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Toward comprehensive measurement of protein hydration dynamics: Facilitation of NMR-based methods by reverse micelle encapsulation

Pamela N. Gallo, Joseph C. Iovine, Nathaniel V. Nucci*

Department of Physics & Astronomy, Department of Molecular & Cellular Biosciences, Rowan University, 201 Mullica Hill Road, Glassboro, NJ 08028, United States

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ABSTRACT

Protein-water interactions are a fundamental determinant of protein structure and function. Despite their importance, the molecular details of water orientations and dynamics near protein surfaces remain poorly understood, largely due to the difficulty of measuring local water mobility near the protein in a site-resolved fashion. Solution NMR-based measurement of water mobility via the nuclear Overhauser effect was presented as a method for performing comprehensive, site-resolved measurements of water dynamics many years ago. Though this approach yielded extensive insight on the dynamics and locations of waters buried within proteins, its promise for measuring surface hydration dynamics was impeded by various technical barriers. Over the past several years, however, this approach has been pursued anew with the aid of reverse micelle encapsulation of proteins of interest. The confined environment of the reverse micelle resolves many of these barriers and permits site-resolved measurement of relative water dynamics across much of the protein surface. Here, the development of this strategy for measuring hydration dynamics is reviewed with particular focus on the important remaining challenges to its widespread application.

1. Introduction

Interactions with water provide a thermodynamic driving force that heavily influences the structure and functions of biological macromolecules. The hydrophobic effect is a well-known manifestation of these interactions, and its importance is firmly established [1]. Despite decades of recognition regarding the fundamental relationships between water and biological molecules, many aspects of biomolecular hydration remain poorly understood. Continuum representations of water's impact on proteins, for example, have existed for many years and are known to accurately reproduce a wide range of experimentally observable phenomena. Similarly, measurements of bulk water properties (dielectric relaxation, scattering profiles, etc.) in the presence of proteins have provided vast insight [2–4]. An atomistic understanding of local hydrogen bonding and dynamics of water near protein surfaces, however, has remained elusive in large part due to the paucity of experimental data against which to benchmark theoretical results such as those provided by molecular dynamics simulations. An experimentally-verified atomic view of protein hydration is particularly important for developing a complete understanding of the subtle, local influences that drive molecular recognition. The critical experiment should provide access to comprehensive, temporally- and spatially-resolved water mobility across a protein surface. Solution NMR was argued many years ago to be the optimal method for gaining such insight via measurement of nuclear Overhauser enhancements between protein and water [5–7]. Many technical barriers were identified that challenged the utility of this approach, so much so that the method was all but abandoned for many years. Important steps have been taken in recent years, however, to overcome these barriers and realize the promise of this approach; these advancements are the focus of the present review.

1.1. The hydration layer

The water molecules whose mobility is impacted by protein surface interactions are collectively known as the hydration layer (Fig. 1A). This layer is typically argued to consist of the nearest 2–4 layers of water molecules from the protein surface due to the magnitude of motional retardation near the surface, though some studies have indicated motional restriction up to 9 water layers from the surface, suggesting that the hydration layer may extend much further [3,4,8–10]. Water molecules in the hydration layer have been measured to move ~2 to 10 times slower on average than bulk water. A minority of water molecules interact so strongly with the protein surface that they are effectively bound, resulting in their dynamic motions being slowed to the nanosecond range. Bound waters that are buried within the protein structure, frequently called structural waters, exhibit

* Corresponding author.
E-mail address: Nucci@rowan.edu (N.V. Nucci).

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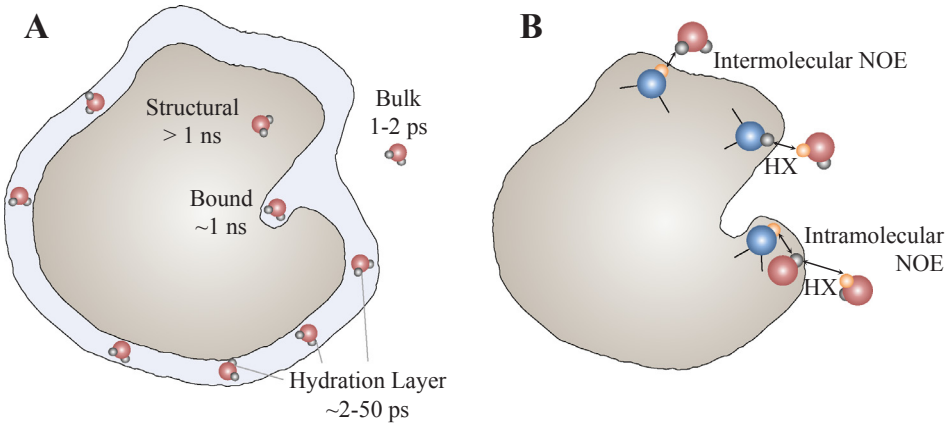

