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Site-Specific Hydration Dynamics of Globular Proteins and the Role of Constrained Water in Solvent Exchange with Amphiphilic Cosolvents

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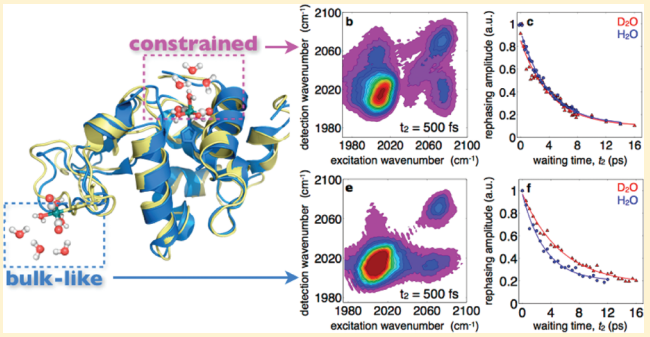
Supporting Information

ABSTRACT: The thermodynamic driving forces for protein folding, association, and function are often determined by protein−water interactions. With a novel covalently bound labeling approach, we have used sensitive vibrational probes, site-selectively conjugated to two lysozyme variants—in conjunction with ultrafast two-dimensional infrared (2D-IR) spectroscopy—to investigate directly the protein−water interface. By probing alternatively a topologically flat, rigid domain and a flexible domain, we find direct experimental evidence for spatially heterogeneous hydration dynamics. The hydration environment around globular proteins can vary from exhibiting bulk-like hydration dynamics to dynamically constrained water, which results from stifled hydrogen bond switching dynamics near extended hydrophobic surfaces. Furthermore, we leverage preferential solvation exchange to demonstrate that the liberation of dynamically constrained water is a sufficient driving force for protein−surface association reactions. These results provide an intuitive picture of the dynamic aspects of hydrophobic hydration of proteins, illustrating an essential function of water in biological processes.



1. INTRODUCTION

Biological processes, from DNA replication to enzyme catalysis, occur in the presence of water. Water's indispensible role in biology has motivated efforts to uncover the degree to which it actively participates in chemical events.[1] As the universal solvent of living organisms, water has a remarkable ability to accommodate both hydrophilic solutes through strong electrostatic interactions as well as hydrophobic solutes through subtle modifications to the hydrogen bonding network.[2] The hydration of large solutes (>1 nm), such as membranes and proteins, requires significant rearrangements of the hydrogen bonding network, leading to the sacrifice of hydrogen bonds. Hydration water—water directly solvating the large solute—is thus structurally and dynamically constrained, restricting the configuration space as well as limiting dynamical flexibility. These constraints endow interfacial water with properties that are different from the bulk liquid.[1−17] Whether or not one adopts a picture of protein dynamics as being "slaved" to the solvent, it is nevertheless clear that the preponderance of free energy changes attributable to the solvent arise from the relatively thin hydration layer of water solvating the protein.[18,19]

The interest in studying and characterizing the properties of interfacial water arises from the extensive role that the protein−water interface plays in influencing such processes as small ligand binding,[20,21] protein−protein recognition,[15,22,23] and protein−DNA interactions.[24] Studies of orientational and spectral dynamics of water near lipid bilayers,[5] within reverse micelles,[7] or in the presence of small solutes[8,17] indeed support the picture that limiting the configuration space can impose constraints on water's dynamics. Additionally, molecular dynamics simulations have been used extensively to study dynamics that may be difficult to access experimentally, such as in the immediate hydration environments of proteins.[9,25,26] Experimental evidence of water confinement near protein surfaces has been found by studying solvation dynamics of site-specific fluorescent probes of protein surfaces via ultrafast fluorescence upconversion.[11−13] Recently, the combined constraining influence of both protein and lipids has enabled NMR measurements of local water structure and its mobility using reverse micelle-encapsulated ubiquitin.[14,15] Terahertz absorption spectroscopy has also been demonstrated to be a powerful technique for studying the hydration environments of proteins.[27,28] Though these experiments provide evidence for constrained water, it remains unclear precisely which aspects of water's motion are most strongly affected by the interface.

