Proteins Take up Water Before Unfolding

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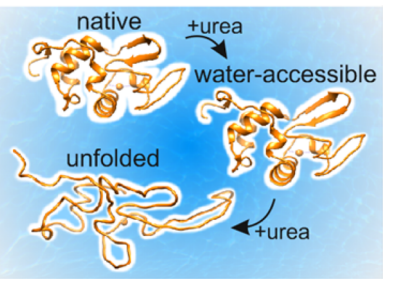
Supporting Information

ABSTRACT: Proteins perform specific biological functions that strongly depend on their three-dimensional structure. This three-dimensional structure, i.e. the way the protein folds, is strongly determined by the interaction between the protein and the water solvent. We study the dynamics of water in aqueous solutions of several globular proteins at different degrees of urea-induced unfolding, using polarization-resolved femtosecond infrared spectroscopy. We observe that a fraction of the water molecules is strongly slowed down by their interaction with the protein surface. By monitoring the slow water fraction we can directly probe the amount of water-exposed protein surface. We find that at mild denaturing conditions, the water-exposed surface increases by almost 50%, while the secondary structure is still intact. This finding indicates that protein unfolding starts with the protein structure becoming less tight, thereby allowing water to enter.



The interaction between proteins and water is crucial for protein folding and stability.[1] However, what happens locally at the protein−water interface at different degrees of unfolding remains largely unexplored. A common technique to observe the process of unfolding is circular dichroism (CD), which is sensitive to the macromolecular structure of the protein.[2] The macromolecular structure of proteins can also be probed with magnetic relaxation dispersion of water ^{17}O, as this technique is very sensitive to the dynamics of the internal water molecules that are released upon unfolding.[3,4] These methods show that the unfolding of globular proteins involves a sharp cooperative transition, a property that sets them apart from nonfunctional random polypeptides. It is unclear, however, what happens at the protein−water interface upon unfolding, in particular whether the macromolecular structural transition corresponds to a similarly sharp change in the intermolecular interactions between the water molecules and the protein surface.

The dynamics of water near protein surfaces has been studied with several theoretical and experimental techniques. MD calculations predict that the dynamics of water slow down near protein surfaces, and that the amount of slowing down strongly depends on the protein surface topology.[5−8] NMR studies also find a slowdown effect,[3,4,9] but cannot determine the number of slow water molecules and their reorientation rates independently.[3] Time-resolved fluorescence[10,11] and Nuclear Overhauser Effect[12,13] studies find a wide distribution of water reorientation times in the protein hydration layer, but both techniques require the embedding of specific probes in the protein (or protein encapsulation[12]). The experimental information on the properties of the hydration shell of proteins thus remains limited, in particular regarding the number and dynamics of the water molecules that are in direct contact with the protein surface.

Here we study the dynamics of water in aqueous solutions of bovine α-lactalbumin, hen egg-white lysozyme, bovine β-lactoglobulin, and bovine serum albumin at different degrees of unfolding, using polarization-resolved femtosecond infrared spectroscopy.[14] This technique directly probes the picosecond reorientation dynamics of water molecules, via the decay of the anisotropy R of the vibrational excitation. We (partially) unfold the proteins by adding the denaturant urea.

Figure 1A presents the frequency-averaged anisotropy decay as a function of delay time for different concentrations of native α-lactalbumin in isotopically diluted water. For water without added protein, the anisotropy decays exponentially with a time constant of 2.45 ± 0.1 ps. This means that in neat HDO in H_2O, water molecules reorient with this time constant, in good agreement with the value of 2.5 ± 0.1 ps reported earlier.[14] Addition of α-lactalbumin leads to a slow reorientation component of which the amplitude increases with concentration. The time constant of this slow component is >10 ps. As the experimental time window amounts to 8 ps, this component can be modeled well as an offset in the anisotropy decay. We thus fit the decay of the anisotropy $R(t)$ of the vibrational excitation to a single exponential with an offset: $R(t) = R_0 \exp(-t/\tau_r) + R_{slow}$. The reorientation time constant τ_r is 2.45 ± 0.15 ps for all protein concentrations. The fact that τ_r does not change with protein concentration shows that a fraction of the water molecules reorients as in neat water, even for highly concentrated protein solutions.