Fig. 1. (A) Water mobility within and near a protein (tan) is illustrated for water molecules integrated into the protein structure (structural), surface waters exhibiting long-lived interactions with the protein surface (bound), hydration waters, and waters in bulk solution. (B) Mechanisms of magnetization exchange between protein hydrogens (orange) and water hydrogens (gray) are illustrated schematically (following Otting) [7]. Water dynamics may be evaluated by measurement of dipolar exchange via intermolecular NOEs (top), but such measurements are complicated by hydrogen exchange (HX)-mediated interactions (middle and bottom). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

exchange with bulk water on the order of tens of nanoseconds or longer. These bound waters are typically resolved in protein crystallographic structures and are well characterized [11,12]. The local properties of the hydration layer, however, remain largely a mystery. Molecular dynamics simulations predict the importance of surface electrostatics and geometry in dictating water mobility [13,14]. Experimental data to validate these predictions remain limited, however.

Perhaps the most comprehensive experimental approach has been fluorescence relaxation measurement using tryptophan scanning in model protein systems [8,15,16]. These studies have generally argued that the mobility of the side-chain correlates with that of the nearby solvent. Minimal evaluation of solvent mobility with respect to surface curvature or electrostatics is available from such data, however, because all measurements result from solvent interacting with the tryptophan side chain, thus the native protein surface is necessarily modified from wild-type. A broad array of NMR-based approaches has also been applied to this problem, and in recent years Overhauser dynamic nuclear polarization has offered the most wide-ranging insight [17–23].

1.2. NMR-based measurements of protein-water interactions

NMR has been brought to bear on the protein hydration problem in a wide variety of ways over the past three decades. Most studies have explored averaged behavior of water in the presence of proteins by monitoring relaxation properties of the water signal itself. Perhaps the most historically dominant approach for such measurement has been nuclear magnetic relaxation dispersion (NMRD) which been recently reviewed in detail [24]. This approach interrogates protein-water interactions through differences in the magnetic field dependence of water nuclear spin relaxation. Due to the degeneracy of the water spin resonance, general relaxation properties of the water near proteins is obtained using hydrated protein powders. Important insight has been gained by comparing proteins with different surface character (charge, hydrophobicity, etc.) In cases where detailed structural insight is available from crystal structures or simulations, the observed relaxation may be interpreted with site-specificity for motionally restricted structural waters or bound waters in deep surface pockets [25]. NMRD, however, offers minimal spatially-resolved surface hydration insight with the exception of instances where the interference of water in respect to ligand binding may be measured [26]. In such cases, the hydration of ligand binding sites may be directly probed.

Recently, solid state NMR has also been shown to offer insight into the nature of protein hydration [27–29], especially with respect to illuminating hydration of membrane proteins. In many cases, linewidth analysis of the water resonance or of protein signals under varying conditions of hydration has been used to draw general conclusions about the mobility of solvent near the protein surface [30]. More site-resolved insight may be gained by monitoring magnetization transfer from water to proteins [29]. In one recent example, Hong and coworkers demonstrated site-resolved measurement of magnetization exchange between different frozen water structures and regions of various amyloid fibrils, thereby deriving functional insight regarding water and proton mobility through the M2 channel [28]. These findings suggest exciting potential future facilitation of protein-water measurements from solid-state approaches.

Over the past decade, Overhauser dynamic nuclear polarization (ODNP) has become the dominant NMR technique for site-specific measurement of water mobility on picosecond to nanosecond time scales near macromolecular surfaces [21]. Amplification of the NMR signal of water due to polarization transfer from an excited electron spin probe, typically of a stable nitroxide radical, is interpreted in terms of the local water mobility. The spin probe transfers polarization from an unpaired electron to a water molecule within 5–10 Å thereby amplifying the ^{1}H signal of the water through the Overhauser effect. [20,22,31] Because this enhancement results from a time-dependent polarization transfer, the dipolar coupling constant offers a measure of the effective residence time of the water molecules near the probe [23]. Nitroxide radicals may be incorporated into a protein via cysteine mutation to allow site-specific labeling [22] or they may be incorporated into a lipid or other amphiphilic construct for embedding in a membrane to interrogate membrane hydration [20]. ODNP has yielded important new knowledge regarding hydration for a variety of proteins, such as chaperonins, cytosolic and membrane proteins [18,19,22,31–33]. Measurement of water dynamics within the core of the chaperonin GroEL/ES complex, for example, showed that water mobility within the cage is similar to bulk, thereby supporting a passive cage model for chaperonin function [31].

