JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Communication
pubs.acs.org/JACS

Proton Clouds to Measure Long-Range Contacts between Nonexchangeable Side Chain Protons in Solid-State NMR

Tessa Sinnige, Mark Daniëls, Marc Baldus,* and Markus Weingarth*

NMR Spectroscopy, Bijvoet Center for Biomolecular Research, Department of Chemistry, Faculty of Science, Utrecht University, 3584 CH Utrecht, The Netherlands

Supporting Information

ABSTRACT: We show that selective labeling of proteins with protonated amino acids embedded in a perdeuterated matrix, dubbed 'proton clouds', provides general access to long-range contacts between nonexchangeable side chain protons in proton-detected solid-state NMR, which is important to study protein tertiary structure. Proton-cloud labeling significantly improves spectral resolution by simultaneously reducing proton line width and spectral crowding despite a high local proton density in clouds. The approach is amenable to almost all canonical amino acids. Our method is demonstrated on ubiquitin and the β-barrel membrane protein BamA.

Over the last years, solid-state NMR (ssNMR) spectroscopy has evolved as an established technique to study insoluble biomolecules, driven by impressive technical and methodological progress.[1−6] The use of ssNMR could be further boosted by the realization of ^{1}H-detection in solid biomolecules,[7−10] which by virtue of the high ^{1}H gyromagnetic ratio can enhance spectral sensitivity by more than 1 order of magnitude in comparison to heteronuclear detection. Labile amino protons can be accessed by means of perdeuteration and subsequent back-exchange of deuterons by protons,[7,9,11−14] possibly in combination with fast magic-angle spinning (MAS) and high magnetic fields.[15] High ^{1}H resolution has been reported with these approaches for backbone H_N in microcrystalline proteins, membrane proteins, and amyloid fibrils.[16] H_N−H_N distance measurements[17,18] were shown to allow the rapid determination of protein fold.[18]

Protein structures are defined by side chain−side chain contacts,[19−21] and protein structure determination by ^{1}H-detection hence requires access to aliphatic protons. Due to their high gyromagnetic ratio and peripheral location they are ideal to probe molecular distances in ssNMR, which has been extensively employed in heteronuclear-detected experiments.[1,2,6,22] As shown recently,[23] the use of fully protonated proteins is principally a straightforward mean to the assignment of aliphatic protons, although the measurement of long-range (i.e., separated by > 4 sequential residues) contacts between nonexchangeable side chain protons in such systems is challenging due to spectral overlap and has not been reported thus far. Another approach, coined reduced adjoining protonation (RAP),[24] that relies on random incorporation of aliphatic protons in a deuterated matrix was shown to yield very high resolution. RAP was reported to give access to long-range contacts between methyl protons. Such contacts could also be obtained using precursors with $^{13}C^1H_3$ groups.[19,20] Contacts between methyl protons are precious structural probes. However, to determine high-resolution structures it is principally desirable to collect distance information for all side chains and chemical groups. Here we present a general avenue to measure long-range contacts between nonexchangeable protons by selective protonation of amino acid types. Such 'proton clouds' in a perdeuterated background provide a significant improvement in ^{1}H-spectral resolution compared to fully protonated samples.

Proton-cloud samples are prepared by addition of uniformly ^{1}H,^{13}C,^{15}N-labeled amino acids to D_2O minimal medium containing ^{2}H,^{12}C-glucose. As a test case we prepared a sample of perdeuterated ubiquitin labeled with protonated valine and leucine (V,L ^{1}H-cloud ubiquitin, see also Supporting Information, SI). Figure 1a shows a superposition of ^{1}H-detected 2D ^{13}C−^{1}H correlation spectra of (Figure 1b,c) fully protonated (FP) ubiquitin (in blue) and V,L ^{1}H-cloud ubiquitin (in red). It is readily visible that spectral congestion is much reduced in the ^{1}H-cloud spectrum, which is a combined effect of a narrower ^{1}H line width (Figure 1d), about a factor of 2−3 better than in the FP sample (meaning 0.12−0.26 ppm), and a stark reduction in signal crowding. The improvement in ^{1}H line width is most significant for protons of CH and CH_2 groups and almost a factor of 2 for methyl protons. The spectral quality in the ^{1}H-cloud sample allowed, based on available ^{1}H solution[25] and ^{13}C solid-state[26] NMR assignments, to identify all C_α−H_α groups (except for the highly mobile sites L8, L71, and L73) and most of the side chain resonances. In all ssNMR experiments the MISSISSIPI[27] scheme was used for water suppression, and low-power PISSARRO decoupling was applied in both ^{13}C and ^{1}H dimensions.[28] Further experimental parameters can be found in the SI.

