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FRONTIERS ARTICLE

Biological water: A critique

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ABSTRACT

In this overview, we provide a critique of the hydration dynamics of macromolecules, particularly those of protein and DNA. Only in the past decade has femtosecond spectroscopy enabled direct access to the ultrafast dynamical motion of surface water. With the wealth of results from this spectroscopic technique, NMR, and neutron scattering, it is now established that hydration is indeed an ultrafast phenomenon, and in this sense the 'iceberg model' is invalid. Here, we overview the experimental and the theoretical studies, hoping to clarify the confusion resulting from some recent MD simulations. We maintain that there are two types of water hydration, those that reorient in the vicinity of the surface and those which are ordered, however in dynamic interaction with the protein.

1. Introduction

Water is essential to life, yet we still do not fully understand many aspects of its involvement at the molecular level. It plays a wide variety of roles at different levels of complexity, from molecules and cells to tissues and organisms [1–4]. Increasingly evident is its participation as an active matrix, not simply as a solvent [4]. Almost all proteins and nucleic acids are inactive in the absence of water, and hydration determines their structural stability, flexibility, and the function [5–12]. Specifically for proteins, the dynamics of water–protein interactions govern various activities, including: the facilitation of protein folding, maintenance of structural integrity, mediation of molecular recognition, and acceleration of enzymatic catalysis. Thus, it is important to characterize the dynamic behavior of a biomolecule-associated water – biological water – at the molecular level.

The presence of biological water was confirmed in the 1950s from protein structural determination by Perutz [13], Kendrew [14], and others [5], and for DNA, water is similarly involved in the Watson and Crick structure [15]. Earlier, water bound to proteins was recognized by Bernal and Crowfoot [16]. One important realization of water's involvement in protein folding came from the work of Kauzmann [17] who introduced the hydrophobicity concept and iceberg model, building on the work of Frank and Evans [18] for the hydration around a hydrophobic group with a structured patch of water. Experimentally, various techniques and strategies have been invoked to study hydration and these primarily include: dielectric relaxation, X-ray diffraction, nuclear magnetic resonance, neutron scattering, and ultrafast laser spectroscopy. A number of reviews and books have been published [1–12] and the recent article by Ball [4] provides a comprehensive overview. In general, there are two classes of water in hydration, those molecules that are surface 'adsorbed' in the hydration shell, and those which are 'trapped' inside the protein. The former is more mobile and the latter is relatively rigid. For decades, the question was: how labile is surface water?

Dielectric relaxation measurements [19] gave a clue for the difference between bulk and protein water behavior. The frequency-dependent dielectric function was found to exhibit distinct regions of response, in contrast to bulk water, suggesting different types of water dielectric properties; e.g., the relaxation times of 8.3 ps, 40 ps, 10 ns and 80 ns were measured for myoglobin, in contrast to the 8.2 ps for bulk water at 298 K. The longer relaxation times, together with the Stokes expression of friction (from hydrodynamics), also suggest a larger radius for the protein as a result of hydration. X-ray diffraction provides a static structural picture of buried bound water molecules, but more recently it was shown that a solvation layer includes well-ordered sites as well as a diffuse partially–disordered solvent shell [20]. Neutron scattering experiments reveal, by means of Fourier deconvolution, the mean square displacement of water protons on the picosecond time scale [21].

Using the NMR technique of nuclear Overhauser effect (NOE), Wüthrich and coworkers, in a seminal contribution [22], measured the residence time of water at protein surfaces and found the time scale to be sub-nanoseconds, 300–500 ps. Later, Halle and coworkers, using the water ^{2}H and ^{17}O nuclear magnetic relaxation dispersion (NMRD) method, reported in 1996 that surface water has a

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relaxation time of 10–50 ps [23] and, in 2003, a 3–7 ps [24]. On the other hand, Bryant and colleagues, using the NMRD ^{1}H spin–lattice relaxation method, reported that the translational motion of surface water around a protein surface has a characteristic time of 30–40 ps [25]. Due to limited temporal resolution of these methods, neither the ultrafast (femtosecond and longer) relaxation time nor the real temporal response of the dynamics were available. Furthermore, the reported relaxation times represent an average behavior around the surface of the protein, i.e., not site specific.

A decade ago, we published in a series of papers [6] our time-resolved studies of surface hydration dynamics probed using femtosecond spectroscopy and a single intrinsic site on a variety of proteins. Earlier studies used extrinsic dyes to probe the protein interior [26,27]; in all our studies the 'active' center was a surface tryptophan or an attached probe molecule of a native protein. For surface probing, we observed a robust biphasic behavior in the correlation function, providing the first study of the ultrafast dynamics at a local surface site and for a number of proteins, including *Subtilisin Carlsberg* [28], monellin [29] and phospholipase A_2 [30]. By comparing with the behavior of tryptophan in bulk water, which has a solvation time on the femtosecond (fs) time scale, the first component, observed for the protein (one to a few ps) was identified with 'labile water' of the hydration layer. The second component, which has ten or more picoseconds decay, was interpreted as due to ordered water at the surface interacting with the protein and in exchange with bulk water. These results of picosecond (ps) response are consistent with neutron scattering data, earlier NMR work, and the earlier MD simulations as discussed below.

More recently, however, MD simulations reported by Halle [31] have suggested that water is even more labile around a protein, thus making his earlier report and our longer-time component an issue to revisit. This article addresses the various experimental approaches and critically examines evidences of ultrafast hydration dynamics from the experimental and theoretical point of view. We maintain, as do the neutron scattering experiments by Doster and colleagues [21,32] and the NMR studies by Bryant and colleagues [25], that this ps behavior reflects protein hydration due to water–protein interactions. We suggest that in view of controversies among MD simulations that the integrity of the force field and nonequilibrium correlations be scrutinized.

2. Experimental evidences of ultrafast hydration

2.1. Biphasic dynamic behavior: various wild-type proteins

Over the years, we have studied protein surface hydration in a series of wild-type proteins. The technique of femtosecond-resolved fluorescence upconversion was the method of choice to follow in time energy relaxation of site-selected solvation (for early references see [6]). The intrinsic tryptophan was found to be a powerful optical probe due to its minimum local perturbation and site specificity [6,33–35]. In these experiments, from the authors' laboratories, we follow dynamic Stoke shifts of the excited probe by monitoring a series of wavelength-resolved fluorescence transients. The observed energy relaxation with time provides solvation times and energies. The proteins studies are different in structure and property: *Subtilisin Carlsberg* [28], monellin [29], phospholipase A_2 [30], melittin [36,37], human serum albumin [38], staphylococcus nuclease [39], human thioredoxin [40], and leucine-zipper protein A1 [41]. In a separate study, we used a probe (dansyl), attached to the protein to examine the effect of distance away from the protein surface [28], and in another we investigated the effect of solvent composition and pH on hydration dynamics and enzyme function [42]. Expression of functionality and domain solvation has also been explored by using extrinsic probes [43,44].