2. Comprehensive, site-resolved measurement of water dynamics via the nuclear overhauser effect

Wuthrich and Otting proposed spatiotemporal resolution of protein-water interactions via measurement of dipolar magnetization exchange between protein and water hydrogens many years ago [6,7]. The nuclear Overhauser enhancement (NOE) cross-relaxation rate between protein and water hydrogens is compared between the longitudinal and rotating frames to extract dynamic information about water mobility. By performing this measurement in conjunction with multi-dimensional resolution of protein resonances, the promise of site-resolved mapping of water dynamics across a protein surface was offered. As the method was applied to various systems, extensive measurement of structural water mobility was achieved, but it failed in comprehensively interrogating the hydration layer for multiple reasons [7]. In bulk solution, water motion in the hydration layer is too rapid to permit detectable build-up of protein-water magnetization exchange, and rapid hydrogen exchange between water and labile hydrogens of the protein obfuscate interpretation of protein-water NOE data in bulk solution. In addition to these issues, long-range dipolar relaxation to the bulk water spin bath

was argued to compromise the local nature of the measurement [34,35]. Each of these complications is mitigated, in part or in total, by encapsulation of the protein in a reverse micelle.

2.1. Theory of dipolar magnetization exchange between water and solute hydrogens

Determination of water dynamics by solution NMR relies on the comparison of dipolar magnetization exchange via the nuclear Overhauser effect in both the laboratory (NOE) and rotating (ROE) frames. The rate of magnetization transfer (σ) in the two frames is dependent upon the power spectral densities, $J(\omega)$, describing the motion of the internuclear vector of the two interacting nuclei [7].

$$\sigma_{NOE} = K[-J(0) + 6J(2\omega)]; \quad \sigma_{ROE} = K[2J(0) + 3J(2\omega)] \tag{1}$$

where ω is the proton (hydrogen) Larmor frequency and K is a prefactor composed of fundamental constants. In the case of an intramolecular internuclear vector that is rigid within the molecular frame of a protein molecule tumbling isotropically with a correlation time τ_m, the spectral density is:

$$J(\omega) = \frac{1}{2\pi}\frac{\tau_m}{1 + \omega^2\tau_m^2} \tag{2}$$

This ensures that the σ^{ROE} is always positive. The σ^{NOE}, conversely, will be positive for short τ_m, pass through zero at $\omega\tau_m \sim 1.1$, and remain negative at longer τ_m. In the slow-tumbling regime, where $\omega\tau_m \gg 1$, the ratio of the NOE and ROE relaxation rates approaches a limit of -0.5. In the fast tumbling regime, where $\omega\tau_m \ll 1$, σ_{NOE} and σ_{ROE} are equal, giving a ratio of 1. Proteins are most often in the slow-tumbling regime. Here, the NOE cross peaks are of identical phase to the diagonal peaks in the NOESY spectrum while ROE cross peaks are of opposite phase to the diagonal peaks in the ROESY spectrum. Thus, for intramolecular dipolar exchange, the ratio of relaxation rates is modulated by rotational dynamics of the whole molecule. The case for intermolecular dipolar magnetization exchange is rather more complex due to convolution of the internuclear distance and the angle between the internuclear vector and the external magnetic field. For *inter*molecular Overhauser exchange, $J(\omega)$ is given by the Fourier integral of the autocorrelation function describing the motion of the internuclear vector [36].

$$J(\omega) = \int_{-\infty}^{+\infty} C(t)\cos(\omega t)dt \tag{3}$$

The autocorrelation function is generally given by:

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

where r_j is the internuclear vector and $Y_{20}(r_j) = \sqrt{5/16\pi}(3\cos^2\theta - 1)$, which is a normalized spherical harmonic containing the angle θ between r and the external magnetic field. In the case of protein-water dipolar exchange, variance of r and θ is dominated by the mobility of water. A quantitative relation between $\sigma^{NOE}/\sigma^{ROE}$ and water mobility requires assumption of a model to describe the motion, thereby imposing a model-dependence to interpretation. Various models have been presented in previous works [7,36,37], but they have generally shown that the predicted relationship between the limits of the $\sigma^{NOE}/\sigma^{ROE}$ and the relative local mobility of water hold with the exception that the time-dependence of the interaction may be considered in terms of an effective correlation time, τ_{eff}, given by:

$$\frac{1}{\tau_{eff}} = \frac{1}{\tau_{res}} + \frac{1}{\tau_m} \tag{5}$$

Here, the effective correlation time of the interaction depends on the residence time of the water molecule, τ_{res}, at a given site on the protein surface (or interior) and the rotational behavior of the protein.

