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Proteins

Exploring the Piezophilic Behavior of Natural Cosolvent Mixtures**

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Proteins are only marginally stable and are hence very sensitive to environmental conditions, such as high and low temperatures or high hydrostatic pressures.[1] In nature, living organisms are able to compensate for extreme environmental conditions and hence rescue proteins from denaturation by using osmolytes. Organic osmolytes are accumulated under anhydrobiotic, thermal, and pressure stresses. Among those osmolytes are amino acids, polyols and sugars (e.g., glycerol and trehalose), methylamines such as trimethylamine-*N*-oxide (TMAO), and urea.[2] TMAO has been found to enhance protein folding and ligand binding most efficiently. On the other hand, urea, a highly concentrated waste product in mammalian kidneys, is a perturbant. It is also a major organic osmolyte in marine elasmobranch fishes. Interestingly, TMAO has been found to counteract perturbations imposed by urea and hydrostatic pressure in deep-sea animals, most effectively at a 2:1 urea:TMAO ratio.[2] In the deep sea, hydrostatic pressures up to the 1 kbar (100 MPa) range prevail, and living organisms have to cope with such extreme environmental conditions. High hydrostatic pressure generally destabilizes the protein structure, inhibits polymerization of proteins and ligand binding.[3,4] Interestingly, TMAO has been shown to largely offset these pressure effects. In fact, it was found that the amount of TMAO in the cells of a series of marine organisms increases linearly with the depth of the ocean. For that reason, TMAO is thought to serve as pressure counteractant. The term "piezolyte" has been coined for such kind of cosolute.[2]

About the underlying mechanism of stabilization by TMAO at ambient pressure conditions several experimental and theoretical (molecular dynamics simulations) articles have been published in recent years.[5–9] TMAO is largely excluded from the protein surface and enhances the water structure causing greater organization through more and stronger hydrogen bonding among water molecules. However, the mechanism of this "chemical chaperon" at high hydrostatic pressure (HHP) conditions is still unclear. To yield a deeper understanding of this phenomenon, we determined the intermolecular interaction of dense protein solutions in the absence and presence of cosolvent mixtures of TMAO and urea also under HHP conditions. Small-angle X-ray scattering (SAXS) experiments on dense lysozyme solutions have been carried out in the pressure range from 1 bar up to 4 kbar. The SAXS technique accurately monitors structural alterations of the protein solution and yields quantitative information on the state-dependent protein–protein interaction potential.[10–13] As lysozyme is a highly stable protein, pressure-induced effects will only be attributed to changes in the protein–protein interaction of the native protein and how this is influenced by osmolytes. No pressure-induced unfolding of the protein occurs in the pressure range covered. Complementary thermodynamic data, that is, the temperature of unfolding and the volume change upon unfolding of the protein, were obtained by differential scanning (DSC) and pressure perturbation calorimetry (PPC), respectively.

To verify that the protein is folded at all solution conditions studied, SAXS measurements on diluted lysozyme solutions ($c_P = 10\ \mathrm{mg\,mL^{-1}}$) were carried out in the whole pressure range covered. For diluted protein solutions, the scattering intensity $I(q)$ is proportional to the form factor $P(q)$ ($q = (4\pi/\lambda)\sin(\Theta/2)$ is the wave vector transfer, λ the wavelength of the X-rays, and Θ the scattering angle), which depends on the structure and size of the protein. For dilute lysozyme solutions, the radius of gyration of the particle, R_g, could be determined. We found a constant R_g value of (15.1 ± 0.4) Å up to 4 kbar, indicating the absence of unfolding even at the highest pressure applied.

