Solid-State NMR

DOI: 10.1002/anie.201203595

Hydrogen Bonding in Alzheimer's Amyloid-β Fibrils Probed by $^{15}N\{^{17}O\}$ REAPDOR Solid-State NMR Spectroscopy**

Oleg N. Antzutkin, Dinu Iuga, Andrei V. Filippov, Robert T. Kelly, Johanna Becker-Baldus, Steven P. Brown, and Ray Dupree*

Hydrogen bonding is of utmost importance in stabilizing molecular and supramolecular structures in biological systems. One of the key atoms actively involved in such H-bonding is oxygen, such as in C=O···H–N H-bonding in proteins and peptides. However, although the magnetic isotope oxygen-17 is potentially an attractive NMR probe for H-bonding in biological systems, this method is still poorly developed because of the low natural abundance of ^{17}O (0.037%) and the quadrupolar nature of this nucleus (nuclear spin 5/2), which causes low sensitivity and poor resolution in ^{17}O solid-state NMR spectra of organic and biological samples. Recently, $^{15}N\{^{17}O\}$ REAPDOR NMR spectroscopy has been used to measure ^{15}N–^{17}O dipolar couplings in hydrogen-bonded small model compounds, namely between the selectively labeled amino acid glycine and nucleic acid uracil.[1] Herein, we explore further this novel approach to determine the hydrogen-bonding contacts between the carbonyl oxygen and the amide nitrogen atoms in fibrils of the amyloid beta (Aβ) peptides associated with Alzheimer's disease.

[*] Prof. O. N. Antzutkin, Dr. D. Iuga, R. T. Kelly, Dr. J. Becker-Baldus, Prof. S. P. Brown, Prof. R. Dupree
Department of Physics, University of Warwick
CV4 7AL, Coventry (UK)
E-mail: O.N.Antzutkin@warwick.ac.uk
Homepage: http://go.warwick.ac.uk/nmr
Prof. O. N. Antzutkin, Dr. A. V. Filippov
Chemistry of Interfaces, Luleå University of Technology
SE-971 87, Luleå (Sweden)
Dr. A. V. Filippov
Kazan Federal University
420008, Kazan (Russian Federation)

[**] We acknowledge Dr. A. Kukol for preparation of Fmoc-Val(^{17}O), Dr. S. Afonin for electrospray mass-spectrometry analysis of peptides, Dr. A. T. Petkova and Dr. R. Tycko for seeds of $A\beta_{(11-25)}$ (pH 2.4) fibrils, reagents and access to the peptide synthesizer at LCP, NIDDK, NIH, where $A\beta_{(11-25)}$ was synthesized, and I. Portman at the Electron Microscopy Facility, School of Life Sciences, University of Warwick (Wellcome Trust reference 055663/Z/98/Z) for technical support. This work was supported by the Foundation in memory of J.C. and Seth M. Kempe and the Alzheimer's Foundation of Sweden (funds for a peptide synthesizer and HPLC at LTU, Sweden, reagents for synthesis of $A\beta_{(16-22)}$ and stipends for A.V.F.). The UK 850 MHz solid-state NMR Facility used in this research was funded by EPSRC and BBSRC, as well as the University of Warwick including partial funding through Birmingham Science City Advanced Materials Project 1 and 2 supported by Advantage West Midlands (AWM) and the European Regional Development Fund (ERDF).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201203595.

Different polymorphs of amyloid fibrils[2] and in particular small intermediate aggregates (oligomers)[3–7] have been shown to cause death of nerve-cell cultures in vitro. Therefore, these species may be key toxic species for brain neurons causing a variety of neurodegenerative diseases. It has been suggested that toxic amyloid species are rich in different types of β-sheet and/or β-hairpin structures, having different inter- and/or intramolecular hydrogen-bonding patterns. Because these systems do not form single crystals, it is a challenge to determine how peptides or proteins are packed against each other and whether parallel or anti-parallel β-sheets or β-hairpin structures are formed in the toxic aggregates. Knowing their structure, inhibitors or monoclonal antibodies could be developed for treatment of amyloid related neurodegenerative diseases.