2.2. Interpretation of protein-water dipolar magnetization exchange

In the case of a very long-lived protein-water interaction, the NOE behaves essentially as an intramolecular interaction, thus a $\sigma^{NOE}/\sigma^{ROE}$ ratio of -0.5 indicates a protein-water interaction which is long-lived on the NMR time scale [7,36]. Such an interaction must be rigid within the molecular frame for a time corresponding to the τ_m or longer. In the limit of short-lived protein-water interaction, such as seen for a site with very fast hydration dynamics, σ^{NOE} tends towards zero because the slow rotation of the protein fixes the system in the slow-tumbling regime, producing a $\sigma^{NOE}/\sigma^{ROE}$ ratio of zero. Theoretical relations between predicted $\sigma^{NOE}/\sigma^{ROE}$ and water mobility have argued that fast hydration should result in positive $\sigma^{NOE}/\sigma^{ROE}$ values, but this would require experimental observation of NOE signals of opposite phase to the diagonal. In practice, such signals are typically only seen for small molecules or peptides. Brüschweiler and Wright have detailed the $\sigma^{NOE}/\sigma^{ROE}$ dependence on protein tumbling and relative water diffusion, illustrating the complexities for quantitative interpretation of experimental data [36]. Otting predicted an effective correlation time of ~70 ps for the zero-crossing point of σ^{NOE} for a water-protein interaction measured at 600 MHz [7]. Given the average retardation of water mobility in the hydration layer predicts reorientational times on the order of ~2 to 50 ps, longitudinal dipolar exchange between protein and hydration water should be generally inefficient. Indeed, bulk solution measurements of NOESY spectra for proteins in solution at room temperature do not show widespread cross-relaxation to the water resonance.

The absence of general longitudinal cross-relaxation to water from surface protein hydrogens was argued to result from long-range relaxation to the water spin-bath [34,35]. In short, the number-averaging of water spins as a function of angle and distance was argued to compromise the local nature of the dipolar relaxation. Recently, quantitative relation between local water mobility and predicted $\sigma^{NOE}/\sigma^{ROE}$ were presented from molecular dynamics simulations of ubiquitin in solution [10]. This analysis included consideration of the pair correlation function between each protein spin and all solvent spins [38]. Inclusion of this critical parameter shows that the local nature of the NOE is maintained and is modulated by water dynamics for protein-water intermolecular dipolar exchange. Importantly, long-range coupling to the water spin-bath was argued to be significant only for non-surface-exposed protein hydrogens. Thus, previous theoretical objections regarding the value of protein-water dipolar exchange as a measure of hydration dynamics seem unfounded. The practical problem of rapid reorientation within the hydration layer that leads to minimal measurable exchange, however, still precludes comprehensive measurement for proteins in bulk aqueous solution.

Further complicating interpretation of observed $\sigma^{NOE}/\sigma^{ROE}$ are contributions from hydrogen exchange between water hydrogens and labile hydrogens of the protein itself (Fig. 1B) [7]. Hydrogen exchange (HX) between observed protein hydrogens and water produce ROE signals at the water resonance with identical phase to the diagonal, thus these are easily distinguished from dipolar exchange peaks. HX-relayed dipolar exchange, however, produces signals that are indistinguishable from direct protein-water dipolar exchange. This mechanism involves HX between water and a labile protein hydrogen followed by subsequent *intramolecular* NOE between the protein hydrogen probe and the protein HX site. Many previous NMR studies of protein hydration in bulk solution have been questioned due to potential contamination from such mechanisms, so much so that the approach was all but abandoned except in cases where buried waters were far enough from the protein surface to minimize the probability of HX-relay as a mechanism for observed signals or other approaches could be utilized to minimize such contributions [39,40].

3. Facilitation of comprehensive hydration dynamic measurement via reverse micelle encapsulation

Encapsulation of a protein in the aqueous interior of a reverse micelle has been shown to facilitate comprehensive measurement of hydration dynamics for the model protein ubiquitin [41,42]. These proof-of-principle studies illustrated the utility of reverse micelle encapsulation to overcome the primary impediments to measurement of water dynamics in the hydration layer via the NOE. First and foremost, confinement of the protein and its associated hydration layer produced extensive cross-relaxation between protein hydrogens and the water resonance. Secondly, the reverse micelle mixture used in these studies, composed of bis(2)-ethylhexylsulfosuccinate (AOT), was shown to reduce the rate of amide hydrogen-water HX by at least two orders of magnitude [42]. The data obtained in these studies provided hydration dynamic information for approximately three-quarters of the protein surface and showed surprising heterogeneity, particularly as compared to similar hydration maps calculated from molecular dynamics simulations [13,43]. The NMR data were interpreted as qualitatively representative of relative water mobility and showed an intriguing apparent correlation between retarded hydration and the region of ubiquitin that engages in protein–protein interactions [41]. This correlation suggested the potential for a role of local hydration mobility in modulating the desolvation energetics of such interactions. Ubiquitin is unique in its capacity to encapsulate in AOT reverse micelles with structural fidelity [44]. The vast majority of proteins denature when dissolved in AOT-based reverse micelle mixtures, thus general application of this approach for measurement of hydration dynamics requires use of alternative surfactant mixtures [45].

