Love and fear of water: Water dynamics around charged and apolar solutes
van der Post, S.T.

Citation for published version (APA):

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Osmolytes and Denaturation

Some of the work in previous chapters was considered in the context of protein denaturation. In chapter 9 we saw that cations with a large surface charge density have a preference to form hydration complexes with strongly hydrated anionic groups. Weakly hydrated anions, on the other hand, like to pair-up with weakly hydrated cationic groups. In proteins most cationic groups are weakly hydrated and most anionic groups are strongly hydrated, explaining the Hofmeister series in which most denaturing power is assigned to a combination of a weak cation and strong anion. However, there exist also non-ionic osmolytes that are able to denature proteins, like urea and tetramethylurea (TMU). **In this chapter we investigate the hydration dynamics around guanidinium chloride and tetramethylguanidinium chloride and compare their properties to those of urea and TMU.** The replacement of four protons in guanidinium and urea by methyl groups (yielding tetramethylguanidinium and TMU, respectively), has a strong effect on the reorientation dynamics of their solvating water molecules. We find that guanidinium chloride is the strongest denaturant and urea the weakest, but the methyl substitution puts them both in the middle. These results suggest a different mechanism of denaturation for the denaturants containing hydrophobic groups.
11.1 Introduction

The conformation of macromolecules in aqueous media is crucial for their functioning in biological systems. Many studies have been devoted to the understanding of how these structures are formed and how this formation can be influenced by co-solutes like salts and sugars. These studies go back to the pioneering work of Franz Hofmeister [136]. In his 1888 paper Hofmeister discussed the influence of different salts on the precipitation of proteins, a process called salting out. Currently, the well known Hofmeister series comprises two series of cations and anions, ordered by their ability to salt out proteins. It has been argued that salts affect the conformation of proteins by changing the denaturants work indirectly by affecting the properties of water [192, 193, 194]. This effect has been assigned to structure-making and structure-breaking concepts [137], i.e. a presumed ion-induced strengthening or weakening of the hydrogen-bond network of water over long distances. Although these concepts offer an explanation of the observed (de)naturation effects according to the Hofmeister series, experimentally no evidence was found of the supposed long range structuring influence of ions on water [119, 120, 121, 122]. Most ions influence the structure and dynamics of only their first solvation shell [118] and only in the (rare) case of very strongly hydrated ions, water molecules were found to be affected over a somewhat larger distance [123]. A more detailed consideration of ion-water interaction and its possible relation to denaturation and the stabilization of conformations can be found in chapter 9.

The guanidinium cation (see inset Fig. 11.1) is on the far end of the Hofmeister series. Its chloride salt (GndCl) is among the strongest denaturants known, causing almost all proteins to unfold completely at a concentration of 6 mol/l [195]. There is an ongoing debate about the underlying microscopic principles of its working mechanism. Dielectric relaxation spectroscopy and neutron diffraction studies on the water structure around the guanidinium ion suggest that the ion lacks a significant hydration shell [196, 174]. From these results it was concluded that the denaturation process likely involves a direct interaction with proteins, as has been proposed for urea [174, 175]. However, molecular dynamics (MD) simulations show surprising differences between urea and guanidinium chloride [197, 192, 193, 198]: it was found that urea binds to peptide groups but guanidinium does not [193]. In line with this finding, it was shown in other studies that the interaction between guanidinium and water might be crucial in understanding its denaturing properties [194]. Finally, it should be noted that both microscopic mechanisms for denaturation are not mutually exclusive and might in fact both be active [199].

The substitution of the hydrogen atoms of urea by methyl groups yields the osmolyte tetra-methyl urea (TMU). Compared to urea, TMU is an even more effective denaturant [200] and shows a profound (hydrophobic) effect on the water dynamics of its solvation shell [17]. It is therefore not surprising that previous work indicated that denaturation induced by TMU may have another microscopic origin than urea [200]. Given the fundamental differences in the nature of guanidinium chloride and urea it is interesting to study the effects
of the substitution of the hydrogen atoms of the guanidinium ion by methyl groups on the molecular properties of water and the denaturation of proteins.