The intermolecular hydrogen-bonded arrangement of peptides within a β-sheet is described by considering two questions: first, are the chains are packed parallel or anti-parallel to each other and, second, what is the orientation (registry) of the amino acids in adjacent chains with respect to each other, that is, between which amino acids are intermolecular hydrogen bonds formed. Pioneering work by Griffin and co-workers,[8] Benzinger et al.,[9,10] and Tycko and co-workers[11–14] have shown that these two considerations can be determined by homonuclear ^{13}C and heteronuclear ^{13}C–^{15}N magic-angle spinning (MAS) solid-state NMR experiments that probe C–C and C–N distances of 4–5 Å between atoms in adjacent chains. Specifically, ^{13}C–^{13}C (rotational resonance,[15] DRAWS,[16] fpRFDR-CT,[17] and PITHIRDS[18]) and ^{13}C–^{15}N (REDOR)[19] dipolar recoupling and ^{13}C multiple-quantum (MQ) spin-counting[20] experiments have been applied. The close approach of Cα carbon atoms on adjacent chains have also been probed by two-dimensional ^{13}C–^{13}C (CHHC) NMR spectroscopy.[21] These approaches have identified anti-parallel arrangements with different registries for short Aβ peptides,[8,11,22] while the longer fragment of Aβ, $A\beta_{(10-35)}$,[9,10,14] and the full length wild-type Alzheimer's $A\beta_{(1-40)}$ and $A\beta_{(1-42)}$ peptides were shown to exhibit parallel in-registry β-sheet supramolecular structures.[12–14,23] Recently, it was also found from solid-state NMR spectroscopy that the Iowa mutant (D23N) of the $A\beta_{(1-40)}$ peptide forms different polymorphs of fibrils exibiting both parallel (stable) and antiparallel (metastable) β-sheet structures.[24] In combination with other multi-dimensional solid-state NMR methods, extended sets of structural constraints have been obtained and molecular level models for amyloid fibrils of a variety of peptides have been presented.[2,13,22–30]

A more direct approach for identifying N–H···O=C hydrogen-bonding contacts between the carbonyl oxygen

© 2012 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

and the amide nitrogen atom would be to probe the ^{15}N–^{17}O dipolar coupling between the hydrogen-bonding-donor nitrogen atom and the hydrogen-bonding-acceptor oxygen atom where, for a typical N–H···O=C hydrogen bond of 2.8 Å, the ^{15}N–^{17}O dipolar coupling is 75 Hz. Herein, we demonstrate the applicability of $^{15}N\{^{17}O\}$-(^{1}H-decoupled, ^{15}N-detected, ^{17}O-adiabatic pulse) REAPDOR[31] solid-state NMR for probing intermolecular hydrogen bonding in fibrils formed by the amyloid-β peptides, Ac-$A\beta_{(16-22)}$-NH_2 and $A\beta_{(11-25)}$, which are the key amyloidogenic central fragments of the full-length Alzheimer's amyloid-β peptides.

Putative models describing anti-parallel β-sheets with different registries have been proposed using the ^{13}C–^{13}C and ^{13}C–^{15}N experiments (that probe distances over 4 Å) described above. These models suggested specifically a $17+k\leftrightarrow21-k$ ($k=-1, 0, \ldots, 5$) registry for Ac-$A\beta_{(16-22)}$-NH_2 (see Figure 1a)[11] while, depending on the pH of the incubated solution, it has been proposed that $A\beta_{(11-25)}$ forms fibrils adopting either a $17+k\leftrightarrow22-k$ ($k=-3, -2, \ldots, 8$; at pH 2.4; Figure 1b) or a $17+k\leftrightarrow20-k$ ($k=-5, -4, \ldots, 8$; at pH 7.4; Figure 1c) registry.[22] Peptides with selective ^{17}O and ^{15}N labeling have been synthesized to validate the novel REAPDOR method with amyloid-β fibrils and to confirm the hydrogen bonding pattern in Ac-$A\beta_{(16-22)}$-NH_2 and $A\beta_{(11-25)}$ (pH 2.4) fibrils with ^{17}O enrichment, at the Val18 carbonyl group for all peptides, but with ^{15}N labeling of Phe20 for Ac-$A\beta_{(16-22)}$-NH_2 or Ala21 for $A\beta_{(11-25)}$. In this study, 20% enriched $H_2{}^{17}O$ was used, but higher sensitivity and accuracy could have been achieved using 90% $H_2{}^{17}O$.

