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Observation of Heteronuclear Overhauser Effects Confirms the $^{15}N-^{1}H$ Dipolar Relaxation Mechanism in a Crystalline Protein

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Many of the key functional aspects of proteins are determined by their flexibility. NMR relaxation measurements have long been established as the primary tool to probe protein dynamics in solution.[1] Recently, we have shown that in microcrystalline proteins nitrogen-15 spin−lattice relaxation rates (R_1s) provide a very sensitive probe of the variations in molecular dynamics along the backbone.[2] We proposed a quantitative framework to analyze ^{15}N R_1s with an Explicit Average Sum (EAS) approach[2] which extends pioneering early work, by Torchia and Szabo[3] and by McDowell and co-workers,[4] to the case of restricted N−H bond motions in microcrystalline proteins. Our interpretation of ^{15}N R_1 data assumed that the N−H dipolar interaction was the dominant mechanism for ^{15}N longitudinal relaxation in this type of sample. However, other mechanisms of relaxation are conceivable. Notably, it has been proposed that paramagnetic oxygen may be present in the hydrophobic regions of crystalline proteins, thereby contributing significantly to carbon-13 R_1s.[5] If an analogous mechanism exists for ^{15}N, associated R_1s could not be interpreted in terms of the internal reorientational properties of the dipolar interaction. It is therefore crucial to identify the mechanism for ^{15}N relaxation in crystalline proteins, to put subsequent dynamic analysis on a solid footing. Here we present the observation of ^{1}H to ^{15}N cross-relaxation (heteronuclear nOe) in a microcrystalline protein and provide an analysis of the different pathways that could lead to the observed results. The analysis confirms the N−H dipolar relaxation mechanism.

All experiments were carried out on a Bruker Avance 700 MHz SB spectrometer using a 4 mm double tuned CP-MAS probe, at a sample spinning speed of 10 kHz, on a microcrystalline, uniformly labeled [^{15}N, ^{13}C] sample of the protein Crh.[6] The effective sample temperature was about +7 °C.

The spectra of Figure 1a were obtained with a solid-state version of the proton to nitrogen-15 transient nOe pulse sequence.[7] We observe clear heteronuclear nOe enhancements of both backbone and side chain nitrogens. We also note that the amplitude and time dependence of the enhancements (Figure 1b and c) are consistent with the cross-relaxation rates predicted for a dipolar relaxation mechanism due to diffusion of a N−H vector in a cone using parameters for typical restricted dynamics, measured for Crh through an EAS[2] analysis. In this way, we predict the expected heteronuclear nOe enhancements to be about 20%, in good qualitative agreement with the spectra (see Supporting Information).

Figure 1d shows a ^{1}H−^{15}N solid-state HOESY (Heteronuclear Overhauser Enhancement SpectroscopY)[8,9] correlation spectrum, obtained to determine where the magnetization leading to the nOe enhancements originates from. In the 2D spectrum we observe that each nitrogen signal essentially only gives rise to a correlation with the water signal in the proton dimension. Although at first sight this may appear surprising, it can be fully explained in terms of nOe, fast chemical exchange with the high concentration of water protons and rapid spin diffusion among the protein protons.

