Orientation of polar molecules near charged protein interfaces†

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We study the orientation of water, urea molecules and protein amide vibrations at aqueous α-lactalbumin and α-lactalbumin/urea interfaces using heterodyne-detected vibrational sum-frequency generation. We vary the net charge of the protein by changing the pH. We find that the orientation of the water and urea molecules closely follows the net charge of the protein at the surface of the solution. In contrast, the net orientation of the amide groups of the backbone of the protein is independent of pH. We discuss the implications of these results for the mechanism by which urea denatures proteins.

1 Introduction

Water plays a crucial role in the functioning and dynamics of proteins. Several recent experimental and theoretical studies indicate that water not only determines the conformation of proteins, but can also play an active role in their function[1]. For instance, water molecules can form essential parts of enzymatic pockets[2,3], and the structuring of water layers adjacent to the ice-binding site of an antifreeze protein can provide the protein with a high affinity for nascent ice crystals[4]. Protein-water interfaces further play an important role in colloid research and the food industry. The macroscopic material properties of aqueous-protein mixtures can often be tuned by subtle changes in the composition, because these changes can induce interfacial rearrangements that in turn strongly affect the intermolecular interactions[5]. A detailed investigation of the structural and dynamical properties of water molecules at protein surfaces is therefore of great interest for both the understanding of biomolecular processes and for industrial applications.

Over the last decade vibrational sum-frequency generation spectroscopy (VSFG) has become an important spectroscopic tool to investigate the properties of water molecules next to biomolecular surfaces. In VSFG spectroscopy an infrared and a visible light pulse are combined to generate their sum frequency. The generated electric field E_{VSFG} is proportional to $\chi^{(2)}E_{IR}E_{VIS}$, where $\chi^{(2)}$ is the second-order nonlinear susceptibility. $\chi^{(2)}$ is enhanced in case the infrared light field is resonant with a vibration at the interface. For most systems VSFG is highly surface-specific as the generation of sum-frequency light is symmetry-forbidden in the bulk. The phase of $\chi^{(2)}$, in particular the sign of $\mathrm{Im}\chi^{(2)}$, reflects the orientation of the probed vibration at the interface, and therefore provides direct information on the molecular structure and orientation[6]. In conventional VSFG spectroscopy the intensity of the sum-frequency light is measured, which is proportional to the absolute square of $\chi^{(2)}$. As a result, conventional VSFG does not allow for an independent measurement of the real (Re) and imaginary (Im) parts of $\chi^{(2)}$.

The real and imaginary parts of $\chi^{(2)}$ can be determined by heterodyne-detected VSFG (HD-VSFG). In this technique the sum-frequency light generated by the sample is interfered with reference sum-frequency light of a well-known phase. Using heterodyne-detected VSFG it has been shown that charged lipid monolayers and organic molecules can orient interfacial water molecules[7,8]. For proteins, conventional SFG studies showed that a change of the pH induces a strong change in the signal of the water molecules, and this change can be explained from the pH-dependent net charge of the protein[9,10]. In an VSFG study of aqueous protein solutions by the group of Paul Cremer, it was proposed that these changes can be well explained from a change of the orientation of the water molecules in response to the variation of the total charge on the protein as a function of pH[11].

Urea is a small, highly polarizable molecule with similar properties as water, and it is widely used to denature proteins. The underlying mechanism of the denaturation is still under debate[12–14]. Denaturation can either occur through a direct or an indirect mechanism. In the direct mechanism, urea accumulates at the protein surface and replaces water molecules solvating the protein, meaning that urea directly binds to the backbone of the protein[12,15,16]. In the indirect mechanism the denaturation results from a urea-induced change in the water structure[13,17]. The effects of urea on the structure and dynamics of water are observed to be small, which points at a direct denaturation mecha-