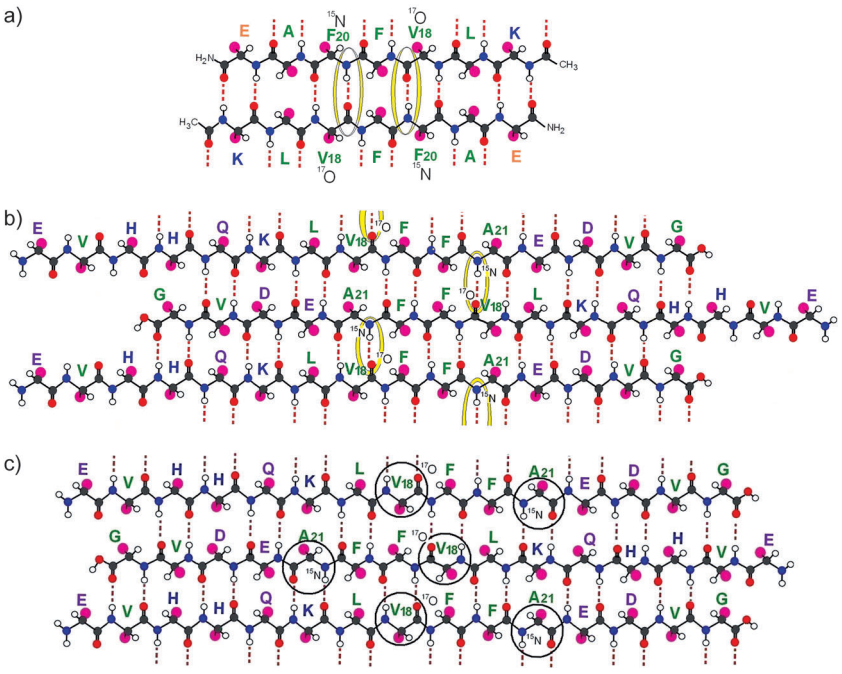

Figure 1. Schematic representation of hydrogen bonding in putative models of amyloid fibrils of Ac-$A\beta_{(16-22)}$-NH_2 (a) and $A\beta_{(11-25)}$ incubated in an aqueous solution at pH 2.4 for 80 days with seeding (b) and at pH 7.4 for 20 days (c). Three anti-parallel arrangements with different registries are shown: a) $17+k\leftrightarrow21-k$; b) $17+k\leftrightarrow22-k$; c) $17+k\leftrightarrow20-k$. Experiments were performed for samples with ^{17}O labelling of the Val_{18} carbonyl group and ^{15}N labelling of the a) Phe_{20} (Ac-$A\beta_{(16-22)}$-NH_2) or b,c) Ala_{21} ($A\beta_{(11-25)}$) amide group. Selectively isotopically labeled fragments involved in C=^{17}O···H-^{15}N hydrogen bonds are highlighted by yellow ellipsoids and black circles.

Figure 2 compares ^{15}N NMR spectra recorded using the $^{15}N\{^{17}O\}$ REAPDOR experiment with 7 ms of dephasing (red lines) and without the ^{17}O adiabatic pulse (blue lines). For Ac-$A\beta_{(16-22)}$-NH_2 and $A\beta_{(11-25)}$ (pH 2.4) samples, a narrow resonance is observed at approximately 127 ppm from the amyloid fibrils together with a broad peak at approximately 120 ppm, attributed to amorphous aggregates. For the narrow resonance, a dephasing of 8% is observed, while there is little or no dephasing for the broad resonance. No dephasing was observed for $A\beta_{(11-25)}$ (pH 7.4) fibrils (see Supporting Information, Figure S11 and discussion below Figure 3b).

