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High-Accuracy Distance Measurement between Remote Methyls in Specifically Protonated Proteins

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NMR structure determination of biological macromolecules in solution has relied on the use of semiquantitative NOE distance restraints and dihedral angle restraints. The introduction of orientational information derived from residual dipolar couplings has allowed a significant improvement in the precision of structural models of globular proteins.[1] Nevertheless the lack of long-range distances remains a major limiting factor for the accuracy of structure determination of modular and elongated biomolecules as well as for biomolecular complexes. The use of perdeuterated samples and improvement in spectrometer hardware have allowed in the last years the measurement of distance restraints between protons separated by up to 8 Å.[2] Detection of ^{1}H−^{1}H NOEs between more remote protons has been hampered by the small magnitude of such effects due to the r^{-6} dependence. Here we demonstrate that observation of ^{1}H−^{1}H NOEs over distances of up to 12 Å is feasible using a highly deuterated protein in which protons have been selectively incorporated into isoleucine δ_1-methyl sites. By reducing the proton density to 2−3% compared to fully protonated samples, the measurement of accurate long-range distance restraints becomes possible. The detection of NOEs between such remote protons benefits from three major effects. First, because of the proton multiplicity, the choice of methyls for selective protonation enhances signal transferred between two sites by a factor of 9, in the limit of long intermethyl distances. Second, in highly deuterated samples, methyl protons are characterized by favorable transverse and longitudinal relaxation rates providing high resolution and signal-to-noise ratio of the NMR spectra.[3] And last, by reducing the proton density, spin diffusion is considerably reduced, a prerequisite for accurate distance measurements. Long mixing times can then be used to transfer substantial amounts of magnetization through very weak NOEs.

The efficiency of the method for observing the long distance NOE is demonstrated on two proteins for which high resolution solution structures are available: ubiquitin and γS-crystallin, a 19 kDa protein comprising two domains.[4] Highly deuterated samples with selective incorporation of protonated methyls on Ile-δ_1 methyl sites of ubiquitin and γS-crystallin, have been prepared using 2-keto-3,3-d_2-butyrate as the unique proton source during protein overexpression in *E. coli* as previously proposed by Gardner and Kay.[5] A protocol has been implemented to incorporate selectively protonated methyls on Ile-δ_1 sites with an efficiency larger than 98% while the average deuteration level at all other sites in the overexpressed proteins remains larger than 98% (Supporting Information S1). As all the ^{1}H resonances of Ile-δ_1 methyls (7 for ubiquitin and 9 for γS-crystallin) are well resolved, all the NOESY spectra have been recorded using ^{1}H edited 2D-experiments (Supporting Information S2).

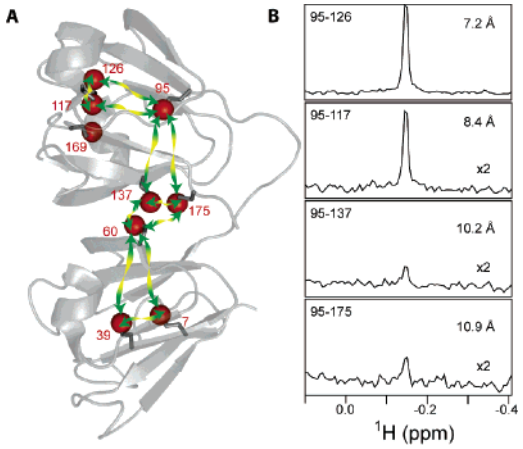

Figure 1. (A) Ile-δ_1 intermethyl NOEs observed in γS-crystallin are indicated by green arrows. Ile-169 does not give rise to detectable intermethyl NOEs owing to extensive line broadening. (B) Intermethyl NOEs detected for Ile-95-δ_1. F_1 cross-sections at δ_1 methyl frequency of Ile 126, 117, 175, and 137 are displayed. The 800 MHz NOESY spectrum has been acquired in 14 h on a 1 mM sample of U-[^{2}H],[CH_3]-Ile-δ_1 γS-crystallin.