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nism of urea[18]. However, in a recent conventional VSFG study by the group of Paul Cremer evidence was found for an indirect denaturation mechanism of urea[11]. In this study it was shown that the orientation of urea molecules at the protein interface depends on the net charge of the protein and, moreover, that this orientation follows the orientation of the interfacial water molecules. This finding was interpreted as urea molecules being completely enclosed by the hydrogen-bond network of water thus closely following the orientation of the water molecules[11]. However, the net orientation of the dipolar amide groups of the protein may show a similar dependence on the protein charge state and thus on the pH as the urea and water molecules. Hence, a change in the orientation of the urea molecules could be induced by the flipping of the net orientation of the water molecules (pointing at an indirect denaturation mechanism) or by the flipping of the amide groups. In the latter case the urea molecules can be hydrogen bonded to the amide groups, which would point at a direct denaturation mechanism. A distinction between these two scenarios can be made by probing not only the orientation of the urea and water molecules, but also the orientation of the amide groups of the protein. Here we study the structure and orientation of water, urea and the amide groups of α-lactalbumin using heterodyne-detected VSFG (HD-VSFG). α-lactalbumin is a common protein that is present in the milk of almost all mammalian species used in industrial food products. The structure of α-lactalbumin is known and has been widely investigated[19,20]. The protein consists of 123 amino acids and its structure contains α-helical ($\sim$34%), β-sheet ($\sim$12%) and disordered ($\sim$50%) elements.[21,22]. The molecular weight is 14 kDa and the isoelectric point (IEP) is $\sim$4.9.

2 Results and Discussion

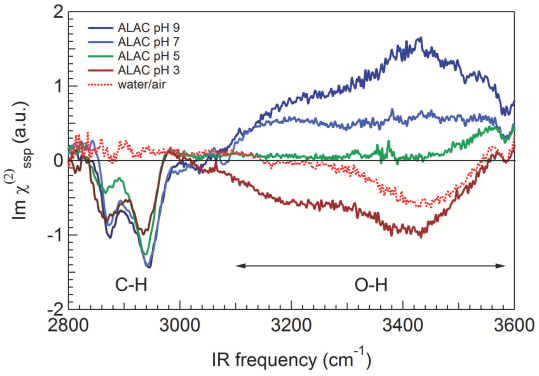

Fig. 1 Imaginary $\chi^{(2)}$ spectra of aqueous α-lactalbumin at different pH values in the region of 2800 - 3600 cm^{-1}.

Figure 1 shows HD-VSFG spectra of water and aqueous α-lactalbumin at the air/water interface at different pH values, measured in ssp polarization (s-SFG, s-VIS, p-IR). The Im$\chi^{(2)}$ spectrum of the pure water/air interface is in excellent agreement with the results of previous studies[23,24]. The spectrum shows a negative band between 3200 cm^{-1} and 3500 cm^{-1} and a weak positive band between 3000 cm^{-1} and 3200 cm^{-1}. The sign of the Im$\chi^{(2)}$ spectrum of the OH symmetric vibration depends on the projection of its vibrational transition dipole moment on the surface normal. A negative Im$\chi^{(2)}$ corresponds to OH groups that on average point to the bulk of the liquid, while a positive Im$\chi^{(2)}$ corresponds to OH groups pointing away from the bulk, i.e. to air. The presence of a weak positive signal at frequencies <3200 cm^{-1} is under debate[25–27]. Recently, the Tahara group showed that there is no significant positive signal in the low-frequency region of the SFG spectrum of the OH stretch vibrations[27]. In this study it was also shown that previous reports of such a positive signal may well be due to an error in the phase of the experimentally determined $\chi^{(2)}$. Recent calculations also did not find evidence for the presence of a positive feature in the imaginary $\chi^{(2)}$ spectra at 3100 cm^{-1}[28]. The phase uncertainty of our experiments is such that we cannot exclude that the small positive signal observed for the water/air interface at frequencies <3200 cm^{-1} in Fig. 1 results from a small phase error (see SI).

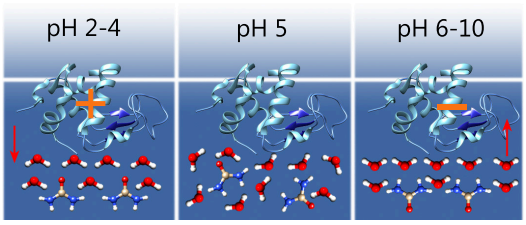

Fig. 2 Schematic representation of α-lactalbumin and the water subphase for neutral, negatively and positively charged proteins at the water/air interface.