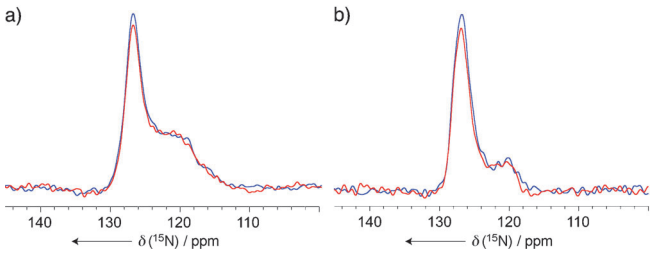

Figure 2. $^{15}N\{^{17}O\}$ REAPDOR solid-state NMR (850 MHz, 8 kHz MAS) spectra of amyloid fibrils formed by the Alzheimer's peptides a) Ac-$A\beta_{(16-22)}$-NH_2 (with selective labeling at Val18-^{17}O and Phe20-^{15}N) and b) $A\beta_{(11-25)}$ fibrils at pH 2.4 (with selective labeling at Val18-^{17}O and Ala21-U-^{13}C, ^{15}N). Spectra show REAPDOR dephasing with a mixing time of 7 ms (red lines) and without dephasing (no adiabatic ^{17}O pulse, blue lines). A broad spectral feature in the range 115–125 ppm corresponds to non-fibrillar (amorphous) aggregates, which show only a minor decrease in the ^{15}N NMR signal in the $^{15}N\{^{17}O\}$ REAPDOR spectra.

The dephasing observed in $^{15}N\{^{17}O\}$ REAPDOR experiments for times up to 9 ms is plotted in Figure 3. Considering the squares that represent the narrow resonance at approximately 127 ppm from the amyloid fibrils for Ac-$A\beta_{(16-22)}$-NH_2 (Figure 3a) and $A\beta_{(11-25)}$ (pH 2.4) (Figure 3b), good fits are found to REAPDOR dephasing simulated using SIMPSON[32] for an ^{15}N–^{17}O dipolar coupling of 75 Hz (long dashed lines), corresponding to the typical NH···O hydrogen-bond distance of 2.8 Å. In Figure 3a, the crosses correspond to the broad peak at approximately 120 ppm owing to amorphous aggregates for Ac-$A\beta_{(16-22)}$-NH_2, and reasonable agreement with simulated dephasing (short dashed line) for a ^{15}N–^{17}O dipolar coupling of 15 Hz is observed. This corresponds to an intramolecular closest N–O distance in Ac-$A\beta_{(16-22)}$-NH_2 of 5 Å. In Figure 3b, no dephasing is observed for $A\beta_{(11-25)}$ (pH 7.4) fibrils (stars). This is consistent with intra- and intermolecular ^{15}N–^{17}O distances of more than 10 Å for a $17+$

© 2012 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

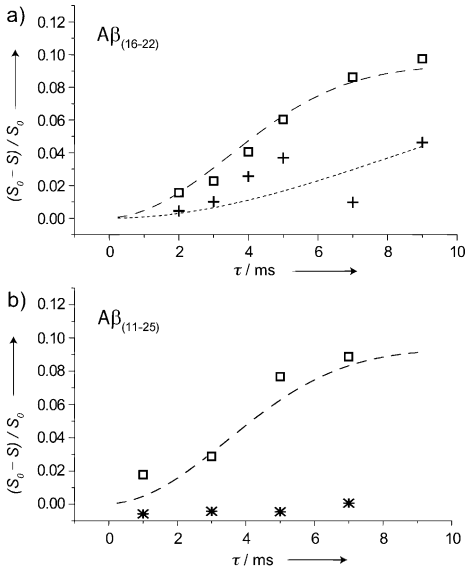

Figure 3. Experimental $^{15}N\{^{17}O\}$ REAPDOR solid-state NMR (850 MHz, 8 kHz MAS) dephasing for a) the Ac-$A\beta_{(16-22)}$-NH_2 sample (squares = 127 ppm signal from amyloid fibrils; crosses = broad 120 ppm signal of amorphous aggregates) and b) $A\beta_{(11-25)}$ sample (squares = pH 2.4; stars = pH 7.4). A best-fit simulation (SIMPSON[32]) of REAPDOR dephasing for a ^{15}N–^{17}O dipolar coupling of 75 Hz (long dashed lines = fibrils) and a simulation for a 15 Hz ^{15}N–^{17}O dipolar coupling (short dashed line = amorphous) are also shown. The simulated curves were scaled by a factor of 0.13 corresponding to the degree of ^{17}O labeling.

