Dynamics of water interacting with biomolecules

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6 Water dynamics in aqueous protein solutions

Proteins perform specific biological functions that strongly depend on their three-dimensional structure that results from the folding of the polypeptide chain. A crucial factor for protein folding is the interaction between the protein and the water solvent. In this chapter, we study the dynamics of water in aqueous solutions of several globular proteins in their native state and at different degrees of temperature and urea-induced unfolding. We observe that a fraction of the water molecules is strongly slowed down by their interaction with the protein surface. The slow water fraction is a measure of the amount of water-exposed surface. In the case of urea-denatured proteins, we observe that the wetted protein 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.
6.1 Introduction

The interaction between proteins and water is crucial for protein folding and stability\textsuperscript{23}. 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\textsuperscript{103}. The macromolecular structure of proteins can also be probed with magnetic relaxation dispersion of water \textsuperscript{17}O, as this technique is very sensitive to the dynamics of the internal water molecules that are released upon unfolding\textsuperscript{104,105}. These methods show that the unfolding of globular proteins constitutes a sharp cooperative transition, a property which sets them apart from non-functional random polypeptides. It is unclear, however, what happens at the protein-water interface during 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\textsuperscript{106–109}. NMR studies also find a slowdown effect\textsuperscript{104,105,110}, but cannot determine the number of slow water molecules and their reorientation rates independently\textsuperscript{105}. Time-resolved fluorescence\textsuperscript{111,112} and Nuclear Overhauser Effect\textsuperscript{113,114} 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\textsuperscript{114}). 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.

In this chapter 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. We (partially) unfold the proteins by increasing the temperature or adding the denaturant urea.

6.2 Experimental

Spectroscopy The measurements described in this chapter are performed with the single-color setup described in section 3.2. The pump and probe pulses are centered around 2500 cm\textsuperscript{-1}, in resonance with the OD stretch vibration.

Sample preparation 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 4% D₂O in H₂O. The protein concentrations are determined using their molar extinction coefficient (\( \epsilon \)) at 280 nm: \( \epsilon = 2.01 \text{ g}^{-1}\text{cm}^{-1} \), \( 0.958 \text{ g}^{-1}\text{cm}^{-1} \), \( 0.6606 \text{ g}^{-1}\text{cm}^{-1} \) and \( 2.67 \text{ g}^{-1}\text{cm}^{-1} \) for \( \alpha \)-lactalbumin, \( \beta \)-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 \( \alpha \)-lactalbumin and \( \beta \)-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 unless stated otherwise.

6.3 Results and discussion

6.3.1 Vibrational relaxation

Figure 6.1A presents the isotropic transient absorption spectra at different delay times for a concentrated solution of \( \alpha \)-lactalbumin in isotopically diluted water. At early delay times, the transient response shows a bleach around 2500 cm\(^{-1}\), due to the fundamental transition of the OD stretch vibration, and an induced absorption at frequencies below 2420 cm\(^{-1}\). These signals decay to the spectral response of a thermal difference spectrum, indicative of a heated ground state. To calculate the anisotropy decay that exclusively represents the reorientation of excited OD stretch vibrations, the transient spectra have to be corrected for the time-dependent heating contribution.

For isotopically diluted water, the vibrational decay and subsequent rise of the heat signal are well described with the cascade model (eq. 4.6), which describes the relaxation of the OD stretch vibrations via an intermediate state to a thermalized, heated ground state\(^{74}\). Applying this model to solutions of \( \alpha \)-lactalbumin in isotopically diluted water, we find that it very well describes the transient spectral response (solid lines in fig. 6.1A). The extracted vibrational lifetime, \( T_1 \), and the thermalization time, \( T^* \), are shown in fig. 6.1D as a function of protein concentration. It can be seen that both times depend only weakly on the concentration of \( \alpha \)-lactalbumin. The spectral signatures of the OD vibration and the thermalized ground state do not change with protein concentration.