Proton-cloud labeling hence provides attractive spectral resolution. However, the ^{1}H line width in RAP samples, that was demonstrated to be on the order of 25−60 Hz,[24,29] is narrower than in ^{1}H-cloud samples, which we suspected to result from residual homogeneous line broadening in ^{1}H clouds.[30] Numerical simulations indeed indicate that very high magnetic fields (≥1000 MHz) and MAS frequencies (≥90 kHz) are required to suppress the local proton couplings of a single valine residue (Figures 2a,b and S1).

Received: December 18, 2013
Published: January 27, 2014

dx.doi.org/10.1021/ja412870m | J. Am. Chem. Soc. 2014, 136, 4452−4455

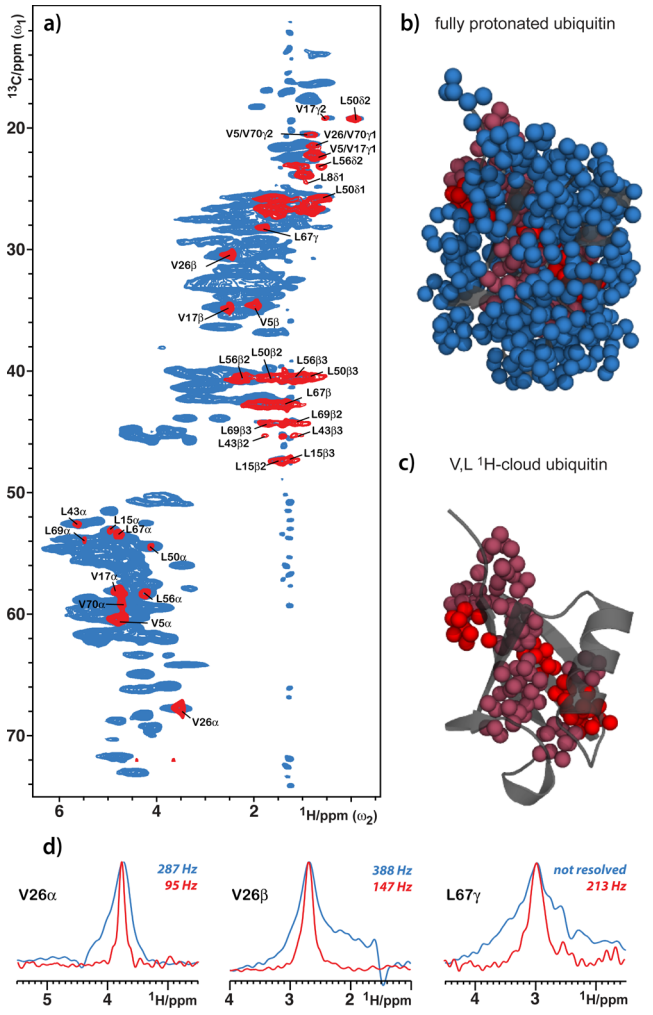

Figure 1. (a) Superposition of 2D ^{13}C−^{1}H spectra of perdeuterated V,L ^{1}H cloud ubiquitin (in red) and fully protonated (FP) ubiquitin (blue). Spectra were recorded at 18.8 T (800 MHz ^{1}H frequency) and 60 kHz MAS. (b,c) Protonation pattern of (b) FP and (c) V,L ^{1}H-cloud ubiquitin. Leucine and valine residues are colored in dark and light red, respectively. (d) Superposition of cross-sections extracted from (a) without application of a window function in the ^{1}H dimension.