The inset of Figure 1A shows the slow water fraction, given by the offset R_{slow}, as a function of α-lactalbumin concentration. From the slope of R_{slow} it follows that, on average, 342 ± 20 water molecules are strongly slowed down in their reorientation

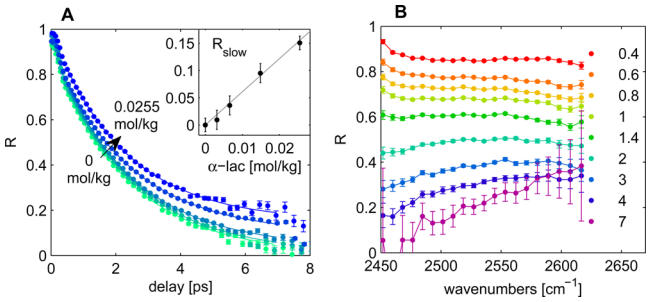


Figure 1. Water reorientational dynamics in aqueous α-lactalbumin solutions. (A) Anisotropy decay of the OD stretch vibration for solutions of α-lactalbumin in isotopically diluted water with concentrations up to 0.0255 mol/kg, averaged over the frequency range 2450−2600 cm^{-1}, revealing the appearance of a slow reorientation component with the addition of α-lactalbumin. The solid lines are fits to a monoexponential with an offset R_{slow}. The inset shows R_{slow} as a function of α-lactalbumin concentration. (B) Anisotropy decay for 0.0255 mol/kg α-lactalbumin in isotopically diluted water as a function of frequency at different picosecond delay times.

per α-lactalbumin molecule. For solutions of lysozyme, β-lactoglobulin, and serum albumin in isotopically diluted water, we observe a distinct slow component in the anisotropy dynamics as well (see Figures S1−S3), that corresponds to 292 ± 20, 433 ± 20, and 1310 ± 150 slowly reorienting water molecules per lysozyme, β-lactoglobulin, and serum albumin molecule, respectively.

The slow component of the anisotropy decay will also contain a small contribution of protein hydroxyl groups. The number of protein OH groups can be exactly calculated from the protein sequence and amounts to 38, 29, 45, and 180 for α-lactalbumin, lysozyme, β-lactoglobulin, and serum albumin, respectively. This is a small amount compared to the measured number of slow waters, which correspond to 684 ± 40, 584 ± 40, 866 ± 40, and 2620 ± 300 slow OD groups. Assuming that all protein hydrogens can exchange with water, and that the anisotropy decay of these groups is infinitely slow, we can thus calculate the maximum contribution of the protein OH groups to the slow fraction R_{slow} of the anisotropy decay: this is about 5% for all studied proteins, which is comparable to the error bar of the amplitude of R_{slow}.

The number of slowly reorienting water molecules per protein molecule is proportional to the number of water molecules in the first hydration layer of the protein. This hydration number can be estimated by computing the solvent accessible surface area of the protein (with a probe radius of 1.7 Å),[15] where the protein structure is obtained with crystallography, and dividing this surface by 10.75 $Å^2$ (the mean surface area per water molecule).[16,17] With this approach, we calculate hydration numbers of 629, 610, 769, and 2335 for α-lactalbumin, lysozyme, β-lactoglobulin and serum albumin, respectively (see Table 1). This implies that the effect of the proteins on the reorientation dynamics of water is quite local: the number of slow water molecules corresponds to about half the water molecules in the first hydration layer of the protein.

The hydration layer can be subdivided into water molecules hydrating hydrophilic and hydrophobic groups (Table 1). Previous femtosecond infrared and dielectric relaxation experiments on small amphiphilic molecules showed that hydrophobic methyl groups have a stronger slowing down effect on the reorientation of nearby water molecules than hydrophilic

Table 1. Number of Slowly Reorienting Water Molecules N_{slow} and the Number of Water Molecules in the First Hydration Layer of the Protein N_h (Calculated According to Refs 15 and 16)

	N_{slow}	N_h (N_h hydrophobic)
bovine α-lactalbumin	342 ± 20	629 (367)
hen egg-white lysozyme	292 ± 20	610 (335)
bovine β-lactoglobulin	433 ± 20	769 (476)
bovine serum albumin	1310 ± 150	2335 (1560)

groups.[18,19] One can therefore expect that the local water reorientation dynamics are mainly governed by the exposed hydrophobic part of the protein surface. This is indeed what we observe. Comparing the hydration numbers for lysozyme and α-lactalbumin, two proteins with very similar secondary and tertiary structures,[20] the difference in the number of slow water molecules (292 ± 20 versus 342 ± 20) agrees within the error bar with the calculated number of waters hydrating hydrophobic groups of the protein (335 versus 367).

To further investigate the nature of the slow water molecules hydrating the protein, we measure the frequency dependence of the anisotropy decay, which is shown in Figure 1B for a concentrated solution of α-lactalbumin in isotopically diluted water. It is seen that the anisotropy decays slower at high frequencies than at low frequencies. This frequency dependence is absent for neat water and becomes more apparent with increasing protein concentration. We observe a similar frequency dependence of the anisotropy decay for solutions of lysozyme, β-lactoglobulin, and serum albumin (see Figures S1−S3). Since this frequency dependence is not observed for solutions of small amphiphilic solutes,[18,19] it likely originates from the three-dimensional folded protein structure. A higher vibrational frequency of the water OD stretch vibration corresponds to a weaker donated water hydrogen bond. Thus, the water molecules that are most strongly slowed down by the protein form on average weaker hydrogen bonds. This combination of weak hydrogen bonding and slow reorientation is characteristic for confined water molecules. It was demonstrated by Laage and Hynes that water molecules reorient by rapidly switching hydrogen-bond partners.[21] The reorientation rate of a water molecule is thus strongly determined by the rate at which a bifurcated hydrogen-bond configuration can be formed, since this configuration forms the transition state for reorientation. The formation of this configuration requires the approach of another water molecule, which will be hindered near surfaces and in nanoconfinement, thus leading to a slowing down of the reorientation compared to bulk water. This indicates that the slow water is located in nanopockets of the protein and in grooves on the protein surface, in agreement with the results of MD simulations.[5−8]

As we have established that a fraction of the water is slowed down by interacting with the surface of native proteins, we can study how this fraction changes upon protein unfolding. Figure 2A presents the anisotropy decay for a concentrated solution of α-lactalbumin in isotopically diluted water with different amounts of added urea, which is a well-known and widely used protein denaturant. With increasing concentration of urea, the fraction of slow water increases. To quantify this, we again fit the anisotropy to a single exponential decay with an offset. We find that the reorientation time of the bulk-like water fraction remains 2.45 ± 0.15 ps at all urea concentrations, while

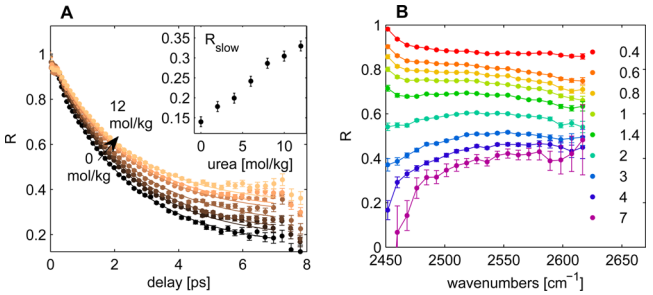


Figure 2. Effect of urea on the water reorientational dynamics in aqueous α-lactalbumin solutions. (A) Anisotropy decay of the OD stretch vibration for 0.0255 mol/kg α-lactalbumin in isotopically diluted water with different concentrations of added urea, averaged over the frequency range 2450−2600 cm^{-1}. The solid lines are fits to a monoexponential with an offset R_{slow}. The inset shows R_{slow} as a function of urea concentration. (B) Anisotropy decay for 0.0255 mol/kg α-lactalbumin in 10 mol/kg urea solution as a function of frequency at different picosecond delay times.

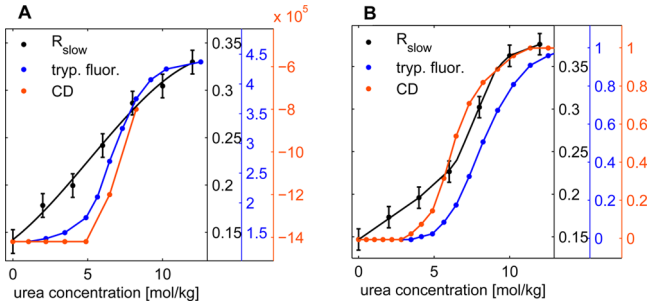


Figure 3. Comparison between different probes of unfolding. The slow water fraction, the intrinsic tryptophan fluorescence[26,27] (a.u.) and the CD signal[27,28] at 222 nm (deg·cm^2/dmol or a.u.) for (A) α-lactalbumin and (B) serum albumin in urea solutions as a function of urea concentration, revealing that that there is a substantial increase of the slow water fraction before the change in secondary structure occurs.

the amplitude of the slow water fraction increases up to a factor 2.5, as shown in the inset of Figure 2A.

Since urea itself has a very small effect on the dynamics of water[22] (see also Figure S6), the increase in slow water fraction results from a change in the spatial structure of the protein that leads to higher exposure of protein surface to water. A higher exposure of the protein surface to water could also imply a higher accessibility for urea. However, the accumulation of urea at the protein interface would lower the amount of water interacting with the protein,[23−25] which would in fact lead to a decrease of the slow water fraction. The fact that we observe an increase of the slow water fraction with urea concentration shows that there is no strong accumulation of urea at the surface of the unfolding protein.

To investigate the nature of the slow water molecules, we measured the frequency-dependence of the anisotropy decay, presented for a concentrated solution of α-lactalbumin in 10 mol/kg urea in Figure 2B. Just as in the case without any added urea, the anisotropy decays slower at high frequencies compared to low frequencies. Comparing Figure 2B with Figure 1B, we observe that the frequency dependence is enhanced at high urea concentration, which indicates that the slowly reorienting water molecules are still located in nanopockets or grooves of the largely unfolded protein.

The slow water fraction shows a quite different dependence on the urea concentration than the change in secondary structure that is characteristic for unfolding, and that is observed with CD and fluorescence techniques. This can be clearly seen in Figure 3, where we present the slow water fraction and the CD and tryptophan fluorescence signals as a function of the urea concentration for α-lactalbumin and serum albumin. For both proteins, the slow water fraction increases already at low urea concentrations and continues to increase up to 12 mol/kg urea. At a urea concentration of ∼10 mol/kg, the slow water fraction starts to saturate, indicating that the unfolding transition is almost complete. For lysozyme, we find a similar increase of the slow water fraction with urea concentration (see Figure S4). In contrast, both the CD and tryptophan fluorescence signals hardly change at low urea concentrations and then show a relatively abrupt transition at a urea concentration of ∼7 mol/kg. We thus find that the exposure of the protein surface to water is a much more gradual process than the change in macromolecular structure as monitored by CD or by the fluorescence response of tryptophan residues. Hence, at mild denaturation conditions, the protein is already more accessible to water, even though the secondary structure is still intact.

A higher accessibility of the protein to water can be either dynamical or the result of expansion of the protein. Expansion of the protein is reflected in an increase of the hydrodynamic volume. For a large number of globular proteins in their native and partially unfolded states, it was found that the hydrodynamic volume and secondary structure content are strictly related; they change simultaneously.[29] Pulsed field gradient NMR measurements and ion exchange chromotography show that the hydrodynamic radii of lysozyme,[30,31] α-lactalbumin[32] and serum albumin[33] hardly increase at low urea concentrations, which is thus consistent with the lack of change in secondary structure as monitored by circular dichroism. These results indicate that proteins do not show a well-defined expansion at low urea concentrations, but rather that they become less tight, showing larger conformational fluctuations and thus dynamical access to water molecules. This notion is consistent with amide hydrogen exchange studies, where very slow hydrogen exchange indicates stable protein backbone hydrogen-bonding structure and low solvent accessibility. α-Lactalbumin forms a "molten globule" at mild denaturing conditions,[34] which is a conformational ensemble of compact states[35,36] with native-like secondary structural motifs but no specific tertiary structure. The molten globule has faster hydrogen exchange with water.[36,37] Lysozyme does not form a clear equilibrium molten globule like α-lactalbumin;[38] however, in the refolding pathway of lysozyme, similar states with fast hydrogen exchange are observed.[39]

Previous studies showed that for many proteins, equilibrium unfolding intermediates are identical to kinetic unfolding intermediates in terms of their secondary and tertiary structure content, hydrogen exchange protection, collision cross-section, and stability toward unfolding.[40−45] Hence, mild denaturation conditions can be associated with earlier stages of unfolding. This means that our observation that proteins become more accessible to water at low urea concentrations, indicates that unfolding starts with the protein becoming less tight, allowing water to enter.

The above results show that the reorientation dynamics of water provides direct information on the amount of protein surface that is exposed to water. We find that the sharp

cooperative unfolding of the macromolecular structure of globular proteins is preceded by a substantial increase of the protein surface exposed to water. This finding presents a new view on the process of protein unfolding: the unfolding starts with the protein structure becoming less tight, thereby allowing water to enter. The measurement of the molecular reorientation dynamics of water thus provides a new way to study protein unfolding, and could prove an exciting avenue to study conformational transitions of proteins.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpclett.6b00708.

Experimental methods; anisotropy decays for aqueous solutions of lysozyme, β-lactoglobulin, and serum albumin with and without added urea, and for urea only. (PDF)

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Notes

The authors declare no competing financial interest.

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Supporting information of the paper

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Contents

1. Experimental methods

The reorientation dynamics of water are studied using polarization-resolved femtosecond infrared spectroscopy[1]. The infrared pulses are generated by frequency conversion of the output of a Ti:sapphire regenerative amplifier producing 900 μJ, 100 femtosecond pulses with a central wavelength of 800 nm at a repetition rate of 1 kHz. Part of the light is used to pump a β-bariumborate (BBO)-based optical parametric amplifier that produces 1.3 μm signal and 2 μm idler pulses. The idler pulses are frequency-doubled in another BBO crystal and subsequently mixed with the remaining 800 nm light in a lithiumniobate crystal to produce 10 μJ pulses centered at 4 μm (2500 cm^{-1}), with a bandwidth of 100 cm^{-1} and a pulse duration of 200 fs. These pulses are split into pump, probe and reference beams by a wedged CaF_2 window and focused into the sample by a parabolic mirror. The sample consists of a protein solution in between two CaF_2 windows that are held apart by a 50 μm spacer layer. After passing through the sample, the probe and reference beams are recollimated using a second parabolic mirror, dispersed by a grating, and detected by a 3x32 mercury-cadmium-telluride (MCT) array.

Bovine α-lactalbumin (purity >90%, Davisco foods), bovine β-lactoglobulin (purity >90%, mixture of type A and B, Davisco foods), bovine serum albumin (purity >96%, Sigma) and hen egg-white lysozyme (70000 U/mg, Fluka) are used without further purification. Each protein is dissolved in isotopically diluted water, consisting of ultrapure milli-Q grade H_2O and 4% D_2O (99.9%D, Cambridge Isotope Laboratories). The protein concentrations are determined using their molar extinction coefficient (ε) at 280 nm: ε=2.01 $g^{-1}cm^{-1}$, 0.958 $mg^{-1}cm^{-1}$, 0.6606 $mg^{-1}cm^{-1}$ and 2.67 $mg^{-1}cm^{-1}$ for α-lactalbumin, β-lactoglobulin, serum albumin and lysozyme respectively. In the experiments with urea, the proteins are dissolved with urea (purity>98%, Sigma-Aldrich) in isotopically diluted water. The solution pH is left unadjusted and ranges between 7.0 and 7.2 for α-lactalbumin and β-lactoglobulin, between 6.6 and 6.8 for serum albumin and between 4.2 and 4.8 for lysozyme, depending on the urea concentration. Control experiments with small added amounts of NaOH or HCl showed that there is no dependence of the water dynamics on pH within this range. All measurements are conducted at 24°C.

To calculate the number of slowly reorienting water molecules from the measured anisotropy decay, the anisotropy at each protein concentration is fitted to an exponential decay and an offset: $R(t)=R_0 \exp(-t/\tau_r)+R_{slow}$. Here R_{slow} is the fraction of slowly reorienting water molecules (> 10 ps). This fraction increases linearly with the protein concentration in mol/kg. Hence, the number of slowly reorienting water molecules per protein molecule is given by the slope of R_{slow} against the protein concentration, R_{slow}/c, multiplied by the number of moles of water in a kilogram (which is 55.257 for 4% D_2O in H_2O):

$$N_{slow} = \frac{R_{slow}}{c} \cdot 55.257$$

References

(1) Rezus, Y. L. A.; Bakker, H. J. On the Orientational Relaxation of HDO in Liquid Water. *J. Chem. Phys.* **2005**, *123*, 114502.

2. Supporting figures

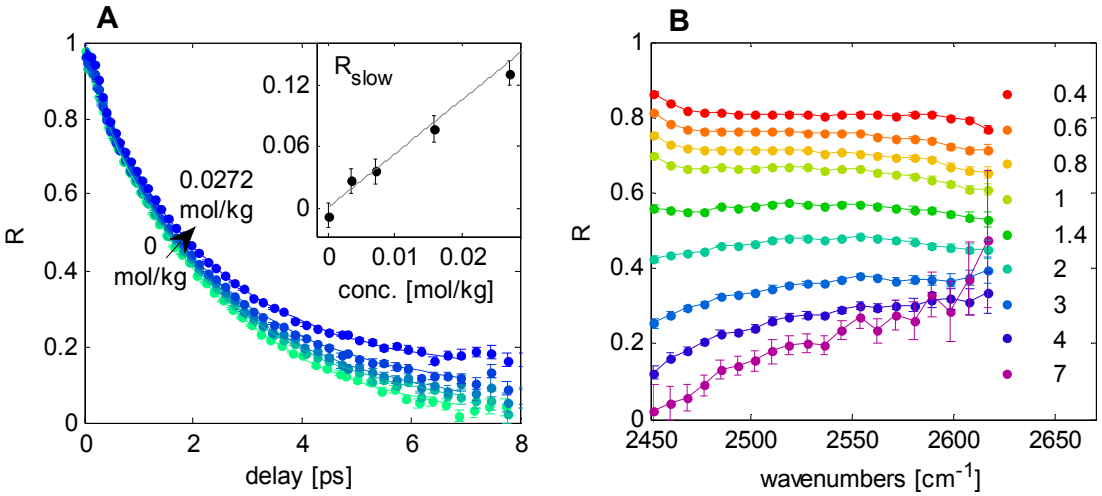

Figure S1. Water reorientational dynamics in aqueous lysozyme solutions. (A) Anisotropy decay of the OD stretch vibration for solutions of lysozyme in isotopically diluted water with concentrations up to 0.0272 mol/kg, averaged over the frequency range 2450-2600 cm^{-1}. The solid lines are fits to a mono-exponential with an offset R_{slow}. The inset shows R_{slow} as a function of lysozyme concentration. (B) Anisotropy decay for 0.0272 mol/kg lysozyme in isotopically diluted water as a function of frequency at different picosecond delay times.

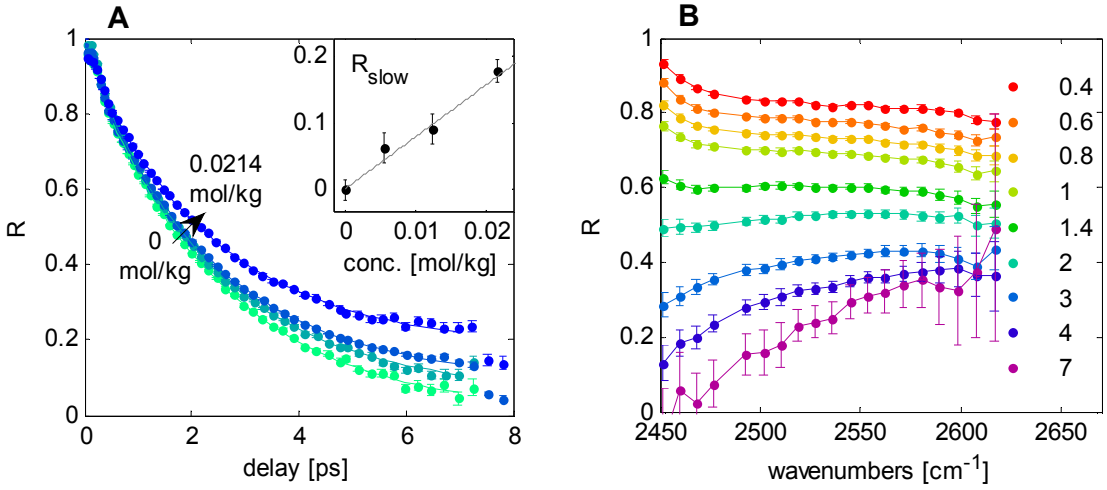

Figure S2. Water reorientational dynamics in aqueous β-lactoglobulin solutions. (A) Anisotropy decay of the OD stretch vibration for solutions of β-lactoglobulin in isotopically diluted water with concentrations up to 0.0214 mol/kg, averaged over the frequency range 2450-2600 cm^{-1}. The solid lines are fits to a mono-exponential with an offset R_{slow}. The inset shows R_{slow} as a function of β-lactoglobulin concentration. (B) Anisotropy decay for 0.0214 mol/kg β-lactoglobulin in isotopically diluted water as a function of frequency at different picosecond delay times.

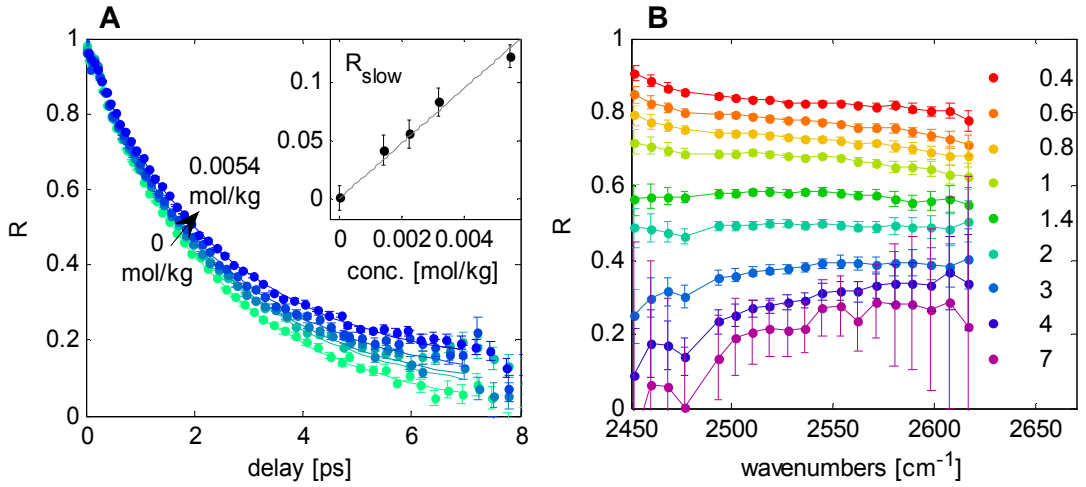

Figure S3. Water reorientational dynamics in aqueous serum albumin solutions. (A) Anisotropy decay of the OD stretch vibration for solutions of serum albumin in isotopically diluted water with concentrations up to 0.00544 mol/kg, averaged over the frequency range 2450-2600 cm^{-1}. The solid lines are fits to a mono-exponential with an offset R_{slow}. The inset shows R_{slow} as a function of serum albumin concentration. (B) Anisotropy decay for 0.00544 mol/kg serum albumin in isotopically diluted water as a function of frequency at different picosecond delay times.

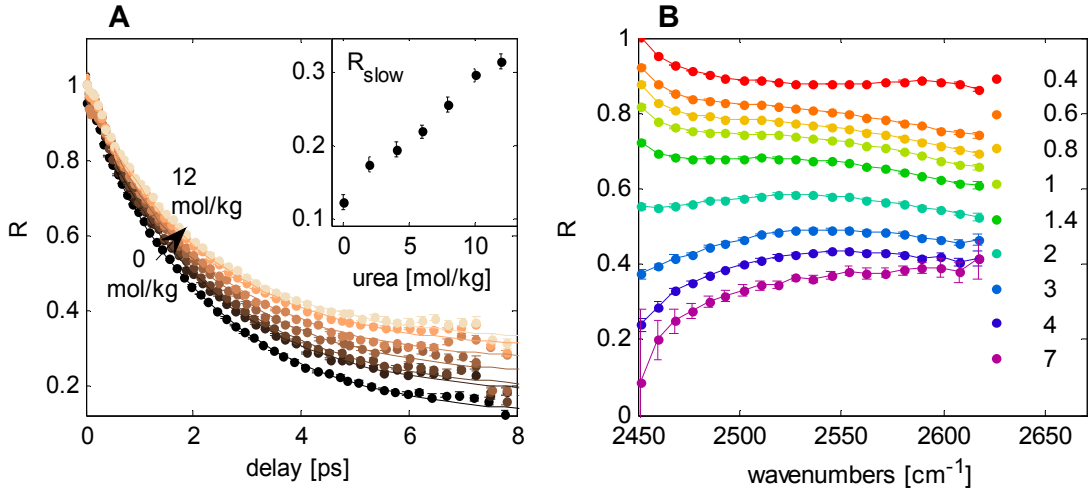

Figure S4. Effect of urea on the water reorientational dynamics in aqueous lysozyme solutions. (A) Anisotropy decay of the OD stretch vibration for 0.0248 mol/kg lysozyme in isotopically diluted water with different concentrations of added urea, averaged over the frequency range 2450-2600 cm^{-1}. The solid lines are fits to a mono-exponential with an offset R_{slow}. The inset shows R_{slow} as a function of urea concentration. (B) Anisotropy decay for 0.0248 mol/kg lysozyme in 10 mol/kg urea solution as a function of frequency at different picosecond delay times.