In this chapter we use THz-DR and fs-IR to study the effects of electrostatic and hydrophobic interactions on the dynamics of water. We compare solutions of the strong denaturing salts guanidinium chloride and tetramethylguanidinium chloride. To relate the results to denaturation, we compare the effectiveness of guanidinium chloride, tetramethylguanidinium chloride, urea and TMU in the denaturation of the model protein Photoactive Yellow Protein.

### 11.2 Experimental

The fs-IR measurements were performed using the one color pump-probe setup that was described in section 3.1.1. The measurements were performed on the OD-stretch vibration in isotopically diluted water (4% D$_2$O in H$_2$O). We tuned the pump and probe spectrum to be centered around 2525 cm$^{-1}$. The dielectric spectra were measured with the THz dielectric relaxation setup described in section 3.3.

Guanidinium chloride (GndCl) and 1,1,3,3-tetramethylguanidine (TMG) were purchased from Sigma Aldrich. TMG is a strong base (pK$_a$ = 13.6) and was neutralized by adding a stoichiometric amount of hydrochloric acid to create the tetramethylguanidinium chloride (TMGndCl) salt. Fig. 11.1 shows a graphical representation of the (tetramethyl)guanidinium cation. Solutions at different concentrations were prepared with a mixture of 4% D$_2$O in H$_2$O (Sigma Aldrich) as the solvent. The measured concentrations range from 0.25 to 4 molal (mol/kg solvent) for the THz-DR experiment, and from 1 to 6 molal for the fs-IR experiments. The deuterium atoms of D$_2$O exchange both with water
Figure 11.2. The imaginary part of the dielectric function of (A) GndCl and (B) TMGndCl solutions of different concentrations. The decrease of the spectrum for increasing concentrations is called depolarization. The depolarization in solutions of TMGndCl is clearly much larger than that in solutions of GndCl.

to form HDO molecules and with the NH$_2$ groups of the guanidinium salts to form NHD. The resonance frequency of the thus formed ND is very close to that of the OD, but its cross section - which is entering quadratically in the pump-probe signal - is twice as small. In addition, for a 4 m GndCl solution the concentration of ND is 3 times lower than the OD concentration. Therefore the contribution of the NHD groups to the pump-probe signal will be small and is neglected. In the THz-DR experiment an Infrasil quartz cuvette was used with a pathlength of 103 ± 0.5 µm. In the fs-IR measurements we used two square CaF$_2$ windows (thickness 4 mm) with a 50 or 100 µm spacer in between. The concentrations of H$_2$O in the solutions are determined using a density meter (Mettler Toledo DM40).

11.3 Results

11.3.1 THz Dielectric Relaxation

In Fig. 11.2 we show the imaginary part of the dielectric function $\varepsilon(\nu)$ measured for different concentrations of GndCl and TMGndCl. A decrease of $\varepsilon(\nu)$ is indicative for a decreased polarization response of water molecules with bulk-like reorientation and is called depolarization. There are a number of effects causing depolarization. In the first place there is the effect of dilution due to the decrease in the number of water molecules in a constant volume contributing to the signal when a solute is added. This is corrected for using the densities of the measured solutions. Second, there is the effect of kinetic depolarization that results from the movement of charges in an electric field. Water molecules close to the moving ion will align preferentially to the local field of the ion.
instead of the externally applied field, leading to a depolarization effect when rotating against this external field. This contribution can also be accounted for [37]. The conductivities of the GndCl solutions required to calculate the kinetic depolarization effect were obtained by interpolation from the data of ref. [196]. Thirdly, the water molecules that weakly bind to the ions may no longer reorient in the applied electric field and therefore no longer contribute to \( \varepsilon(\nu) \). And finally, the reorientation time of the dipoles may increase, thereby shifting the imaginary response to lower frequencies and therefore out of the THz measurement window.