In the case of concentrated protein solutions, the interaction between the particles gives rise to an additional scattering contribution. This SAXS signal can be described as the product of the form factor and an effective structure factor, which is related to the intermolecular structure factor $S(q)$. To relate the structure factor to the protein–protein interaction potential, statistical mechanical model approaches have to be employed. Here, the mean-spherical approximation (MSA) in combination with the DLVO (Derjaguin–Landau–Verwey–Overbeek) potential $V(r)$ has been used. The pair potential $V(r)$ is given as the sum of a hard sphere potential $V_{HS}(r)$, a repulsive screened Coulomb-like potential $V_{SC}(r)$ and an attractive Yukawian potential $V_Y(r)$, which is frequently used to describe protein–protein interactions (for details, see the Supporting Information).[10,11]

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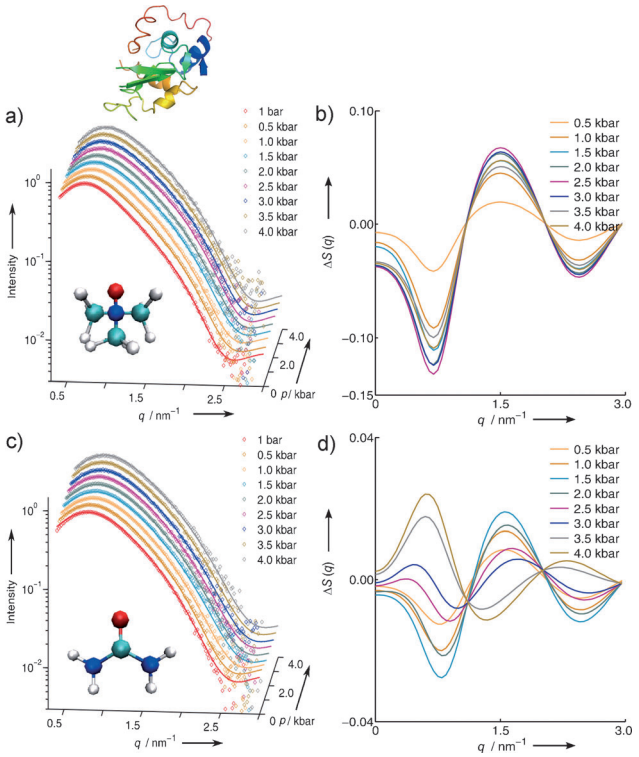

Figure 1. Pressure dependence of X-ray small-angle scattering data of lysozyme solutions in the presence of osmolytes (top: structure of the lysozyme). SAXS curves of a lysozyme solution of $c_P = 100\ mg\,mL^{-1}$ (pH 7) in a) 1.0 M TMAO, and c) in 1.0 M urea at different pressures (at $T = 25\,°C$). Solid lines denote the refinement. Pressure-dependent changes of the structure factor data, that is, $\Delta S(q) = S(q)_p - S(q)_{1\,bar}$, for b) 1.0 M TMAO and d) 1.0 M urea are shown on the right.

High protein concentrations were chosen to mimic the crowded conditions met in a biological cell. As an example, Figure 1 a shows SAXS curves $I(q)$ of a concentrated lysozyme solution ($c_P = 100\ mg\,mL^{-1}$) with 1.0 M TMAO added, together with the refinement of the data. The SAXS intensity curves exhibit a pronounced correlation peak because of the presence of a structure factor reflecting strong protein–protein interactions.[10,11] We observe a slight shift of the peak position q_{corr} to higher q values with increasing pressure up to about 2.5 kbar. A further pressure increase reverses this effect to some extent. The structure factors $S(q)$ were obtained by refinement of the scattering data. To highlight the pressure-induced changes, differences $\Delta S(q)$ between $S(q)$ at high pressures and at 1 bar, that is, $\Delta S(q) = S(q)_p - S(q)_{1\,bar}$, are shown in Figure 1 as well. (Differences in the primary experimental data, $I(q)$, for different osmolyte solutions are given in the Supporting Information).

The corresponding real-space dimension of the correlation peak, d_{corr} ($d_{corr} \approx 2\pi/q_{corr}$), which roughly corresponds to the mean intermolecular separation of the protein molecules in the solution, decreases from (8.6 ± 0.1) nm at 1 bar to (8.0 ± 0.1) nm at 2.5 kbar, and then increases slightly again up to (8.2 ± 0.1) nm at 4 kbar (Figure 2).