In this article, we present evidence from ultrafast two-dimensional infrared spectroscopy that the primary dynamical distinction of hydration water is the protein's suppression of large-angle orientational jumps. The unique dynamics of water has been used previously to sense the presence of water using 2D-IR through its influence on both vibrational lifetimes[29] and

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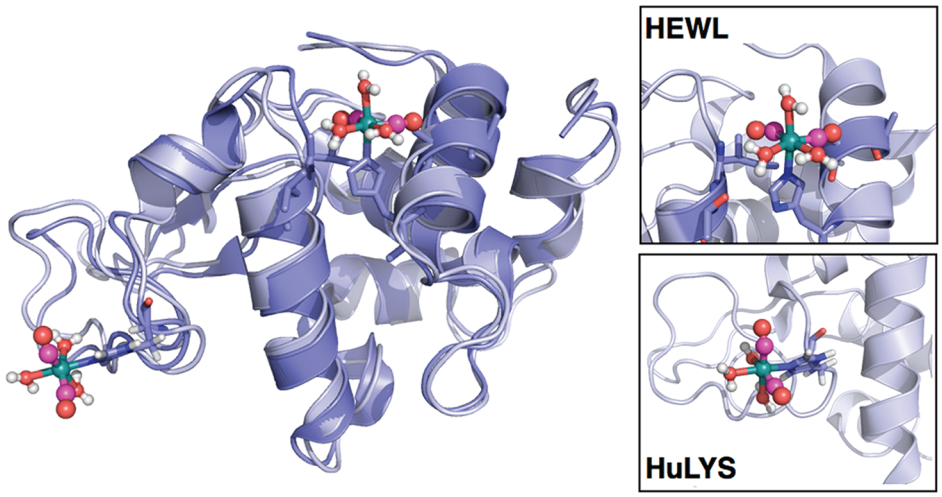

Figure 1. Crystal structures of HEWL-RC (PDB code 2XJW) overlaid with the crystal structure of native HuLys (PDB code 2ZIJ). The binding location of the metal carbonyl on the HEWL protein has been determined by X-ray crystallography. While no crystallographic data are available for the HuLys-RC complex, the binding location is proposed by comparison with the HEWL-RC complex.

spectral dynamics.[30] Since our probe is able to identify regions of hydration while simultaneously distinguishing between constrained hydration water and bulk-like solvation, we are able to determine directly from experiment that an amphiphilic cosolvent (trifluoroethanol) preferentially dehydrates the protein in the region where the protein constrains the water dynamics. Our data also show that the cosolvent associates directly with the protein by replacing water in the hydration shell, rather than indirectly by disrupting the hydration layer from a distance. This detailed picture of the heterogeneous dynamics of "biological water" should provide a microscopic basis for a more complete understanding of interactions between domains in large proteins as well as between proteins in large-scale assemblies including pathological aggregates.

Here, we present experimental evidence for constrained biological water solvating model enzymes, hen egg white lysozyme (HEWL), and human lysozyme (HuLys), using a vibrational probe of the structure and dynamics of the interfacial water solvating the near-native protein. By leveraging the isotope dependence of the probe's vibrational relaxation in water (i.e., H_2O and D_2O), we are able to observe the influence of qualitatively distinct protein surfaces on the associated hydration dynamics. Using a relatively strong IR probe based on a transition metal carbonyl adduct, we are able to record 2D-IR spectra with protein concentrations at the 200−500 μM level, which precludes complications due to the spatial coupling of hydration shells of other protein molecules in solution. The observed slowdown in water's dynamics is the result of protein surface-induced constraints placed on a subset of water's fast dynamics, namely, hydrogen bond switching events that occur through angular jumps,[31,32] which has proven difficult to observe with other spectroscopic techniques.

We study the hydration environment of two homologous proteins, hen egg white lysozyme and human lysozyme. The crystal structure of the hen egg white lysozyme ruthenium carbonyl complex (HEWL-RC) shows a Ru−carbonyl complex bound to the lone His15 residue (Figure 1).[33] While no crystal structure is available for the human lysozyme Ru−carbonyl complex (HuLys-RC), the structure is deduced by imposing the octahedral coordination found in HEWL-RC and by Fourier transform IR spectra which show identical carbonyl stretching frequencies for both HEWL-RC and HuLys-RC. HuLys has a single, solvent-exposed histidine residue (His78) which is the proposed binding location of the metal carbonyl complex. There are several examples of metal carbonyl complexes binding to surface histidines.[34,35] The linear and 2D-IR spectra of HEWL-RC and HuLys-RC in D_2O are shown in Figure 3. The linear spectrum of HEWL-RC shows two small additional bands corresponding to the low-population binding locations (Asp18 and Asp52) found in crystallography,[33] whereas the HuLys-RC shows only a single binding location. There is a slight (~1 cm^{-1}) shift in the vibrational frequencies of the two carbonyl modes, consistent with the metal center being coordinated to a histidine residue in both cases but having different local protein environments. In HEWL, His15 is in the highly structured α domain, whereas His78 of HuLys is located in the unstructured β domain (Figure 1). We note, however, that the unstructured domains of HEWL and HuLys are structurally similar.