In case the temperature is increased, the isotropic transient absorption spectrum of \( \alpha \)-lactalbumin in isotopically diluted water shifts to higher wavenumbers (fig. 6.1B), reflecting a weakening of the water hydrogen-bond network. Nonetheless, we find that the vibrational relaxation can be described with the cascade model at all temperatures. The temperature-dependent vibrational lifetime and thermalization time are presented in figure 6.1E for a concentrated solution of \( \alpha \)-lactalbumin. With increasing temperature, the vibrational lifetime increases, while the thermalization time decreases. This is in agreement with
Figure 6.1. (A-C) Isotropic absorption change as a function of frequency at different picosecond delay times, for a solution of 0.0255 mol/kg α-lactalbumin in isotopically diluted water (A), for the same solution at 90 °C (B), and for a solution of 0.0255 mol/kg α-lactalbumin in 10 mol/kg urea (C). The solid lines represent the result of a model fit (see text). (D-F) Vibrational lifetimes $T_1$ and $T^*$ as a function of α-lactalbumin concentration (D), temperature (E), and urea concentration (F).
previous studies on the temperature dependence of the OD stretch vibrational relaxation in neat isotopically diluted water (HOD in H$_2$O)\textsuperscript{115} and in solutions of 1.5 mol/kg tetramethylurea\textsuperscript{116}. The increase of the vibrational lifetime likely originates from the blueshift of the OD stretch vibration, which increases the energy gap between the OD stretch vibration and the lower energy vibrational modes to which the OD stretch vibration relaxes.

In case urea is added to a concentrated solution of α-lactalbumin in isotopically diluted water (fig. 6.1C), the spectral signature of the isotropic spectrum stays unchanged. We find that the vibrational relaxation can be described quite well with the cascade model at all urea concentrations. The extracted vibrational lifetime and thermalization time are presented in figure 6.1F. The vibrational lifetime is independent of the urea concentration, in good agreement with results reported earlier on solutions of urea in isotopically diluted water\textsuperscript{117}. The thermalization time has a slight tendency to increase with urea concentration, but stays nearly constant as well.

For solutions of lysozyme, β-lactoglobulin and serum albumin in isotopically diluted water we observe similar trends for the vibrational relaxation of the OD stretch vibration. In all cases the relaxation is well described by the cascade model, which allows for an accurate subtraction of the time-dependent heat signal, and subsequent calculation of the OD anisotropy decay.

### 6.3.2 NATIVE PROTEINS

Figure 6.2A presents the anisotropy decay as a function of delay time for different concentrations of native α-lactalbumin in isotopically diluted water. The presented curve represents an average over the frequency range 2450-2600 cm$^{-1}$. For water without added protein, the anisotropy decays exponentially with a time constant of 2.45 ± 0.1 picoseconds. This means that in neat HDO in H$_2$O, water molecules reorient with this time constant, in good agreement with the value of 2.5 ± 0.1 picoseconds reported earlier\textsuperscript{74}. 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 picoseconds, 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 decay plus an offset: $R(t) = R_0 e^{-t/\tau_r} + R_{\text{slow}}$. The fitted reorientation time constant $\tau_r$ is 2.45 ± 0.15 picoseconds for all protein concentrations. The fact that $\tau_r$ 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.

Figure 6.3A shows the slow water fraction, given by the offset $R_{\text{slow}}$, as a function of α-lactalbumin concentration. The slow water fraction increases linearly with the protein concentration in mol/kg. Hence, we can calculate the number of slowly reorienting water molecules per protein molecule from the slope of $R_{\text{slow}}$ against the protein concentration $c$, multiplied by the number of
Figure 6.2. (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$^{-1}$. The solid lines are fits to a single exponential decay plus an offset $R_{slow}$. (B) Anisotropy decay for 0.0255 mol/kg α-lactalbumin in isotopically diluted water as a function of frequency at different picosecond delay times. All curves are divided by 0.4.

Figure 6.3. Slow water fraction $R_{slow}$ obtained from the fit to the anisotropy decay, as a function of the protein concentration, for the proteins α-lactalbumin, lysozyme, β-lactoglobulin and serum albumin.
moles of water in a kilogram (which is 55.257 for 4\% D_2O in H_2O):

\[ N_{\text{slow}} = \frac{R_{\text{slow}}}{c} \cdot 55.257 \]  

(6.1)

It follows that on average 342 ± 20 water molecules are strongly slowed down in their reorientation 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, that corresponds to the slow water fractions as shown in fig. 6.3. From the slow water fraction, we calculate that 292 ± 20, 433 ± 20 and 1310 ± 150 water molecules are slowed down 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 \( R_{\text{slow}} \) of the anisotropy decay: this is about 5\% for all studied proteins, which is smaller than the error bar of the amplitude of \( R_{\text{slow}} \).