Figure 3 depicts the strength of attraction, J, as a function of pressure for all TMAO concentrations measured, which has been obtained from the fit of the DLVO potential to the

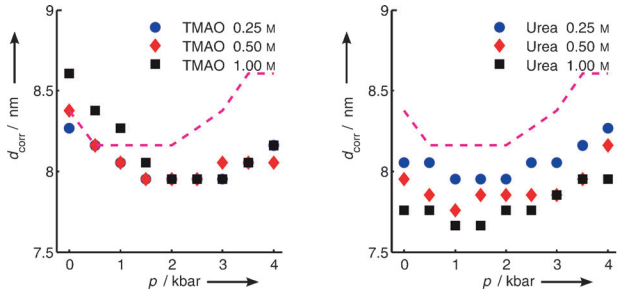

Figure 2. Average intermolecular distance between protein molecules. Intermolecular distance, d_{corr}, of lysozyme molecules obtained from the position of the correlation peak as a function of pressure p for different concentrations of TMAO and urea. The results for the pure buffer solution are shown as well (dashed line).[11]

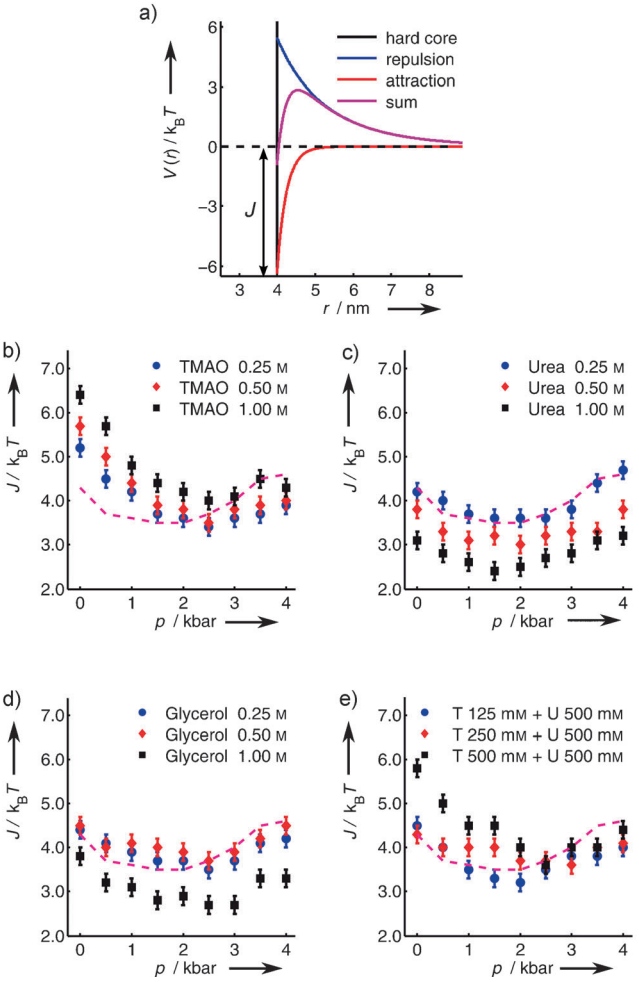

Figure 3. Pressure dependence of the attractive interaction of lysozyme molecules in different osmolyte solutions. Top a): different contributions to $V(r)$. Bottom: strength of the attractive part of the intermolecular protein–protein interaction potential, J, as a function of the pressure, p, for different concentrations of b) TMAO, c) urea, d) glycerol, and e) for different mixtures of TMAO and urea. The results for the pure buffer are shown as well (dashed line).

$I(q)$ data within the MSA approximation. For comparison, the data for the protein in pure buffer solution are included (dashed line).[11] At atmospheric pressure, the presence of TMAO in solution leads to a marked increase of J with

increasing osmolyte concentration ($+2k_BT$ for 1 M TMAO), which is in accord with literature data.[13] An increasing pressure results, as expected, in a drastic decrease of J, up to a pressure of around 2.5 kbar. At higher pressures, no significant change of J is observed anymore. Consistently, J scales with the TMAO concentration.