The Im$\chi^{(2)}$ spectrum of α-lactalbumin at the water/air interface strongly differs from that of pure water. This spectrum shows two negative bands at $\sim$2880 cm^{-1} and $\sim$2950 cm^{-1}. We assign the 2880 cm^{-1} band to the methyl symmetric stretching vibration (CH_3,SS) and the 2950 cm^{-1} band to the Fermi resonance of this vibration and the overtone of the methyl bending vibration (CH_3,FR)[29]. A much weaker positive band is observed at $\sim$2990 cm^{-1} which we assign to the methyl antisymmetric stretching vibration (CH_3,AS)[30]. The negative signs of the CH_3,SS and CH_3,FR bands, and the positive sign of CH_3,AS indicate that the associated methyl groups are oriented towards the air phase[7,31]. We attribute the CH stretch vibrations to the methyl groups of amino acid residues. An additional weak band is observed at 3060 cm^{-1}. This band can be assigned to aromatic CH stretch vibrations of the aromatic amino acids present in the protein (4x tryptophan, 4x tyrosin, 4x phenylalanin, 3x histidin). In a previous conventional SFG study of aqueous β-lactoglobulin the band at $\sim$3060 cm^{-1} was used to deduce the absolute water orientation at the protein/water interface by considering its interference with the broad hydrogen-bonded water band[10].

Changing the pH of the solution does not have a prominent effect on the intensity of the CH vibrational bands at $\sim$2880 and $\sim$2950 cm^{-1}, indicating that the net orientation of the CH groups of the protein does not change when varying the pH. The HD-VSFG signal scales with the number of molecules probed and strongly depends on the net orientation of probed molecules. Hence, the constant intensity of the CH signals indicates that the surface propensity of the protein does not change significantly

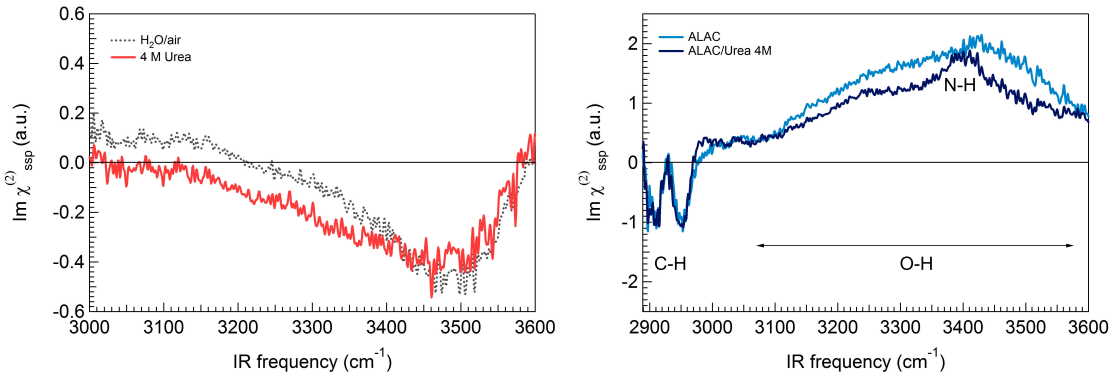

Fig. 3 Left panel: Imaginary $\chi^{(2)}$ spectra of H_2O and 4 M Urea in H_2O interfaces. Right panel: Imaginary $\chi^{(2)}$ spectra of α-lactalbumin in H_2O and in 4 M urea interfaces at pH=9.

when varying the pH. The aromatic CH band at ~3060 cm^{-1} only appears as a small dip in the Im$\chi^{(2)}$ spectra and shows no significant change of its phase upon changing the pH.

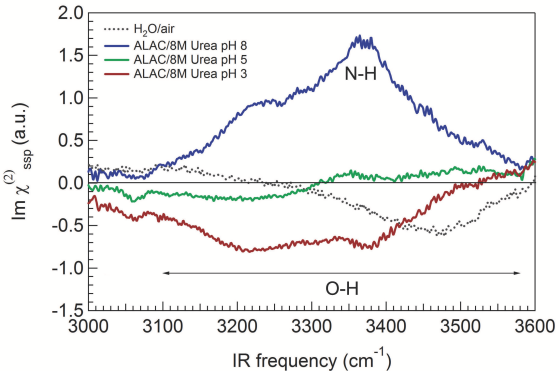

Fig. 4 Imaginary $\chi^{(2)}$ spectra of α-lactalbumin in 8 M urea at different pH values.

The OH region of the Im$\chi^{(2)}$ spectrum of aqueous solutions of α-lactalbumin shows substantial changes when the pH is changed. These changes closely follow the net charge of the protein (see Figure SI 1). At the isoelectric point (IEP) of α-lactalbumin (pH ~4.9), at which the overall charge of the protein is zero, the VSFG signals of the OH stretch vibrations at 3200 cm^{-1} and 3500 cm^{-1} are small, indicating very little net orientation of the OH groups of the water molecules. At pHs above the IEP of α-lactalbumin the signals at 3200 cm^{-1} and 3500 cm^{-1} are both positive, while at pHs lower than the IEP they are both negative. Hence, decreasing the pH changes the orientation of the O-H groups of the water molecules from positive (pointing towards the negatively charged protein at the surface) to negative (pointing away from the positively charged protein). The orientation of the interfacial water molecules at the protein/water interface thus closely follows the electric field induced by the charge state of the protein, as illustrated in Figure 2.