3.1. Reverse micelles for encapsulation of proteins

Reverse micelles are thermodynamically stable nanoemulsions that spontaneously organize to encapsulate an aqueous core in a shell of surfactant molecules [46–48]. These complexes are dissolved in a bulk non-polar solvent, typically an alkane. Aqueous solutes may be incorporated into the aqueous core spontaneously, and for simple solutes such as small molecules or salts, AOT has been the surfactant of choice for decades. In general, the size of the reverse micelle droplet scales with the amount of water added to the system. This parameter is described by the molar ratio of water to surfactant, or water loading (W_0). Application of reverse micelles for protein NMR was originally proposed as an alternative to TROSY methods for facilitating interrogation of large proteins [45,49]. Using low-viscosity solvents such as liquid propane or liquid ethane under moderate pressures, the rotational correlation time of encapsulated proteins may be dramatically reduced, thereby improving linewidths and efficiency of magnetization transfer via scalar coupling [50,51]. The initial proof-of-principle studies were performed using ubiquitin and AOT reverse micelles, showing that encapsulation did not perturb ubiquitin's native fold [44]. Application of the encapsulation approach to subsequent protein systems, however, necessitated identification of alternative surfactant mixtures with less denaturing tendency than AOT.

Mixtures of cetyltrimethylammonium bromide (CTAB) and hexanol were identified as a promising alternative to AOT [52]. These mixtures showed broad applicability for proteins of wide-ranging size and charge character. Integral membrane proteins [53,54], membrane-anchored proteins [55], and soluble proteins may be encapsulated with structural fidelity. Maintenance of structural fidelity may be readily assessed by comparison of ^{15}N-HSQC spectra. An example is shown in Fig. 2 for the M1.1 mutant of the type III antifreeze protein from ocean pout [56]. The extent of chemical shift perturbation upon encapsulation indicates the degree to which the native fold has been preserved. Further confirmation may be obtained from inventory of intramolecular NOEs for the encapsulated protein. Encapsulation of novel proteins in CTAB/hexanol typically requires optimization of reverse micelle pH and W_0.

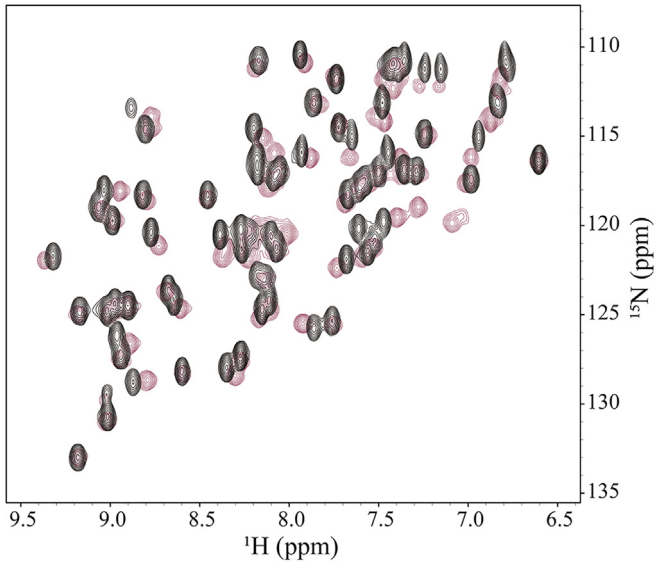

Fig. 2. ^{15}N-HSQC spectra of the M1.1 mutant of the type III antifreeze protein from ocean pout in aqueous solution (red) versus confined in CTAB/hexanol reverse micelles (black). Observed chemical shift perturbations arise primarily from side chain amine resonances and result from differences in hydrogen exchange under encapsulated conditions. The minimal perturbation of backbone resonance chemical shifts indicates general maintenance of the native protein fold in the reverse micelle. Local chemical shift perturbations require further characterization using typical approaches [58]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In most cases, optimized conditions yield samples that are stable and produce identical NMR spectra for weeks to months with storage at room temperature. More recently, a novel mixture composed of decyl-1-monoacylglycerol (10MAG) and lauryldimethylamine-N-oxide (LDAO) was described that has even more general applicability for encapsulation of macromolecules [57]. This mixture has the added advantage of dramatically reducing the need for optimization of pH and W_0.

3.2. Important considerations for measuring hydration dynamics by reverse micelle NMR

While the recently described 10MAG/LDAO mixture is the most broadly applicable reverse micelle mixture for encapsulation of proteins described to date, it has an important weakness that impedes its utility for measurement of hydration dynamics. Resolution of protein-water dipolar exchange relies on unique identification of cross peaks in the indirect ^{1}H-dimension of NOESY and ROESY-HSQC spectra. Because the hydroxyl protons of the 10MAG glycerol headgroup are degenerate with the water resonance, observed cross peaks between protein hydrogens and the water resonance may arise from interaction with water or with the surfactant headgroup. This complication precludes unambiguous measurement of hydration dynamics using this mixture.