$k \leftrightarrow 20-k$ registry, corresponding to a negligibly small dipolar coupling, less than 2 Hz, for which no REAPDOR dephasing is to be expected. In conclusion, the observed $^{15}N\{^{17}O\}$ REAPDOR dephasing for the Ac-$A\beta_{(16-22)}$-NH_2 and $A\beta_{(11-25)}$ (pH 2.4) fibrillar samples is due to intermolecular hydrogen bonds (Val18-C=O···HN-Phe20 and Val18-C=O···HN-Ala21, respectively), which unambiguously confirms the previously published structural models based on ^{13}C–^{13}C and ^{13}C–^{15}N solid-state NMR measurements on Ac-$A\beta_{(16-22)}$-NH_2 and $A\beta_{(11-25)}$ amyloid fibrils.[11,22]

In summary, we have demonstrated the use of the ^{17}O-isotope for probing hydrogen bonding and specifically the robustness of the $^{15}N\{^{17}O\}$ REAPDOR solid-state NMR method for biologically important systems such as amyloid fibrils with anti-parallel β-sheet structures formed by fragments of Alzheimer's amyloid-β peptide. This method can also be used for probing hydrogen bonding in β-hairpin fragments of globulomers[33] and toxic oligomers.[6,7] For amyloid fibrils of peptides and proteins that form in-registry parallel β-sheet structures, 50:50 molecular mixtures of a peptide selectively labeled with ^{17}O at one specific carbonyl site and a peptide selectively (or uniformly-^{15}N) labeled at NH sites along the peptide backbone were prepared. Multiple ^{15}N sites could be resolved by 2D ^{1}H–^{15}N correlation NMR spectroscopy (and assigned using a variety of 2D ^{15}N–^{13}C correlation techniques) as shown for some membrane proteins and amyloid fibrils of $A\beta_{(1-40)}[U\text{-}^{2}H, ^{15}N, ^{13}C]$.[34] The 2D ^{1}H–^{15}N HETCOR NMR experiment could be combined with the $^{15}N\{^{17}O\}$ REAPDOR method, whereby dephasing would only be observed for ^{15}N signals of amine site(s) hydrogen bonded to the ^{17}O labeled site. Further work could show the applicability of $^{15}N\{^{17}O\}$ or $^{17}O\{^{15}N\}$ spin-echo experiments that probe hydrogen-bond mediated $^{2h}J_{^{15}N-^{17}O}$ couplings.[1]

We believe that $^{15}N\{^{17}O\}$ REAPDOR NMR has the potential to be a useful complimentary technique to the well-established and highly successful ^{13}C–^{13}C and ^{13}C–^{15}N solid-state NMR methods developed for structural biology, in particular, for systems where ambiguities in structural modeling of hydrogen bonding still remain. The inherent advantage is the ability to probe shorter (less than 3 Å) nitrogen–oxygen distances as opposed to longer (more than 4 Å) indirect carbon–carbon and carbon–nitrogen distances. Using higher levels of ^{17}O enrichment to increase the amplitude of REAPDOR dephasing, $^{15}N\{^{17}O\}$ REAPDOR could be readily applied to study hydrogen bonding in key fragments of toxic oligomers of the full length $A\beta_{(1-40)}$ or $A\beta_{(1-42)}$ peptides and their mutations; work along these lines is currently in progress.

Experimental Section

The ^{17}O and ^{15}N selectively labeled peptides were synthesized using Fmoc-protected solid-phase peptide synthesis and purified by HPLC: (1) a seven-residue peptide Ac-$A\beta_{(16-22)}$-NH_2: *N*-acetyl-Lys-Leu-$Val_{18}(^{17}O)$-Phe-$Phe_{20}(^{15}N)$-Ala-Glu-NH_2 and (2) a fifteen-residue peptide $A\beta_{(11-25)}$: Glu-Val-His-His-Gln-Lys-Leu-$Val_{18}(^{17}O)$-Phe-Phe-Ala_{21}(U-^{13}C, ^{15}N)-Glu-Asp-Val-Gly. Fmoc-Val(^{17}O) was prepared by exchanging ^{16}O to ^{17}O in valine dissolved in $H_2{}^{17}O$ (ca. 20% enrichment) at elevated temperature followed by Fmoc protection. It was found by electrospray mass spectrometry (see Supporting Information) that the final ^{17}O enrichment in the peptides after the synthesis and purification was 15 ± 5%.

