Effects of Chaotropic and Kosmotropic Cosolvents on the Pressure-Induced Unfolding and Denaturation of Proteins: An FT-IR Study on Staphylococcal Nuclease[†]

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ABSTRACT: FT-IR spectroscopy was used to study the effects of various chaotropic and kosmotropic cosolvents (glycerol, sucrose, sorbitol, K_2SO_4, $CaCl_2$, and urea) on the secondary structure and thermodynamic properties upon unfolding and denaturation of staphylococcal nuclease (Snase). The data show that the different cosolvents have a profound effect on the denaturation pressure and the Gibbs free energy ($\Delta G°$) and volume ($\Delta V°$) change of unfolding. Moreover, by analysis of the amide I′ infrared bands, conformational changes of the protein upon unfolding in the different cosolvents have been determined. An increase, a reduction, or an independence of the volume change of unfolding is observed, depending on the type of cosolvent, which can at least in part be attributed to the formation of a different unfolded state structure of the protein. The data are compared with the corresponding thermodynamic values of $\Delta V°$ for the temperature-induced unfolding process of Snase as obtained by pressure perturbation calorimetry, and significant differences are observed and discussed.

The in-depth knowledge of thermodynamic properties of proteins in solution has been drawing the attention of many researchers as these properties form the basis for understanding the biological functions of the proteins, but also for purposes of drug design and formulations. Basically, the stability of globular proteins depends on their solvent properties, temperature, and pressure (*1*−*4*). Pressure is an important factor, and in recent years, the study of the effects of pressure on biological systems has drastically expanded (*5*−*8*). In particular, pressure has been used for studying pressure-induced un/refolding of proteins and for understanding protein stability and its folding pathways, since pressure can stabilize partially unfolded states and molten globules. However, to date, very little is known about the pressure stability of proteins in the presence of particular reagents (in the present context, a cosolvent) in comparison to water or buffer (*19*, *20*).

Polyhydric alcohols and sugars have been used as protein stabilizers for many years, but it was not until recently that the mechanism of stabilization was established. Timasheff and co-workers demonstrated that sugars and other polyhydric alcohols are preferentially excluded from the surface of the protein (*21*−*25*). Thus, the folded state of the protein is stabilized relative to the unfolded state because it exposes less surface area from which the cosolvent must be excluded. Thus, the relative stabilization does not involve contact with the cosolvent. Rather, the hydration layer around the protein is altered. On the other hand, denaturation is induced by using cosolvents that directly bind to particular groups on the protein, for example, to hydrophobic residues in the case of alcohols (*25*) or to peptide groups in the case of urea (*27*, *28*) and guanidinium hydrochloride (*29*).

The Hofmeister series characterizes some properties of ions as cosolvents in protein solutions. In this series, the ions are divided into two groups: kosmotropic and chaotropic ions. The protein is salted out, i.e., stabilized, by kosmotropic ions and salted in, i.e., destabilized, by chaotropic ions (*3*, *30*). Reduction of the net charge of proteins, via salts acting as counterions (screening effect), is an additional important effect of salts on protein stability (*31*).

In this work, we have explored the effect of different cosolvents on the high-pressure stability of a well-characterized monomeric protein, staphylococcal nuclease (Snase).[1] Changes in unfolding were obtained in the presence of different concentrations of polyhydric cosolvents (glycerol and sorbitol), sugars (sucrose), urea, chaotropic ions (calcium chloride), and kosmotropic ions (potassium sulfate). Additionally, FT-IR difference spectra were recorded and analyzed in an effort to detect conformational changes in the native and unfolded state of the protein in the presence of the different cosolvents.

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[1] Abbreviations: Snase, staphylococcal nuclease; FT-IR, Fourier transform infrared spectroscopy; DAC, diamond anvil cell; p_m, transition pressure of pressure denaturation; $\Delta V°$, volume change of pressure denaturation; $\Delta G°$, standard Gibbs free energy change of pressure denaturation at 1 bar; T_m, transition temperature of denaturation at ambient pressure.