Experimental data (Figure 2c), measured at 40−60 kHz MAS frequency and 16.4 T (700 MHz ^{1}H frequency) magnetic field, showed an approximately linear decrease in ^{1}H line width with increasing MAS frequency, which is in line with previous studies[10,30] and confirms the presence of homogeneous contributions. We observed broad ^{1}H line shapes at medium MAS frequencies, which further illustrates strong local dipolar couplings in ^{1}H-cloud preparations (Figure S2).

In ^{1}H-cloud samples, the closest ^{1}H neighbors do usually not correspond to long-range contacts and dipolar truncation curtails the potential of first-order recoupling methods to bring about nontrivial distance information.[33] However, the presence of pronounced residual homogeneous proton couplings at high MAS frequencies opens up the possibility to resort to second-order recoupling methods including spin diffusion. It was demonstrated that such methods are not very sensitive to dipolar truncation,[22,34,35] which provides a potential way to transfer magnetization between ^{1}H clouds. We probed whether ^{1}H-cloud samples gave rise to long-range contacts by means of

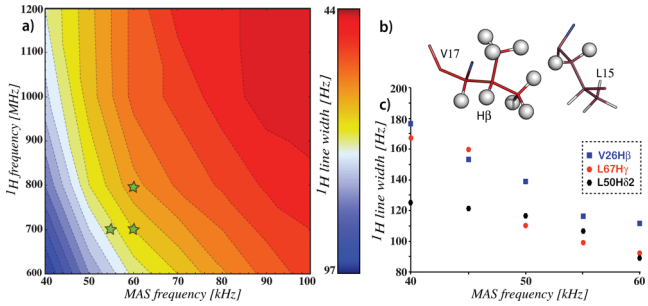

Figure 2. Numerical simulations[31] were carried out to analyze the homogeneous contribution to the ^{1}H line width in ^{1}H-cloud samples. (a) ^{1}H line width of V17H$_\beta$ shown as a function of MAS frequency and magnetic field. Green stars indicate the experimental conditions used in Figures 1 and 3. Note that experimental and simulated values differ due to the finite proton network in simulations and possibly inhomogeneous broadening effects seen in our experiments. Simulations were carried out using a (b) 10 spin system. (c) Experimental ^{1}H line width as a function of the MAS frequency, measured at 700 MHz with the ubiquitin ^{1}H-cloud sample described in Figure 3.

^{1}H−^{1}H spin diffusion,[22] using a ubiquitin sample in which only ^{1}H-valine was supplemented to the perdeuterated medium, while leucine C$_\gamma$ and C$_\delta$ positions became ^{13}C labeled and protonated due to long incubation times (Figure S3). The protein was precipitated in D$_2$O-based buffers and ^{2}H-MPD to prevent re-introduction of fast relaxing precipitant signal during the mixing block. 2D ^{13}C−(^{1}H)^{1}H spectra (Figure 3) acquired