The optimal NOE mixing time for transfer between protonated Ile-δ_1 methyls was determined from the build-up of cross-peak intensities in a series of short 2D NOESY spectra. Maximum cross-peak intensities were obtained for mixing times of 1.25 and 0.8 s for ubiquitin and γS-crystallin, respectively (data not shown). 2D NOESY spectra with optimized mixing times have been acquired at 25 °C in 14 h for each protein on a 800 MHz spectrometer equipped with a cryogenic probe. A total of ten pairs of intermethyl cross-peaks for ubiquitin and eleven for γS-crystallin has been detected. All the intermethyl NOEs observed for γS-crystallin are displayed in Figure 1A. 1D traces showing the four observed NOEs involving methyl δ_1 of Ile-95 are displayed in Figure 1B. Comparison of the distance distribution of Ile-δ_1 methyls predicted from the ubiquitin and γS-crystallin structures[4] with cross-peaks detected in the NOESY spectra, revealed that all observed NOEs correspond to intermethyl distances ranging from 4 to 12 Å (Figure 2). All methyl pairs distant by less than 10.5 Å give rise to observable NOEs. For intermethyl distances ranging from 10.5 to 12.5 Å, more than 60% of the expected NOEs are still detected on both sides of the NOESY diagonal with an average signal-to-noise ratio of 5:1.

To evaluate the accuracy of the method, a full-relaxation-matrix analysis was employed to extract distance restraints from NOESY spectra.[6] Given the high resolution of the spectra and the simplicity of the spin system in Ile-δ_1 methyl specific protonated samples, the relaxation matrix σ can be directly determined from diagonal- and cross-peak volumes in NOESY spectra (Supporting Information S3). For the simplified case of fast rotating methyl groups in an isotropically tumbling macromolecule,[7] the average distance be-

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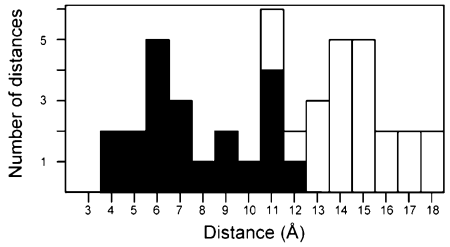

Figure 2. Distribution of Ile-δ_1 intermethyl distances *versus* average interproton distance calculated from structures of ubiquitin and γS-crystallin (except for Ile-169).[4] The experimentally detected NOEs are displayed by black bars.

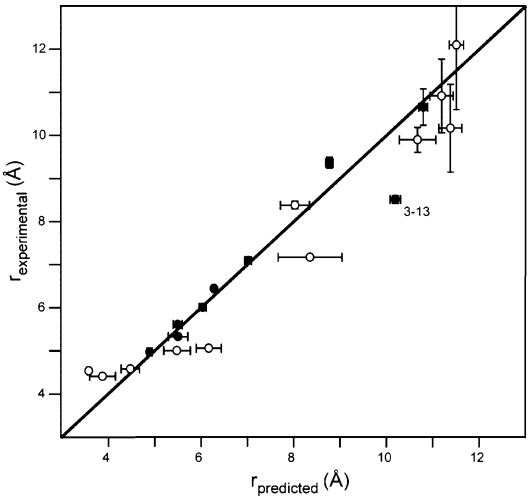

Figure 3. Comparison of the experimental distances calculated from NOE peak volumes using full-relaxation-matrix analysis with distances predicted from ubiquitin (filled circles) and γS-crystallin (open circles) structures.[4] The standard deviation of the distances predicted from the structure ensemble are represented by a horizontal error bar, with $r = (\langle r^{-3}\rangle)^{-1/3}$. A thousand sets of noise-corrupted NOESY peak volumes have been generated (using experimental noise) and used to extract the standard deviation on $r_{\text{experimental}}$ (displayed as vertical error bars). The largest discrepancy occurs between residues 3 and 13 of ubiquitin, which have been shown to populate different side-chain rotamers.[11]

tween methyls i and j can be estimated directly from the relaxation matrix elements σ_{ij}. Comparison of experimental distances with distances extracted from the ubiquitin and γS-crystallin structures shows a very good agreement (Figure 3), with a Pearson's correlation coefficient $R_P = 0.96$. The distances exceeding 5 Å can be determined with an average accuracy of 9% without the use of any internal distance reference. Despite the simplicity of the dynamic model, the NOE-derived distances remain accurate. Because of the $\langle r^{-3}\rangle^2$ dependence of the NOE, the extracted distances are relatively insensitive to error on τ_C. In the slow tumbling limit, an error of 30% in τ_C will result in an error of 5% in the experimentally determined distances.