The CTAB/hexanol mixture, however, provides a broadly applicable reverse micelle condition in which the hydroxyl hydrogens of the hexanol may be distinguished from water hydrogens under appropriate conditions. In addition to identifying conditions that preserve the native structure of the encapsulated protein, the pH and W_0 of the sample must yield resolution of water and hexanol hydroxyl peaks. As shown in Fig. 3, this relationship is complex and depends on both parameters, but maximal resolution may be achieved by using the minimum concentration of hexanol needed for formation of a uniform reverse micelle sample (indicated by optical clarity) and by using an interior pH of between 5 and 6. The pH of a reverse micelle mixture is a complex

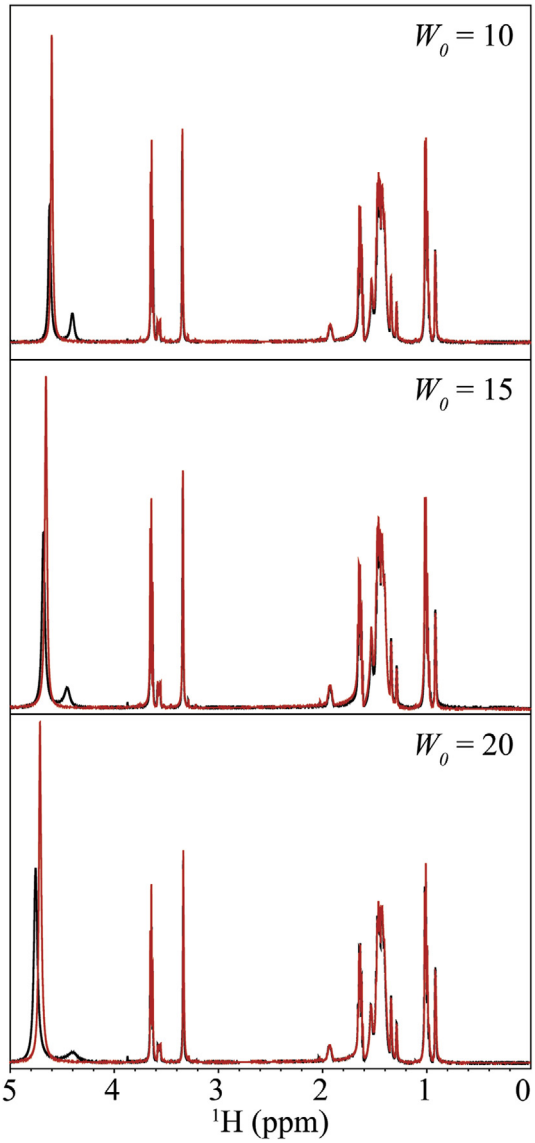

Fig. 3. ^{1}H spectra of 75 mM CTAB, 450 mM hexanol reverse micelles in perdeuterated pentane are shown at varied W_0 at pH 5 (black) and pH 7.5 (red). The resolution of the water (~4.7 ppm) and hexanol hydroxyl (~4.4 to 4.7 ppm) depends on both parameters of the reverse micelle sample. Complete resolution is important for unambiguous assignment of protein-water dipolar relaxation cross peaks. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

aspect of these systems that has been widely studied [59,60], but a practical ^{1}H NMR-based approach for manipulation and measurement of the pH of the aqueous phase has been described [61]. Incorporation of buffer molecules with pH-dependent chemical shifts that are well resolved in reverse micelle spectra permit direct monitoring of interior pH. In addition to these advantages, measurement of rotational correlation times for a wide range of soluble proteins using solvents of various viscosities illustrated that the optimal W_0 in CTAB/hexanol mixtures corresponds with a reverse micelle aqueous core that correlates closely with the hydrated radius of the encapsulated protein as measured in bulk aqueous solution [62]. This finding indicates that CTAB/hexanol reverse micelles maintain a similar thickness of the protein hydration layer as found in bulk solution, thus perturbations of the hydration layer itself are likely to be minimized.

3.3. Measurement of hydration dynamics by reverse micelle NMR

Once an optimal sample condition is identified, the NMR experiments for measuring hydration dynamics are relatively straightforward. Determination of $\sigma^{NOE}/\sigma^{ROE}$ values is achieved by measurement of ^{15}N- or ^{13}C-resolved NOESY-HSQC and ROESY-HSQC spectra using identical recycle delay times, signal averaging, and receiver gain settings. [63] These may be optimized for the sample, but should be identical in each NOESY/ROESY pair to simplify data analysis. Reverse micelle samples include small concentrations of water, thus water suppression is unnecessary for these samples and may cause artefactual signals if employed in hydration dynamics measurements. To prevent carrier artifacts from interfering with interpretation of protein-water cross peaks, it is recommended to move the carrier frequency to a relatively empty region of the spectrum. Typically 3.5 ppm or 5.5 ppm works well for most proteins. Interference from organic solvent may be easily eliminated by using deuterated solvent without negatively impacting spectral performance or sample longevity. For dipolar mixing times in the linear regime of cross peak build-up, the $\sigma^{NOE}/\sigma^{ROE}$ ratio is equivalent to the ratio of NOE to ROE cross peak intensities. Short mixing times (< 50 ms) must be used to ensure that the NOE is measured in the linear regime and to minimize potential contributions from spin diffusion. Measurement of the ROE in the linear regime, however, is problematic with proteins due to the rapid $T_{1\rho}$ relaxation in the slow tumbling regime. As a result, ROE intensities may be corrected for $T_{1\rho}$ relaxation to extrapolate the linear regime intensity before calculation of $\sigma^{NOE}/\sigma^{ROE}$ ratios [7,41,42]. Efficiency of protein-water cross peak detection is markedly improved by deuteration of all protein hydrogens save those that serve as hydration probes. Perdeuteration and back-exchange of backbone amides for ^{15}N-detected experiments is particularly advantageous.

