NMR Spectroscopy

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Polarization Transfer over the Water–Protein Interface in Solids**

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Interactions between proteins and water play an important role in the determination of the structure, folding, and dynamics of biomolecules and are thus an essential factor in chemical processes in living organisms. The characterization of water–protein interactions is generally difficult, but necessary for understanding of the above processes. Moreover, the contrast in anatomical magnetic resonance images is based on the fact that water molecules have different relaxation times in different tissues.[1] Although the properties of water may be altered by the addition of paramagnetic agents,[2] interactions between water and cellular components form the principal determinant of the relaxation behavior of water in tissue and cells.

Much information about protein–water interactions has been provided by solution-state NMR spectroscopic studies. Magnetic resonance offers two complementary approaches to the monitoring of water–protein interactions, namely NMR spectroscopy[3] and magnetic resonance dispersion[4] (MRD). These two methods today provide a consensus interpretation of phenomena observed in solution.[3–7] Recently, the availability of isotopically enriched microcrystalline protein samples has provided a new platform for the study of site-specific water–protein interactions in solid proteins.[8–12] However, no thorough understanding of polarization-transfer pathways yet exists for hydrated solid samples. Notably, in solids, the presence of secular dipolar interactions adds extra polarization-transfer pathways to the already complex network of direct and indirect water–protein polarization transfer by chemical exchange and nuclear Overhauser effect (NOE) pathways identified in solution by NMR spectroscopy. The NOE pathway in solid proteins was described by Zilm and coworkers,[9] and chemical exchange in references [8, 11, 12].

Herein we describe experiments that enable us to resolve the contributions of the individual transfer pathways in microcrystalline protein samples by determining water–protein polarization-transfer behavior either in supercooled samples over a relatively broad temperature range or by using methods based on high-frequency magic-angle spinning. We found that at room temperature, or under slow sample spinning, interactions are dominated by fast chemical exchange followed by spin diffusion. At low temperatures, or at high-frequency spinning, contributions from these pathways are decreased, and Overhauser effects can be detected.

One key to the observations reported herein is that we found that we could supercool the water solvent in the microcrystalline protein preparation and thus extend the observation range for the study of water–protein interactions without inducing the significant line broadening that occurs at the freezing point of the solvent.[13,14] To achieve this effect, we exploit the fact that the sample contains 10% poly(ethylene glycol) (PEG) 6000: PEG has been shown to influence ice nucleation in aqueous solutions, whereby an increase in the molar mass of the PEG leads to increased supercooling.[15] Figure 1 illustrates this effect with freezing/melting points in a freeze–thaw cycle that indicate marked hysteresis. Over a range of temperatures, down to about −20 °C, the supercooled state is stable long enough for two-dimensional NMR spectra to be recorded. Water–protein interactions in the supercooled state are reflected in 2D ^{13}C–^{1}H HETCOR spectra in the cross-peaks at the water frequency in ω_1.[8–11,17] Figure 2a shows cross-sections at the water frequency in ω_1 of 2D HETCOR spectra recorded at various temperatures (all indicated temperatures are sample temperatures; see also the Experimental Section).

At 20 °C, the cross-peaks corresponding to E70 Cδ, Y80 Cζ, His Cε1 and Cδ2, Thr Cβ and Cγ, Ser Cβ, N-ter Met1 Cα, and Lys Cε and Cδ can be clearly identified.[8,11,18] These cross-peaks all decrease in intensity when the sample is cooled, with the exception of the His resonances (all located in the 6×His tag). The His resonances at first increase in intensity as a result of slower molecular dynamics at lower temperatures, which favor polarization transfer by cross-polarization (CP). Most of the positive signals in the spectra clearly arise from chemical exchange[8,11,12] (Figure 2b, left). Some signal intensity is also observed at frequencies at which no carbon nuclei near to fast-exchanging protons resonate. These signals have their origin in exchange-relayed polarization transfer through proton-spin diffusion (Figure 2b, center).