TMGndCl shows a much stronger depolarization effect than GndCl. This cannot be due to electrostatic alignment of the water molecules in the solvation shell of the cation, since the electric field exerted by the TMGnd\(^+\) cation is not stronger than the field exerted by the Gnd\(^+\) cation. More likely, a large fraction of water in TMGndCl solutions reorients slowly because of the presence of the hydrophobic methyl groups, as has been observed before for other amphiphilic solutes [103, 17] and was elaborated on in chapters 7 and 10 in this thesis. For these water molecules the frequency response has shifted to lower frequencies. In addition to the two Debye modes for pure water (described in section 4.5) we therefore add another Debye mode for the slowly reorienting water to the model. Including the term for conductivity the model function can thus be written as,

\[
\varepsilon(\nu) = \frac{S_{\text{slow}}}{1 + 2\pi i\nu\tau_{\text{slow}}} + \frac{S_{\text{bulk}}}{1 + 2\pi i\nu\tau_{\text{bulk}}} + \frac{S_{\text{fast}}}{1 + 2\pi i\nu\tau_{\text{fast}}} + \frac{\sigma}{2\pi\varepsilon_0\nu} + \varepsilon_\infty. \tag{11.1}
\]

As pointed out in section 4.5, the THz-DR experiment is not very sensitive to the frequency dependence of the dielectric relaxation modes that peak at much lower frequencies. These modes are rather present as a decrease in the amplitude. Hence in fitting the observed dielectric response of the TMGndCl, we fix the values for \( \tau_{\text{slow}} \) of the slow water mode and \( \tau_{\text{bulk}} \) of the bulk water to the parameters found for TMU in the GHz regime [17]. The fits to the data are presented as the solid lines in Figs. 11.2.

After correcting for dilution effects and the kinetic depolarization, we obtain the values of the slow water fractions for the different solutions, shown in Fig. 11.3A. For comparison we also show the slow fractions observed for TMU from Ref. [17].

### 11.3.2 FS-IR Pump-Probe

Chapters 8, 9 and 10 already extensively elaborated on how to treat fs-IR pump-probe data of aqueous halide salt solutions. Also for the chloride salts studied in this chapter we expect that two separate bands can be resolved with different dynamics. The transient spectra of GndCl in fact showed the presence of a third spectral component (in addition to the OD\(\cdots\)O and OD\(\cdots\)Cl\(^-\) components) on the red side of the OD\(\cdots\)O spectrum (<2470 cm\(^{-1}\)) with a very fast vibrational decay. This fast relaxing mode is possibly due to coupling of the OD-stretch vibration to vibrational modes in the guanidinium cation [194, 201]. We minimize
A B

**Figure 11.3.** (A) Slow water fractions obtained from the THz-DR measurements. For both experiments hardly any effect is seen for GndCl, while the effects for TMGndCl and TMU are almost identical. The slow water fractions for GndCl and TMU were calculated with data from [196] and [17] respectively. (B) Slow water fractions of the OD···O anisotropy decays obtained from the fs-IR measurements. The data for TMU are taken from ref. [24].

The data is modeled following the same approach as was used for solutions of tetra-\(n\)-alkylammonium salts in chapter 10. We assumed a kinetic model for the vibrational decay that includes two excited states that decay with different time constants to an intermediate state. The intermediate state subsequently decays to the thermalized ground state. A graphical representation of this model was presented in Fig. 4.7. For the anisotropy data we assume the functional forms of (10.1) and (10.2), for which all made assumptions were the same except for the slow reorientation time \(\tau_s^O\) in the OD···O component. The values for \(\tau_s^O\) were fixed to the reorientation times that were found for solutions of TMU using GHz dielectric relaxation [17]. We performed a self-consistent fit in which both the kinetic model and the functional forms of the OD···O and OD···Cl\(^-\) reorientation dynamics are simultaneously fitted to the isotropic data and the anisotropy data, respectively.

Fig. 11.5 shows the total anisotropy measured for solutions of GndCl and TMGndCl of different concentrations. The curves shown in Fig. 11.5 represent the averaged reorientation behavior of all OD···O and OD···Cl\(^-\) groups in solution, weighted by their respective time-dependent populations. For the GndCl solutions the anisotropy decays are observed to be only slightly different from the anisotropy decays of pure HDO:H\(_2\)O. In contrast, the anisotropy curves of the TMGndCl solutions show a much slower decay, indicating that a large
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Figure 11.4. Transient absorption spectra measured for a 4 molal GndCl solution. For increasing pump-probe delay times the transient absorption spectra decay to the thermal difference spectrum (blue).

amount of the water molecules have very slow orientational dynamics. The retardation effect strongly increases with concentration.