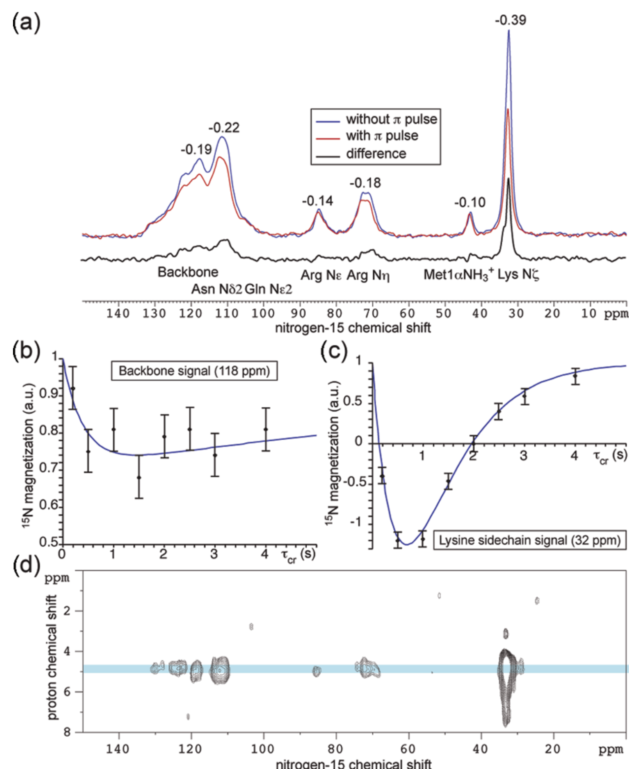

Figure 1. (a) The reference spectrum (blue), the transient nOe spectrum (red), and the difference spectrum (black) are shown with the assignment of the resonances[6] as well as the value of the nOe enhancement for each signal (see Supporting Information for the pulse sequences). The ^{1}H and ^{15}N rf field strengths for the pulses were 100 kHz and 50 kHz, respectively. The proton decoupling field was 80 kHz with SPINAL64.[10] Data were zero-filled and subject to a 50 Hz exponential line broadening. Each spectrum was recorded in 13 h using about 8 mg of protein, with a recycle delay d_{rec} = 2.5 s and a cross-relaxation delay τ_{cr} = 2 s. In (b) and (c), we show buildups of the magnetization as a function of the cross relaxation delay. In (d) the proton indirect dimension was acquired under homonuclear decoupling, using the eDUMBO-1_{22} pulse sequence[11] with ν_1^{H} = 100 kHz. Each of the 30 increments in t_1 were acquired with 4608 scans and a 3 s recycle delay between scans, with maximum acquisition times of 1.44 ms in t_1 and 25 ms in t_2. Data were processed with an exponential line broadening in t_1 (100 Hz) and t_2 (200 Hz). The cross-relaxation delay τ_{cr} was set to 2 s. (The pulse sequences and phase cycles are available on our web site[12] or upon request.)

Figure 2 summarizes the different magnetization transfer pathways which include both a ^{1}H−^{15}N cross relaxation step, essential to achieve heteronuclear transfer here, as well as an interaction

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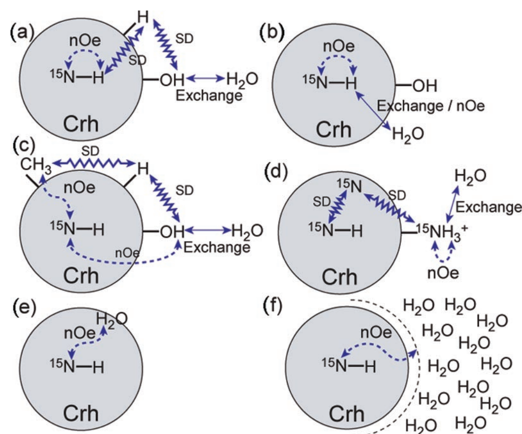

Figure 2. Schematic representation of possible mechanisms accounting for both $^{1}H-^{15}N$ cross relaxation and magnetization transfer involving water protons and protein ^{15}N. SD denotes spin diffusion. (a) Magnetization from water protons is transferred to the protein through a fast exchanging group and then spin diffuses to a proton in an amide group whose motion induces cross relaxation. In (b) water is involved in a direct exchange (or nOe) process with the amide proton that undergoes cross relaxation. (c) After transfer from water to a labile group, magnetization diffuses among the protons to mobile groups (such as side chain methyls or NH_3^+) whose motion causes cross relaxation between their protons and the closest nitrogens. (d) nOe occurs between the proton and nitrogen within a labile group (e.g., Lysine NH_3^+), and magnetization is then transferred to backbone nitrogens through ^{15}N spin diffusion. (e) Magnetization is transferred by a direct nOe between a nitrogen and a trapped water molecule and a (f) direct nOe with a layer of bulk water within the crystal lattice.[13,14]

between water protons and the protein (either chemical exchange or direct nOe), and which could lead to cross-peaks between backbone nitrogens and water protons.

Only schemes (a) and (b) are consistent with the amide N−H dipolar relaxation mechanism. Both these schemes appear reasonable, since (i) the site resolved observation of hydrogen exchange between protein protons and water protons in microcrystalline Crh, with exchange times on the order of milliseconds, has been reported[15,16] and (ii) spin diffusion among the protons occurs on a time scale of the order of a few hundreds of microseconds. Pathway (c) can be discounted as the direct nOe between mobile protons in side chains, and amide nitrogens should be negligible, since σ_{NH} decays very rapidly with distance and since the shortest N−H distance is much shorter than any other N−H distance. This term is also considered negligible in liquid-state studies. The heteronuclear nOe within a mobile labile side chain group, shown in pathway (d), is reasonable. However, pathway (d) can also be discounted since we have measured the degree of homonuclear spin diffusion among the ^{15}N nuclei and find it to be minor under these conditions: limited to weak transfers between neighboring backbone amide nitrogens, with no side chain to backbone cross-peaks (see Supporting Information).