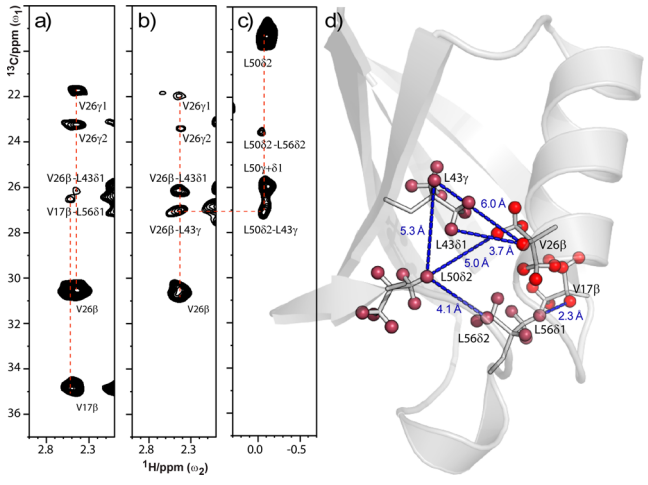

Figure 3. (a−c) Cut-outs of 2D ^{13}C−(^{1}H)^{1}H spectra measured at (a) 55 kHz and (b,c) 60 kHz MAS with 25 and 75 ms ^{1}H−^{1}H spin diffusion mixing time, respectively, using V,L* ^{1}H-cloud ubiquitin (*only C$_\gamma$ and C$_\delta$ positions of leucine were protonated and ^{13}C labeled). Long-range contacts involving (a,b) V17$_\beta$ and V26$_\beta$ and (c) L50$_{\delta2}$ are annotated. (d) Illustration of the long-range contacts annotated in measurements (a−c) in the crystal structure of ubiquitin (PDB: 1UBQ).[32]

at 55 and 60 kHz MAS with 25 and 75 ms ^{1}H−^{1}H mixing, respectively, showed numerous long-range contacts between ^{1}H clouds, spanning distances of 2−6 Å in the hydrophobic core of ubiquitin. Long-range contacts including H$_\beta$ protons of valine (V17H$_\beta$−L56H$_{\delta1}$ and V26H$_\beta$−L43H$_{\delta1}$) and H$_\gamma$ of leucine (L43H$_\gamma$−L50$_{\delta2}$ and V26H$_\beta$−L43H$_\gamma$) demonstrate that ^{1}H-cloud sample preparations bear the capacity to provide a

dx.doi.org/10.1021/ja412870m | J. Am. Chem. Soc. 2014, 136, 4452−4455

general access to long-range contacts between nonexchangeable side chain protons. It is also apparent from Figure 3 that the access to chemical groups other than methyl alleviates spectral degeneracy.

^{1}H-cloud labeling does not require reprotonation of exchangeable sites, which is especially challenging for membrane proteins, that are shielded by the bilayer.[36] To probe the scope of ^{1}H-cloud labeling, we prepared a V,L,K ^{1}H-cloud sample of membrane-embedded BamA, which is the main component of the β-barrel assembly machinery.[37,38] We could readily identify separated spectral regions that group signature correlations of the three amino acid types in a 2D ^{13}C−^{1}H spectrum (Figure 4a). In comparison to FP BamA, spectral

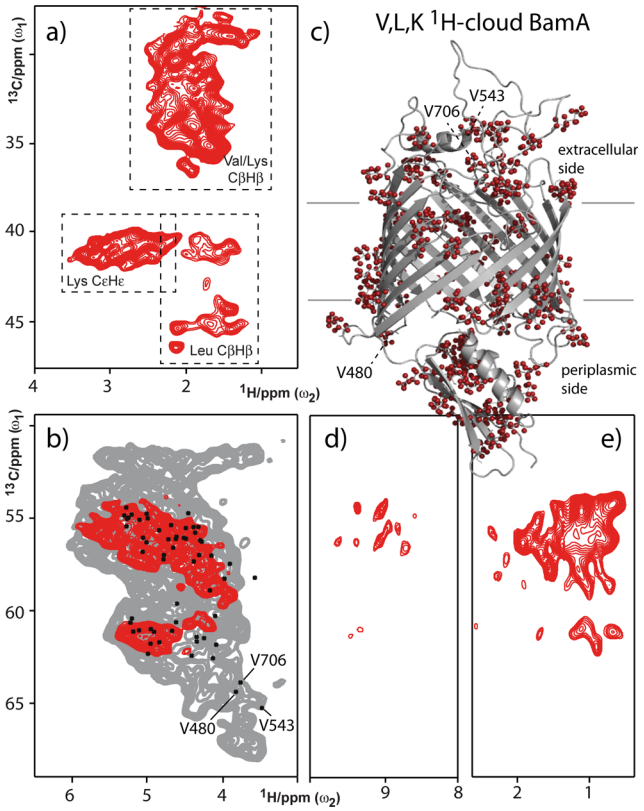

Figure 4. (a,b) Cut-outs of a 2D ^{13}C−^{1}H spectrum (in red) measured at 55 kHz MAS and 800 MHz (c) using a V,L,K ^{1}H-cloud sample of membrane protein BamA, washed in D_2O. A spectrum measured with FP BamA (in gray) is superposed in (b). The FANDAS[40] spectral predictions plotted in (b) (black crosses) were obtained with SHIFTX2[39] using the (c) homology model for *E. coli* BamA,[38] in which Val, Leu, and Lys residues are highlighted by red spheres. (d,e) Cut-outs of a 2D ^{13}C−(^{1}H)^{1}H spectrum showing transfer from H_α to (d) H_N and (e) side chain protons.