Amyloid fibrils of Ac-$A\beta_{(16-22)}$-NH_2 (in a PBS buffer at pH 7) and $A\beta_{(11-25)}$ (at both pH 2.4 and pH 7.4) were prepared following methods described in detail by Balbach et al.[11] and Petkova et al.,[22] respectively. To ensure formation of the correct polymorph of fibrils, solutions of $A\beta_{(11-25)}$ (pH 2.4) were seeded with sonicated fibrils kindly provided to us by Petkova and Tycko and further confirmed by the ^{15}N chemical shift of Ala21.[22]

Solid-state NMR experiments were performed on a Bruker Avance III 850 MHz spectrometer using a triple-resonance 2.5 mm double broadband probe (see Supporting Information for additional experimental details). Experiments were performed with approximately 2 mg of Ac-$A\beta_{(16-22)}$-$NH_2[Val_{18}(^{17}O), Phe_{20}(^{15}N)]$ amyloid fibrils and approximately 10 mg $A\beta_{(11-25)}[Val_{18}(^{17}O), Ala_{21}(U\text{-}^{13}C,^{15}N)]$ of amyloid fibrils prepared at either pH 2.4 or pH 7.4.

Received: May 9, 2012
Published online: September 13, 2012

Keywords: $^{15}N\{^{17}O\}$ REAPDOR NMR · amyloid-β fibrils · hydrogen bonds · NMR spectroscopy · solid-state NMR

[1] I. Hung, A.-C. Uldry, J. Becker-Baldus, A. L. Webber, A. Wong, M. E. Smith, S. A. Joyce, J. R. Yates, C. J. Pickard, R. Dupree, S. P. Brown, *J. Am. Chem. Soc.* **2009**, *131*, 1820 – 1834.
[2] A. T. Petkova, R. D. Leapman, Z. Guo, W.-M. Yau, M. P. Mattson, R. Tycko, *Science* **2005**, *307*, 262 – 265.
[3] S. Chimon, Y. Ishii, *J. Am. Chem. Soc.* **2005**, *127*, 13472 – 13473.
[4] S. Chimon, M. A. Shaibat, C. R. Jones, D. C. Calero, B. Aizezi, Y. Ishii, *Nat. Struct. Mol. Biol.* **2007**, *14*, 1157 – 1164.