Finally we consider pathways (e) and (f) which invoke the possibility of direct cross-relaxation through longer-range dipole couplings between amide nitrogens and water protons, with the water being either a single trapped molecule (e) or a layer of bulk water (f).[14] We have crudely modeled both possibilities, using parameters that favor these processes to the maximum reasonableness, and find that they can both be clearly excluded. Even if a resident water molecule (e) was tumbling isotropically around an amide nitrogen (the most favorable case, which is clearly not physically reasonable), it would have to be closer than 2.5 Å to the nitrogen to contribute more than 10% in comparison to the dominant dipolar relaxation mechanism within the amide group. Furthermore, only one internal water molecule has been observed by X-ray crystallography for Crh. Similarly, (f) there is a potential influence of diffusion of bulk water throughout the spaces in the crystal lattice, similarly to that observed for water to protein nOe in solution.[14] In the schematic (most favorable) case of an amide group at the center of a spherical protein, we find that a significant effect (i.e., a contribution to the estimated cross relaxation rate of at least 10%) would be detected, for typical diffusion times,[14] and for a water layer extending to infinity, only for distances of closest approach less than 4 Å, which is significantly shorter than the typical distances of closest approach in Crh (see Supporting Information for details of the calculations).

In conclusion, we confirm the presence of $^{15}N-^{1}H$ heteronuclear Overhauser effects between amide nitrogens and their bound protons in microcrystalline Crh. This unambiguously confirms that the main contribution to ^{15}N longitudinal relaxation is the fluctuation of the N−H bond. Other mechanisms, such as relaxation by paramagnetic impurities, cannot give rise to Overhauser effects and can therefore be considered as making only a minor, if any, contribution to ^{15}N relaxation. This is primarily due to the fact that the amide N−H distance is much shorter than any other N−H distance, and to the low gyromagnetic ratio of ^{15}N, which reduces the influence of homonuclear $^{15}N-^{15}N$ effects. This makes ^{15}N an ideal, very local relaxation probe of protein dynamics. Finally, quantitative site-specific heteronuclear nOe measurements, if possible in the future, would provide an excellent parameter to complement the R_1s to constrain more efficiently spectral densities and probe a wider range of motional time scales and dynamical models.

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Supporting Information Available: Pulse sequences used in this work, nitrogen-15 2D PDSD spectrum, and details of the calculations for the different models invoked in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Mittermaier, A.; Kay, L. E. *Science* **2006**, *312*, 224−228.
(2) Giraud, N.; Blackledge, M.; Goldman, M.; Böckmann, A.; Lesage, A.; Penin, F.; Emsley, L. *J. Am. Chem. Soc.* **2005**, *127*, 18190−18201.
(3) Torchia, D.; Szabo, A. *J. Magn. Reson.* **1982**, *49*, 107−121.
(4) Naito, A.; Ganapathy, S.; Akasaka, K.; McDowell, C. A. *J. Magn. Reson.* **1983**, *54*, 226−235.
(5) Morcombe, C. R.; Gaponenko, V.; Byrd, R. A.; Zilm, K. W. *J. Am. Chem. Soc.* **2005**, *127*, 397−404.
(6) Böckmann, A.; Lange, A.; Galinier, A.; Luca, S.; Giraud, N.; Juy, M.; Heise, H.; Montserret, R.; Penin, F.; Baldus, M. *J. Biomol. NMR* **2003**, *27*, 323−339.
(7) Neuhaus, D.; Williamson, M. P. *The Nuclear Overhauser Effect in Structural and Conformational Analysis*, 2nd ed.; John Wiley & Sons: New York, 2000.
(8) Ashbrook, S. E.; Dowell, N. G.; Prokes, I.; Wimperis, S. *J. Am. Chem. Soc.* **2006**, *128*, 6782−6783.
(9) Lesage, A.; Emsley, L.; Penin, F.; Böckmann, A. *J. Am. Chem. Soc.* **2006**, *128*, 8246−8255.
(10) Fung, B. M.; Khitrin, A. K.; Ermolaev, K. *J. Magn. Reson.* **2000**, *142*, 97−101.
(11) Elena, B.; de Paepe, G.; Emsley, L. *Chem. Phys. Lett.* **2004**, *398*, 532−538.
(12) http://www.ens-lyon.fr/CHIMIE/Fr/Groupes/NMR/Pages/library.html.
(13) Modig, K.; Liepinsh, E.; Otting, G.; Halle, B. *J. Am. Chem. Soc.* **2004**, *126*, 102−114.
(14) Halle, B. *J. Chem. Phys.* **2003**, *119*, 12372−12385.
(15) Lesage, A.; Böckmann, A. *J. Am. Chem. Soc.* **2003**, *125*, 13336−13337.
(16) Böckmann, A.; Juy, M.; Bettler, E.; Emsley, L.; Galinier, A.; Penin, F.; Lesage, A. *J. Biomol. NMR* **2005**, *32*, 195−207.

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