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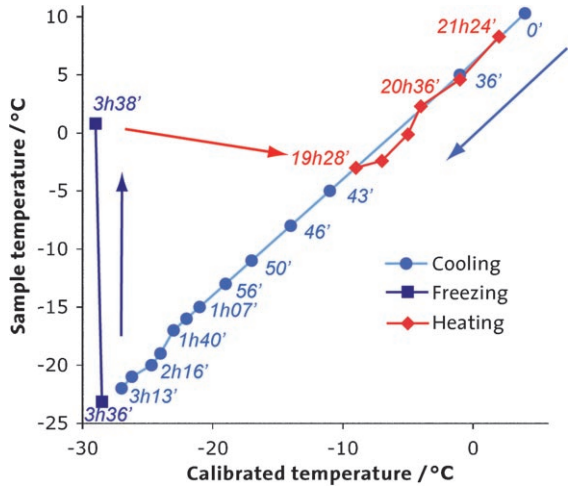

Figure 1. Temperature of a microcrystalline sample of the Crh protein during a freeze–thaw cycle. The sample temperature (determined from the frequency of the water resonance) is plotted against the temperature calibrated with respect to the ^{119}Sn NMR signal of the chemical-shift thermometer $Sm_2Sn_2O_7$.[16] A temperature difference of about 5 K is observed for the points at which the sample is in thermodynamic equilibrium. This difference is probably due to the fact that calibration has to be carried out with a different rotor, which generates a different frictional heat. Blue circles correspond to signals recorded on lowering the temperature; dark-blue squares indicate freezing of the sample. Red squares correspond to signals in spectra recorded during heating of the sample. As expected, the temperature of the sample decreases with decreasing environmental temperature. In the spectra, the frequency of the water resonance (blue circles) is shifted downfield to a chemical shift of about 5.21 ppm (the corresponding spectra are shown in Figure S1 of the Supporting Information), which corresponds to a sample temperature of about −21 °C (see the Experimental Section). Further lowering of the calibrated temperature by one degree leads to a sudden rise in the sample temperature to approximately 0 °C, which indicates ice formation. On further cooling, the water resonance diminishes further in intensity, but remains at the same chemical shift (see Figure S1 in the Supporting Information). When the sample is heated (red data points), its temperature can not be determined from the chemical shift until melting occurs.

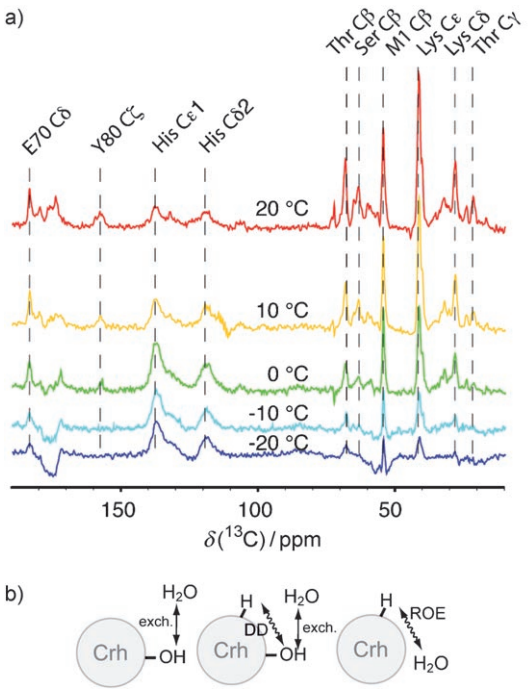

Figure 2. a) ω_2 rows extracted at the water frequency from conventional HETCOR spectra recorded at different temperatures. A contact time of 2 ms was used, and the spinning speed was set to 9 kHz. The temperatures indicated correspond to sample temperatures determined from the water chemical shift (see the Experimental Section). The pulse sequence for the HETCOR experiment and the resulting 2D spectra are shown in the Supporting Information (Figures S2a and S3). b) Different modes of polarization transfer that lead to the peaks in the spectra in (a).

At −20 °C, the strongest positive signals come from carbon atoms in histidine side chains. Protons in histidine side chains have been shown to exchange very rapidly[19a] (>100 000 s^{-1} at room temperature in ubiquitin[19b]), which explains their presence in the spectra even at −20 °C. The exchange rates of protons in lysine residues are slower by an order of magnitude at around 10 000 s^{-1} at room temperature (in ubiquitin[20]); this value can be extrapolated to about 1000 s^{-1} at −20 °C. These exchange rates support our observations that the His and several Lys residues still show exchange peaks within a few milliseconds at −20 °C. The progressive disappearance of positive cross-peaks in the spectra with decreasing temperature clearly identifies these cross peaks as resulting from chemical exchange.

Another interesting feature is revealed in the spectra in Figure 2. As peaks due to water–protein exchange disappear progressively with decreasing temperature, negative cross-peaks become evident in the spectra, and at −20 °C, negative cross-peaks are clearly visible in the CO and Cα regions of the spectrum. The pattern observed is strongly reminiscent of similar observations in liquid-state ROESY NMR spectra (Figure 2b, right). Indeed, in such spectra, in which polarization transfer occurs during a spin-lock period, transfer by rotating-frame NOEs (ROEs) gives rise to negative cross-peaks, whereas cross-peaks due to chemical exchange are always positive. At −20 °C, the majority of the ^{13}C resonances in the spectrum are inverted, except those from carbon atoms in the vicinity of exchanging protons, which suggests that ROE effects with water can occur in a non-site-specific manner or that such effects are relayed by spin diffusion.

To confirm the observation of ROE-type interactions, we carried out ROESY–HETCOR experiments that include a proton-spin-lock period before the CP step (see Figure S2b in the Supporting Information for the pulse sequence). Figure 3a shows the traces at the water frequency in ω_1 extracted from 2D spectra recorded at −18 °C for various spin-lock periods (see Figure S4 in the Supporting Information). Their similarity to the traces in Figure 2 at low temperatures confirms that the negative peaks originate from polarization exchange during the proton-spin-lock period. Figure 3b shows the polarization buildup as a function of the mixing time. The nonzero intensity of the signal at zero mixing time originates from an ROE and chemical exchange during CP. Negative signals show the same evolution in the carbonyl and aliphatic regions, with a minimum at around 4 ms. Positive signals show different behavior as a reflection of the different exchange rates of the exchanging protons.

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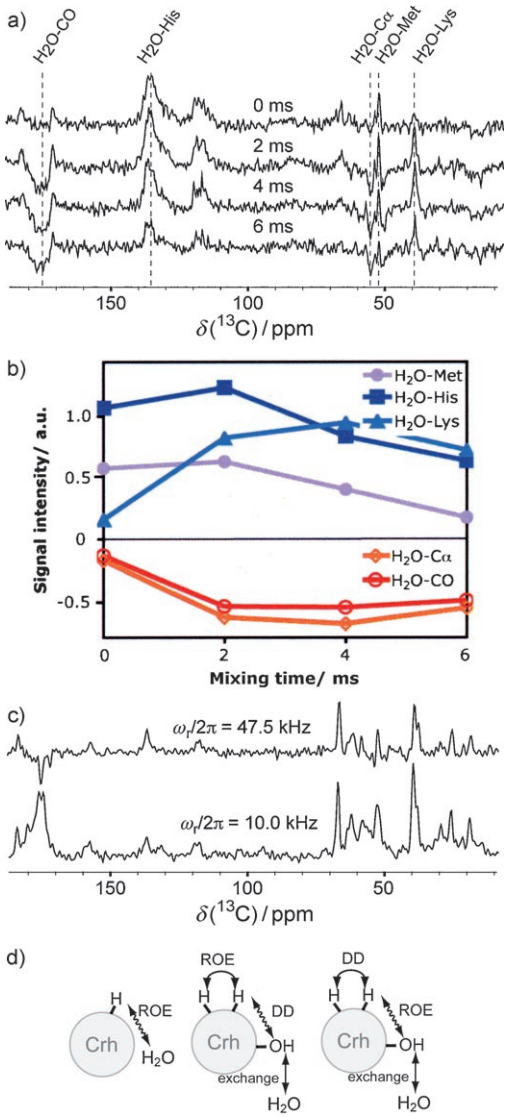

Figure 3. a) ω_2 rows extracted at the water frequency from conventional ROESY–HETCOR spectra recorded at a sample temperature of −18 °C for different proton spin-lock times. The pulse sequence for the ROESY–HETCOR experiments, and the 2D spectra, are shown in the Supporting Information (Figures S2b and S4, respectively). b) Buildup curves for positive signals from carbon nuclei near exchanging protons (Met1 Cα, His Cε1, Lys Cε) and negative signals, indicative of intermolecular ROEs, for resonances in the carbonyl and Cα regions. c) ROESY–HETCOR spectra recorded at two different spinning speeds at a sample temperature of 10 °C. d) Possible pathways for water–protein polarization.

The observation of negative signals indicates that at some point during the pulse sequence, proton–proton magnetization transfer by ROEs takes place: either direct ROE magnetization transfer from water protons to protein protons (Figure 3d, left) or intramolecular ROE polarization transfer from a fast-exchanging proton to another protein proton (exchange-relayed transfer; Figure 3d, center). The latter mode of transfer is, however, only efficient if the fluctuation of the dipolar interaction with a nearby spin as a result of internal motion is so pronounced as to provide a dominant mechanism.[21,22] Finally, ROE signals relayed by chemical exchange could also be transferred by spin diffusion to the remainder of the protein protons (Figure 3d, right). A contribution from direct spindiffusion from an immobile waterlayer to the protein is judged unlikely here since no double-quantum coherence between water and protein-protons could be excited under similar conditions, butcan not rigidly be excluded.[12]

Pathways that involve spin diffusion (exchange–spin diffusion or exchange–ROE spin diffusion) are expected to be less efficient at higher spinning frequencies. We therefore recorded ROESY–HETCOR spectra at two different spinning frequencies (10 and 47.5 kHz), with a spin-lock time of 6.25 ms and at a temperature of 10 °C. The resulting cross sections at the water frequency are shown in Figure 3c. Negative signals are clearly visible in the carbonyl and Cα regions at high spinning frequencies, whereas the corresponding signals are positive under slow spinning but otherwise identical experimental conditions. We conclude that at a magic-angle-spinning (MAS) frequency of 10 kHz, the spectrum is dominated by chemical exchange, which is relayed by spin diffusion. The ROE polarization transfer is masked by polarization transfer that is relayed by fast spin diffusion from the exchanging sites. Therefore, at low spinning frequencies, negative cross-peaks are only observed at low temperatures, at which chemical exchange and relays thereof are less efficient. At faster MAS, proton-spin diffusion is slowed down, and the exchange–spin diffusion pathway becomes less efficient than ROE transfer. Therefore, negative cross-peaks are observed. As an alternative to fast MAS, spin diffusion can also be slowed down by using a spin lock at the magic angle (for example, through homonuclear dipolar decoupling, e-DUMBO). Under these conditions, the same pattern of negative cross-peaks is observed in the spectra, which confirms that spin diffusion becomes secondary to direct polarization transfer (for more details, see the Supporting Information).

In conclusion, we have demonstrated for the Crh protein that the dominant signals observed at the water frequency in ^{1}H–^{13}C HETCOR spectra originate from chemical exchange between protons in the protein side chain and water. The exchange process is slowed down by lowering the temperature. This polarization may then be transferred to other protein protons by spin diffusion, a pathway that is less efficient under fast MAS. Intermolecular water–protein ROEs are also detectable in solid proteins, even if the signals are weaker than those that arise from chemical exchange. Our results could form a basis for similar investigations on noncrystalline systems, for example, amyloid fibrils or proteins of the cytoskeleton. Furthermore, this study is of great relevance to the development of new pulse sequences with better water management for solid-state NMR experiments, including water-suppression and proton-detection techniques.

Experimental Section

Sample preparation: Crh was overexpressed as described previously.[23] The bacteria were grown in >98% ^{2}H-,^{13}C-,^{15}N-labeled medium (Silantes). Exchangeable protons were re-exchanged under

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denaturing conditions (8 M guanidinium chloride). The protein was crystallized[18] in a crystallization plate over a 2 M NaCl solution by using 20% PEG 6000 as the precipitant. The crystallization solution containing the protein had a pH value of 7. The rotor was filled with microcrystals derived from about 8 mg of the protein. The protein showed residual protonation, mainly on methyl groups. The protein sample used for the experiments with high-frequency spinning was expressed in ($^2H^{13}C^{15}N$)S9 medium (Spetra Stable Isotopes) and showed a higher degree of deuteration than the protein described above. The sample was placed in a 1.8 mm rotor.

Temperature calibration: The position of the H_2O proton signal in the NMR spectrum has a significant temperature dependency. As a consequence, the chemical shift of the water resonance can be used to measure the sample temperature. By external referencing to DSS (2,2-dimethyl-2-silapentane-5-sulfonic acid), which defines a chemical shift of 0 ppm, the temperature can be determined by using the relationship $\delta(H_20) = 7.83 - T/96.9$ ppm, with the temperature measured in Kelvin.[24]

NMR spectroscopy: NMR experiments were performed with a Bruker Avance DSX 700 MHz standard-bore spectrometer equipped with a double-resonance 4 mm MAS probe, and with a Varian Infinity + 500 MHz spectrometer equipped with a double-resonance 1.8 mm MAS probe (manufactured by A. Samoson and J. Past, Tallinn). Experimental procedures are described in detail in the Supporting Information.

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