The binding motif of the vibrational label to the proteins is shown in Figure 2. We also rely on comparisons with the small

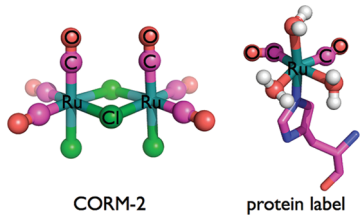

Figure 2. Structures of the vibrational chromophores used in this study. CORM-2 is used throughout the study as a model small molecule metal carbonyl. The key feature of the molecule that allows this comparison is the presence of multiple CO modes that are coupled, allowing for water-assisted vibrational relaxation.

molecule dichloro-ruthenium(II) dimer (a so-called "carbon monoxide releasing molecule" often denoted CORM-2), which is the precursor to the labeling complex (referred to as CORM-3).[36] Because of the scarcity of water-soluble metal carbonyls, we rely on comparisons between the labeled proteins and the CORM-2 complex in both aqueous and organic solvents. While the molecules are clearly different, the comparison between these molecules is both robust and instructive. The crucial

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properties shared between these molecules are the presence of coupled CO chromophores as well as the presence of numerous low-frequency modes. The anharmonic coupling between the spectroscopic modes and the lower frequency modes of the molecule results in water-assisted relaxation in aqueous environments, a key aspect of the results and interpretations presented here.[36] The side chains that are in the immediate vicinity of the CO oscillators on HEWL-RC are isoleucine, phenylalanine, and alanine residues, which are nonpolar residues, as well as an arginine residue. The HuLys-RC probe is mostly exposed to the solvent, though it is neighbored by cysteine, leucine, and alanine. While the environment presented by the protein is an important aspect of the dynamics felt by the vibrational probes, the observed lifetimes are dominated by the hydration water.

Ultrafast 2D-IR spectroscopy is used to study the hydration environments of HEWL-RC and HuLys-RC in pure water solvent (either H_2O or D_2O), as well as in solvent mixtures of D_2O and 2,2,2-trifluoroethanol (TFE) ranging from 0 to 20% TFE v/v. Because of the structural similarities of HEWL and HuLys (60% sequence homology, Cα rmsd = 1.1 Å), the two labeling locations, though occurring on different proteins, sample the heterogeneous protein structure as well as distinct solvation environments. The vibrational lifetime (T_1) of the metal carbonyl probe is used as a reporter of the local solvation environment at the interfacial region of the protein. The lifetime is sensitive to the presence of water and has been shown to be an order of magnitude shorter in water (H_2O or D_2O)[37] than in either proteinaceous environments[38] or in polar organic solvents.[37,39] Thus, the vibrational lifetime effectively acts as a water sensor positioned at the protein−water interface.

2. RESULTS AND DISCUSSION

2.1. Water-Assisted Vibrational Relaxation. The sub-5-ps absolute vibrational lifetime of the CO modes reports on the presence of liquid water as the principal pathway for vibrational relaxation. We have previously shown that the vibrational lifetimes of metal-bound carbonyls are on the order of 50−100 ps.[39] Even in the highly polar solvent methanol, we find the vibrational lifetime of the small CORM-2 complex to be 42.25 ± 3 ps.[37] In water, however, the vibrational lifetime of CORM-2 is an order of magnitude smaller, an effect attributed to the high density of vibrational states in which to dissipate energy as well as the extremely rapid fluctuations of charge, both hallmarks of water solvation.[37] Water acts to facilitate the intramolecular coupling of the solute vibrational degrees of freedom. The observed vibrational lifetimes (see Supporting Information for experimental methods) of the protein-bound metal carbonyls also exhibit lifetimes on the order of 3−4 ps (Figure 3), suggesting that the chromophores are sensitive to the interfacial water, which provides the dominant relaxation pathway. This conclusion, that the vibrational relaxation is sensitive mainly to the water hydrating the protein, was further verified using D_2O−TFE solvent exchange discussed in detail below, where we find that replacing the hydration water with an alcohol cosolvent results in a pronounced increase in the vibrational lifetime.

2.2. Constrained Water at the Protein Surface. The thermodynamic driving forces for hydrating small and large hydrophobic cavities differ according to the relative significance of enthalpic and entropic contributions. Small hydrophobes and small ions generally sustain water's local hydrogen bonding network through subtle rearrangements, so that free energy