From the fitting results we reconstruct the reorientation dynamics of the OD⋯O and OD⋯Cl\(^-\) components separately. The results for TMGndCl are shown in Fig. 11.6, the solid lines are the results of the fits. In both components a strong retardation effect of the reorientation dynamics is present. The slower anisotropy dynamics in the OD⋯Cl\(^-\) component is an indirect result of the hydrophobic groups of the cation. Anion-bound OD groups display a wobbling motion that leads to a fast (\(\sim\)2 ps) anisotropy decay (see chapter 9). This wobbling motion is hindered by the hydrophobic cation, causing the dynamics of the hydrogen-bond network surrounding the anion solvation shell to become slower (10). In this chapter we mainly on the OD⋯O component. The fraction of water molecules with slower dynamics is calculated from the fitted amplitudes \(A_f\) and \(A_s\) in \(R_{OD⋯O}(t)\) and plotted for different concentrations in Fig. 11.3B. The slow water fractions for TMGndCl are a bit higher than what we found for tetramethylammonium bromide, as presented in chapter 10. This is mainly caused by a different choice of the slow water reorientation time \(\tau_s^O\): Here we assumed \(\tau_s^O\) of TMGndCl to be equal to \(\tau_s^O\) of TMU, whereas in the analysis of the data obtained for different tetra-\(n\)-alkylammoniumbromide solutions, we set \(\tau_s^O\) equal to infinity. This latter choice was based on the fact that we observed quite different behavior for tetra-\(n\)-alkylammoniumbromide salts with \(n = 2, 3, 4\). Since we have a good estimate of \(\tau_s^O\) for TMGndCl from GHz-DR data on TMU [17], this time constant was not set to infinity. This finite values of \(\tau_s^O\) yield slightly higher values for the slow water fractions.
11.4 Discussion

The addition of GndCl to water is observed to have a very small effect on the reorientation dynamics of the water molecules. Guanidinium is a relatively large cation which means that the charge density of the positive charge will be rather low. As previously discussed (chapter 9), the electrostatic interaction between water and this ion will thus be weak. The interaction of water with guanidinium will thus be dominated by hydrogen bonding to the NH$_2$
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These hydrogen-bond interactions are quite similar to the hydrogen bonds between water molecules [174, 196]. An analog can be drawn here with aqueous solutions of urea (CH$_4$N$_2$O). Hydrogen-bonds between water and urea are also quite like water-water hydrogen-bonds, and the reorientation dynamics of water in such solutions was observed to be largely unaffected by the presence of urea [111]. Apparently, urea and guanidinium both fit very well in the hydrogen-bond structure of water, allowing the water molecules to reorient with a similar rate as is observed in bulk liquid water. The small slowing down of the total anisotropy decay observed for solutions of GndCl, as shown in Fig. 11.5A, can be fully assigned to the presence of the chloride anion. Water molecules solvate the Cl$^-$ anions by forming a OD/OH···Cl$^-$ hydrogen-bond. This limits the reorientation freedom of those hydroxyl groups, whereas the static dipole moments of these water molecules remain quite free in their reorientation. The reorientation of the static dipoles of the water molecules is measured in the THz-DR experiment (Fig. 11.3A). This notion explains why the THz-DR measurements show no difference in water dynamics between solutions of GndCl and bulk water. This effect is more extensively discussed in chapter 9.

We observe that TMGndCl has a large effect on the dynamics of water in both the THz-DR and in the fs-IR experiments. The fs-IR measurements show a strong retardation effect of the water dynamics in both the OD···O and the OD···Cl$^-$ component (Fig. 11.6). The slower dynamics in the OD···O component are due to water molecules in the hydrophobic solvation shell of the TMGnd$^+$ cation similar to the effect of TMU on water dynamics [28]. With regard to the water dynamics, the hydrophobic nature of the TMGnd$^+$ cation is thus dominant over its charged nature, as was also found for tetramethylammonium bromide. We also see a strong increase of the slow fraction of the OD···Cl$^-$ component of TMGndCl compared to that of GndCl. This increase can be assigned to the effects of the hydrophobic molecular groups of TMGnd$^+$ on the dynamics of the wobbling component of the chloride-bound OD groups. This is analogous to the results for tetra-$n$-methylammonium bromide that were presented in chapter 10.