congestion is much reduced in the ^{1}H cloud BamA spectrum (Figure 4b), which, drawing on chemical shift predictions,[39,40] allows the observation of the apparent absence of low-field valine residues located in periplasmic (V480) and extracellular (V543 and V706) loops, suggesting increased mobility of these elements (Figure 4b,c). Spectral congestion is increased in comparison to V,L ^{1}H-cloud ubiquitin, due to the residue degeneracy (31 × V, 25 × L, 21 × K) in BamA (52 kDa for the construct used in this study) and due to a residual ^{1}H line width of 0.3−0.4 ppm. Causes for the increased ^{1}H line width are presumably that BamA is not microcrystalline and that the hydrophobic side chains in BamA point to the protonated membrane (Figure 4c), which may be remedied by the use of deuterated lipids. We observed efficient ^{1}H−^{1}H magnetization transfer in V,L,K ^{1}H-cloud BamA including transfer to amino protons, which are presumably water inaccessible since the sample was prepared in D_2O, and aliphatic protons (Figure 4e,f). This bodes well for future structural studies of BamA and other large membrane proteins, especially since the spectral quality of ^{1}H-cloud samples will considerably benefit from emerging magnetic fields (≥1000 MHz) and MAS frequencies (≥80 kHz) (see Figures 2 and S1).

Importantly, our approach is not restricted to the presence of methyl groups but renders most canonical amino acids amenable to measure contacts between nonexchangeable protons (Figure S4). This includes positively charged (Lys, Arg, His) and aromatic (Phe, Trp, Tyr) amino acids, which are all devoid of methyl functions. To avoid incorporation of protons in undesired amino acids ('scrambling'), certain amino acids, such as serine, cysteine and glycine, must be simultaneously labeled, while most amino acids can be added independently to mix the desired ^{1}H-cloud pattern. Alternatively, auxotrophic strains can be used to avoid scrambling (e.g., see ref 41). Generally, ^{1}H-cloud labeling can be readily designed based on the nature of the research problem. For structure calculations, combining restraints from different mixtures of aliphatic ^{1}H clouds will be most informative, while ^{1}H-cloud labeling with charged, polar, or aromatic residues can be useful to refine protein catalytic sites or study protein−membrane interactions.[42] In conclusion, we have shown that fully protonated amino acids in a deuterated background give rise to favorable spectral resolution despite a high local proton density. Such sample preparations provide a general and straightforward approach to obtain long-range distance information between nonexchangeable protons in ^{1}H-detected experiments, which we assume to increase the use of ssNMR in structural biology.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Details of ssNMR experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

m.baldus@uu.nl

m.h.weingarth@uu.nl

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We acknowledge financial support by NWO (grants 700.26.121 and 700.10.443 to M.B. and 722.012.002 to M.W.) and a FEBS long-term fellowship (to M.W.).

■ REFERENCES

(1) Lange, A.; Giller, K.; Hornig, S.; Martin-Eauclaire, M. F.; Pongs, O.; Becker, S.; Baldus, M. *Nature* **2006**, *440*, 959.

(2) Wasmer, C.; Lange, A.; Van Melckebeke, H.; Siemer, A. B.; Riek, R.; Meier, B. H. *Science* **2008**, *319*, 1523.

(3) Shahid, S. A.; Bardiaux, B.; Franks, W. T.; Krabben, L.; Habeck, M.; van Rossum, B. J.; Linke, D. *Nat. Methods* **2012**, *9*, 1212.