 © 2012 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

[5] D. M. Walsh, I. Klyubin, J. V. Fadeeva, W. K. Cullen, M. S. Anwyl, M. S. Wolfe, M. J. Rowan, D. J. Selkoe, *Nature* **2002**, *416*, 535 – 539.
[6] A. Sandberg, L. M. Luheshi, S. Söllvander, T. Pereira de Barros, B. Macao, T. P. J. Knowles, H. Biverstål, C. Lendel, F. Ekholm-Petterson, A. Dubnovitsky, L. Lannfelt, C. M. Dobson, T. Härd, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 15595 – 15600.
[7] M. Ahmed, J. Davis, D. Aucoin, T. Sato, S. Ahuja, S. Aimoto, J. I. Elliot, W. E. Van Nostrand, S. O. Smith, *Nat. Struct. Mol. Biol.* **2010**, *17*, 561 – 568.
[8] P. T. Lansbury, Jr., P. R. Costa, J. M. Griffith, E. J. Simon, M. Auger, K. J. Halverson, D. A. Kocisko, Z. S. Hendsch, T. T. Ashburn, R. G. S. Spencer, B. Tidor, R. G. Griffin, *Nat. Struct. Biol.* **1995**, *2*, 990 – 998.
[9] 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 – 13412.
[10] 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 – 3499.
[11] J. J. Balbach, Y. Ishii, O. N. Antzutkin, R. D. Leapman, N. W. Rizzo, F. Dyda, J. Reed, R. Tycko, *Biochemistry* **2000**, *39*, 13748 – 13759.
[12] O. N. Antzutkin, J. J. Balbach, R. D. Leapman, N. W. Rizzo, J. Reed, R. Tycko, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 13045 – 13050.
[13] A. T. Petkova, Y. Ishii, J. J. Balbach, O. N. Antzutkin, R. D. Leapman, F. Delagio, R. Tycko, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 16742 – 16747.
[14] O. N. Antzutkin, R. D. Leapman, J. J. Balbach, R. Tycko, *Biochemistry* **2002**, *41*, 15436 – 15450.
[15] D. P. Raleigh, F. Creuzet, S. K. Das Gupta, M. H. Levitt, R. G. Griffin, *J. Am. Chem. Soc.* **1989**, *111*, 4502 – 4503.
[16] M. A. Mehta, D. M. Gregory, S. Kiihne, D. J. Mitchell, M. E. Hatcher, J. C. Shiels, G. P. Drobny, *Solid State Nucl. Magn. Reson.* **1996**, *7*, 211 – 228.
[17] Y. Ishii, J. J. Balbach, R. Tycko, *Chem. Phys.* **2001**, *266*, 231 – 236.
[18] R. Tycko, *J. Chem. Phys.* **2007**, *126*, 064506.
[19] T. Gullion, J. Schaefer, *J. Magn. Reson.* **1990**, *90*, 330 – 340.
[20] O. N. Antzutkin, R. Tycko, *J. Chem. Phys.* **1999**, *110*, 2749 – 2752.
[21] R. Tycko, Y. Ishii, *J. Am. Chem. Soc.* **2003**, *125*, 6606 – 6607.
[22] A. T. Petkova, G. Buntkowsky, F. Dyda, R. D. Leapman, W.-M. Yau, R. Tycko, *J. Mol. Biol.* **2004**, *335*, 247 – 260.
[23] A. K. Paravastu, R. D. Leapman, W.-M. Yau, R. Tycko, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 18349 – 18354.
[24] W. Qiang, W.-M. Yau, Y. Luo, M. P. Mattson, R. Tycko, *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 4443 – 4448.
[25] R. Verel, I. T. Tomka, C. Bertozzi, R. Cadalbert, R. A. Kammerer, M. O. Steinmetz, B. H. Meier, *Angew. Chem.* **2008**, *120*, 5926 – 5929; *Angew. Chem. Int. Ed.* **2008**, *47*, 5842 – 5845.
[26] C. Wasmer, A. Lange, H. Van Melckebeke, A. B. Siemer, R. Riek, B. H. Meier, *Science* **2008**, *319*, 1523 – 1526.
[27] J. T. Nielsen, M. Bjerring, M. D. Jeppesen, R. O. Pedersen, J. M. Pedersen, K. L. Hein, T. Vosegaard, T. Skrydstrup, D. E. Otzen, N. C. Nielsen, *Angew. Chem.* **2009**, *121*, 2152 – 2155; *Angew. Chem. Int. Ed.* **2009**, *48*, 2118 – 2121.
[28] S. Parthasarathy, F. Long, Y. Miller, Y. Xiao, D. McElheny, K. Thuber, B. Ma, R. Nussinov, Y. Ishii, *J. Am. Chem. Soc.* **2011**, *133*, 3390 – 3400.
[29] I. Bertini, L. Gonnelli, C. Luchinat, J. Mao, A. Nesi, *J. Am. Chem. Soc.* **2011**, *133*, 16013 – 16022.
[30] V. S. Mithu, B. Sarkar, D. Bhowmik, M. Chandrakesan, S. Maiti, P. K. Madhu, *Biophys. J.* **2011**, *101*, 2825 – 2832.
[31] T. Gullion, *Chem. Phys. Lett.* **1995**, *246*, 325 – 330.
[32] M. Bak, J. T. Rasmussen, N. C. Nielsen, *J. Magn. Reson.* **2000**, *147*, 296 – 330.
[33] L. Yu, R. Edalji, J. E. Harlan, T. F. Holzman, A. P. Lopez, B. Labkovsky, H. Hillen, S. Barghorn, U. Ebert, P. L. Richardson, L. Miesbauer, L. Solomon, D. Bartley, K. Walter, R. W. Johnson, P. J. Hajduk, E. T. Olejniczak, *Biochemistry* **2009**, *48*, 1870 – 1877.
[34] R. Linser, M. Dasari, M. Hiller, V. Higman, U. Fink, J.-M. Lopez del Amo, S. Markovic, L. Handel, B. Kessler, P. Schmieder, D. Oesterhelt, H. Oschkinat, B. Reif, *Angew. Chem.* **2011**, *123*, 4601 – 4605; *Angew. Chem. Int. Ed.* **2011**, *50*, 4508 – 4512.

 © 2012 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim