Published in final edited form as:

Methods Enzymol. 2019 ; 615: 77–101. doi:10.1016/bs.mie.2018.09.040.

Characterizing protein hydration dynamics using solution NMR spectroscopy

Christine Jorge^{1,2}, Bryan S. Marques^{1,2}, Kathleen G. Valentine², and A. Joshua Wand^{1,2}

¹Graduate Group in Biochemistry and Molecular Biophysics, Perelman School of Medicine, University of Pennsylvania, Philadelphia PA, 19104

²Johnson Research Foundation and Department of Biochemistry and Biophysics, Perelman School of Medicine, University of Pennsylvania, Philadelphia PA, 19104

Abstract

Protein hydration is a critical aspect of protein stability, folding and function and yet remains difficult to characterize experimentally. Solution NMR offers a route to a site-resolved view of the dynamics of protein-water interactions through the nuclear Overhauser effects between hydration water and the protein in the laboratory (NOE) and rotating (ROE) frames of reference. However, several artifacts and limitations including contaminating contributions from bulk water potentially plague this general approach and the corruption of measured NOEs and ROEs by hydrogen exchange relayed magnetization. Fortunately, encapsulation of single protein molecules within the water core of a reverse micelle overcomes these limitations. The main advantages are the suppression hydrogen exchange and elimination of bulk water. Here we detail guidelines for the preparation solutions of encapsulated proteins that are suitable for characterization by NOE and ROE spectroscopy. Emphasis is placed on understanding the contribution of detected NOE intensity arising from magnetization relayed by hydrogen exchange. Various aspects of fitting obtained NOE, selectively decoupled NOE and ROE time courses are illustrated.

Keywords

protein hydration; hydration dynamics; nuclear Overhauser effect; rotating frame Overhauser effect; hydrogen exchange; relayed Overhauser effect

1 Introduction

Water is critical to many aspects of protein function (Bellissent-Funel, Hassanali, Havenith, Henchman, Pohl, Sterpone et al., 2016) including folding (Tanford, 1979), stability (Adrover, Martorell, Martin, Urosev, Konarev, Svergun et al., 2012), catalysis (Dey, Jenny, Adams, Babini, Takahashi, Fukuyama et al., 2007), and potentially dynamics (Zaccai, 2004) and molecular recognition and binding. It is known that motion of water is heterogeneously slowed near the protein surface but the precise origins of the dynamic character of the protein hydration layer remain obscure (Sterpone, Stirnemann, & Laage, 2012). A site

resolved view of protein hydration dynamics has historically been very difficult to obtain experimentally. Solution nuclear magnetic resonance spectroscopy (NMR) would seem to provide a route to detailed site-resolved information about the structural and dynamical interaction between protein molecules and hydration water in solution and at room temperature (Otting, & Wuthrich, 1989; Otting, Liepinsh, & Wuthrich, 1991; Otting, 1997).

The primary solution NMR tools for probing protein-water interactions are the laboratory frame nuclear Overhauser effect (NOE) and its rotating frame counterpart (ROE) of through-space dipolar magnetization transfer (Bothner-By, Stephens, Lee, Warren, & Jeanloz, 1984; Macura, & Ernst, 1980). For an isolated spin pair, the limiting behavior with respect to the dynamics of the interaction results in NOE/ROE ratios approaching a limit of -0.5 in the slow tumbling limit and $+1$ in the extreme narrowing limit. Unfortunately, it has been recognized that several potential artifacts can corrupt the NOE/ROE approach. In this chapter we describe samples that are designed to largely eliminate these artifacts and provide experimental and analytical strategies to obtain quantitative information about hydration water dynamics.

2 Theory

2.1 Foundation theory

The rates of NOE and ROE mediated magnetization transfer have different reference frames and accordingly have different dependence on the spectral density functions describing the motion of the internuclear vector (Macura, et al., 1980):

$$\sigma^{NOE} = q[6J(2\omega_o) - J(0)] \quad (1)$$

$$\sigma^{ROE} = q[3J(\omega_o) - 2J(0)] \quad (2)$$

Where q is a prefactor of fundamental constants including the gyromagnetic ratio of protons and Planks constant, ω is the proton Larmor frequency, and $J(\omega)$ is the power spectral density function defined as:

$$J(\omega) = \int C(t) \cos(\omega t) dt \quad (3)$$

where $C(t)$ is the sum of the auto-correlation function of the magnetic dipole-dipole interactions between the two spins. Note, that the spectral densities of Equations 1 and 2 are general. The autocorrelation function is defined as:

$$C(t) = \sum_j \left\langle \frac{Y_{20}(r_j(0))}{r_j^3(0)} \frac{Y_{20}(r_j(t))}{r_j^3(t)} \right\rangle \quad (4)$$

Where $Y_{20}(r_j) = \sqrt{5/16\pi} \times (3\cos^2\theta - 1)$ is a normalized spherical harmonic function that describes the internuclear vector between the two spins. The auto-correlation function (Eq. (4)) is therefore a convolution of the time dependence and length and orientation of the two interacting spin systems, and makes a detailed interpretation challenging (Bruschweiler, & Wright, 1994).

Nevertheless, one can obtain considerable insight by treating the cross relaxation between two rigidly connected spins, with one associated with the protein and the other associated with a water molecule. In this case, which assumes no relative motion between the protein and water molecule, the spectral density function is simply:

$$J(\omega) = \frac{1}{r^6} \frac{\tau}{1 + \omega^2 \tau^2} \quad (5)$$

where r is the distance between two interacting spins and the effective correlation time (τ) is often interpreted to include the rotational motion of the protein-water “complex” and the mean residence time of the water (Otting, et al., 1991). Using this spectral density in the expressions for the rate of cross relaxation in the laboratory (σ^{NOE}) and rotating (σ^{ROE}) frames gives a smooth dependence of the $\sigma^{NOE}/\sigma^{ROE}$ ratio on the effective time constant that ranges from +1 to -0.5 (Figure 1). It is important to note that the effective correlation time for water in bulk is on the order of a few picoseconds. This implies that all but the strongest interactions of water with protein will be in the extreme narrowing limit and many will be near the null for the NOE.

2.2 Overcoming artifacts and limitations

There are two main artifacts that impede the use of the NOE/ROE ratio as a measure of protein hydration dynamics: long-range spatial averaging and the effects of hydrogen exchange (Halle, 2004). The simple theoretical description of Figure 1 does not take into account that, in bulk solution, the number of waters surrounding protein molecules is enormous and these waters are moving very quickly. The motion has two prominent effects. On the one hand, the waters are averaged spectrally. On the other hand, all waters in a bulk solution experiment contribute to the dipolar relaxation between a protein hydrogen and water. Halle has argued that consideration of this effect can completely corrupt the distance selectivity of the NOE and ROE suggested by Equations 4 and 5 (Halle, 2003). However, Steinhäuser and workers have recently re-examined the effect of distance and dynamical averaging of long-range dipole-dipole interactions between bulk water and protein. They find that the pair correlation function describing this interaction is highly dependent on the depth of burial of the involved protein hydrogens. For protein hydrogens at the surface of the

protein the contribution from the pair correlation function is much higher due to the higher incidence of nearby waters and leads to less of a contribution to the protein-water NOE from bulk solvent. It is concluded that that NOE/ROE ratio is an excellent tool for the detection of hydration dynamics near the surface of a protein without fear of contamination from bulk solvent (Braun, Schmollngruber, & Steinhauser, 2017).

The primary concern for using the NOE and ROE to characterize local hydration water dynamics is the corruption of measured values by the transfer of magnetization to the detection spin via hydrogen exchange directly from solvent water (Figure 2A). In bulk solution, catalysis of hydrogen exchange between water and protein amide hydrogens can be fast enough to contribute significantly to a measured NOE by indirect magnetization transfer (Bai, Milne, Mayne, & Englander, 1993). Generally, however, under normal NMR sample conditions (i.e. neutral or low pH and/or temperatures below 40 °C), the amide exchange rate is such that only non-intraprotein hydrogen bonded amides are susceptible to this artifact. However, many amino acid side chains have sufficiently high pKa values to be (partially) protonated under conditions of the NMR experiments but have sufficiently low pKa values for hydrogen exchange to be efficient enough to move magnetization by relayed NOE(ROE) from solvent water to the detection protein spin (Liepinsh, & Otting, 1996). It is this artifact that is of the most concern here.

To avoid these various potential artifacts we have turned to encapsulation of individual protein molecules within the water core of a reverse micelle (RM) (Nucci, Pometun, & Wand, 2011; Nucci, Pometun, & Wand, 2011). The RM is composed of about ~3,000 water molecules contained within a spherical shell comprised of ~300 surfactant molecules with their polar head groups pointed inwards and their hydrophobic tails pointed outwards (Figure 2B). Reverse micelles optimal for NMR (i.e. small & spherical) are created under water-limited conditions (Dodevski, Nucci, Valentine, Sidhu, O'Brien, Pardi et al., 2014). The “water loading” or W_0 , is the molar ratio of water to surfactant and is typically between 10 and 20. The absolute concentration of surfactant(s) is usually optimal between 75 and 150 mM. They form spontaneously and are highly uniform (Fuglestad, Gupta, Wand, & Sharp, 2016). We have developed a surfactant system for encapsulation that is a combination of the non-ionic surfactant 1-decanoyl-rac-glycerol (10MAG) and the zwitterionic surfactant dimethyldodecylamine N-oxide (LDAO) (Dodevski, et al., 2014). A variety of proteins, ranging from 9 to 80 kDa in size and 5 to 10 in pI, have been encapsulated with high structural fidelity (Dodevski, et al., 2014). The capabilities of reverse micelle NMR have been recently reviewed (Nucci, Valentine, & Wand, 2014) and a practical guide is presented by Fuglestad et al. in a Chapter in this volume.

With respect to the study of protein hydration using the NOE and ROE, the reverse micelle has several critical advantages over bulk solution. Under compositions optimal for solution NMR spectroscopy the water core of the reverse micelle is largely restricted to the protein hydration layer and contains little “bulk” water (Nucci, Marques, Bédard, Dogan, Gledhill, Moorman et al., 2011). The absence of bulk water largely eliminates the long-range averaging effects noted by Halle (Halle, 2003). In addition, the small volume of the water core results in a low probability that hydroxide or hydronium ions are present in any given reverse micelle even though their distribution conforms to the pH of a bulk measurement

(Marques, Nucci, Dodevski, Wang, Athanasoula, Jorge et al., 2014). Thus the constant collisional averaging of reverse micelles, as evidenced by the single high-resolution protein NMR spectrum that is obtained, becomes rate limiting for specific acid or base catalysis of hydrogen exchange. Indeed, the amide hydrogen exchange rates are slowed by over two orders of magnitude in the reverse micelle (Nucci, et al., 2011). Furthermore, the motion of encapsulated water is greatly slowed relative to bulk water (Fayer, & Levinger, 2010), which results in enhanced dipolar contact between hydration water and the encapsulated protein (Nucci, et al., 2011). Additionally, the rate of general catalysis of hydrogen exchange is also slowed considerably and puts the resonances of most exchangeable side chain hydrogens into slow exchange on the NMR chemical shift time scale. The hydroxyl resonances of glucose, which have relatively moderate pKa values ~12, clearly indicate this condition (Wiebenga-Sanford, DiVerdi, Rithner, & Levinger, 2016)(Figure 3A). The most relevant amino acid side chains here are tyrosine, serine, and threonine and have pKa's of 10.5, 13, and 13 respectively (Liepinsh, et al., 1996). In the absence of buffer, the glucose hydrogen exchange rates are ~50 s⁻¹ compared to bulk aqueous solution with rates of ~2000 s⁻¹. These HX rates will vary depending on the surfactant, pH, and buffer.

For example, the head group of AOT has a pKa ~5 and acts as a catalyst of hydrogen exchange in the acid regime with a log₁₀ *k*_{HX} per unit pH slope of unity. The base form of the AOT sulfate headgroup does not catalyze HX (Figure 3C). The CTAB/hexanol surfactant mixture also does not catalyze HX. The CTAB headgroup is an obligatory cation and the pKa ~20 of hexanol is too high to be an effective catalyst of hydrogen exchange under reverse micelle conditions, especially in the context of slowed water dynamics in general (Figure 3C). Buffers also have the potential to act as general catalysts of hydrogen exchange. Acetate has a modest capacity for catalysis of HX while phosphate is an extraordinary catalyst (Figure 3D). Therefore phosphate buffer should be avoided for hydration studies. It has been shown that HX rates vary depending on the water loading used (Wiebenga-Sanford, et al., 2016) and is presumably due to an increased dynamics of water in the RM at higher water loadings. However, it should be noted, that protein-containing reverse micelles tend to establish a specific water loading as thermodynamically preferred. This amount of water seems to conform to the hydration layer and little bulk water is present (Nucci, et al., 2011). In summary, the hydrogen exchange rates of T/S/Y residues are anticipated to be on the order of or slower than the time scale of NOE and ROE cross relaxation rates. Fortunately, these rates will generally be in the slow exchange time regime on the NMR chemical shift time scale.

There are three types of magnetization transfer that can contribute to the NOE and ROE (Figure 2). The first is the direct dipolar magnetization transfer from the protein to the water, which is the interaction of interest. The second is the case of direct hydrogen exchange between the protein and water. The observed cross relaxation rate then takes the form:

$$\sigma_{NOE}^{obs} = \sigma_{NOE} + k_{HX} \quad (6)$$

$$\sigma_{ROE}^{obs} = \sigma_{ROE} + k_{HX} \quad (7)$$

Where k_{HX} is the hydrogen exchange rate. The sign of σ^{NOE} and k_{HX} term are both negative whereas the sign of σ^{ROE} is positive. Dominance of direct hydrogen exchange to magnetization transfer giving rise to an ROE is discriminated by a change in sign causing the ROE cross-peak to be the same sign as the NOE (i.e. same sign as the diagonal peak) (Deverell, Morgan, & Strange, 1970; Dobson, Lian, Redfield, & Topping). This drives the choice of probe for characterization of protein hydration to be restricted to the amide hydrogen and the non-exchangeable carbon bonded hydrogens such as methyl groups.

Magnetization transfer occurring from exchange-relayed NOEs is the most nefarious of the mechanisms. In this case water hydrogens exchange with a labile protein hydrogen, which in turn gives rise to an NOE to a nearby detection hydrogen of the protein. This type of exchange is spectrally indistinguishable from direct dipolar magnetization exchange and is the major artifact of NOE detected hydration experiments (Bax, Sklená, & Summers, 1986; van de Ven, Janssen, Gräslund, & Hilbers, 1988). The $\sigma^{NOE}/\sigma^{ROE}$ ratio is generally made more negative than in the absence of exchange. This may cause the $\sigma^{NOE}/\sigma^{ROE}$ ratio to be outside of the theoretical limits but in many cases the value distorted by hydrogen exchange may remain within these limits and the distortion is hidden. Below we show how to identify and quantify the contribution of hydrogen exchange to apparent NOE (ROE) rates.

In the absence of hydrogen exchange, the cross relaxation rate can be calculated from the time dependence of the intensity (I_{NOE}) of the cross-peak. For a two-spin system, the intensity takes the form (Macura, et al., 1980):

$$I_{NOE} = A_o [e^{-R_l \tau_m} (1 - e^{-\sigma_{NOE} \tau_m})] \quad (8)$$

$$I_{ROE} = A_o [e^{-R_l \tau_m} (1 - e^{-\sigma_{ROE} \tau_m})] \quad (9)$$

Where R_l is the longitudinal relaxation rate and τ_m is the longitudinal magnetization mixing time. A_o is a prefactor that is specific to the spectrometer settings. This factor is key to subsequent data analysis as emphasized below. The goal is to choose sample conditions, spectrometer settings, experimental and analytical strategies that allow the isolation of true σ^{NOE} and σ^{ROE} values between effectively non-exchanging protein hydrogens used for detection and water associated with the surface of the protein.

3 Preparation of protein encapsulated reverse micelle samples

3.1 Protein labeling and purification

As suggested by the introduction above and analysis described below, the most optimal situation is to isolate hydrogen spin pairs as much as a possible. Detection of hydration is most easily carried out using amide H and, occasionally, with methyl H and/or aromatic H in an otherwise perdeuterated background. A perdeuterated background simplifies the kinetics of magnetization transfer and results in the additional benefit of more favorable relaxation by suppressing spin diffusion and reducing longitudinal relaxation and thereby opening access to longer NOE/ROE mixing times. The latter is helpful for obtaining higher signal-to-noise (S/N) cross peaks and a broader sampling of the time dependence. The ROE experiment requires transverse magnetization and is generally limited by $R_{1\rho}$ relaxation, which is also significantly reduced by perdeuteration. Additionally, some H_{α} protein hydrogens may resonate at frequencies close to the water resonance and may contribute magnetization to the protein-water cross peaks by an unfortunate combination of close spatial proximity to the detection spin and accidental (near) degeneracy with the water resonance. It is important to note that because of the differential dynamics and averaging effects intra-protein molecular NOEs are usually much stronger than an inter-molecular NOE with water. Accordingly, very high (~99%) deuteration is recommended to maximally suppress these types of artifactual contributions to apparent NOEs (ROEs) to water. If sufficiently high perdeuteration levels cannot be achieved, analysis of the protein structure in combination with H_{α} resonance assignments can be used to identify potential artifacts. If a high-resolution crystal structure is not available more complicated pulse schemes may also be used to remove signals from H_{α} 's as will be discussed below.

The simplest isotopic labeling scheme uses uniform ^{15}N -labelling in an otherwise perdeuterated background. ^{15}N -labelling is relatively inexpensive and provides a probe on every residue except proline. This provides good detection coverage of protein-water NOEs across the protein. However, it is noted that many amide Hs may be too far from the protein surface or internal cavities to generate cross peaks to water of detectable intensity. Appropriate selective methyl (Proudfoot, Frank, Ruggiu, Mamo, & Lingel, 2016) and aromatic labeling (Kasinath, Valentine, & Wand, 2013) can provide additional coverage both on the surface of the protein and especially in the hydrophobic core of the protein (see also Chapters by Lingel and Wieninger in this Volume).

3.2 Reverse micelle encapsulation and considerations

Determination of encapsulation conditions that maintain optimal structural integrity can be achieved using the protocols described in the Chapter by Fuglestad and co-workers in this Volume. The final pH of the reverse micelle sample (buffer and surfactant) should be kept relatively low in order to reduce hydrogen exchange. In general a pH of ~5 is optimal for low hydrogen exchange rates of amides and side chains (Bai, et al., 1993). It is important to verify and monitor pH once the sample is made (Marques, et al., 2014). Protein resonances sensitive to pH are often the most reliable indicators of the effective pH. Protein should be free of salt and buffers prior to concentration. Care should be taken to avoid excess salt being co-purified with the protein due to various effects of the Donnan equilibrium. High

salt concentrations can interfere with reverse micelle encapsulation (Fathi, Kelly, Vasquez, & Graeve, 2012). Buffer components must be carefully chosen to avoid corrupting contributions from buffer catalyzed hydrogen exchange as described above.

Three-dimensional ^{15}N -NOESY-HSQC spectra should be collected on solutions of encapsulated proteins to verify that the protein is not interacting with the surfactant shell. A well-solubilized protein with its native hydration shell will not have any detectable NOEs between the protein and the surfactant. If protein-surfactant NOEs are detected the surfactant mixture or encapsulation conditions should be changed. If the protein cannot encapsulate in other mixtures only buried or structural waters should be studied. If CTAB/hexanol is used care should be taken to resolve the water and hexanol hydroxyl peaks in the one-dimensional ^1H -spectrum. Small variations in the water loading, pH and temperature are often sufficient to resolve the hexanol hydroxyl and water resonances. Decreasing the hexanol concentration, increasing the temperature, or reducing the concentration of surfactant can also accomplish this. ^{15}N -NOESY-HSQC spectra can also verify that no exchange cross peaks exist between the protein and the hexanol hydroxyl. Protonated surfactants can be used for ^{15}N -detected experiments. For ^{13}C detected experiments, deuterated surfactants should be used to avoid streaking and artifacts in the spectrum.

The NOE/ROE hydration experiments are relatively insensitive and so the reverse micelle sample must be stable for several weeks in order to collect data of sufficient quality. RM sample instability often arises from evaporation of either the pentane or water and can be mitigated by using moderate experimental temperature (e.g. 20 °C) and using a vortex plug. One-dimensional ^1H spectra should be recorded periodically through the course of the experiments to make sure no changes occur in the peak height or frequency of the water peak.

4 NMR spectroscopy and experimental setup

4.1 NOESY and ROESY experiments

The NOE between protein and water can be measured using two- or three- dimensional heteronuclear NOESY-HSQC experiments (Figure 4). Both ^{15}N and ^{13}C experiments can be used, with either standard single quantum or TROSY (Hernández, & LeMaster, 2003) detection schemes. Homonuclear TOCSY experiments (Dalvit, 1996) have been reported but are not recommended for use in this context. Gradient selected quadrature detection is highly recommended to suppress signals from residual ^1H resonances of surfactants. Since the water content of the reverse micelle system is reduced to ~1 M no water suppression schemes are usually necessary. For ^{13}C detected experiments the WET-suppression scheme can be employed to further suppress residual alkane resonances (Smallcombe, Patt, & Keifer, 1995). For simplicity of analysis (see below), all experimental parameters including receiver gain, mix times, pulse length and power, interscan-delays, and increments collected in each dimension should be the same for both the NOE and ROE experiment.

The ROE experiment replaces the laboratory frame NOE mixing period with a continuous wave spinlock bracketed by 90°_y pulses (Bax, & Grzesiek, 2007) (Figure 4). A weak CW spinlock pulse with a bandwidth of ~10 kHz ensures a sufficient excitation bandwidth and

suppresses contributions from Hartman-Hahn transfers. The 90° hard pulses bracketing the spinlock remove any off resonance effects on the edge of the bandwidth. This scheme does not require complicated phase cycling.

4.2 Two dimensional versus three-dimensional experiments

Three-dimensional experiments require long data collection times. Two-dimensional projection variants can be used to reduce data collection time and simply involves selective excitation of the water resonance prior to the mixing time. Many water selection schemes have been reported but the e-PHOGSY water excitation scheme is robust, easy to implement, and can be used for both aqueous and reverse micelle experiments. The e-PHOGSY scheme is a spin-echo sequence that begins with a 90°-G1-180°_{wat}-G1-90° scheme, which renders the water magnetization longitudinal (Dalvit, 1996). The type of water-selective 180° pulse should be chosen to reflect the needs of selective bandwidth of excitation versus the duration of the pulse length. If fully perdeuterated protein is used a relatively short (100 Hz excitation bandwidth) water selective (e.g. sinc/x , Gaussian) pulse is recommended to prevent excessive relaxation of the water during the spin-echo. The shorter selective pulse will help increase signal-to-noise in the experiment. If the protein cannot be fully perdeuterated and contributions from H_α are a concern a longer (G3, ReBurp) pulse can be used. The intra-protein hydrogens relax due to T₂ relaxation during this time removing contamination from intramolecular H_α peaks. However, some of the water will also relax in this time period resulting in poorer signal-to-noise. Optimization of water selective pulses can be performed in one-dimensional ¹H-detected experiments. Example pulse sequences for both two-dimensional and three-dimensional ¹⁵N NOESY and ROESY-HSQC's are shown in Figure 4.

4.3 Non-uniform sampling

If three-dimensional experiments are used a significant savings in time can be obtained by using non-uniform sampling. Traditional NMR experiments sample frequencies uniformly in a Cartesian grid in order to satisfy the criteria for the discrete Fourier transform. Non-uniform sampling takes advantage of the fact that only a small subset of the frequencies sampled contains signal information whereas the rest contain noise (Donoho, 2006). While the DFT can't be directly applied to non-uniformly sampled data numerous reconstruction algorithms have been employed that can accurately reproduce the S/N and frequency information of NMR signals (Hyberts, Arthanari, & Wagner, 2012; Hyberts, Robson, & Wagner, 2013; Hyberts, Robson, & Wagner, 2017) (see also the Chapter by Robson and coworkers in these Volumes). NUS has been established for many three-dimensional and four-dimensional experiments. Several aspects of the NOE pose challenges for NUS reconstruction. The dynamic range of auto- and cross-peak intensities in NOESY spectra is very large with many inter-molecular cross peaks being a fraction of a percent of the intensity of the auto peak. The peak height intensity of the cross peaks needs to be quantitatively accurate. As the density of peaks in a spectral plane (e.g. HSQC) increases the fidelity of peak height reconstruction decreases. Therefore, aggressive NUS is not recommended for three-dimensional hydration experiments and Cartesian sampling is recommended for two-dimensional experiments. These difficulties require slightly higher NUS sampling densities than traditional three-dimensional experiments. For sinusoidally

weighted Poisson-gap scheduling and iterative soft thresholding (IST) reconstruction a sampling density of 20 to 25% is recommended (Figure 5) and can result in a 4–5 times savings. If other sampling or reconstruction methods are used these criteria should be carefully considered.

4.4 Identification of hydrogen exchange

As emphasized above, the NOE can potentially contain contributions from direct hydrogen exchange and exchange-relayed hydrogen exchange. A sufficiently large contribution from direct hydrogen exchange of the probe spin (e.g. amide H) with water will result in a reversal of the sign of the ROESY cross-peak. However, this criterion is insufficient and care should be taken to prepare samples of sufficiently low pH to avoid direct exchange. As a rule of thumb, the predicted non-hydrogen bonded exchange rate based on the primary amino acid sequence (Bai, et al., 1993) can be taken as two orders of magnitude slower in the reverse micelle than in bulk solution (Nucci, et al., 2011). Because the general catalysis of exchange of side chain hydrogens with water is also generally slowed, especially if buffer and pH are appropriately set (see above), the resonances of serine, threonine and tyrosine hydroxyls are in slow exchange on the chemical shift time scale with the water resonance. This provides two advantages. In conjunction with extensive resonance assignments and a well-determined structural model of the protein, the NOE can be used to identify the hydroxyl hydrogens of these side chains. In addition, the slow exchange regime provides an opportunity to examine the contribution of NOE intensity arising from a hydrogen-exchange relay between water and an exchangeable hydrogen and then on to the detection spin by the NOE (Figure 2A).

4.5 Quantification of hydrogen exchange relayed magnetization

One of the most pernicious artifacts in employing the NOE to access hydration dynamics arises from the hydrogen exchange relayed NOE (Figure 2A) (Bax, et al., 1986). If a labile hydrogen that is within NOE-distance of the NOE detection spin (e.g. amide hydrogen) exchanges sufficiently rapidly with water, then an NOE cross-peak between the water resonance and the amide hydrogen will be observed, even though water may not actually be close to the amide hydrogen. This exchange relay process is otherwise indistinguishable from an authentic intermolecular NOE. This has been the main barrier to employing the NOE/ROE approach in bulk aqueous solution (Halle, 2004; Otting, 1997). Fortunately, as summarized above, hydrogen exchange chemistry is sufficiently slow to put most sites into the slow exchange regime. Thus, the contribution of hydrogen exchange relay(s) to an observed water-protein NOE can be directly dissected by selective decoupling (Figure 6) (Melacini, Boelens, & Kaptein, 1999; Melacini, Kaptein, & Boelens, 1999).

Ideally, decoupling would be performed by continuously inverting all of the hydroxyl peaks using a broadband selective pulse. For these purposes selective “top hat” pulses such as G3 or BURP pulses are recommended (Geen, & Freeman, 1991). They have a uniform excitation profile and minimal perturbations outside of the bandwidth of excitation. An example pulse sequence is given in Figure 6. Unfortunately, even highly selective decoupling pulses will cause slight excursions of the water magnetization from Z (Melacini, et al., 1999). A correction for this artifact can be determined by following the water selective excitation scheme with an NOE mixing period containing a centered 360° rotation

decoupling pulse. Comparison of the intensity of the water resonance (in one dimensional experiments) or NOESY-HSQC cross peaks (in two-dimensional experiments) with and without the decoupling pulse yields the correction factor (κ) (Figure 7). Determination of κ follows from a distribution of ratios of intensities with and without application of the decoupling pulse (Figure 7).

This is an imperfect approach but as shown below arrives at a sufficiently accurate value. The correction depends on the pulse length and the center of excitation and thus needs to be determined for each decoupling pulse profile and parameter set used. In principle, once the correction factor is determined, the NOE peak intensities for all mixing times can be corrected by using κ^{-n} where n is the number of 360° cycles used (Melacini, et al., 1999). The magnitude of κ needs to be very close to one and pulse profiles that result in κ values less than 0.95 should be avoided. It is therefore often necessary to employ weaker, more selective decoupling pulses at the expense of having to repeat the experiments to cover the entire region of interest. Nevertheless, even for very high κ values, it is generally not feasible to correct the entire NOE evolution curve satisfactorily (Figure 8). Accordingly, quantitative analysis is restricted to the (near) linear region of the NOE buildup.

If hydroxyl frequencies are close to water a broadband pulse cannot be used to decouple due to the relaxation of the water during the pulse. In this case a single frequency low power CW pulse can be used. This allows for increased specificity and less perturbation of the water, but is obviously inefficient. Unfortunately the HX contributions cannot be decoupled in ROESY experiments.

5 Data collection and analysis

5.1 Data Collection

Hydration dynamics experiments can be performed on any protein that is stable in the reverse micelle. Here, we illustrate a general procedure for measuring surface hydration dynamics of ubiquitin in AOT reverse micelles. Ubiquitin is an 8.3 kDa protein that encapsulates stably for up to several years. The structure of ubiquitin encapsulated in AOT has an average rmsd of less than 0.5 Å backbone to bulk aqueous solution of ubiquitin (Babu, Flynn, & Wand, 2001). Here we detail a protocol to examine protein hydration for proteins that can be 100% perdeuterated. For larger proteins TROSY detection schemes should be employed.

- 1) Determine the optimal encapsulation conditions using ^{15}N protein. Please see Fuglestad *et. al.* for a comprehensive description of how to screen and benchmark encapsulation conditions.
- 2) Assign the protein in the reverse micelle using ^{13}C , ^{15}N protein using standard triple resonance experiments (e.g. HNCA, HNCOCA, HNCO, HNCACO, HNCACB, CBCACONH) (Rule, & Hitchens, 2006; Sattler, Schleucher, & Griesinger, 1999).
- 3) Prepare the protein of interest ~99%-deuterated and ^{15}N -labeled. Dialyze extensively against water and lyophilize if possible.

- 4) Dissolve or otherwise transfer the protein in 6.75 μL ($W_0 = 10$, 75 mM surfactant, 500 μL sample) buffer containing 10mM NaAcetate, 10mM NaCl, pH 5.
- 5) Prepare the reverse micelle sample for hydration dynamics using 99.8% deuterated alkane as the bulk reverse micelle solvent.
- 6) Collect a 3D ^{15}N -NOESY-HSQC to assign T/S/Y hydroxyl protons and determine the decoupling bandwidth needed for all slow exchanging hydroxyl hydrogens ($\sim 4.7 - 6.2$ ppm).
- 7) Setup the G3 decoupling pulses using the Bruker program STDISP. A 10 ms decoupling pulse will cover a bandwidth of 0.8 ppm. Define two decoupling pulses centered at 5.4 and 6.1 ppm to cover the bandwidth of 5.0 – 5.8 ppm and 5.7 – 6.5 ppm, respectively. Adjust the excitation profiles as required to satisfy the spectral distribution and performance of your protein.
- 8) Measure the two-dimensional NOESY-HSQC with and without one 360° rotation during the mix period using the calibrated G3 pulses. The mix time should be relatively long (e.g. 70 – 100 ms) to increase the signal-to-noise.
- 9) Determine the κ correction factors for each of the selective pulses. Plot a histogram of the ratio of the decoupled vs non-decoupled peak height intensity (Figure 7). Identify the top 10% of resonance intensities and plot the decoupled versus non-decoupled peak height intensities. The slope of the linear regression line is the κ correction factor. This slope should be > 0.95 .
- 10) Collect the 2D water-selective ^{15}N -NOESY-HSQC, ^{15}N -ROESY-HSQC, and ^{15}N -NOESY-HSQC with G3 decoupling. The mix times for the NOE and the decoupled-NOE experiment are 20, 40, 60, 80, 100, 140, 180, 240, 300, 500 ms. Example mix times for the ROE experiments would be 10, 15, 20, 25, 30, 35, 40, 50, 75, 150 ms. A 15 ms water selective $\sin x/x$ pulse should be used for the water selective excitation scheme. A weak CW spinlock with a bandwidth of 8.3–10 kHz should be used for the ROESY-HSQC experiment.
- 11) Correct the NOE-decoupled peak height intensities with the determined κ value.
- 12) Fit the data as described below and calculate the NOE/ROE rates.

5.2 General fitting strategy

Full buildup curves for the NOE, ROE, and decoupled-NOE should be collected (Figure 8). The curves for the NOE, ROE, and decoupled-NOE experiments need to be individually fit to equations (8) and (9) in general form. Unfortunately the similar timescales of σ^{NOE} and R_1 ($\sigma^{\text{ROE}}/R_{1\rho}$) make these equations difficult to fit directly for all parameters. Fortunately, the A_o term is a constant for all experiments assuming identical acquisition parameters and good sample stability. In order to simplify the data fitting the A_o term can be determined using a global fit of the NOE data and held constant for the remaining curves. A global fit of the A_o term is accomplished by fitting cross peaks with mixing times in the linear regime of the NOE buildup by using a simplified version of equation (6):

$$I_{NOE} = A_o(1 - e^{-\sigma^{NOE}\tau_m}) \quad (10)$$

Once A_o is determined it can then be held constant and each experiment is then fitted to equations (8) and (9) using the full buildup or equation (10) using early mixing time points. Fitting to equation (10) requires fewer parameters, but because it is constrained to data in the linear range the signal-to-noise may be low. In cases where the linear regime is too noisy the full buildup allows sampling at longer mix times and therefore greater signal-to-noise. As a general rule this data fitting method must include at least 5–10 different mixing times per experiment type. In this case, the choice of mixing times does not need to be matched for the different experiments. It is highly recommended that the linear regime be highly sampled. For selectively decoupled NOE profiles, fitting of the initial rate is generally satisfactory to obtain information necessary to identify and correct for NOE intensity relayed by hydrogen exchange.

The fitting of primary NOE and ROE curves results in apparent NOE ($\tilde{\sigma}^{NOE}$) and apparent ROE ($\tilde{\sigma}^{ROE}$) cross relaxation rates. Comparison of $\tilde{\sigma}^{NOE}$ to $\tilde{\sigma}_{dec}^{NOE}$ identifies those detection spins having measured NOEs (ROEs) to water contaminated by hydrogen exchange. For sites that are not contaminated by exchange relayed NOE intensity, the ratio of the fitted σ^{NOE} and σ^{ROE} rates is what is desired. For sites found to have detectable contribution by relayed NOE intensity, the difference of the cross relaxation rates between the NOE experiment and the decoupled-NOE experiment give the contribution from HX relayed magnetization:

$$\tilde{k}_{HX} = \tilde{\sigma}_{dec}^{NOE} - \tilde{\sigma}^{NOE}; \sigma^{NOE} = \tilde{\sigma}^{NOE} - \tilde{k}_{HX} \quad (11)$$

where $\tilde{k}_{HX}$ is the effective rate of magnetization transfer from water through hydrogen exchange to an intermediate spin and subsequently transferred to the detection spin via the NOE. In the rigid limit, the intra-molecular ROE is twice the NOE and therefore the HX contribution is two times greater for the ROE than the NOE. The ROE can be obtained:

$$\sigma^{ROE} = \tilde{\sigma}^{ROE} - 2\tilde{k}_{HX} \quad (12)$$

The NOE/ROE ratios can then be calculated. Ratios should fall between the lower theoretical limit of -0.5 and the effective limit of ~ 0 defined by the dynamics of water in the reverse micelle (Figure 9).

5.3 Simplified analysis in the absence of hydrogen exchange.

In some cases, such as the study of deeply buried “structural” water where hydrogen exchange is ineffective on the NOE and ROE time scales, a simpler analysis is possible. By

collecting NOE and ROE build-ups at matched mixing time points, a linearized ratio can be directly fitted. In the absence of HX relayed magnetization:

$$\frac{I_{NOE}(\tau_m)}{I_{ROE}(\tau_m)} = \frac{\sigma_{NOE} e^{-R_1 \tau_m}}{\sigma_{ROE} e^{-R_{1\rho} \tau_m}} \quad (13)$$

$$\ln \left[-\frac{\sigma_{NOE} e^{-R_1 \tau_m}}{\sigma_{ROE} e^{-R_{1\rho} \tau_m}} \right] = \ln \left[-\frac{\sigma_{NOE}}{\sigma_{ROE}} \right] + (R_{1\rho} - R_1) \tau_m \quad (14)$$

The data fitting relies on linear regression and therefore at least 4 mix time points are recommended. The linear regression fitting is simple and robust.

6 Conclusions

In principle, solution NMR spectroscopy is unique in its potential to characterize the dynamical character of the interaction of protein molecules with solvent water in a site resolved way (Otting, et al., 1991). Unfortunately, a number of artifacts have historically thwarted this approach (Halle, 2004). By taking advantage of various characteristics of the reverse micelle the main barriers are overcome. Most important among these is hydrogen exchange, which is greatly slowed such that it becomes irrelevant for many types of protein detection hydrogens and can be manageably quantified for most others. Careful optimization of protein encapsulation and experimental parameters can lead to robust data that can be confidently employed to characterize the hydration layer of proteins.

Acknowledgements

Supported by the NSF (MCB 1158038). BSM is an NIH pre-doctoral trainee (T32 GM071339). CJ is an NIH pre-doctoral trainee (T32 GM008275) and is a recipient of an NIH NRSA pre-doctoral fellowship (F31 GM116520).

References

- Adrover M, Martorell G, Martin SR, Urosev D, Konarev PV, Svergun DI, et al. (2012). The role of hydration in protein stability: comparison of the cold and heat unfolded states of Yfh1. *Journal of Molecular Biology* 417(5), 413–424. [PubMed: 22342930]
- Babu CR, Flynn PF and Wand AJ (2001). Validation of protein structure from preparations of encapsulated proteins dissolved in low viscosity fluids. *Journal of the American Chemical Society* 123(11), 2691–2692. [PubMed: 11456950]
- Bai Y, Milne JS, Mayne L and Englander SW (1993). Primary structure effects on peptide group hydrogen exchange. *Proteins: Structure, Function, and Bioinformatics* 17(1), 75–86.
- Bax A, Sklenář V and Summers MF (1986). Direct identification of relayed nuclear overhauser effects. *Journal of Magnetic Resonance* 70(2), 327–331.
- Bellissent-Funel M-C, Hassanali A, Havenith M, Henchman R, Pohl P, Sterpone F, et al. (2016). Water determines the structure and dynamics of Proteins. *Chemical Reviews* 116(13), 7673–7697. [PubMed: 27186992]

- Bothner-By AA, Stephens RL, Lee J, Warren CD and Jeanloz RW (1984). Structure determination of a tetrasaccharide: transient nuclear Overhauser effects in the rotating frame. *Journal of the American Chemical Society* 103(3), 811–813.
- Braun D, Schmollgruber M and Steinhauser O (2017). Revival of the intermolecular nuclear overhauser effect for mapping local protein hydration dynamics *Journal of Physical Chemistry Letters* 8(14), 3421–3426. [PubMed: 28686451]
- Bruschweiler R and Wright PE (1994). Water self-diffusion model for protein-water NMR cross-relaxation. *Chemical Physics Letters* 229(1–2), 75–81.
- Dalvit C (1996). Homonuclear 1D and 2D NMR experiments for the observation of solvent–solute interactions. *Journal of Magnetic Resonance, Series B* 112(3), 282–288. [PubMed: 8812917]
- Deverell C, Morgan RE and Strange JH (1970). Studies of chemical exchange by nuclear magnetic relaxation in the rotating frame. *Molecular Physics* 18, 553–559.
- Dey A, Jenny FE, Adams MWW, Babini E, Takahashi Y, Fukuyama K, et al. (2007). Solvent tuning of electrochemical potentials in the active sites of HiPIP versus ferredoxin. *Science* 318(5855), 1464–1468. [PubMed: 18048692]
- Dobson CM, Lian L-Y, Redfield C and Topping KD (1986). Measurement of hydrogen exchange rates using 2D NMR spectroscopy. *Journal of Magnetic Resonance* 69(2), 201–209.
- Dodevski I, Nucci NV, Valentine KG, Sidhu GK, O'Brien ES, Pardi A, et al. (2014). Optimized reverse micelle surfactant system for high-resolution NMR spectroscopy of encapsulated proteins and nucleic acids dissolved in low viscosity fluids. *Journal of the American Chemical Society* 136(9), 3465–3474. [PubMed: 24495164]
- Donoho DL (2006). Compressed sensing. *IEEE Transactions on Information Theory* 52(4), 1289–1306.
- Fathi H, Kelly JP, Vasquez VR and Graeve OA (2012). Ionic concentration effects on reverse micelle size and stability: Implications for the synthesis of nanoparticles. *Langmuir* 28(25), 9267–9274. [PubMed: 22642604]
- Fayer MD and Levinger NE (2010). Analysis of water in confined geometries and at interfaces. *Annual Review of Analytical Chemistry* 3(1), 89–107.
- Fuglestad B, Gupta K, Wand AJ and Sharp KA (2016). Characterization of Cetyltrimethylammonium Bromide/Hexanol Reverse Micelles by Experimentally Benchmarked Molecular Dynamics Simulations. *Langmuir* 32(7), 1674–1684. [PubMed: 26840651]
- Geen H and Freeman R (1991). Band-selective radiofrequency pulses. *Journal of Magnetic Resonance* 93(1), 93–141.
- Halle B (2003). Cross-relaxation between macromolecular and solvent spins: The role of long-range dipole couplings. *Journal of Chemical Physics* 119(23), 12372–12385.
- Halle B (2004). Protein hydration dynamics in solution: a critical survey. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* 359(1448), 1207–1223. [PubMed: 15306377]
- Hernández G and LeMaster DM (2003). Relaxation compensation in chemical exchange measurements for the quantitation of amide hydrogen exchange in larger proteins. *Magnetic Resonance in Chemistry* 41(9), 699–702.
- Hyberts SG, Arthanari H and Wagner G (2012). Applications of non-uniform sampling and processing. *Topics in Current Chemistry* 316, 125–148. [PubMed: 21796515]
- Hyberts SG, Robson SA and Wagner G (2013). Exploring signal-to-noise ratio and sensitivity in non-uniformly sampled multi-dimensional NMR spectra. *Journal of Biomolecular NMR* 55(2), 167–178. [PubMed: 23274692]
- Hyberts SG, Robson SA and Wagner G (2017). Interpolating and extrapolating with hmsIST: seeking a tmax for optimal sensitivity, resolution and frequency accuracy. *Journal of Biomolecular NMR* 68(2), 139–154. [PubMed: 28332026]
- Kasinath V, Valentine KG and Wand AJ (2013). A ¹³C labeling strategy reveals a range of aromatic side chain motion in calmodulin. *Journal of the American Chemical Society* 135(26), 9560–9563. [PubMed: 23767407]
- Liepinsh E and Otting G (1996). Proton exchange rates from amino acid side chains—implications for image contrast. *Magnetic resonance in medicine* 35(1), 30–42. [PubMed: 8771020]

- Macura S and Ernst RR (1980). Elucidation of cross relaxation in liquids by two-dimensional N.M.R. spectroscopy. *Molecular Physics* 41(1), 95–117.
- Marques BS, Nucci NV, Dodevski I, Wang KW, Athanasoula EA, Jorge C, et al. (2014). Measurement and control of pH in the aqueous interior of reverse micelles. *Journal of Physical Chemistry B* 118(8), 2020–2031.
- Melacini G, Boelens R and Kaptein R (1999). Band-selective editing of exchange-relay in protein-water NOE experiments. *Journal of Biomolecular NMR* 13(1), 67–71. [PubMed: 21080264]
- Melacini G, Kaptein R and Boelens R (1999). Editing of chemical exchange-relayed NOEs in NMR experiments for the observation of protein–water interactions. *Journal of Magnetic Resonance* 136(2), 214–218. [PubMed: 9986764]
- Nucci NV, Marques BS, Bédard S, Dogan J, Gledhill JM, Moorman VR, et al. (2011). Optimization of NMR spectroscopy of encapsulated proteins dissolved in low viscosity fluids. *Journal of Biomolecular NMR* 50(4), 421–430. [PubMed: 21748265]
- Nucci NV, Pometun MS and Wand AJ (2011). Mapping the hydration dynamics of ubiquitin. *Journal of the American Chemical Society* 133(32), 12326–12329. [PubMed: 21761828]
- Nucci NV, Pometun MS and Wand AJ (2011). Site-resolved measurement of water-protein interactions by solution NMR. *Nature Structural and Molecular Biology* 18(2), 245–249.
- Nucci NV, Valentine KG and Wand AJ (2014). High-resolution NMR spectroscopy of encapsulated proteins dissolved in low-viscosity fluids. *Journal of Magnetic Resonance* 241, 137–147. [PubMed: 24656086]
- Otting G and Wuthrich K (1989). Studies of protein hydration in aqueous solution by direct NMR observation of individual protein-bound water molecules. *Journal of the American Chemical Society* 111(5), 1871–1875.
- Otting G, Liepinsh E and Wuthrich K (1991). Protein hydration in aqueous solution. *Science* 254(5034), 974–980. [PubMed: 1948083]
- Otting G (1997). NMR studies of water bound to biological molecules. *Progress in Nuclear Magnetic Resonance Spectroscopy* 31(2), 259–285.
- Proudfoot A, Frank AO, Ruggiu F, Mamo M and Lingel A (2016). Facilitating unambiguous NMR assignments and enabling higher probe density through selective labeling of all methyl containing amino acids. *Journal of Biomolecular NMR* 65(1), 15–27. [PubMed: 27130242]
- Sattler M, Schleucher J and Griesinger C (1999). Heteronuclear multidimensional NMR experiments for the structure determination of proteins in solution employing pulsed field gradients. *Progress in Nuclear Magnetic Resonance Spectroscopy* 34(2), 93–158.
- Smallcombe SH, Patt SL and Keifer PA (1995). WET solvent suppression and its applications to LC NMR and high-resolution NMR spectroscopy. *Journal of Magnetic Resonance, Series A* 117(2), 295–303.
- Sterpone F, Stirnemann G and Laage D (2012). Magnitude and molecular origin of water slowdown next to a protein. *Journal of the American Chemical Society* 134(9), 4116–4119. [PubMed: 22335572]
- Tanford C (1979). Interfacial free energy and the hydrophobic effect. *Proceedings of the National Academy of Sciences of the United States of America* 76(9), 4175–4176. [PubMed: 16592699]
- van de Ven FJM, Janssen HGJM, Gräslund A and Hilbers CW (1988). Chemically relayed nuclear overhauser effects. Connectivities between resonances of nonexchangeable protons and water. *Journal of Magnetic Resonance* 79(2), 221–235.
- Wiebenga-Sanford BP, DiVerdi J, Rithner CD and Levinger NE (2016). Nanoconfinement's Dramatic Impact on Proton Exchange between Glucose and Water. *The Journal of Physical Chemistry Letters* 7(22), 4597–4601. [PubMed: 27779880]
- Zaccai G (2004). The effect of water on protein dynamics. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* 359(1448), 1269–1275. [PubMed: 15306381]

Figure 1:

A) Dependence of σ^{NOE} in black and σ^{ROE} in red as a function of the correlation time of the protein water interaction. The ratio of the σ^{NOE} and σ^{ROE} rates is shown in blue.

Fortuitously, the dynamics of water in the reverse micelle core is on the time scale of the null of the NOE, which reduces the expected range of the inter-molecular protein-water NOE in the reverse micelle to between 0 to -0.5 . Thus the shaded region is not accessible in the reverse micelle because of slowed dynamics of the water. Rates were calculated using a distance of $2.2 \text{ \AA}$, a magnetic field strength of 14.6 T (^1H 600 MHz) and the rigid rotor spectral density.

Figure 2:

A) Examples of magnetization transfer that can contribute to the measured NOE. Top - Direct NOE between a protein hydrogen and water. Middle - NOE relayed to the detection amide H by hydrogen exchange with an intermediate hydroxyl hydrogen. Bottom - Direct hydrogen exchange between the detection amide hydrogen and water. B) Schematic illustration of a protein molecule encapsulated within a reverse micelle.

Figure 3:

Hydrogen exchange in the reverse micelle. A) Expansion of the one-dimensional ^1H spectrum of glucose in CTAB/hexanol reverse micelles showing that the hydrogen exchange has slowed sufficiently to place the hydroxyl hydrogens into the slow-exchange regime via NMR. B) Example exchange spectroscopy (NOESY) curve for the 1β hydroxyl hydrogen of glucose peak in CTAB/hexanol RM at pH 5. C) pH dependence of exchange in AOT RM (red triangles) and in CTAB/hexanol (blue circles). D) Buffer catalysis of HX of the 1β hydroxyl hydrogen of glucose in CTAB/hexanol reverse micelles by sodium acetate (blue circles) and sodium phosphate (red squares).

Figure 4:

Pulse sequences used for hydration measurements. A) Water-selective two-dimensional NOESY-HSQC pulse sequence. The boxed region indicates the mix with the ROE counterpart illustrated to the right. Narrow and wide bars indicate 90° and 180° pulses respectively. The water selective 180° shaped pulse is shaded. All phases are x unless otherwise indicated. The phase cycling schemes are $\Phi_1 = 4(x), 4(y), 4(-x), 4(-y)$, $\Phi_2 = x, -x$, $\Phi_3 = 2(x), 2(-x)$, $\Phi_4 = 2(x), 2(-x)$, $\Phi_5 = 2(y), 2(-y)$, Rec = $x, -x, -x, x, x, -x, x, x, -x, x, -x, x, x, -x, x, -x$. B) Non-selective 3 dimensional NOESY-HSQC pulse sequence with ROESY spinlock in boxed region. All pulses are described above. The phase cycling of schemes are: $\Phi_1 = 4(x), 4(-x)$, $\Phi_2 = 4(y), 4(-y)$, $\Phi_3 = 16(x), 16(-x)$, $\Phi_4 = x, -x$, $\Phi_5 = 2(x), 2(-x)$, $\Phi_6 = 2(x), 2(-x)$, $\Phi_7 = 2(y), 2(-y)$, Rec = $x, -x, -x, x, x, -x, x, x, -x, x, x, x, -x, -x, x, x, -x, -x, -x, x, x, -x, -x, -x, x, x, -x, -x, x, x, -x, -x, x, x, -x$.

Figure 5:

Effect of NUS sampling density on accuracy of NOE cross-peak intensity. Comparison of peak height intensities between uniformly and non-uniformly sampled data sets for a bulk aqueous ^{15}N -NOESY-HSQC using fully protonated ubiquitin. A) uniform sampling reproducibility B) uniform versus 25% NUS-sampling C) 25% NUS-sampling reproducibility.

Figure 6:

A) Pulse sequence used to decouple relaying hydroxyls from the NOE spectrum. Black rectangle pulses are 90° hard pulses, open shaped pulse is a water-selective 180° shaped pulse, and black pulses are 180° broadband selective pulses centered on the hydroxyl peaks. The phase cycling and continuation of the HSQC are as described in Figure 4. The selective decoupling pulse is looped n times for the duration of the mixing time. See (Melacini, et al., 1999) B) Schematic illustration of selective decoupling of an intermediate spin to remove exchange relayed NOE intensity to the detection spin. See (Melacini, et al., 1999)

Figure 7:

Determination of κ . A) Histogram of the ratio of NOESY-HSQC cross peak height intensities with and without application of a decoupling pulse. The highest ratio of decoupled/non-decoupled are those that do not have a relayed NOE contribution and are scaled solely by the affects of the pulse. The top 10% of peaks are used to determine κ . B) The peak height intensity of the NOE with decoupling versus without a decoupling pulse for all peaks (gray circles). The highest 10% ratios (black circles) give $\kappa = 0.965$.

Figure 8:

Example buildup curves for the various hydration experiments. A) NOE buildup showing best fitted line. B) ROE buildup curve showing the best fitted line. C) The effect of κ on peak height intensity of the buildup curves. The measured decoupled NOE buildup curve is shown as black squares and the κ corrected is shown in solid circles. The dashed lines represent one standard deviation in κ . The long-time sensitivity to κ renders fitting of the full time course meaningless. Fortunately, the short mixing time regime is not sensitive to this effect and quantitative build-up rates can be obtained. D) Detection of NOE intensity relayed by hydrogen exchange. The NOE buildup curve is shown as black squares. The κ -corrected NOE buildup with the decoupling centered at 6.3 ppm is shown as blue triangles and the κ -corrected buildup with the decoupling at 5.7 ppm is shown as green circles. Decoupling at 6.3 ppm does not affect the initial NOE rate whereas the NOE rate is decreased by ~50% when the decoupling is centered at 5.7 ppm confirming the presence of hydrogen exchange relayed NOE contribution from a hydroxyl resonating in the bandwidth of the decoupling (5.3 to 6.1 ppm).

Figure 9:

Distribution of corrected $\sigma^{\text{NOE}}/\sigma^{\text{ROE}}$ ratios for encapsulated ubiquitin based on amide hydrogen detection. Hydrogen exchange relayed contributions were determined using two band selective decoupling experiments. Determined relayed NOE rates were used to correct measured raw NOE and ROE cross relaxation rates. Five amide hydrogens show $\sigma^{\text{NOE}}/\sigma^{\text{ROE}}$ ratios more negative than the theoretical limit of -0.5 . Four are located in the N- and C-termini of the protein. No amides show positive $\sigma^{\text{NOE}}/\sigma^{\text{ROE}}$ ratios, which is consistent with an upper bound determined by the slowed dynamics of water in the reverse micelle. See text for further details.