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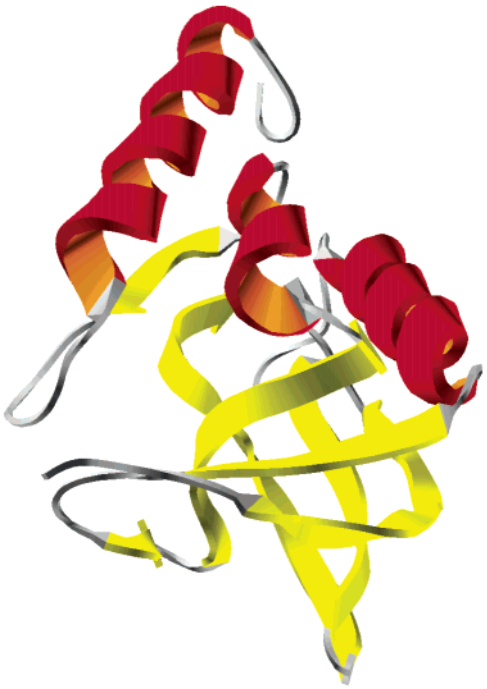

FIGURE 1: Ribbon diagram of native Snase as obtained from X-ray diffraction experiments at a resolution of 1.7 Å (*32*).

Snase is a small 17.5 kDa protein containing 149 amino acid residues and no disulfide bonds (Figure 1). The crystal structure of Snase reveals that the protein contains 26.2% α-helices, 24.8% β-sheets, 7.4% extended chains, 24.8% turns and loops, and 8.7% unordered structure (*32*). It has an extraordinarily high fraction of ionizable groups (~39%). The thermodynamic properties for the pressure-induced unfolding transition, such as the denaturation pressure, the volume, and standard Gibbs free energy change of denaturation, in the presence of different cosolvents and the comparison with the corresponding data for the temperature-induced unfolding transition are still largely unknown (*19*, *20*). To yield valuable data for a series of different cosolvents is the objective of this paper.

MATERIALS AND METHODS

Purification of Snase. Recombinant Snase with the sequence of nuclease A from the V8 strain of *Staphylococcus aureus* was obtained using the λ expression system in *Escherichia coli* strain Arλ9 as described by Shortle and Lin (*33*). The cells were grown according to the procedure described by Shortle *et al.* (*34*), except that SB rather than Mops medium was employed. The protein purification was carried out according to the method described by Shortle *et al.* (*35*) with modifications described by Frye *et al.* (*36*).

Preparation of the Snase/Cosolvent/Buffer Solutions. Infrared spectroscopic studies on proteins generally call for D_2O as the solvent. This is due to the strong absorption of H_2O in the amide I spectral range. With regard to structural properties, the difference between D_2O and H_2O as a solvent is very slight; D_2O has a slightly larger hydrogen bonding strength only, but the structure of the liquid is essentially the same within the accuracy of existing X-ray and neutron scattering data. Hence, we assume that the Hofmeister series has the same sequence in D_2O as in H_2O. To deuterate the *d*-sorbitol (Fluka, Taufenkirchen, Germany) and sucrose (Fluka), 50 mg portions were dissolved in 1.5 mL of D_2O (Aldrich, Taufenkirchen, Germany). After the solution had been stored for 48 h, the D_2O was evaporated using an SC 110 speed vac from Savant (Vaterstetten, Germany), and the procedure was repeated. The d_8-glycerol (LGC Promochem, Wesel, Germany), d_4-urea (Aldrich), K_2SO_4 (Aldrich), and $CaCl_2$ (Aldrich) were used without any modification. The corresponding amount of cosolvent was dissolved in 10 mM BisTris (Sigma, Taufenkirchen, Germany) buffer. Snase was dissolved at a concentration of 5% (w/w) in the cosolvent/buffer solution, and the pD value of the solution was adjusted to 5.5 using DCl. The pD was measured using a pH-meter calibrated with standard buffers in H_2O taking the isotope effect into account (pD = pH + 0.4) (*37*, *38*).