The THz-DR results also show a slowing down effect of the water dynamics (Fig. 11.2). The fact that slower reorientation dynamics is observed in both experiments implies that the TMGnd$^+$ cation affects the dynamics of the OD groups and the water dipole moments in a similar manner. For solutions of TMU it was also observed that the slowing down effect is quite similar in dielectric relaxation and in femtosecond mid-infrared measurements [17]. It is illustrative to compare the results obtained for TMGndCl with the results obtained for solutions of TMU at similar concentrations, as TMU also possesses four methyl groups in a similar configuration as the TMGnd$^+$ cation. The THz-DR data of TMGndCl agree very well with the results of TMU (Fig. 11.3). This agreement strongly supports the interpretation that the effects of TMGnd$^+$ and TMU on the dynamics of water are completely dominated by the methyl groups and their hydrophobic group or charged nature is less relevant.

In NMR studies it is also observed that hydrophobic molecular groups have a strong effect on the reorientation dynamics of liquid water that increases with
increasing size of the hydrophobic group [99, 100, 101, 102]. For instance, in the NMR study by Shimizu et al. [101], the slowing down of the water reorientation dynamics was found to scale with the number of substituted methyl groups of the urea molecule, urea showing only a negligible effect and TMU the strongest effect. In the same study GndCl was also found to have a negligible effect on the reorientation dynamics of water [101], in line with the present observations.

In order to relate the effects of GndCl and TMGndCl on the dynamics of water to their role in the denaturation of proteins, we measured the fraction of unfolded Photoactive Yellow Protein (PYP) as a function of concentration GndCl, TMGndCl, urea and TMU. The measurements, of which the results are shown in Fig. 11.7, were performed according to the procedure described in Ref. [202] at pH 7 (using a potassium phosphate buffer). GndCl is clearly most effective, as it completely unfolds all PYP at a concentration of 3.5 mol/l. Urea is by far the least effective.

TMU completely denatures PYP at much lower concentrations compared to urea. Apparently, the substitution of the four hydrogen atoms of urea by methyl groups enhances the effectiveness of the denaturant. One may expect a similar enhancement for TMGndCl relative to GndCl, but TMGndCl is in fact less effective than GndCl. TMGndCl rather shows a similar denaturation behavior as TMU. The denaturation mechanisms of urea and GndCl compounds appear to be completely replaced by denaturation effects associated with the methyl groups of TMU and TMGndCl. We also found that TMGndCl and TMU show a quite similar retardation effect on the dynamics of water. Both observations can be explained by the limiting effect of the methyl groups on the configurational space of the solvating water. As a result, the entropy of the solution is decreased and the reorientation dynamics slows down. Entropy changes are believed to be the main driving force for the denaturation of proteins by TMU. Both the denaturation effects and the retardation of water dynamics thus both find a common origin in the hydrophobic moieties of TMU and TMGndCl.

11.5 Conclusions

We studied the effects of guanidinium chloride (GndCl) and tetramethylguanidinium chloride (TMGndCl) on the reorientation dynamics of water using THz dielectric relaxation (THz-DR) and polarization-resolved femtosecond mid-infrared spectroscopy (fs-IR). THz-DR probes the reorientation of the water dipole moments while fs-IR probes the reorientation of the water OH/OD groups.

For GndCl solutions there is a small slow component in the reorientation dynamics associated with water molecules that are hydrogen bonded to the Cl$^{-}$ anion. The guanidinium ion has no effect on the reorientation dynamics of water. In the case of TMGndCl the effect on the dynamics of water are dominated by the hydrophobic methyl groups of the TMGnd cation. The four methyl groups of TMGnd$^{+}$ lead to a strong slowing down of the reorientation dynamics.
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Figure 11.7. Fraction of folded protein PYP as a function of denaturant concentration for different denaturants. The measurements were performed for a buffered solution at pH 7.

dynamics of water, that is quite similar to what is observed for solutions of tetramethylurea (TMU). The water molecules that form hydrogen bonds to Cl\(^{-}\) show a strong slowing down of their wobbling motion due to the nearby presence of the hydrophobic TMGnd\(^{+}\) cations.

Finally, we put these results in the perspective of the capabilities of GndCl, TMGndCl, urea and TMU to denature proteins. While urea and GndCl differ strongly in their effectiveness of denaturation, TMGndCl and TMU both show very similar behavior. The denaturation mechanism of TMGndCl and TMU may thus well be associated with their hydrophobic moieties.