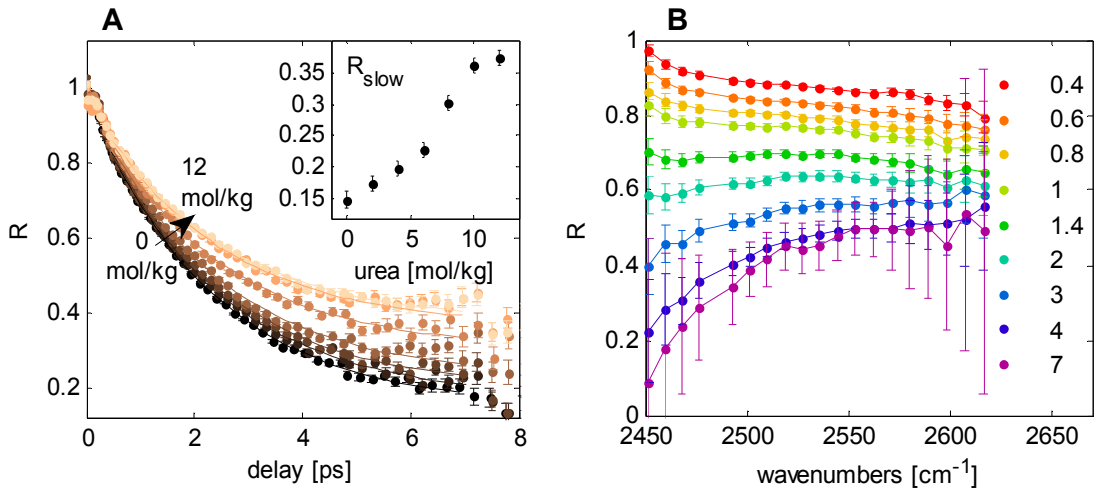

Figure S5. Effect of urea on the water reorientational dynamics in aqueous serum albumin solutions. (A) Anisotropy decay of the OD stretch vibration for 0.00544 mol/kg serum albumin in isotopically diluted water with different concentrations of added urea, averaged over the frequency range 2450-2600 cm^{-1}. The solid lines are fits to a mono-exponential with an offset R_{slow}. The inset shows R_{slow} as a function of urea concentration. (B) Anisotropy decay for 0.00544 mol/kg serum albumin in 10 mol/kg urea solution as a function of frequency at different picosecond delay times.

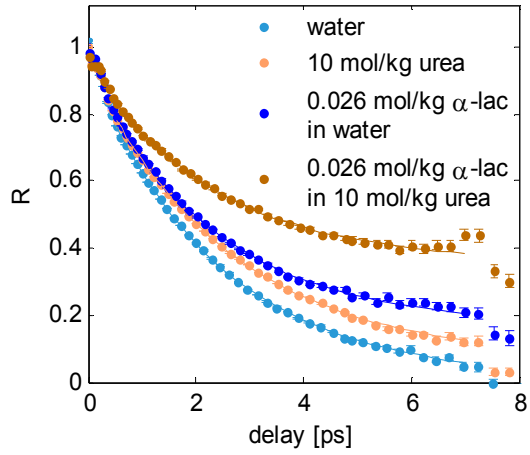

Figure S6. Effect of urea on the water reorientational dynamics. Anisotropy decay of the OD stretch vibration averaged over the frequency range 2450-2600 cm^{-1}, for water, 10 mol/kg urea, 0.0255 mol/kg α-lactalbumin in water and 0.0255 mol/kg α-lactalbumin in 10 mol/kg urea (all isotopically diluted). The slow water fraction for α-lactalbumin in 10 mol/kg urea is much larger than the added slow water fractions for 10 mol/kg urea and α-lactalbumin in water.