Recent HHP SAXS data of dense lysozyme solutions in pure buffer solution[11] revealed that for pressures up to 2 kbar, the solution is continuously compressed, resulting in a shift of d_{corr} to lower values and a concomitant decrease of J as a consequence of an increased effective repulsion of the proteins. Remarkably, at higher pressures, above about 2 kbar, an increase of the attraction sets in, which can be explained by a significant change of the water structure, that is, a collapse of the second water hydration shell.[14–17] As can be seen in Figure 3 (dashed curve), for the protein in pure buffer solution, the decrease of J upon pressurization is less pronounced, whereas the increase at higher pressures is significant. Furthermore, the broad minimum in $J(p)$ is shifted to higher pressures when TMAO is added to the buffer solution. Thus, the collapse of the second hydration shell seems to be suppressed by the addition of TMAO.

The influence of the chaotropic agent urea on the pressure-dependent protein–protein interaction is different. The SAXS data for a lysozyme solution in 1.0 M urea are shown in Figure 1c. In contrast to the data of the protein in 1.0 M TMAO solution (Figure 1b), $\Delta S(q)$ shows a biphasic pressure dependence, and the amplitude changes are less pronounced (Figure 1d). The pressure-induced shift of d_{corr} to lower values is less pronounced (Figure 2b). The d_{corr} value is about (7.8 ± 0.1) nm at 1 bar, (7.7 ± 0.1) nm at 1.5 kbar, and increases up to about (7.9 ± 0.1) nm at 4 kbar upon further pressurization. Figure 3b exhibits the pressure dependence of the strength of attraction, J, for different urea concentrations. With increasing urea concentration, the interaction becomes more repulsive. This decrease of J is in accord with previous ambient-pressure studies.[13,18] The pressure dependence of J is similar to that in the pure buffer solution. An increase in the amount of urea results in a systematic shift of J towards smaller values only.

For comparison, the effect of the kosmotropic cosolvent glycerol on the pressure dependence of J is shown in Figure 3c as well. As reported previously,[10,19] glycerol leads also to a decrease of the protein–protein attraction, similar to urea, but to a lesser extent. The effect of pressure on J is of similar magnitude to that for the pure buffer solution. A slight shift of the minimum of $J(p)$ to 2.5 kbar can be seen for the glycerol solutions. Hence, in contrast to urea and glycerol, which reduce only the strength of attraction but have no significant effect on the pressure sensitivity of J, TMAO drastically changes the pressure dependence of the intermolecular interaction of the protein.

Mimicing the conditions met in deep-sea organisms, SAXS data of mixtures of TMAO and urea were measured. As an example, the results for a 0.5 M urea solution at different amounts of TMAO are shown in Figure 3d. The data show that even in the presence of TMAO concentrations as low as 0.125 M, the tendency of urea to foster the repulsive part of the intermolecular protein interactions is largely compensated by the addition of TMAO at ambient pressure. Up to a pressure of 2.5 kbar, the 0.5 M urea/0.125 M TMAO mixture displays a $J(p)$ dependency which is similar to that of the pure buffer solution. At higher pressures, J is slightly below the value of the pure buffer solution, which is also observed for the pure TMAO and urea cosolvent solutions. An increase of the TMAO concentration in the urea–TMAO cosolvent mixture to 0.25 M causes a shift of the minimum of $J(p)$ from about 1 to 2 kbar. For the equimolar mixture of 0.5 M TMAO and 0.5 M urea, the $J(p)$ data look similar to those of the pure 0.5 M TMAO solution. These data thus reveal a strong and counteracting influence of TMAO on the intermolecular protein–protein interaction potential also under HHP conditions.

To compare these data with corresponding stability data of the lysozyme, complementary thermodynamic measurements have been carried out (for PPC, see the Supporting Information). Table 1 displays the unfolding temperatures T_m of the lysozyme in selected cosolvent mixtures. In accord with literature data,[20] we notice a significant decrease of the T_m value upon addition of urea, reflecting its destabilizing effect. Conversely, a marked increase of T_m is observed upon addition of TMAO. For the 1:1 and 2:1 urea/TMAO mixture, a counteracting behavior is observed.