4. Advantages and challenges to the reverse micelle approach for hydration dynamics measurement

The reverse micelle NMR method discussed here provides the only comprehensive approach for site-resolved, experimental measurement of hydration dynamics for a wild type protein reported to date. Alternative approaches using 2D-infrared spectroscopy [64], fluorescence relaxation [8,15,16], or ODNP [23] require incorporation of non-native probes that are typically bulky and/or quite polar. These methods have yielded vast insight in a broad array of systems and are largely responsible for advancement in the field over the past decade, but each includes the possible caveats associated with the potential for the non-native probe to influence the measured solvation behavior. The present method suffers from the obvious and important caveat that encapsulation in a reverse micelle likely impacts the mobility of the detected hydration water.

The dynamic impact reverse micelle confinement on encapsulated water (in the absence of proteins) is quite well understood [65,66] and while the reverse micelle interface retards water mobility, this effect is uniform across the surfactant interfacial surface and decreases with distance toward the center of the water core. The most effective model for explaining encapsulated water behavior, the core-shell model [67], separates the water into an interfacial layer that interacts with the surfactant and a bulk-like water core. For encapsulated proteins, the radius of the reverse micelle particle corresponds closely with the sum of the hydrated radius of the protein and the thickness of the hydrated surfactant layer, strongly suggesting that the thickness of the protein hydration layer is not impacted by encapsulation [62].

The rapid mobility of hydration water measured in bulk solution by other methods includes both pseudo two-dimensional diffusion of water within the hydration layer across the protein surface and exchange between the hydration layer and bulk solution. It is important to note that dipolar magnetic exchange between water and protein is substantially more distance-dependent than other experimental

approaches. This distance dependence means that observed protein-water NOEs will be heavily impacted by both types of diffusive motion. In bulk solution, the rapid exchange of waters in the hydration layer with bulk may be the primary driving factor that prohibits detectable magnetization exchange for most surface sites. Encapsulation of the hydrated protein in a reverse micelle practically eliminates the exchange of hydration waters with a bulk solvent population. It should be noted that even protein-saturated reverse micelle samples achieve only ~20 to 25% occupancy, thus for each protein-containing reverse micelle, there are four to five protein-free reverse micelles that contain a bulk-like water pool. Interparticle exchange of contents is known to occur in reverse micelle mixtures on the millisecond to microsecond timescale [68], thus hydration layer exchange with bulk-like water must occur but on a much slower timescale than in bulk aqueous solution. It is also important to point out that only membrane-associated proteins show spectral evidence of long-lived interaction with the surfactant interface upon reverse micelle encapsulation. The uniformity of the surfactant interface, the preservation of protein structure, and the absence of protein-surfactant cross peaks in NOE spectra strongly suggest that the localized variance in water mobility seen for ubiquitin is driven by details of the protein surface interactions rather than by reverse micellar confinement. The highly collective nature of water dynamics, however, raise the question of whether dynamics within the hydration layer are truly decoupled from exchange with the bulk. Going forward, comparison of reverse micelle hydration data with experimental data obtained via alternative approaches will be helpful in more clearly defining the differences in hydration water mobility that result from encapsulation.

In addition to potential confinement-induced modulation of hydration dynamics, some aspects of the measurements themselves may be improved upon. In particular, the extrapolation of measured ROE intensities via correction for longitudinal relaxation in the rotating frame merely provides an estimation of the true σ^{ROE}. A more accurate determination of $\sigma^{NOE}/\sigma^{ROE}$ values would require direct measurement of cross peak intensity build-up curves. The limited signal-to-noise of NOESY and ROESY spectra using short mixing times makes collection of such data using traditional Cartesian sampling prohibitive in terms of instrument time. Application of approaches such as those used to measure quantitative intramolecular NOEs [69] may be useful for future measurements of hydration dynamics, and precise measures will almost certainly be required to derive quantitative dynamic information from obtained $\sigma^{NOE}/\sigma^{ROE}$ values, as previous data have been only qualitatively interpreted in terms of relative water mobility.