The Im$\chi^{(2)}$ spectrum of a concentrated aqueous solution of urea does not significantly differ from that of pure water, as seen in Figure 3a (also Figure SI 2). This result illustrates that urea is not very surface active. The observed spectrum is consistent with the previously reported notion that urea molecules fit well into the hydrogen-bond network of liquid water, and that they have very little effect on the strength of the hydrogen bonds[32].

Upon addition of urea to an α-lactalbumin solution the overall amplitude of the Im$\chi^{(2)}$ spectrum decreases and a new band at ~3380 cm^{-1} appears, as seen in Figure 3b. We assign this new band to the N-H stretch vibrations of urea, which is consistent with the IR and Raman spectra of urea, and which agrees with the conventional VSFG data of Cremer and coworkers[11,33,34]. The decrease in amplitude of the Im$\chi^{(2)}$ spectrum probably originates from the reduction of the number of water molecules at the protein/water interface due to the additional presence of urea molecules.

In Figure 4 we show the Im$\chi^{(2)}$ spectra of α-lactalbumin in 8 M urea at different pH values. Upon changing the pH of the solution, the sign of the NH_2 vibrational band changes, and follows the same trend as the broad OH vibrational band. At the IEP of α-lactalbumin, only a very weak negative signal at lower frequencies arising from the OH vibrations and a small positive peak of the NH vibrations are observed, indicating that nearly equal amounts of urea molecules have their NH vibrations pointing up as pointing down. At pH values above the IEP (protein negatively charged), the NH vibrations are pointing towards the proteins at the surface, while at pH values below the IEP (protein positively

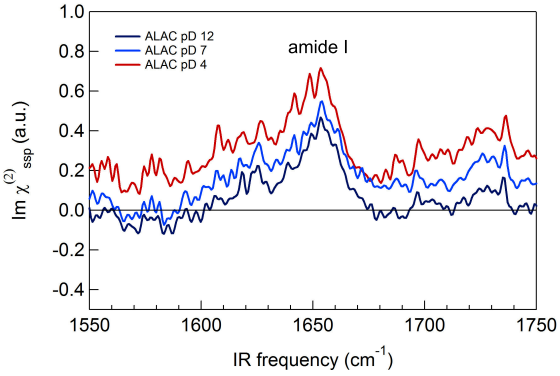

Fig. 5 Imaginary $\chi^{(2)}$ spectra of α-lactalbumin in D_2O at different pD values.

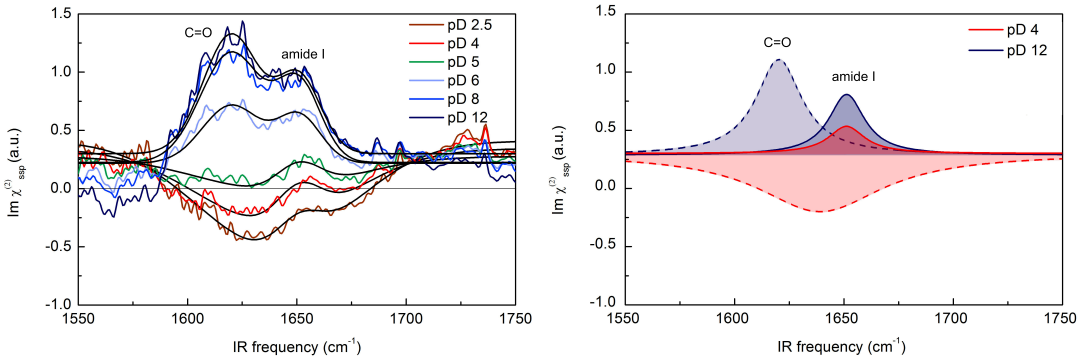

Fig. 6 Left panel: Imaginary $\chi^{(2)}$ spectra of α-lactalbumin in 6.5 M urea at different pD values in the region of amide I vibration. The lines are the result of a fit of the spectra to two bands, one representing the response of urea at $\sim$1620 cm^{-1} (of which the sign depends on the pD), one representing the response of the amide I modes of α-lactalbumin at $\sim$1650 cm^{-1} (of which the sign does not depend on the pD). Right panel: The bands of urea and α-lactalbumin resulting from the fit.

charged) the NH vibrations are pointing downwards into the liquid, in agreement with their expected dipolar orientation.

