sinclair, M. Lind, D. J. Selkoe, *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 6953; c) P. E. Fraser, J. T. Nguyen, W. K. Surewicz, D. A. Kirschner, *Biophys. J.* **1991**, *60*, 1190; d) L. C. Serpell, J. M. Smith, *J. Mol. Biol.* **2000**, *299*, 225.
[7] a) D. A. Kirschner, C. Abraham, D. J. Selkoe, *Proc. Natl. Acad. Sci. USA* **1986**, *83*, 503; b) P. T. Lansbury, *Biochemistry* **1992**, *31*, 6867; c) H. Inouye, P. Fraser, D. Kirschner, *Biophys. J.* **1993**, *64*, 502; d) S. B. Malinchik, H. Inouye, K. E. Szumowski, D. A. Kirschner, *Biophys. J.* **1998**, *74*, 537.
[8] a) L. O. Tjernberg, D. J. E. Callaway, A. Tjernberg, S. Hahne, C. Lilliehöök, L. Terenius, J. Thyberg, C. Nordstedt, *J. Biol. Chem.* **1999**, *274*, 12619; b) N. D. Lazo, D. T. Downing, *Biochemistry* **1998**, *37*, 1731; c) L. Li, T. A. Darden, L. Bartolotti, D. Kominos, L. G. Pedersen, *Biophys. J.* **1999**, *76*, 2871; d) A. R. George, D. R. Howlett, *Biopolymers* **1999**, *50*, 733.
[9] P. E. Fraser, J. T. Nguyen, H. Inouye, W. K. Surewicz, D. J. Selkoe, M. B. Podlisny, D. A. Kirschner, *Biochemistry* **1992**, *31*, 10716.
[10] a) F. Dousseau, M. Pezolet, *Biochemistry* **1990**, *29*, 8771; b) K. Janek, J. Behlke, J. Zipper, H. Fabian, Y. Georgalis, M. Beyermann, M. Bienert, E. Krause, *Biochemistry* **1999**, *38*, 8246; c) W. Qian, J. Bandekar, S. Krimm, *Biopolymers* **1991**, *31*, 193.
[11] R. Tycko, *Curr. Opin. Chem. Biol.* **2000**, *4*, 500.
[12] P. T. Lansbury, P. R. Costa, J. M. Griffiths, E. J. Simon, M. Auger, K. J. Halverson, D. A. Kocisko, Z. S. Hendsch, T. T. Ashburn, R. G. S. Spencer, *Nat. Struct. Biol.* **1995**, *2*, 926.
[13] J. J. Balbach, Y. Ishii, O. N. Antzutkin, R. D. Leapman, N. W. Rizzo, F. Dyda, J. Reed, R. Tycko, *Biochemistry* **2000**, *39*, 13748.
[14] a) T. L. S. Benzinger, D. M. Gregory, T. S. Burkoth, H. Miller-Auer, D. G. Lynn, R. E. Botto, S. C. Meredith, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 13407; b) T. L. S. Benzinger, D. M. Gregory, T. S. Burkoth, H. Miller-Auer, D. G. Lynn, R. E. Botto, S. C. Meredith, *Biochemistry* **2000**, *39*, 3491.
[15] O. N. Antzutkin, J. J. Balbach, R. D. Leapman, N. W. Rizzo, J. Reed, R. Tycko, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 13045.
[16] C. L. Shen, G. L. Scott, F. Merchant, R. M. Murphy, *Biophys. J.* **1993**, *65*, 2383.
[17] J. H. Davis, M. Auger, R. S. Hodges, *Biophys. J.* **1995**, *69*, 1917, and references therein.
[18] H. Puchtler, F. Sweat, M. Levine, *J. Histochem. Cytochem.* **1962**, *10*, 355.
[19] K Wüthrich, *NMR of Proteins and Nucleic Acids*, Wiley, New York, **1986**.
[20] C. Hilbich, B. Kisters-Woike, J. Reed, C. L. Masters, K. Beyreuther, *J. Mol. Biol.* **1991**, *218*, 149.
[21] S. B. Prusiner, M. P. McKinley, K. A. Bowman, D. C. Bolton, P. E. Bendheim, D. F. Groth, G. G. Glenner, *Cell* **1983**, *35*, 349.

  1433-7851/01/4019-3605 $ 17.50+.50/0