Figure 1 shows four representatives of the protein structures we studied. The dynamics of all four, and others to be discussed, display a distinct biphasic behavior on the ps time scale. As shown in Figure 2A–D, solvation times vary somewhat but the behavior is robust: monellin, 1.3 and 16 ps; staphylococcus nuclease, 5.1 and 153 ps; and thioredoxin, 0.67 and 13.2 ps. For monellin and thioredoxin, the probe tryptophan is surface exposed, but for staphylococcus nuclease, the probe is partially buried and is encompassed by three charged residues. These wild-type proteins are of different size, flexibility, local topology and chemical property, yet they display similar behavior with different time constants. The transient behavior is entirely different from that of tryptophan in bulk water, without the protein, which has a solvation time of less than 1 ps, as mentioned above. Moreover, when the synthetic probe (dansyl) is attached to a protein surface at a distance of $\sim$7 Å, its solvation time reduces essentially to that of bulk behavior [28]. Finally, as shown in Figure 2A, denaturation of the protein has serious consequences on the solvation dynamics [29,45], and so are (not shown here) solvent composition [42], pH [42,43], and domain solvation [44].

2.2. Anisotropy dynamics of local probe motions

For each protein studied, the corresponding local probe anisotropy dynamics, obtained from polarized fluorescence intensities as a function of time [6,12], was studied in order to examine the time scale of the motions involved, and hence the degree of local protein flexibility. For all studied proteins, the dynamics of tryptophan wobbling occurs on a time scale that is longer than solvation time. For example, the anisotropy decays with 200 ps for staphylococcus nuclease, and more than one nanosecond for thioredoxin, giving a wobbling angle of 18° and nearly zero, respectively. Thus, on the tens of picoseconds, a protein sidechain, in this case tryptophan, undergoes relatively small local motions. For thioredoxin, the local structure is relatively rigid and the wobbling time is longer than one nanosecond, but solvation relaxation occurs in 13 ps. However, such a picture considers sidechain motions and, in principle, electrostatic solvation may contribute as tryptophan changes its charge distribution upon excitation. As shown below, this is not the case as evidenced from experiments made by varying the local charge distribution (by mutation) around the probe.

2.3. Mutation of amino acids around the tryptophan probe

Using site-directed mutagenesis, we have recently differentiated the contribution of water and protein electrostatic solvation by altering the neighboring residues around the probe tryptophan in two proteins, S-nuclease [39] and thioredoxin [40] (Figure 1). Within 7 Å of the probe tryptophan, there are three important surface-charged residues (K110, K133 and E129) in S-nuclease and one charged neighboring residue (D60) in thioredoxin. An earlier MD simulation study [46] of the emission spectra predicted the dominant contribution to the total Stokes shift from these charged residues, and that water molecules in the hydration shell make either a negative or very minor contribution to the dynamic Stokes shifts.

For S-nuclease, we used alanine scan around the probe to replace three charged residues with hydrophobic alanine, one at a time, and also designed one extra mutant K110C. Significantly, we found nearly no change of the fluorescence emission peak (332.5 ± 0.4 nm) for all mutants. Thus, the observed Stokes shift cannot be mainly from neighboring charged-residue solvation and instead it is from protein hydration dynamics. Figure 2B depicts the obtained solvation correlation functions of the wild type

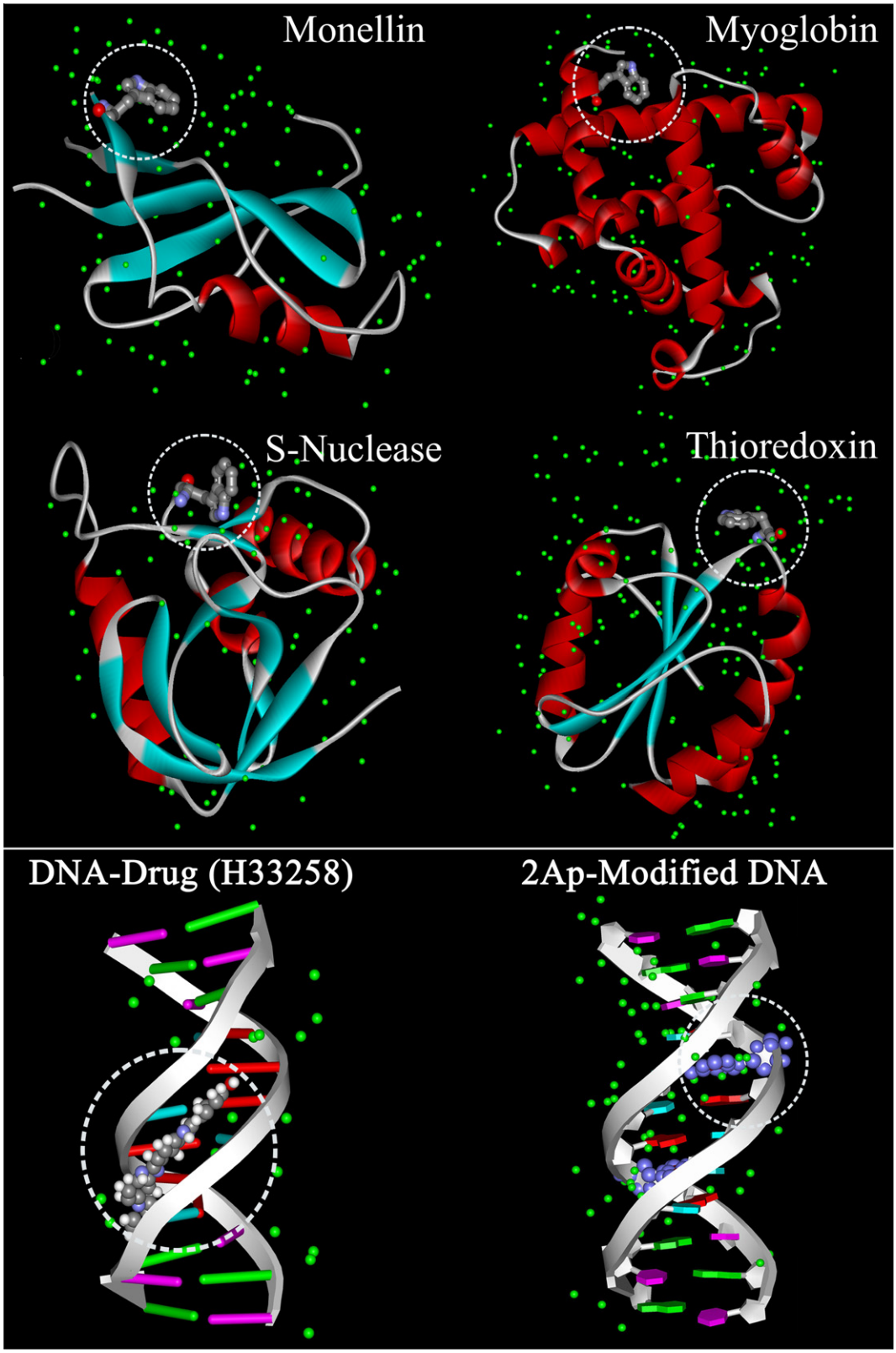

Figure 1. *Top panel:* X-ray structures of four representative proteins studied: monellin, staphylococcus nuclease (S-Nuclease), human thioredoxin (Thioredoxin) and horse heart myoglobin (Myoglobin). The probe tryptophan residue is indicated by dashed circles. *Bottom panel*: Two studied DNAs with the probe indicated by a dashed circle. The small green balls are water molecules.

and its four mutants. Overall, the dynamics are similar and all transient responses exhibit a biphasic decay behavior. More details can be found in reference [39].

For thioredoxin, we mutated the only charged neighboring residue (D60) to a hydrophobic one, glycine (D60G), and to polar asparagine (D60N). The probe (W31) lies at the protein surface

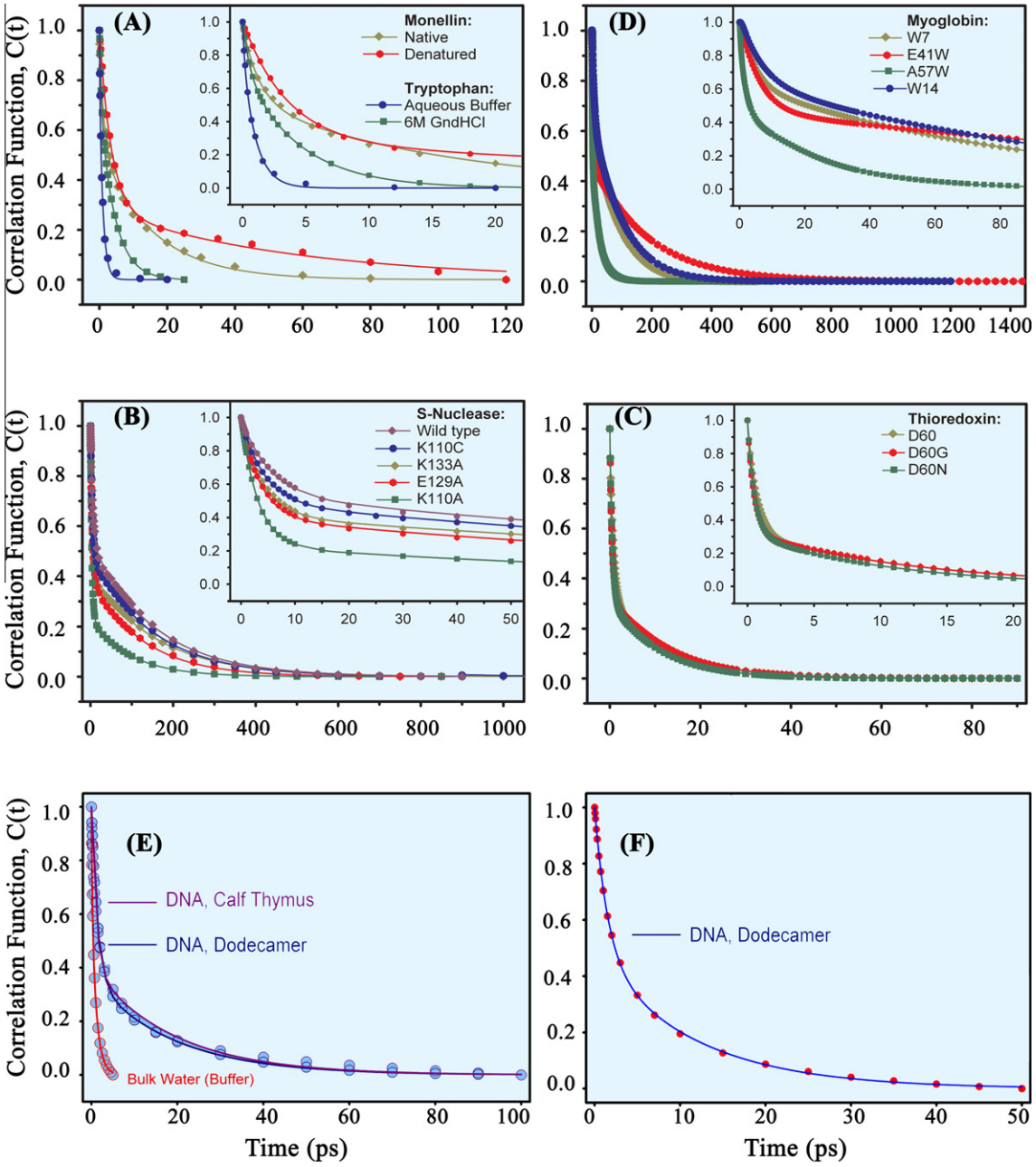

Figure 2. Correlation functions of probe solvation in the representative proteins and in DNAs shown in Figure 1. (A) Monellin, (B) S-Nuclease and four mutants, (C) Thoredoxin and two mutants, (D) Apomyoglobin and four typical mutants among total sixteen mutations, (E) DNA-drug (Hoescht 33258) complex (Calf and Dodecamer) and (F) 2-Ap modified DNA. The correlation function for free tryptophan (no protein) in bulk-water is shown in (A) and (E) for comparison. Panel A also shows the correlation functions for denatured protein, monellin, and free tryptophan in a denaturant solution (6M GndHCl). All correlation responses follow a biphasic decay behavior.

and is mainly enclosed by a hydrophobic environment, fully exposed to water molecules, and emits with a peak at 339 nm. Similarly, we did not observe a noticeable change of the total Stokes shift for the two mutants. Figure 2C displays the obtained solvation dynamics of the wild type and the two mutants. Strikingly, the three responses are nearly the same and they can be best described again by a biphasic decay profile with the time constants of 0.47–0.67 and 10.8–13.2 ps. Comparing with S-nuclease (5 and 150 ps), these solvation dynamics are significantly faster, reflecting the presence of the probe near a more hydrophobic environment. Thus, the three mutants, with a charged, polar, or hydrophobic residue around the probe, suggest that solvation is dominantly from local water–protein dynamics and not from the neighboring protein polar/charged residues. MD simulations predicted the opposite.

2.4. Scanning tryptophan around a protein surface

In another series of experiments, we further examined the origin of the two distinct solvation time scales and the relationship to protein property. For this purpose, we systematically scanned the probe tryptophan around a globular protein surface of apomyoglobin (Figure 1), making 16 mutants by means of site-directed mutagenesis. Solvation dynamics of the native and molten globule state were examined, obtaining a total of 29 correlation-function re-

sponses [47,48]. All 29 solvation responses exhibit the robust, biphasic relaxation profile, and Figure 2D depicts the behavior for typical mutants, W7, W14, E41W and A57W. The solvation times vary, from 1 to 8 ps for the first component and from 20 to 200 ps for the second. This change reflects variations in surface topology and electrostatic binding of water. Generally, it was found that the dynamics slow down around charged areas or rigid structures, but become faster around the hydrophobic patches or flexible structure regions. Moreover, when the anisotropy of transient profiles was obtained, we found that the wobbling angle is nearly constant around the value of 17° (Figure 3B).

2.5. Evolution of solvation energies

For the biphasic behavior there are the corresponding two solvation energies, ΔE_1 and ΔE_2, and we examined their behavior in

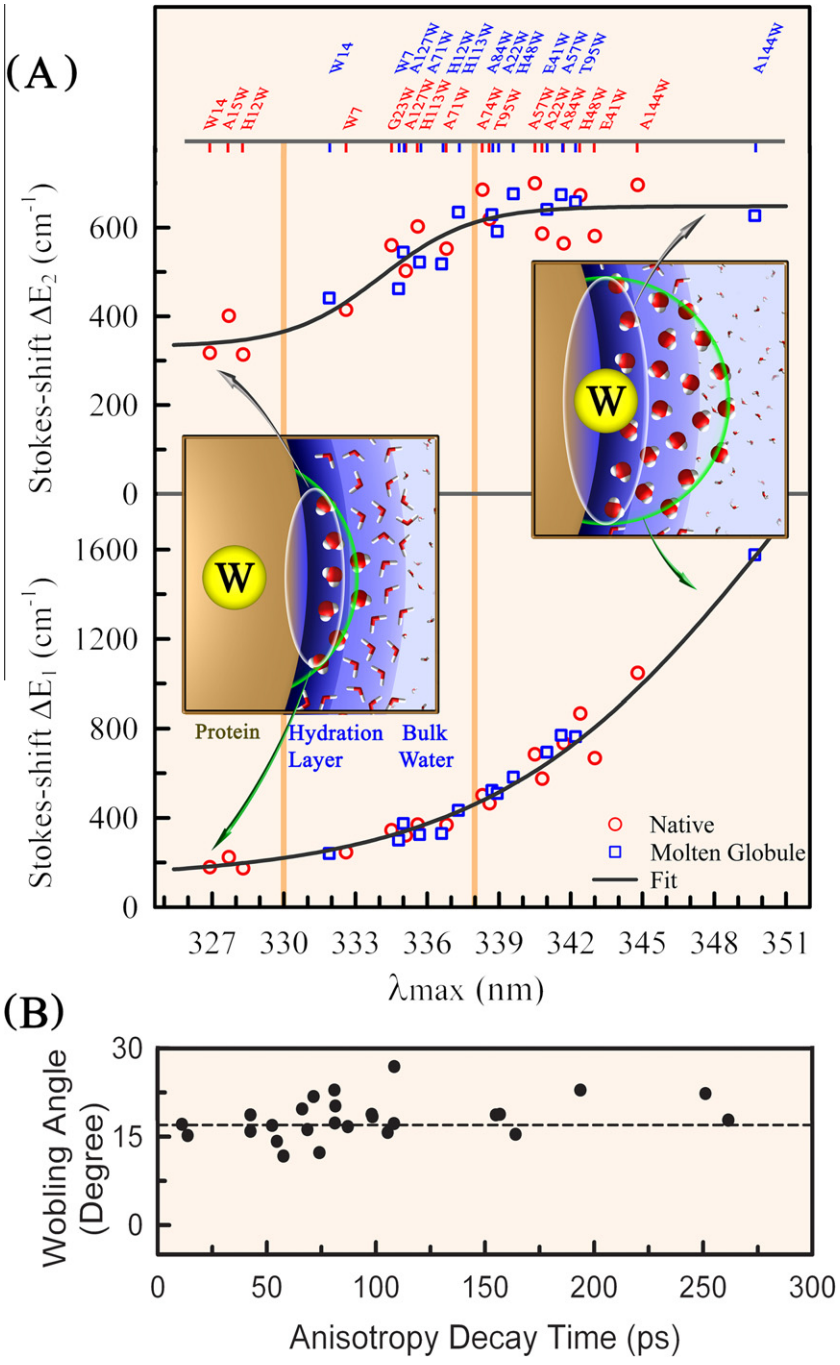

Figure 3. The Stokes shifts and tryptophan wobbling angels from sixteen mutations and in native (N) and molten globule (M) states. (A) Separated dynamic Stokes shifts (ΔE_1 and ΔE_2) plot against the emission maxima (λ_{max}) for all sixteen mutants of apomyoglobin in the two states. Red circles (N) and blue squares (MG) are the original experimental data, and the black lines show the trends with two very different behaviors. Vertical yellow bars divide λ_{max} into three regions of tryptophan locations (buried, partially buried, and exposed). All mutants are shown on the top and the ticks correspond to their data points below. The insets describe different contributions of surface water hydration to ΔE_1 (green arcs and arrows) and ΔE_2 (white ellipses and gray arrows) when tryptophan is buried (left) or exposed (right). Water molecules in the big arcs are within ~10 Å around tryptophan (W) and in the small ellipses water is directly interacting with the protein at the water–protein interface; see text (B) The wobbling angles of 16 mutants in two states, derived from the anisotropy studies, showing a small range of angles with an average value ~17° (dashed line).

apomyoglobin with 16 mutants as tryptophan water exposure increases, which is reflected in the wavelength of the emission peak (λ_{max}) shown in Figure 3A. The two Stokes shifts display distinct relationships with λ_{max}. For the first component, the energy ΔE_1 increases monotonically from 180 to 1600 cm^{-1}, with λ_{max} shifting from the blue to the red, reflecting the increased degree of polarity due to the exposure of the probe to the water environment. For the second component, the energy ΔE_2 increases gradually from an initial 340 cm^{-1} at λ_{max} = 327 nm to reach a plateau of ~650 cm^{-1} at λ_{max} = 338 nm. This observation is significant, as it indicates that the longtime relaxation is that of surface hydration at the water–protein interface, which is clearly identified by the wavelength at λ_{max} = 338 nm [49]. As long as the probe 'moves' to the protein surface (above 338 nm), ΔE_2 is nearly constant and interfacial water is detected; in contrast ΔE_1 displays an accelerated increase as λ_{max} shifts to the red and when the labile water relaxation in the outer layers can be probed. We note that even though the protein surface has topological roughness and hydrophilic/hydrophobic property, ΔE_2 in the asymptote only fluctuates to ±75 cm^{-1}, i.e., about 12% of the solvation energy of ΔE_2. This observation again indicates that ΔE_2 contribution is not mainly from protein solvation, specifically those predicted by MD simulations [31,46] for the neighboring charged residues. For example, T95W and A144W have a very different charge distribution around the probe, but give similar ΔE_2 values [48].

Boxer and coworkers [50,51] have recently measured the temporal responses for seven different sites of the protein GB1, from the interior to the surface, using a synthetic amino acid (Aladan). In this tour de force work [50], they reported two relaxation times of a few ps and a longer one of tens of ps. Later, they showed that more components [51] can be resolved on the fs and at a longer ns scale (Aladan has a long fluorescence lifetime). Compared to our site-directed mutagenesis work discussed above, the fs and ns components may be the result of the protein uniqueness. The GB1 is a small, flexible protein with 56 amino acids whereas apomyoglobin is a large, globular protein with 146 amino acids. There is also a possibility for fs intramolecular electron-transfer dynamics within the probe itself, Aladan. For dynamics on the ps time scale, similar to those reported above for apomyoglobin, we believe they reflect water–protein interactions, as electrostatic responses must include both.

2.6. Fluorinated protein surfaces modified by synthetic amino acids

We recently investigated, in collaboration with the Tirrell's group [41], the hydration dynamics of fluorinated protein surfaces. The protein is an oligomer (trimers or hexamers) assembled from a single α-helical leucine-zipper protein A1; among the main 42 residues, there are six leucines. The probe tryptophan is placed in the middle of the helix at the water-exposed position 34 (D34W mutation). Two neighboring residues (S31 and A37) near the probe were further mutated into leucines, one at a time. Then, all leucines in the three proteins were modified into synthetic trifluorinleucines or similar-volume homoisoleucines. A total of nine proteins, 'native' and modified, were investigated for comparison. Interestingly, we observed similar relaxation behavior, the first two components occur around 1 ps while the longer one exhibits tens of ps decay. Overall, when the fluorinated residues are near the probe, the tens of picoseconds decay becomes longer. Consistent with the above studies, it was found that fluorinated sidechains exert electrostatic drag on neighboring water molecules, slowing the motion of water at the protein surface, but without major change in the solvation temporal profile. Whether we lowered the polarity around the probe, as in S-nuclease and thioredoxin, or increased the polarity, as in fluorinated protein A1, the behavior is robust.

2.7. DNA hydration

In our early review [6], we summarized studies of hydration dynamics of DNA in the minor groove of the dodecamer B-DNA and Calf Thymus DNA using the drug Hoechst 33 258 as a local probe [52], and the same dodecamer using the synthetic base 2-aminopurine (2-Ap) as an 'intrinsic' fluorescence probe [53]. Again, the biphasic behavior was observed and related to hydration dynamics. Berg and coworkers covalently attached the dye coumarin into a 17-mer DNA near an abasic site and measured the relaxation dynamics over a wide time range, from femtoseconds to nanoseconds, using a combination of various optical methods [54]. Although the transient over six orders basically follows a power-law behavior, the dynamics on the ps time scale are similar to ours. On a longer time scale DNA undergoes various motions [55] and, thus, could make contributions to solvation, but not on the time scale of a few to tens of picoseconds. We note that although molecular modeling [56] may show that the dye is inside DNA (with no base on the other side), in solution the dye could cause a bulge or even reside on the outside.

2.8. NMR studies of hydration dynamics

Over the past 20 years, NMR has been utilized in the study of solvation, and the relevant results are summarized in Figure 4A. As mentioned above, the Wüthrich's group used the nuclear Overhauser effect (NOE) to observe the residence time of water at the protein surface and obtained relaxation times of 300–500 ps [22,57]. Later, Denisov and Halle reported that the time scale of water motion for a series of globular protein surfaces, including myoglobin, is shorter, being of 30–50 ps, and their assertion was made using the rotationally-sensitive ^{17}O and ^{2}H NMR relaxation method [23]. More recently, the same group characterized the rotational correlation time of surface hydration and reported that solvation time is even shorter, being retarded by a factor of 2–5 when compared with bulk water [24]. The implication by Halle is that, in contrast with his earlier work, rotation dynamics of hydration occurs in a few picoseconds [58]. This time scale would be consistent with our observed ultrafast hydration dynamics except that the results of his MD simulations imply that the longer decay component (10 ps or longer) is caused solely by protein motion. Further, he called for the reinterpretation of all previous NMR, NOE and NMRD, data made before 2003, asserting that the previous experiments were probing trapped long-lived water molecules [58–60].

However, Halle's claims are in contradiction with another NMR thorough and recent investigation by the Bryant's group [25]. The diffusional motion of surface-water molecules was examined using the ^{1}H spin–lattice relaxation method, and the authors reported [25] an average translational correlation time of 30 ps with a characteristic reduced two-dimensional diffusion of water in the protein–water interfacial region. The NMR technique of Overhauser dynamic nuclear polarization (DNP) [61] can directly measure, through spin labeling, the local translational diffusion dynamics with site specificity, when the time resolution is brought to the shorter ps scale. The average hydration-layer diffusion time of tens of picoseconds observed in Bryant's NMR experiments is consistent with the long-time decay of the solvation correlation function being primarily due to a translational motion, as discussed in Section 3.

2.9. Neutron scattering and other studies

The earlier work by neutron scattering did show the anomalous diffusion of water molecules on the ps time scale [21]. To further confirm that the dynamics is due to hydration, not induced solely

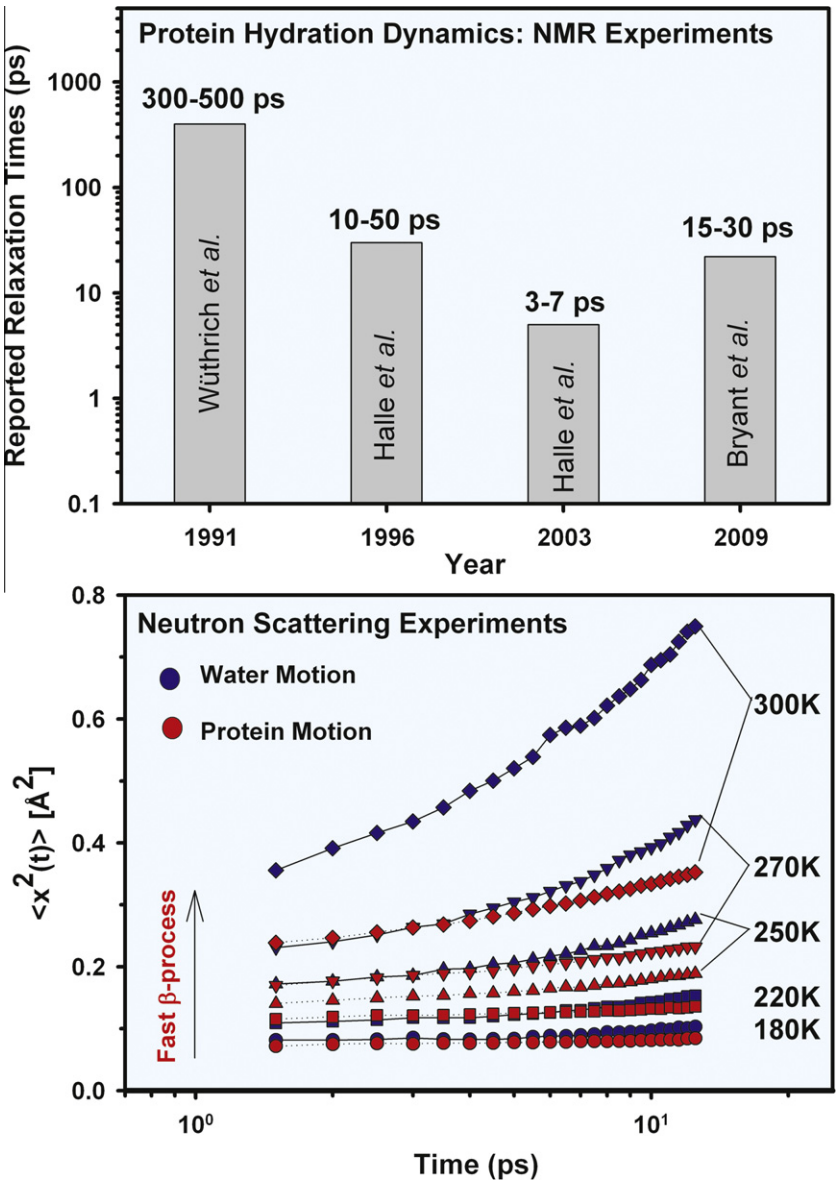

Figure 4. *Top panel*: The reported hydration times from NMR experiments, with the chronology of the work discussed here. Note that the time on the vertical axis is on the log scale. The values given represent the range obtained; e.g. the 3–7 ps is from the statement that hydration is 2–5 times retarded when compared to the values of bulk water. *Bottom panel*: Reported neutron scattering amplitudes obtained by Doster and colleagues. Plotted is mean square fluctuations as a function of time. The water mean square displacements were shifted down by 0.055 Å^2 for comparison. Note that at room temperature, the water amplitude is significantly higher than that of the proteins, and that there is some correlation, particularly at longer times (see text).

by protein motions, the mean square displacements of water motion and that of the protein have recently been separately measured by the Doster's group [32]. Figure 4B shows their comparison of the protein and water displacements in fully hydrated myoglobin over the time range from 1.5 to ~15 ps (from structural factors [62]) and temperature change from 180 to 300 K. At room temperature, the water has a much larger change in amplitude than the protein. Interestingly, even though the water amplitude is significantly larger than that of the protein, there seem to be some correlation between the two. More importantly, on the short time scale of 1.5–15 ps, water motion in the shell is dominant and even 'assists' protein fluctuations [32]. Water at interfaces exhibits retarded relaxations and there are many examples of such studies. The recent theoretical–experimental study of quasi-elastic neutron scattering of a peptide by the Berkeley group showed the retardation of water molecules in the hydration shell [63]. Electron diffraction studies of interfacial hydration at hydrophilic surfaces show a high degree of orientation with the dynamics being on the ps time scale, different from the behavior at the hydrophobic interfaces [64,65], and is consistent with work for interfaces in solution [66].

3. Theory and MD simulations

3.1. Analytical models

Nandi and Bagchi [67] considered a simple two state model of free and bound water near a surface. By using the diffusion equations they expressed the rate constants for a barrier crossing process. The extension of the dielectric relaxation approach for solvation [68] was reported by the Berkeley group [26] who found that on the ultrafast time scale solvation appears to be

dominated by the surrounding water and not by internal motion of the protein; only on longer time scales would such motions contribute. Pal et al. [69], in an analytical model, considered the biphasic behavior and expressed the fast and slow decay of correlation function in relation to bulk hydration rate and bound-to-free (residence time) solvation dynamics; the 4–5 kcal/mol barrier due to hydrogen bonding results in residence time of picoseconds. In collaboration with Wang [70], the solvation models [67,69] were reconsidered in order to account for the exchange of protein-solvation layer with bulk water, including reversibility of bulk/layer population flow. The exchanges between layered labile water, layered bound water, and bulk water give a satisfactory biphasic distribution of residence times of water in the shell. Surface heterogeneity was also taken into account. Figure 5A displays the results. In this regard, quantum dynamics studies made by Mckenzie and colleagues [71] is relevant. They considered the influence of protein and solvent environments on a chromophore, and concluded the direct involvement of both bulk and surface water on the dynamics of the chromophore.

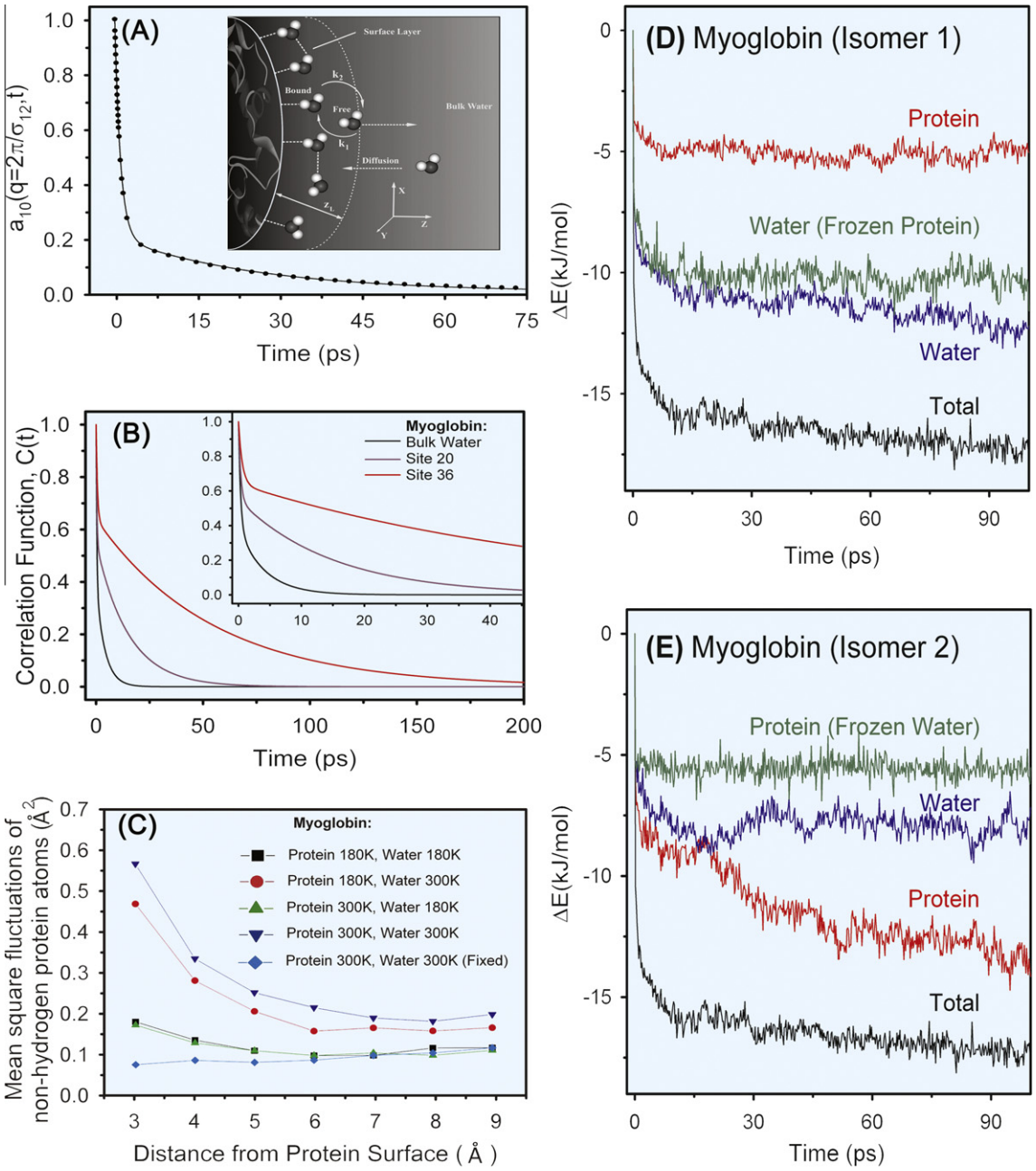

Figure 5. A Hydration dynamics calculated for a heterogeneous surface layer (solid circles) based on the analytical model depicted in the inset, a representation of the hydration-shell model with exchanges between bound, quasi-free, and bulk water molecules [70]. (B) The residence times for two typical hydration sites of myoglobin with a biexponential distributions on the picosecond time scale. Redrawn from the data available in [73]. (C) Mean square fluctuations of (non-hydrogen) myoglobin atoms as a function of distance from the protein surface with different temperature to show the essential role of water mobility. Redrawn from the data available in [74]. (D) The separated stabilization-energy contribution of water and protein (myoglobin at W7) to solvation by nonequilibrium trajectory simulations for one isomer [76], showing the dominant water contribution. With the frozen protein, the long water relaxation disappears. (E) The separated stabilization-energy contributions of water and the protein (myoglobin at W7) to solvation by nonequilibrium trajectory simulations for another isomer [76], showing the dominant protein contribution. With the frozen water, the long protein relaxation disappears.

3.2. MD simulations: residence times and fluctuation displacements

Motivated by the early neutron diffraction/NMR measurements of water residence times, several MD studies were carried out to simulate water motion near protein surfaces [72,73]. Figure 5B shows one such result obtained for myoglobin by the Pettit's group [73]. A biphasic distribution was observed for nearly all sites of the simulated protein. For surface mobile water, the first decay component was in the hundreds of femtoseconds to a few picoseconds, reflecting an ultrafast pathway for water to escape from the shell. The second component was found to be in tens of picoseconds, indicating a slow route for water to diffuse out of the shell. For the bound water molecules in narrow clefts, the residence times are even longer. On these ps time scales, water motion facilitates local protein fluctuations. As shown in Figure 5C [74], which depicts the MD simulations from the Karplus' group, they clearly show that water mobility is essential to controlling protein fluctuations [75].

3.3. MD simulations: linear response and nonequilibrium trajectories

Here, we only focus on recent MD studies which have directly simulated the Stokes shift of fluorescence experiments for proteins and DNA, attempting to separate the contribution of water and protein dynamics. Nilsson and Halle [31] simulated solvation dynamics of monellin (W3) by using linear-response approximation in order to obtain these contributions. They obtained the long correlation time (>10 ps) and ascertained that it is due to protein sidechain motion. Based on this simulation, they claimed that all relaxation longer than 10 ps is from the protein itself. However, Zhong, Singer and coworkers [76] reported both linear-response and nonequilibrium simulations of myoglobin (W7) and concluded that the long component has to be from a coupled water–protein motion. When the water was frozen, the observed long relaxation component disappeared [76]. Even for some isomers (Figure 5D and E), when the protein motion contribution is negligible, water motion persists up to 130 ps, contrary to Halle's assertion that any dynamics beyond 10 ps must be from that of the protein [31,58]. Golosov and Karplus [77] simulated 11 sites of GB1 using linear-response approximation and reported long hydration times, 20–200 ps for certain sites. They also found that the dynamics is heterogeneous and identified a coupled water–protein motion, similar to those observed by Zhong, Singer and coworkers. Toptygin and coworkers [78] simulated W43 of GB1 using nonequilibrium trajectories and found water relaxation times of 5–113 ps; the protein motion was found to have 2.6 ns solvation time.

The simulations for DNA hydration dynamics, using both linear response and nonequilibrium trajectory calculations, share similarity to the pattern observed in proteins [79]; a significant initial component of water motion and a longtime component that has been assigned to DNA motion. However, the MD simulations by Bagchi and Hynes and coworkers have addressed the issue of cross-correlation between water and DNA motions and concluded that the tens of ps component is due to hydration [80]. An interesting simulation by Berg and coworkers [81] has considered the long time scales of water relaxation in DNA and for this purpose used a transformation to eliminate the cross contributions of electric fields due to water, DNA or ions.

Besides these differences in MD simulations there exist some other issues when comparing [12] with the experimental results. First, a relatively small amplitude for the longtime relaxation (<20%) of the tens of ps was found, yet the simulations suggest its dominance; second, the total Stokes shifts obtained from nonequilibrium calculations are significantly larger than the experimental results; third, all simulations, from both linear-response and nonequilibrium considerations, give a significant fs component (>50%), similar to bulk-water initial relaxation, but the experiments mainly show the initial dynamics in 1 ps and longer; fourth, for a number of proteins studied, the simulations suggest that the 10–100 ps component is 'isolated' and solely due to the protein, in contrast to the experimental observations made by neutron scattering, NMR, and ultrafast spectroscopy.

4. Discussion and outlook

In this critique, we overviewed recent studies of protein surface hydration over the past decade, since our own involvement with femtosecond spectroscopy of proteins and DNA. The experimental methods include NMR, neutron scattering, and ultrafast optical spectroscopy. The theoretical methods are those involving simplified analytical models, but the bulk of studies come from MD simulations. Given the robustness of the biphasic profile of hydration observed in ultrafast spectroscopy for different proteins, mutant scanning, probe structures and studies of other macromolecules, there is no doubt that the 'iceberg model', strictly speaking, is not valid – hydration is a dynamical process on the picosecond time scale.

It was femtosecond spectroscopy that first elucidated the biphasic behavior and the time scales involved. Following this ultrafast spectroscopy work, the emerging questions of significance became: what is the nature of motions involved in the water layer, and what determines the coupling between water and the macromolecule? From all experiments made with a single-site (tryptophan) probing, the first hydration time (τ_1) measured was found to typically be between 1 ps and a few picoseconds, noting that free tryptophan in bulk water has a corresponding time of <1 ps [33] (Figure 6). This τ_1 relaxation time was also deduced from studies of rotationally-sensitive NMR [24] and neutron scattering [21], and is understood, from details of theoretical modelings [69,70] and MD simulations [76], to be due to local water-network reorientation motion. On a shorter time scale, all MD simulations gave an overwhelming relaxation component in tens to a hundred of femtoseconds, similar to that of bulk-type water relaxation, which is not observed by experiments.

The second longer measured time (τ_2) is ten (or longer) picoseconds (Figure 6), and the interpretation has been the subject of a debate. As mentioned above, ultrafast spectroscopy experiments measure both the relaxation times and the amount of energy shifts, and as such can determine the degree of participation by the protein in solvation. The behavior of the energy shift (Figure 3A) for different wavelengths (scanning using 16 mutants) clearly indicates the more significant contribution by water to solvation, relative to that of the protein. Further, the fact that mutation of the protein in the vicinity of the probe (site) tryptophan did not alter the rates (and the fluorescence peak position) significantly indicates the minor role of protein electrostatic solvation, at least around the probe. And, all anisotropy measurements reveal a wobbling angle of only $\sim 17^\circ$, suggesting that a protein residue (tryptophan) is incapable of complete large amplitude motion (solvation) on the ps time scale. We note that such measurements of energy shifts, relaxation times, and anisotropies were not examined for a single protein. Studies with ultrafast spectroscopy were made on nearly 10 proteins (and also DNA) and for tens of mutants of the same protein, and for cases exploring the effect of solvent composition, pH, and denaturation on hydration and function of enzymes.

Independent NMR experiments, other than the one reported by Halle, which probed the translational motion of water around the protein support the tens of ps finding. Similarly, neutron scattering probing of the mean square displacement indicates a clear disparity in the amplitude of the protein and water motion, in favor of water dominance. Naturally, the water and protein motions are

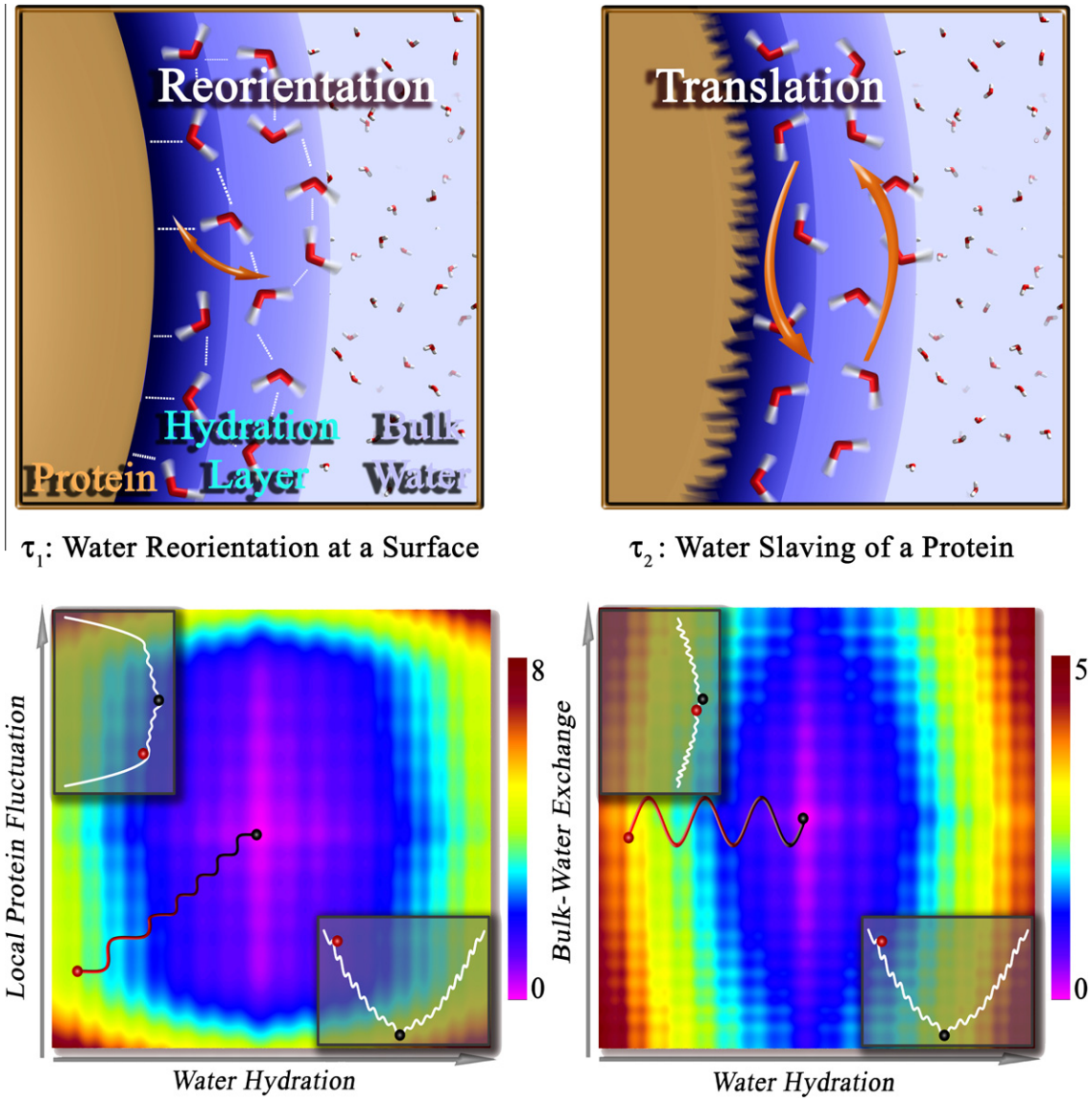

Figure 6. *Top*: Molecular-scale mechanism of protein hydration dynamics and coupled water–protein motions. The initial ultrafast dynamics in a few picoseconds (τ_1) represents local collective water-network reorientation relaxation. For the longer time dynamics (τ_2), water networks undergo structural rearrangements in the inner layers, with coupling to both local protein motions and bulk-water dynamic exchanges. *Bottom*: Schematic contour representation of the energy landscape for the longtime water-network dynamics along three coordinates: water-network arrangement in the hydration layer, coupled local protein motion, and bulk-water dynamical exchange. *Left*: The nonequilibrium hydrating water molecules evolve into a new equilibrated configuration, and along the pathway such water-network rearrangements couple with local protein motions. The protein fluctuates among many substates assisted with the water-network relaxation. *Right*: The water network evolves along the minimum energy path and meanwhile has a dynamic exchange with bulk water on a similar time scale. The bulk water fluctuates around its equilibrium state.

coupled and in fact we believe that water 'slaves' protein motion, and such finding is entirely consistent with the experimental results and model of Frauenfelder and colleagues [11,75]. More work on this slaving concept in relation to water role in protein folding will be forthcoming from the Caltech group.

MD simulations must consider all experimental observables, in this case from ultrafast spectroscopy, NMR, neutron scattering and others. They also should address the discrepancies among these simulation results and address why some support the experimental findings. It is surprising that one uses an MD simulation results (monellin) [31] to adamantly argue, and even sharply criticize, in a series of recent papers [31,58,59], the totality of experimental results by different methods and techniques and the results of other theoretical findings. Once again, in a water medium the protein is not isolated and there must be coupling of motions.

From the summary given above, we believe that the following picture can be painted (Figure 6). All water molecules in the hydration shell are dynamic, not in an iceberg, and that their ultrafast motion is established experimentally to be significantly slower than those of bulk water. The reorientation motion of water in the shell occurs in a few picoseconds in the inner layers and becomes faster in the outer ones. The translational motion (diffusion), measured by the rearrangement times of water networks from a nonequilibrium to an equilibrated state within the layers takes ten (or longer) picoseconds. On this longer time, water motions at the interface couple with protein motions, or fluctuations, and thus correlate to local protein properties, structural and chemical. The notion of water motions and protein fluctuations being 'isolated' because of the way MD simulations are carried out seems too simplistic. The two distinct motions are schematically shown in Figure 6, providing a simplified molecular picture of protein hydration dynamics and coupled water–protein fluctuations at the most fundamental level.

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