(4) Loquet, A.; Sgourakis, N. G.; Gupta, R.; Giller, K.; Riedel, D.; Goosmann, C.; Griesinger, C.; Kolbe, M.; Baker, D.; Becker, S.; Lange, A. *Nature* **2012**, *486*, 276.
(5) Park, S. H.; Das, B. B.; Casagrande, F.; Tian, Y.; Nothnagel, H. J.; Chu, M.; Kiefer, H.; Maier, K.; De Angelis, A. A.; Marassi, F. M.; Opella, S. J. *Nature* **2012**, *491*, 779.
(6) Wang, S.; Munro, R. A.; Shi, L.; Kawamura, I.; Okitsu, T.; Wada, A.; Kim, S. Y.; Jung, K. H.; Brown, L. S.; Ladizhansky, V. *Nat. Methods* **2013**, *10*, 1007.
(7) Reif, B.; Jaroniec, C. P.; Rienstra, C. M.; Hohwy, M.; Griffin, R. G. *J. Magn. Reson.* **2001**, *151*, 320.
(8) Paulson, E. K.; Morcombe, C. R.; Gaponenko, V.; Dancheck, B.; Byrd, R. A.; Zilm, K. W. *J. Am. Chem. Soc.* **2003**, *125*, 15831.
(9) Chevelkov, V.; Rehbein, K.; Diehl, A.; Reif, B. *Angew. Chem., Int. Ed. Engl.* **2006**, *45*, 3878.
(10) Zhou, D. H.; Shah, G.; Cormos, M.; Mullen, C.; Sandoz, D.; Rienstra, C. M. *J. Am. Chem. Soc.* **2007**, *129*, 11791.
(11) Schanda, P.; Meier, B. H.; Ernst, M. *J. Am. Chem. Soc.* **2010**, *132*, 15957.
(12) Ward, M. E.; Shi, L.; Lake, E.; Krishnamurthy, S.; Hutchins, H.; Brown, L. S.; Ladizhansky, V. *J. Am. Chem. Soc.* **2011**, *133*, 17434.
(13) Knight, M. J.; Pell, A. J.; Bertini, I.; Felli, I. C.; Gonnelli, L.; Pierattelli, R.; Herrmann, T.; Emsley, L.; Pintacuda, G. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 11095.
(14) Zhou, D. H.; Nieuwkoop, A. J.; Berthold, D. A.; Comellas, G.; Sperling, L. J.; Tang, M.; Shah, G. J.; Brea, E. J.; Lemkau, L. R.; Rienstra, C. M. *J. Biomol. NMR* **2012**, *54*, 291.
(15) Lewandowski, J. R.; Dumez, J. N.; Akbey, U.; Lange, S.; Emsley, L.; Oschkinat, H. *J. Phys. Chem. Lett.* **2011**, *2*, 2205.
(16) Linser, R.; Dasari, M.; Hiller, M.; Higman, V.; Fink, U.; del Amo, J. M. L.; Markovic, S.; Handel, L.; Kessler, B.; Schmieder, P.; Oesterhelt, D.; Oschkinat, H.; Reif, B. *Angew. Chem., Int. Ed. Engl.* **2011**, *50*, 4508.
(17) Linser, R.; Bardiaux, B.; Higman, V.; Fink, U.; Reif, B. *J. Am. Chem. Soc.* **2011**, *133*, 5905.
(18) Knight, M. J.; Webber, A. L.; Pell, A. J.; Guerry, P.; Barbet-Massin, E.; Bertini, I.; Felli, I. C.; Gonnelli, L.; Pierattelli, R.; Emsley, L.; Lesage, A.; Herrmann, T.; Pintacuda, G. *Angew. Chem., Int. Ed. Engl.* **2011**, *50*, 11697.
(19) Gardner, K. H.; Kay, L. E. *J. Am. Chem. Soc.* **1997**, *119*, 7599.
(20) Huber, M.; Hiller, S.; Schanda, P.; Ernst, M.; Bockmann, A.; Verel, R.; Meier, B. H. *ChemPhysChem* **2011**, *12*, 915.