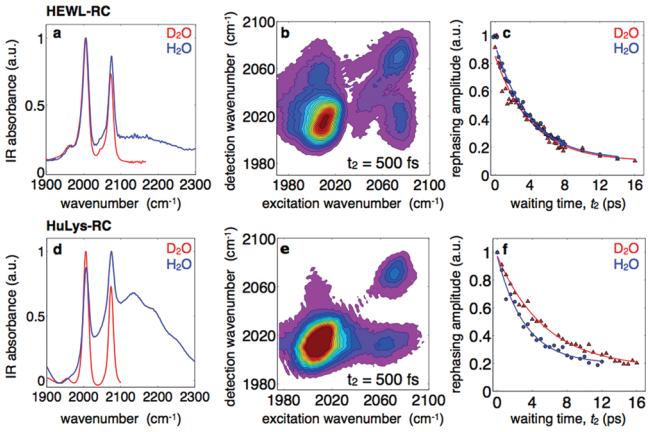

Figure 3. Linear FTIR spectra of HEWL-RC (a) and HuLys-RC (d) in D_2O and H_2O. The broad feature in the H_2O spectrum is the bend-libration combination band, centered at 2150 cm^{-1}. The 2D-IR rephasing spectra for HEWL-RC (b) and HuLys-RC (e) in D_2O are shown for a waiting time of t_2 = 500 fs. Monitoring the amplitude of the 2004 cm^{-1} peak as a function of waiting time, t_2, provides the vibrational lifetime of the mode. For HEWL-RC there is no observable isotope effect in the vibrational relaxation between D_2O and H_2O (c), whereas HuLys-RC shows a very clear isotope effect (f). The lack of an isotope effect suggests solvation by slow constrained water, whereas hydration by bulk-like water leads to an observable isotope effect. These results demonstrate the heterogeneous nature of the water dynamics near a protein, where certain regions are hydrated by slow constrained water while other regions are hydrated by bulk-like water.

gradients arise from changes in entropy.[2,40−43] Conversely, large hydrophobes disrupt hydrogen bonding, leading to driving forces dominated by enthalpic changes.[2,41,43,44] Hence, one expects dynamical perturbations to reflect these distinct underlying free energy landscapes.

Figure 3 shows the Fourier transform IR (FTIR) spectrum of HEWL-RC in H_2O and D_2O. The vibrational probe has two IR-active CO modes located at 2004 and 2080 cm^{-1}. We focus on the low-frequency mode of both HEWL-RC and HuLys-RC for analysis. Using 2D-IR spectroscopy, the vibrational lifetimes of the CO vibrational modes of HEWL-RC in H_2O and D_2O were extracted for the 2004 cm^{-1} mode and found to be 3.60 ± 0.18 and 3.73 ± 0.21 ps, respectively. This result is in stark contrast to what has previously been reported for water-assisted vibrational relaxation, where we observed pronounced isotope differences between water and heavy water.[37]

The loss of the isotope effect can be explained in terms of the restraints that large, hydrophobic surfaces place on water's hydrogen bonding structure and dynamics. Comprising a subset of water's fast dynamics are hydrogen bonding switching events, which have been theoretically predicted[31] and experimentally supported[45] as occurring through abrupt angular jumps that involve large-scale motion of the hydrogen (or deuterium) atoms of the water. Small molecules at low concentrations do not disrupt hydrogen bonding networks and, more importantly, do not significantly limit the configuration space available to hydrogen bond partners, allowing this subset of water's dynamics to occur unperturbed.[10,32,46,47] Because the angular jump dynamics of water involve large displacements of the hydrogen atoms, these dynamics should also be particularly sensitive to isotope substitution. Hence, the solvent fluctuations that drive vibrational relaxation strongly reflect the dynamical differences between H_2O and D_2O. In fact, the water isotope effect on

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solvation dynamics had been successfully modeled from the perspective of Debye relaxation, which relates the macroscopic dielectric constant of water to the microscopic reorientation dynamics,[48] though the angular jump mechanism had not yet been identified.

The dynamical constraints exerted by extended hydrophobic surfaces on the surrounding water arise from the restrictions imposed on the hydrogen bonding network by the surface.[49] Extended surfaces limit both the configuration space available for hydrogen bonding as well as the associated dynamics, causing water to adopt geometries that are not favorable for hydrogen bond coordination while impeding switching events.[10] The structured region surrounding the HEWL His15 label is an excellent example of a natural extended biological surface with low curvature, hence the surface slows down the water dynamics by limiting the available partners for fast hydrogen bond switching while inhibiting the required coordinated reorientation that accompanies large angular jumps. Because the switching events involve large displacements of the hydrogen atoms, these motions contribute significantly to the measured vibrational relaxation isotope effect, which is only observed when the hydrating water exhibits bulk-like dynamics. This interpretation of "hydrophobic slowing" of water's dynamics is consistent with what has been previously observed for small solutes at high concentrations, where neighboring solutes limit hydrogen bonding switching.[8,17,32]

A cartoon depiction of hydrogen bond switching and its modification by the protein surface are shown in Figure 4. Since