Table 1:
Results from DSC (unfolding temperature T_m) data for the unfolding transition of lysozyme (100 mg mL^{-1}, pH 7) for various cosolvent concentrations.

	Lysozyme	Lysozyme +1 M urea	Lysozyme +1 M TMAO	Lysozyme +250 mM TMAO +500 mM urea	Lysozyme +1 M TMAO +1 M urea
T_m [°C]	73.4	71.0	75.2	72.2	73.2

According to experimental and molecular dynamics simulation studies, TMAO seems to enhance the number of strong hydrogen bonds of the water structure, that is, TMAO serves as "water structure maker".[5–9,21] In the presence of proteins, a direct interaction between the protein and the osmolyte is disfavored, and it is the depletion of TMAO from the surface of the protein that gives rise to the increased protein stability as the cosolvent is added. The presence of TMAO increases the attractive part of the interaction potential, J, significantly, which is probably due to the marked change in solvent structural properties (Figure 3b), and the average intermolecular distance between protein molecules increases by around 4% upon addition of 1 M TMAO at ambient pressure (Figure 2). Moreover, our data show that J is more pressure-sensitive for the TMAO solutions than in pure buffer, resulting in a much steeper decay of $J(p)$ up to around 2.5 kbar. Different from the pure buffer solution, above around 2 kbar, no significant changes of J or d_{corr} are observed anymore, as pressure and TMAO

have counteracting effects on the structural properties of water. Conversely, such strong pressure dependence of J is not observed for the urea solution, the $J(p)$ curve is shifted to smaller J values only. This might be due to the fact that the water structure is not significantly perturbed in the presence of urea.[5,8]

Glycerol is a stabilizing osmolyte such as TMAO. For the glycerol solutions studied, only a minor influence on the interaction potential is detectable, that is, the effect of glycerol on the intermolecular interactions is markedly different from TMAO, although both are stabilizing osmolytes. A small shift of the minimum of $J(p)$ is observed for the highest glycerol concentration only. Stronger glycerol–water interactions are observed for higher glycerol concentrations.[22]

The influence of the TMAO–urea mixtures on the protein–protein interactions significantly depends on the mixing ratio. In the case of the equimolar mixture, the influence of strong kosmotrope TMAO on $J(p)$ dominates. For the 1:4 and 1:2 TMAO/urea solutions, their influence on the pressure dependence on J and the mean intermolecular protein–protein distances are similar to the corresponding data for the pure buffer solution, that is, they largely counteract. The cancellation of interactions is in good agreement with neutron scattering data indicating a direct interaction of urea with TMAO.[21] Interestingly, a 1:2 TMAO/urea mixture has also been found to be most effective in avoiding pressure-induced cellular stresses in deep-sea animals.[2]

In summary, protein–protein interactions in dense protein solutions are affected by pressure in a nonlinear way. At pressures above 2 kbar, the coordination number of water has increased markedly because of a collapsed second hydration shell. Here, protein–protein interactions are modified, leading to a relaxation of the interparticle repulsion and hindrance of a closer approach of the proteins, thus preventing them from aggregation.[11] Addition of urea increases the repulsive interaction between lysozyme molecules ($\Delta J = -1\,k_B T$/M urea). Conversely, the addition of TMAO increases the attractive interaction ($\Delta J = +2\,k_B T$/M TMAO). TMAO increases the amount of strong H bonds of water, leads to a strengthening of the H-bond network structure, and the second hydration shell of water moves slightly outward—contrary to the effect of HHP. As a result, no increase of J is observed at pressures above 2 kbar. For urea–TMAO mixtures, a large counteracting effect on the intermolecular interactions, qualitatively similar to the effect on protein stability (T_m), is observed. Hence, indirect, that is, solvent-mediated, effects seem to play a major role in the protein stabilization also under HHP conditions, where TMAO and pressure have counteracting effects on the water structural properties. These findings may thus be of importance for understanding the upregulation of TMAO under HHP stress conditions and the compensatory effect of urea–TMAO mixtures in deep-sea organisms.

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