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)\(^{118}\), where the protein structure is obtained with crystallography, and dividing this surface by 10.75 Å\(^2\) (the mean surface area per water molecule)\(^{90,119}\). 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 I). This implies that the number of slow water molecules corresponds to about half the water molecules in the first hydration layer of the protein, which suggest that the effect of the proteins on the reorientation dynamics of water is quite local.

The hydration layer can be subdivided into water molecules hydrating hydrophilic and hydrophobic groups (table I). Previous femtosecond infrared and

<table>
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<tr>
<th></th>
<th>( N_{\text{slow}} )</th>
<th>( N_h ) (( N_h ) hydrophobic)</th>
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<tr>
<td>bovine α-lactalbumin</td>
<td>342 ± 20</td>
<td>629 (367)</td>
</tr>
<tr>
<td>hen egg-white lysozyme</td>
<td>292 ± 20</td>
<td>610 (335)</td>
</tr>
<tr>
<td>bovine β-lactoglobulin</td>
<td>433 ± 20</td>
<td>769 (476)</td>
</tr>
<tr>
<td>bovine serum albumin</td>
<td>1310 ± 150</td>
<td>2335 (1560)</td>
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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 groups\textsuperscript{101,102}. One can therefore expect that the local water reorientation dynamics are mainly governed by the exposed hydrophobic part of the protein surface. This agrees well with the present observations. Comparing the hydration numbers for lysozyme and α-lactalbumin, two proteins with very similar secondary and tertiary structures\textsuperscript{120}, the difference in the number of slow water molecules (292 ± 20 versus 342 ± 20) agrees within error bars 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 fig. 6.2B 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. Since this frequency dependence is not observed for solutions of small amphiphilic solutes\textsuperscript{101,102}, 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\textsuperscript{13}. 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\textsuperscript{106–109}.

### 6.3.3 Heat-denatured proteins

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. We first unfold the proteins by increasing the temperature. Figure 6.4 presents the anisotropy decay for concentrated solutions of α-lactalbumin and lysozyme in isotopically diluted water at different temperatures (we did not record temperature-dependent spectra for the other proteins). With increasing temperature, the anisotropy decay speeds up and the slow water fraction decreases. To quantify this trend, we again fit the anisotropy decay to a single exponential with an offset. The result is shown as solid lines in figure
6.3 Water dynamics in aqueous protein solutions

Figure 6.4. Anisotropy decay of the OD stretch vibration for 0.0255 mol/kg α-lactalbumin and 0.0248 mol/kg lysozyme in isotopically diluted water at different temperatures, averaged over a frequency range of 150 cm$^{-1}$. The solid lines are fits to a single exponential decay plus an offset $R_{\text{slow}}$. All curves are divided by 0.4.

6.4 and the extracted parameters are presented in figure 6.5. We find that the reorientation time decreases with increasing temperature. Assuming that the water reorientation is an Arrhenius-type activated process, we can describe the temperature dependence of the reorientation by $\tau_r \propto e^{-E_{\text{act}}/k_BT}$. Here $E_{\text{act}}$ is the activation energy for reorientation, $k_B$ is Boltzmann's constant and $T$ is the temperature (in Kelvins). The Arrhenius fit is indicated by the solid line in fig. 6.5A and corresponds to an activation energy of 11 ± 1 kJ/mol. This is in excellent agreement with previous results on the activation energy of bulk water reorientation$^{102,116}$. The fact that $\tau_r$ follows the same temperature dependence as neat water confirms again that a fraction of the water molecules reorients as in neat water, even for highly concentrated protein solutions.

Figure 6.5B presents the slow water fraction as a function of temperature. With increasing temperature, the slow water fraction decreases gradually for both α-lactalbumin and lysozyme. There is no clear change of the slow water fraction at the protein denaturation temperature; in fact, even though α-lactalbumin unfolds around 65 °C and lysozyme unfolds around 80 °C$^{120}$, the temperature dependence of the slow water fraction is quite similar for both proteins. This similarity indicates that not only the protein fold changes with temperature; the effect of the protein surface on the water reorientation dynamics changes as well, and this effect might dominate. Previous dielectric relaxation and NMR experiments on small amphiphilic molecules showed that fewer water molecules are slowed down by hydrophobic groups with increasing temperature$^{102,119}$ (in the current investigated temperature range$^{119}$). Here we observe that this is the case for α-lactalbumin and lysozyme as well.
6.3 Water dynamics in aqueous protein solutions