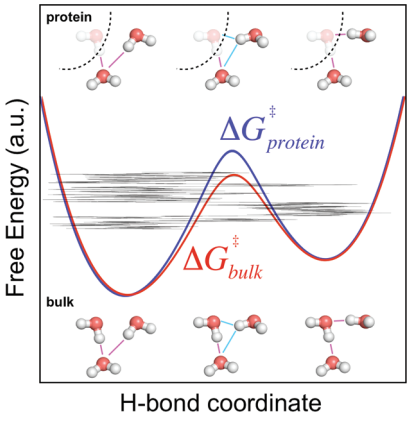

Figure 4. Cartoon depicting the free energy surface for hydrogen bond jumps. The transition state has been identified as a bifurcated hydrogen bond with both initial and final donors (shown with cyan hydrogen bonds). In regions of constrained hydration, the protein limits the availability of final donors, raising the free energy barrier by decreasing its entropy. Besides the relatively rare jumping events, the rapid intrawell fluctuations are able to induce enhanced anharmonic coupling, assisting vibrational relaxation for both water isotopes.

successful hydrogen bond switching events proceed through a bifurcated transition state where the switching hydrogen is fleetingly associated with both the initial and final partner O atoms,[31,50] the free energy barrier is necessarily influenced by the availability of such configurations. Relative to the bulk liquid, the protein interface deprives water molecules of potential partners, which reduces the availability of transition state candidates and lowers the entropy of the transition state. Nevertheless, hydrogen bond jumps are not the only source of environmental fluctuations leading to enhanced anharmonic coupling and the resulting carbonyl vibrational relaxation. The intrawell dynamics comprise the majority of these fluctuations (depicted by the stochastic trajectory in the cartoon), hence resulting in similarly rapid relaxation in both D_2O and H_2O.

The absolute value of the constrained H_2O/D_2O relaxation falls between the values of bulk-like H_2O and D_2O for both CORM-2 as well as HuLys-RC. While the dynamical nature of the solvent can be influential, it is only one component that determines the vibrational lifetime. The electric field generated by the solvent applies the force on the relaxing mode and is thus an important component of vibrational relaxation that we cannot probe directly. Because hydrophobic hydration is accompanied by dynamical and structural changes, the absolute lifetime observed for the constrained H_2O/D_2O will depend on any structural changes that occur in the hydration layer. Thus, the convergence of the H_2O and D_2O relaxation onto a single lifetime and the absolute value of the vibrational lifetime should be considered somewhat separately. Future computational work to investigate the site specificity of hydrogen bond jump dynamics at the protein interface will provide numerical support to this presently qualitative picture.

2.3. Heterogeneous Water Environments. The homologous structures of HEWL and HuLys allow us to investigate the water dynamics near two qualitatively distinct protein–water environments (Figure 1). We have previously discussed the lack of an observable isotope dependence of the vibrational relaxation of the HEWL-RC complex, where the probe is located on a structured, extended protein surface. In the HuLys-RC complex, however, the probe is located in an unstructured and flexible region of the protein. In contrast to HEWL-RC, the isotope effect is clearly observed in HuLys-RC, where the relaxation time constants for the 2004 cm^{-1} mode are 3.12 ± 0.26 ps and 4.70 ± 0.38 ps in H_2O and D_2O, respectively. Despite being located at the protein surface, the measured solvation dynamics appears more consistent with small molecule hydration. Given that the vibrational probe is attached to a histidine residue in both proteins, the data indicate that some degree of collectivity at each site leads to the protein's heterogeneous influence on the hydrating water, as well as highlighting the role of surface topology on local hydrophobicity.[49]

The water dynamics surrounding the unstructured region of the HuLys-RC complex resembles what was previously observed for a small metal carbonyl, CORM-2, at low concentrations (~2 mM). This similarity suggests that the solvation of the unstructured region of the protein is similar to what is seen for a small molecule, namely, that the hydration environment is essentially bulk-like. The picture that emerges from these measurements is that a protein's ability to constrain hydration water dynamics is determined not only by the availability of solvent-exposed side chains capable of forming hydrogen bonds but also by the presence of a low-curvature surface topology. Though this view is consistent with the prevailing model of hydrophobic solvation,[2] our work shows clearly how a single, relatively compact globular protein can exhibit both extremes of hydration structure and dynamics.

The heterogeneous nature of the hydration dynamics of a protein raises interesting questions regarding the role, if any, of the dynamically constrained water in biological processes. It has long been speculated that hydrophobic hydration—hydration environments that constrain water—leads to entropic driving forces for surface processes, all of which require protein dehydration as the initial step.[15,20−24] A region of hydrophobic

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hydration can act as a "thermodynamic reservoir", where entropy is created by relaxing constraints on the hydrating water, in turn enabling greater participation in enthalpically favorable hydrogen bonding. We examine hydrophobic assembly below using an amphiphilic alcohol cosolvent.

2.4. D_2O–TFE Solvent Exchange. The tight interplay between protein dynamics and the hydration environment suggests that modulations can significantly impact a protein's dynamics, structure, and stability. The properties of a protein can be manipulated by adding small amounts of cosolvents, such as alcohols.[51,52] Low concentrations of 2,2,2-trifluoroethanol (TFE), for example, can stabilize protein secondary structure through a mechanism that is generally attributed to preferential solvation of the protein by TFE, promoting intramolecular hydrogen bonding within the protein by alleviating competition with external hydrogen bonding partners from hydrating water.[51,52] At higher concentrations, however, lacking the driving force of hydrophobicity, the protein becomes unstable and partially denatures into an unfolded state characterized by a loosening of the helix packing even as the helices themselves remain stabilized.[53] Partial unfolding in lysozyme has been observed at TFE concentrations near 15% (v/v). The linear FTIR spectra of HEWL-RC in D_2O/TFE mixtures (shown in the Supporting Information) show no significant changes in either the amide region of the spectrum or the metal carbonyl stretch bands.

To investigate the thermodynamic connection between constrained water and the driving force for surface processes such as preferential dehydration, we studied the influence of the amphiphilic cosolvent TFE on the vibrational lifetime of the protein-bound vibrational probe. We have shown that the dominant pathway of vibrational relaxation for the protein-bound probes is driven by the interfacial water dynamics. Therefore, dehydrating the protein surface surrounding the probe should result in measurable changes to its vibrational lifetime.

As a control experiment, we measured the vibrational lifetimes of CORM-2 in a series of D_2O/TFE mixtures (including pure D_2O, 10, 20, 50, and 75% TFE, and pure TFE). Figure 5 shows the vibrational relaxation of CORM-2 in the D_2O/TFE mixtures. At low concentrations, the vibrational lifetime remains dominated by water-assisted vibrational relaxation, only increasing from 4 to 6 ps over a range of 0–50% TFE. At higher concentrations the relaxation becomes dominated by the TFE cosolvent, increasing to 25 and 50 ps at 75% and pure TFE, respectively. It is clear that there is a nonlinear dependence of the vibrational lifetime on the solvent composition, likely due to the dominance of water-assisted vibrational relaxation as the most efficient relaxation pathway. Hence, significant changes in the vibrational lifetime are only observed when water is at a very low concentration. These data provide a baseline for vibrational lifetimes in D_2O/TFE mixtures in the absence of preferential solvation which can be applied to the study of HEWL-RC and HuLys-RC in the presence of TFE.

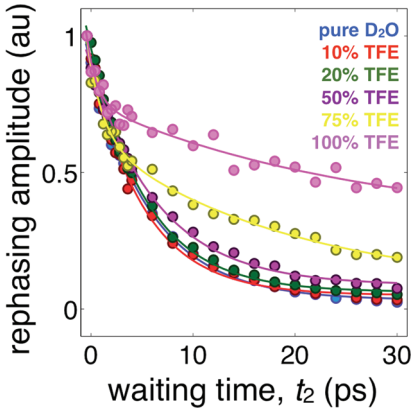

Figure 5. Vibrational relaxation of CORM-2 in D_2O/TFE mixtures, demonstrating the lifetime dependence on the cosolvent in the absence of preferential solvation.

Figure 6a shows the vibrational relaxation of HEWL-RC for four different TFE concentrations (0, 10, 15, 20% v/v). In pure D_2O, the vibrational lifetime is 3.73 ± 0.21 ps. Upon addition of 10% TFE the vibrational lifetime increases to 32.76 ± 1.15 ps (see Supporting Information for discussion of fits to the data), suggesting that at low concentrations the alcohol dehydrates the protein near the vibrational probe in exchange for a preferred alcohol environment. Lacking water, the vibrational relaxation becomes significantly slower and resembles relaxation observed in CORM-2 in TFE environments (Figure 6c). In comparison to the T_1 times for CORM-2 in D_2O/TFE mixtures, the HEWL-RC surface surrounding the vibrational probe has a solvation composition that resembles a solution between 75% TFE and pure TFE, clearly showing there is a lack of water at the protein surface. The vibrational lifetime achieved through only the addition of 10% cosolvent provides clear evidence that the TFE is preferentially drawn to the protein at the hydrophobic region.

Further addition of TFE induces a decrease of the vibrational lifetime, resulting in relaxation times that reflect a homogeneous solution of water and TFE (15% TFE T_1 = 3.99 ± 0.60, 20% TFE T_1 = 4.41 ± 0.48 ps). This decrease in vibrational lifetime, which returns to characteristic time scales for water-assisted relaxation by 20% TFE, warrants additional discussion. This experimental observation, that preferential solvation at low TFE concentration is not sustained at higher TFE concentrations, suggests the emergence of structural instability of the protein at TFE concentrations above 10%. Previous reports using a combination of spectroscopic techniques have shown that TFE concentrations near 15% can promote significant structural changes, including some destabilization of protein tertiary structure.[53] Early work by Dobson using circular dichroism found TFE enhanced the overall helical content of the protein, but at the cost of destabilization of tertiary structure.[54] Our experimental results are consistent with helical portions of the protein being susceptible to dehydration and interactions with the hydrophobic portions of TFE. The decreased lifetime is consistent with the following scenario: As the constrained water is relieved and the protein alters its structure, the solvation environment becomes a mixture of D_2O/TFE as the collective influence of the extended hydrophobic surface is disrupted due to the loosened helix packing.

The mechanism by which small molecules denature proteins has been, and remains, an area of intense research.[52,53,55−60] The present data suggest that there is a direct interaction between the protein surface and TFE, leading to the formation of a dehydrated interface between the protein and the cosolvent. This cosolvent shell in turn can modify the limited water dynamics at the surface by supplying hydrogen bonding partners through the alcohol's hydroxyl group. This interpretation would be consistent with a mixed direct[57,58] and

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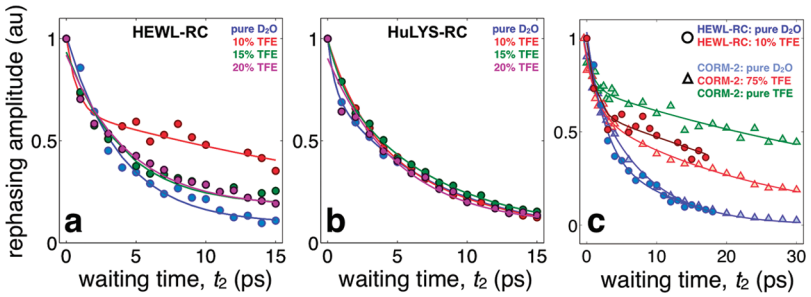

Figure 6. Vibrational relaxation for HEWL-RC (a) and HuLys-RC (b) in D_2O/TFE mixtures ranging from 0% to 20% TFE v/v. The addition of small amounts of TFE results in a large increase in the vibrational lifetime of HEWL-RC, followed by a monotonic decrease upon further addition. The increase in lifetime at low concentrations is the result of preferential solvation, and the subsequent decrease in lifetime is the result of the onset of partial protein destabilization. In contrast, HuLys-RC shows no sensitivity to TFE, suggesting this region of the protein resists solvent exchange with TFE and remains hydrated. (c) A comparison of the cosolvent-dependent relaxation for HEWL-RC (cirlces) and CORM-2 (triangles) shows that at 10% TFE HEWL-RC indicates a local solvation environment with nearly no water, with a relaxation time scale similar to other metal carbonyls in alcohol environments.

indirect[59,60] mechanism, where the cosolvent, directed to regions of constrained water, essentially coats the protein surface, promoting intraprotein hydrogen bonding and stabilizing secondary structure. Cosolvent association destabilizes the tertiary contacts between helices once the protein becomes so dehydrated that it loses the hydrophobic driving force to fold, resulting in partial denaturation, effectively reducing the cooperativity. This picture of TFE-modulated lysozyme stability is consistent with thermodynamic measurements based on calorimetry and structural studies using NMR spectroscopy.[50,57]

While we observe that the structured region of the HEWL-RC complex leads to constrained water that can drive solvent exchange, the unstructured β-region of HuLys is solvated by bulk-like water, suggesting that this region would not experience substantial solvent exchange. Figure 6b shows the vibrational relaxation of HuLys-RC in TFE/D_2O solution. Indeed, the vibrational relaxation of the label at this site shows no dependence on TFE, indicating that this location resists preferential dehydration by TFE and shows a solvation environment that might be expected for a simple mixture of D_2O/TFE (Figure 5). Comparing the experimental observations of HEWL-RC and HuLys-RC, it is clear that the interaction of TFE with the protein depends, to some degree, on the extended properties of the surface and not simply on individual amino acid residues since the vibrational probe is attached to a histidine residue in both cases.

The correlation between constrained water and solvent exchange demonstrates how the release of dynamically constrained water can drive hydrophobic association. It is known, however, that for many association processes the entropic contribution is insufficient to account for the total change in free energy.[61] While the hydrophobic interaction between the protein surface and the hydration environment is indeed the driving force for such processes, its influence is not limited to entropic changes associated with liberating the water's constraints[62] since expelling hydration water affords enthalpic gains by restoring hydrogen bonding that is diminished near extended surfaces.[2] Moreover, since many macromolecular assembly processes are kinetically controlled, the time required to allow for the diffusive liberation of constrained water may be too long given that the approaching extended hydrophobic surfaces are both solvated by water with diffusivity that is lower than the bulk.

3. CONCLUSIONS

The results presented here provide a site-specific probe of heterogeneous hydration dynamics of large proteins in pure H_2O and D_2O and, more importantly, provide experimental evidence of the mechanism of the hydration slowdown. A key aspect of this work is the study of labeled proteins at micromolar concentrations, which allows an unobstructed observation of the influence of the protein surface on hydration water. The results indicate that a single lysozyme protein is capable of influencing its hydration environment. This result is to be contrasted to numerous other studies that rely on high concentrations of solute to observe slowed water dynamics, where the crowding of multiple solutes can cooperatively constrain the hydration water. Although crowding is a central aspect of in vivo chemical biology, it is essential to characterize a single protein's influence over its hydration environment in addition to the specific or nonspecific perturbations induced by crowders. Evidence for constrained water is found near the large, structured α domain of HEWL (Figure 7b), where the individual amino acid residues act in a cooperative manner to create an extended hydrophobic surface, depriving water of hydrogen bonding partners. In contrast, bulk-like water is found on the unstructured region of HuLys (Figure 7c), where the residues behave as independent solutes with a hydration environment resembling that of a small molecule. Around these individual residues water retains a bulk-like hydrogen bonding network, and the dynamics are not suppressed. It is important to note that it is precisely this unstructured and flexible region that acts as a flap over the substrate binding site.

In addition to the heterogeneous nature of hydration dynamics surrounding large globular proteins, this study also reveals the correlation between dynamically constrained water and the driving force for site-specific association at the protein surface. The free energy that is released upon dehydration of constrained water appears to be sufficient to drive the association of small molecules to the protein surface. Using a water sensing vibrational probe, we can distinguish between direct cosolvent−protein association and indirect disruption of the hydration layer. On the basis of the marked changes in the vibrational lifetime, our data are consistent with direct displacement of water from the protein surface. This view is further buttressed by the subsequent cosolvent-induced destabilization caused by competing out the water to such an extent that the protein's tertiary structure loosens, evidenced by

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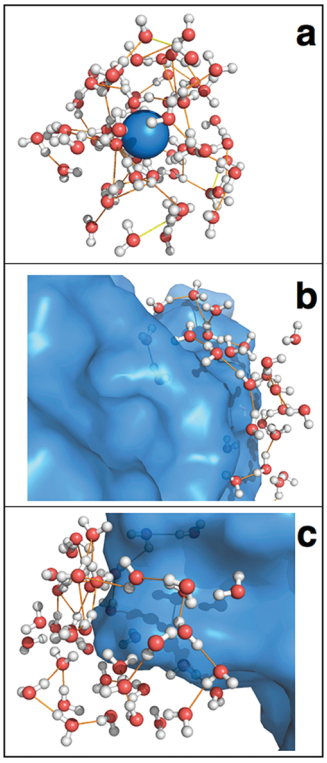

Figure 7. Cartoon demonstrating the effect of extended surfaces on hydrogen bonding switching events. Small molecules do not perturb the hydrogen bonding networks at small concentrations (a), while extended protein surfaces, like the surface found near the vibrational probe on HEWL-RC (b), can limit the hydrogen bonding network and the hydrogen bonding switching events. Loose, unstructured regions of proteins, like that surrounding the probe on HuLys-RC (c), act more like a collection of small molecules, where bulk-like dynamics can be preserved.

the infiltration of water in the absence of the structured, extended hydrophobic surface. Taken together, our data indicate that the spatially heterogeneous dynamics of hydration water is, to a significant degree, responsible for site-directed hydrophobic association, a perspective that should be helpful in rationalizing and perhaps in guiding the controlled disruption of deleterious protein−protein interactions.

Water's importance in biology cannot be overstated, but ample evidence shows that often only a small amount of water is truly necessary for function.[63] Water-sensitive vibrational probes on the surfaces of proteins will enable an experimental platform to systematically map interactions between proteins and other biomacromolecules including DNA and antibodies while simultaneously monitoring the role (or lack thereof) played by the thin layer of hydration water.

■ ASSOCIATED CONTENT

Supporting Information

A detailed description of the sample preparation, linear FTIR spectra of HEWL-RC in D_2O/TFE mixtures, a description of the experimental implementation of two-dimensional infrared spectroscopy, and the experimental fits to the vibrational relaxation data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ NOTE ADDED IN PROOF

Work appearing after the submission of this manuscript provides theoretical support that the constraining effect associated with hydrophobic hydration of proteins is the result of hindered hydrogen bond angular jumps.[64]