Another important challenge to the generalizability of the approach comes from potential residual contributions of hydrogen exchange. Marked reduction in hydrogen exchange rates were demonstrated in AOT reverse micelles with ubiquitin for backbone amides [42]. Reduction in general hydrogen exchange rates with side chain hydroxyls was also illustrated by resolution of intramolecular NOEs to serine and threonine hydroxyl groups. Despite the general reduction in HX shown previously, residual HX of amide side chains may still be seen. As shown in Fig. 4, substantial HX between protein resonances of the M1.1 antifreeze protein [56] and water are evident in bulk solution. Though a majority of these disappeared after encapsulation, a handful of clear HX peaks remained. The spectra shown were collected at pH 7 to illustrate the presence of HX even in the reverse micelle. These signals may be reduced by dropping the internal pH of the reverse micelle sample, but their persistence at pH 7 under confinement suggests that they may not undergo the general reduction in HX rate that has been observed previously for backbone amides. Though they are a minority, the extent to which such sites contaminate measured $\sigma^{NOE}/\sigma^{ROE}$ values needs to be quantitatively determined. If these are significant, efforts to mitigate these effects will also need to be developed before this method can be broadly applied.

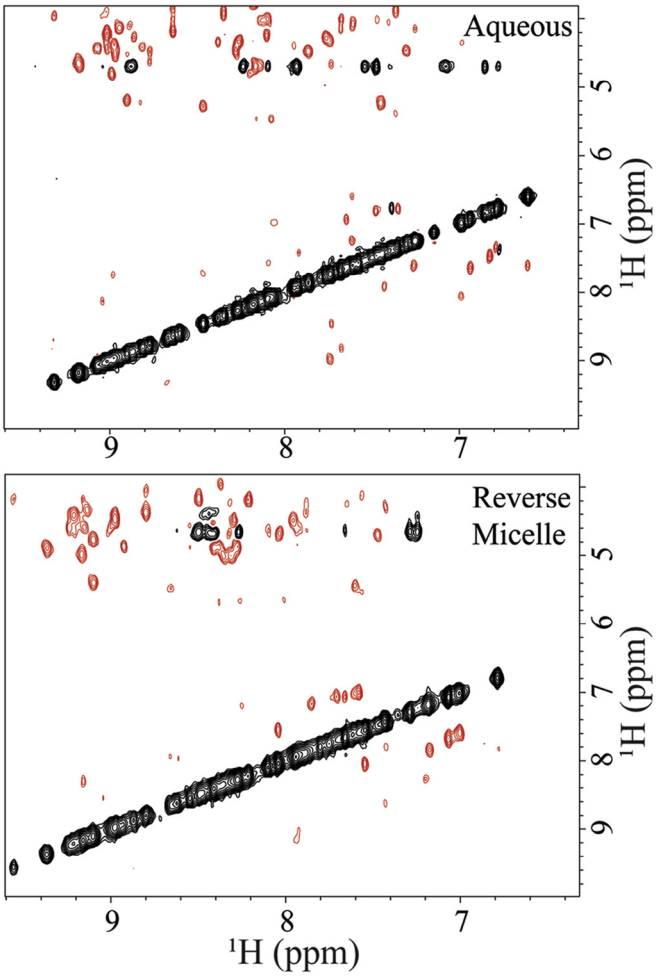

Fig. 4. ^{1}H-^{1}H projections of ^{15}N-HSQC-ROESY spectra collected using a 40 ms mixing time at 600 MHz on a Bruker Avance III spectrometer with a TXI cryoprobe are shown for the M1.1 mutant of ocean pout type III antifreeze protein at 1 mM in aqueous solution at pH 7 or at ~150 μM in CTAB/hexanol reverse micelles. Aqueous and reverse micelle spectra were collected with 16 scans and 1 s recycle delay versus 64 scans and 1.5 s recycle delay, respectively. Cross peaks at the water resonance are visible in both spectra as positive contours (black) at approximately 4.7 ppm.

5. Conclusions

Comprehensive spatiotemporal measurement of hydration water dynamics across protein surfaces has been a long-standing experimental challenge. Solution NMR measurement of dipolar magnetization exchange between proteins and water in reverse micelles is a promising mechanism for performing such measurements. Many of the potential challenges and pitfalls to broad application of this approach have been resolved, yet some remain. Continued advancements in NMR methodologies, especially sparse sampling approaches [70,71], and development of complimentary experimental techniques offer the hope for such measurements to become widely applicable. On a broader scale, our understanding of protein hydration would benefit from a wider effort to perform complimentary experimental measurements in identical systems and to benchmark simulation results against experimental observables. Historically, simulations have yielded molecular level insight regarding local water mobility by comparing trajectories to spherically averaged data like scattering profiles [3]. The recent increase in available site-resolved measurements of hydration dynamics from NMR [29,41], ODNP [20–22], fluorescence relaxation [15,16], and infrared spectroscopy [64] should offer theoreticians a broader range of data points against which to compare simulated trajectories.

Conversely, the experimental community would benefit from more direct comparisons of methods in similar protein systems. In many cases, different measurements will likely prove complimentary and provide the needed insight to improve both predictive models and experimental interpretations.

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