6.3.4 Urea-denatured proteins

Proteins can also be unfolded by adding urea, which is a well-known and widely used protein denaturant. Figure 6.6 presents the anisotropy decay for a concentrated solution of $\alpha$-lactalbumin in isotopically diluted water with different amounts of added urea. With increasing concentration of urea, the fraction of slow water increases. To quantify this finding, we again fit the anisotropy to a single exponential decay plus an offset. We find that the reorientation time of the bulk-like water fraction remains $2.45 \pm 0.15$ picoseconds at all urea concentrations, while the amplitude of the slow water fraction increases by a maximal factor of 2.5, as shown in the inset of figure 6.6A.

Since urea itself has a very small effect on the dynamics of water\textsuperscript{117}, 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\textsuperscript{39,42,121}, 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 $\alpha$-lactalbumin in 10 mol/kg urea in figure 6.6B. Just as in the case without any added urea, the anisotropy decays slower at high frequencies compared to low frequencies. Comparing fig. 6.6B with fig. 6.2B, we observe that the frequency dependence is enhanced at high urea concentration, which indicates that the larger fraction of slowly reorienting water molecules has weak hydrogen bonds, which implies that these 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
6.3 Water dynamics in aqueous protein solutions

Figure 6.6. (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 single exponential decay plus an offset \(R_{\text{slow}}\). The inset shows \(R_{\text{slow}}\) 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. All curves are divided by 0.4.

Figure 6.7. Comparison between different probes of unfolding. The slow water fraction, the intrinsic tryptophan fluorescence\(^{122-124}\) (a.u.) and the CD signal\(^{124-127}\) at 222 nm (deg-cm\(^2\)/dmol or a.u.) for (A) α-lactalbumin, (B) serum albumin and (C) lysozyme in urea solutions as a function of urea concentration.
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 6.7, where we present the slow water fraction and the CD and tryptophan fluorescence signals as a function of the urea concentration for α-lactalbumin, serum albumin and lysozyme. For all 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 for α-lactalbumin and serum albumin, indicating that the unfolding transition is almost complete. 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 (or, in the case of lysozyme, no transition at all, as lysozyme does not fully unfold even at high urea concentrations). 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. This is illustrated schematically in fig. 6.8.

A higher accessibility of the protein to water can be either dynamical or the result of expansion of the protein. Expansion of the protein would lead to 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\(^\text{128}\). Pulsed field gradient NMR measurements and ion exchange chromatography show that the hydrodynamic radii of lysozyme,\(^\text{129,130}\) α-lactalbumin\(^\text{131}\) and serum albumin\(^\text{132}\) 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 together 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\(^\text{133}\), which is a conformational ensemble of compact states\(^\text{134,135}\) with native-like secondary structural motifs but no specific tertiary structure. The molten globule has faster hydrogen exchange with water\(^\text{134,136}\). Lysozyme does not form a clear equilibrium molten globule like α-lactalbumin\(^\text{137}\), however, in the refolding pathway of lysozyme similar states with fast hydrogen exchange are observed\(^\text{138}\).

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 towards unfolding\(^\text{139–144}\). 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 con-
6.4 Water dynamics in aqueous protein solutions

Figure 6.8. Schematic picture of protein unfolding, illustrating the transition from native protein (top) to protein at low urea concentration (middle), to denatured protein (bottom). At low urea concentrations, the protein is more accessible to water while the secondary structure is still intact.

Concentrations indicates that unfolding starts with the protein becoming less tight, allowing water to enter.

6.4 Conclusions

We have investigated the reorientation dynamics of water in solutions of globular proteins at different degrees of unfolding. A fraction of the water molecules is strongly slowed down by their interaction with the protein surface. This fraction amounts to about half of the water molecules in the first hydration shell of the protein. These slow water molecules have on average weaker hydrogen bonds, which implies that they are located in pockets or grooves on the protein surface.

With increasing temperature, the fraction of strongly slowed down water molecules gradually decreases, which can be explained from the weakening of the effect of hydrophobic groups on the dynamics of water with increasing temperature.

At a constant temperature, the slow water fraction is a measure of the amount of water-exposed surface. In the case of urea-denatured proteins, we observe that the water-exposed protein 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.