Full-relaxation-matrix analysis also allows us to evaluate the contribution of spin diffusion to the NOE cross-peaks observed between Ile-δ_1 methyls. Although relatively long mixing times were used (0.8 s for a 17 ns tumbling molecule), spin diffusion only accounts for 12% of the cross-peak volumes, for methyls separated by more than 10 Å in U-[^{2}H], [CH_3]-Ile-δ_1-γS-crystallin (proton density 2%). Protonation of the methyls of Val and Leu residues (proton density 9%) or of all methyl sites in γS-crystallin (proton density 15%) would increase the contribution of spin diffusion to respectively 76% and 92% of the NOEs observed between methyls distant by more than 10 Å (Supporting Information S3), making accurate distance determination difficult. The method proposed here for measurement of accurate long-distance restraints between methyls relies on the reduction of spin diffusion effects by the protonation of only few specific sites in a protein. This specific protonation strategy can be generalized to other methyl-containing residues. For example, specific protonation of only one of the two methyls of Leu and Val residues can be achieved using 2-keto-3-methyl-d_3-3-d_1-butyrate as a precursor during *E. coli* overexpression.[8] The selective protonation of other methyl-containing residues can be obtained alternatively using a cell-free approach.[9] The efficiency of the method was demonstrated on the small model protein ubiquitin as well as on the 19 kDa protein γS-crystallin. The proposed approach can be transposed to larger proteins using ^{13}C,^{1}H-methyl specific labeling. Simulation indicates that in a 45 ns tumbling methyl-specific protonated protein, NOE cross-peaks will still be detectable between methyls separated by up to 10 Å using the recently proposed 4D methyl-TROSY−NOESY experiment.[10] In such large proteins, spin diffusion will contribute for less than 10% of the NOE cross-peak volumes, provided that the total proton density remains lower than 2−3%. The possibility to measure accurate translational restraints between distant methyls offers new opportunities for the study of large proteins and protein complexes by solution NMR.

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Supporting Information Available: Protocol for specific protonation of Ile-δ_1 methyls, experimental details for detection of long-range methyl−methyl NOEs, and equations used to calculate experimental distances from NOESY spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Tjandra, N.; Bax, A. *Science* **1997**, *278*, 1111−1114.
(2) (a) Mueller, G. A.; Choy, W. Y.; Daiwen, Y.; Forman-Kay, J. D.; Venters, R. A.; Kay, L. E. *J. Mol. Biol.* **2000**, *300*, 197−212. (b) Wu, Z.; Bax, A. *J. Am. Chem. Soc.* **2002**, *124*, 9672−9673. (c) Koharudin, L. M. I.; Bonvin, A. M. J. J.; Kaptein, R.; Boelens, R. *J. Magn. Reson.* **2003**, *163*, 228−235. (d) Meier, S.; Häussinger, D.; Jensen, P.; Rogowski, M.; Grzesiek, S. *J. Am. Chem. Soc.* **2003**, *125*, 44−45.
(3) Tugarinov, V.; Hwang, P. M.; Ollerenshaw, J. E.; Kay, L. E. *J. Am. Chem. Soc.* **2003**, *125*, 10420−10428.
(4) (a) Cornilescu, G.; Marquardt, J. L.; Ottiger, M.; Bax, A. *J. Am. Chem. Soc.* **1998**, *120*, 6836−6837. (b) Grishaev, A.; Wu, J.; Trewhella, J.; Bax, A. *J. Am. Chem. Soc.* **2005**, *127*, 16621−16628.
(5) Gardner, K. H.; Kay, L. E. *J. Am. Chem. Soc.* **1997**, *119*, 7599−7600.
(6) Macura, S.; Ernst, R. R. *Mol. Phys.* **1980**, *41*, 95−107.
(7) Tropp, J. *J. Chem. Phys.* **1980**, *72*, 6035−6043.
(8) Tugarinov, V.; Kay, L. E. *J. Biomol. NMR* **2004**, *28*, 165−172.
(9) (a) Kigawa, T.; Muto, Y.; Yokoyama, S. *J. Biomol. NMR* **1995**, *6*, 129−134. (b) Torizawa, T.; Shimizu, M.; Taoka, M.; Miyano, H.; Kainosho, M. *J. Biomol. NMR* **2004**, *30*, 311−325. (c) Kainosho, M.; Torizawa, T.; Iwashita, Y.; Terauchi, T.; Ono, A. M.; Güntert, P. *Nature* **2006**, *440*, 52−57.
(10) (a) Tugarinov, V.; Ollerenshaw, J. E.; Kay, L. E. *J. Am. Chem. Soc.* **2005**, *127*, 8214−8225. (b) Frueh, D.; Vosburg, D. A.; Walsh, C. T.; Wagner, G. *J. Biomol. NMR* **2006**, *34*, 3−172.
(11) Chou, J. J.; Case, D. A.; Bax, A. *J. Am. Chem. Soc.* **2003**, *125*, 8959−8966.

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