(21) Asami, S.; Reif, B. *Acc. Chem. Res.* **2013**, *46*, 2089.
(22) Lange, A.; Luca, S.; Baldus, M. *J. Am. Chem. Soc.* **2002**, *124*, 9704.
(23) Marchetti, A.; Jehle, S.; Felletti, M.; Knight, M. J.; Wang, Y.; Xu, Z. Q.; Park, A. Y.; Otting, G.; Lesage, A.; Emsley, L.; Dixon, N. E.; Pintacuda, G. *Angew. Chem., Int. Ed. Engl.* **2012**, *51*, 10756.
(24) Asami, S.; Schmieder, P.; Reif, B. *J. Am. Chem. Soc.* **2010**, *132*, 15133.
(25) Cornilescu, G.; Marquardt, J. L.; Ottiger, M.; Bax, A. *J. Am. Chem. Soc.* **1998**, *120*, 6836.
(26) Schubert, M.; Manolikas, T.; Rogowski, M.; Meier, B. H. *J. Biomol. NMR* **2006**, *35*, 167.
(27) Zhou, D. H.; Rienstra, C. M. *J. Magn. Reson.* **2008**, *192*, 167.
(28) Weingarth, M.; Bodenhausen, G.; Tekely, P. *J. Magn. Reson.* **2009**, *199*, 238.
(29) Asami, S.; Szekely, K.; Schanda, P.; Meier, B. H.; Reif, B. *J. Biomol. NMR* **2012**, *54*, 155.
(30) Zorin, V. E.; Brown, S. P.; Hodgkinson, P. *J. Chem. Phys.* **2006**, *125*, 144508.
(31) Veshtort, M.; Griffin, R. G. *J. Magn. Reson.* **2006**, *178*, 248.
(32) Vijay-Kumar, S.; Bugg, C. E.; Cook, W. J. *J. Mol. Biol.* **1987**, *194*, 531.
(33) Bayro, M. J.; Huber, M.; Ramachandran, R.; Davenport, T. C.; Meier, B. H.; Ernst, M.; Griffin, R. G. *J. Chem. Phys.* **2009**, *130*, 114506.
(34) Grommek, A.; Meier, B. H.; Ernst, M. *Chem. Phys. Lett.* **2006**, *427*, 404.
(35) Weingarth, M.; Masuda, Y.; Takegoshi, K.; Bodenhausen, G.; Tekely, P. *J. Biomol. NMR* **2011**, *50*, 129.
(36) Shi, L.; Kawamura, I.; Jung, K. H.; Brown, L. S.; Ladizhansky, V. *Angew. Chem., Int. Ed. Engl.* **2011**, *50*, 1302.
(37) Renault, M.; Bos, M. P.; Tommassen, J.; Baldus, M. *J. Am. Chem. Soc.* **2011**, *133*, 4175.
(38) Noinaj, N.; Kuszak, A. J.; Gumbart, J. C.; Lukacik, P.; Chang, H.; Easley, N. C.; Lithgow, T.; Buchanan, S. K. *Nature* **2013**, *501*, 385.
(39) Han, B.; Liu, Y.; Ginzinger, S. W.; Wishart, D. S. *J. Biomol. NMR* **2011**, *50*, 43.
(40) Gradmann, S.; Ader, C.; Heinrich, I.; Nand, D.; Dittmann, M.; Cukkemane, A.; van Dijk, M.; Bonvin, A. M. J. J.; Engelhard, M.; Baldus, M. *J. Biomol. NMR* **2012**, *54*, 377.
(41) Lin, M. T.; Sperling, L. J.; Frericks Schmidt, H. L.; Tang, M.; Samoilova, R. I.; Kumasaka, T.; Iwasaki, T.; Dikanov, S. A.; Rienstra, C. M.; Gennis, R. B. *Methods* **2011**, *55*, 370.
(42) Weingarth, M.; Prokofyev, A.; van der Cruijsen, E. A.; Nand, D.; Bonvin, A. M.; Pongs, O.; Baldus, M. *J. Am. Chem. Soc.* **2013**, *135*, 3983.