In Figure 5 we show the Im$\chi^{(2)}$ spectra of α-lactalbumin at different pD values in the frequency range from 1550 to 1750 cm^{-1}. The amide I vibration strongly overlaps with the bending mode of water, which has a frequency at the water/air interface of $\sim$1643 cm^{-1}. This complication can be overcome by using D_2O as a solvent, for which the bending mode possesses a frequency at the water-air interface of $\sim$1209 cm^{-1}. The most prominent positive spectral feature at $\sim$1650 cm^{-1} originates from the amide I mode, which consists mainly of the C=O stretching vibration with a smaller contribution of the C-N stretching vibration. Interestingly, the Im$\chi^{(2)}$ spectra of the amide I band do not show a change in sign and only small variations in amplitude when the pD is varied. Quantum chemistry calculations showed that the transition dipole moment of the amide I band is approximately parallel to the C=O bond, and thus a positive sign in the Im$\chi^{(2)}$ spectrum corresponds to an orientation of the C=O bond towards the bulk of the liquid (the C atom being closest to the surface)[35]. Hence, we conclude that the amide groups of α-lactalbumin show a net orientation of their C=O groups pointing into the bulk. This orientation does not change when varying the pD. The observed constant amplitude of the amide I band further suggests that the surface propensity of the protein does not change upon varying the pD, agreeing with the observations for the CH stretch vibrational bands. We further investigated the pD dependence of Im$\chi^{(2)}$ of the amide II band of α-lactalbumin. The amide II band is a combination of the C-N stretch and the in-plane bending of N-H. In D_2O the bands of urea are quite well separated from the amide I and amide II vibrations of α-lactalbumin (see Figure SI 3). Changing the pD of the solution did not have a noticeable effect on the intensity of the amide II band (see Figure SI 4), confirming that the amide groups do not change their net orientation when varying the pD.

The Im$\chi^{(2)}$ spectra of α-lactalbumin solution in 6.5 M urea at different pD values are shown in Figure 6. In the frequency region between 1600 and 1700 cm^{-1}, the spectrum shows a band at $\sim$1620 cm^{-1} associated with the carbonyl stretch vibration of urea, and the amide I band of the protein at $\sim$1650 cm^{-1}. The amide band is still located at 1650 cm^{-1}, even though the addition of urea may lead to a partial denaturation of the protein. At pD values higher than the IEP the carbonyl vibrational band of urea is positive (C=O group of urea is pointing to the bulk), and at lower values it becomes negative, indicating that urea changes its orientation (C=O group pointing to the surface). The orientation of the carbonyl vibrational band of urea thus shows the same pD dependence as observed for the NH vibrational band of urea. In the right panel we show a decomposition of the spectra in the left panel. From this decomposition it is clear that the amide I band of the protein at $\sim$1650 cm^{-1} maintains its positive value regardless of the pD value, similar as we observed in the absence of urea (Figure 5). In addition, the urea band shows a blue shift and broadening when the pD of the solution is decreased. These spectral changes could be due to the transient binding of protons to the urea molecules.

From our results we conclude that the orientation of urea closely follows the orientation of the interfacial water molecules, and not the orientation of the protein amide groups. This result strongly indicates that urea is not interacting with the protein directly, and is well embedded in the hydrogen-bond network of water. Thereby we obtain strong experimental evidence that the denaturation of proteins by urea does not rely on the hydrogen-bond interactions between urea and the protein amide groups, which agrees with the results of recent molecular dynamics simulations[36].

3 Conclusions

In conclusion, we performed heterodyne-detected VSFG measurements which enabled us to determine the absolute orientation of water, urea and α-lactalbumin at solution interfaces at different pH values. We find that the orientation of water and urea molecules closely follow the net charge of the protein. We observed that the amide I groups of the protein backbone do not follow the same orientation trend as water and urea, which shows that urea molecules follow more closely the orientation of water than the orientation of the amide groups of the protein back-

bone. Based on our experimental data we conclude that urea is not binding to the protein backbone, thus supporting an indirect mechanism for protein denaturation.

4 Acknowledgment

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