FT-IR Spectroscopy. FT-IR spectroscopy has proven to be a powerful technique for determining the secondary structure elements of proteins (*39*−*42*), also under high-pressure conditions. The amide I′ band (between 1600 and 1700 cm^{-1}) was detected, which is mainly associated with the carbonyl stretching vibration (85%) of the amide groups, and which is directly related to the backbone conformation and hydrogen bonding pattern of the protein. The FT-IR spectra were recorded with a MAGNA 550 spectrometer from Nicolet (Offenbach, Germany) equipped with a liquid nitrogen-cooled MCT (HgCdTe) detector. The infrared light was focused by a spectra bench onto the pinhole of a diamond anvil cell (DAC) from HPDO (Tucson, AZ) with type IIa diamonds (*11*). Each spectrum was obtained by co-adding 256 scans at a spectral resolution of 2 cm^{-1} and was apodized with a Happ−Genzel function. The sample chamber was purged with dry and carbon dioxide free air. Powdered α-quartz was placed in the pinhole of the steel gasket, and changes in pressure were quantified by the shift of the quartz phonon band at 695 cm^{-1} (*43*, *44*). An external ethylene glycol thermostat (RC 6 from Lauda, Lauda-Königshofen, Germany) was used for the pressure- and temperature-dependent measurements to control the temperature to within 0.1 °C. The equilibration time before spectra were recorded at each temperature and pressure was ~20 min. Fourier self-deconvolution of the FT-IR spectra was performed with a resolution enhancement factor of 1.8 and a bandwidth of 15 cm^{-1}; an example is shown in Figure 2A. The denaturation profiles, i.e., intensity I versus p plots, were calculated following the intensity at ~1627 cm^{-1}, which corresponds to the content of β-sheet structures of Snase (*11*, *12*, *39*−*42*). As an example, the denaturation profiles of Snase in solutions with different concentrations of sucrose are depicted in Figure 2B. The standard volume change of unfolding, $\Delta V°$, at the temperature T, was determined from

$$\Delta V° = \left[\frac{\partial \Delta G°(p)}{\partial p}\right]_T \qquad (1)$$

where $\Delta G°(p)$ is the standard free Gibbs energy change at pressure p, which is determined from the denaturation profiles, as shown in Figure 2B, from the equilibrium constant $K_{eq}(p)$:

$$\Delta G°(p) = -RT \ln K_{eq} = -RT \ln \frac{I_N - I(p)}{I(p) - I_D} \qquad (2)$$

where $I(p)$ is the intensity at pressure p and I_N and I_D are

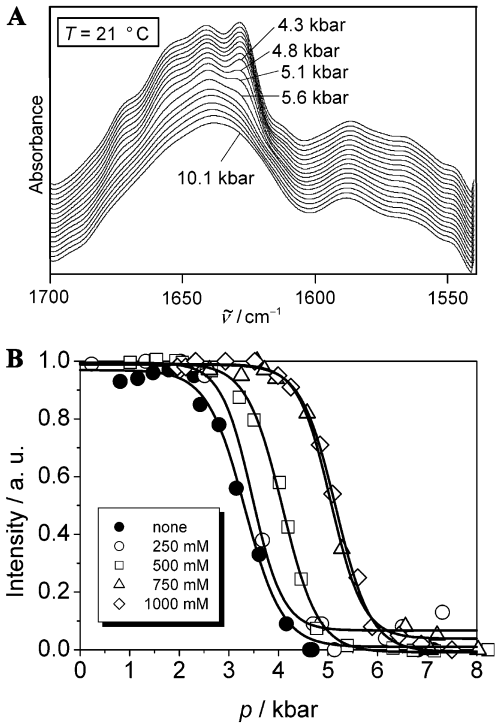

FIGURE 2: (A) Fourier self-deconvoluted infrared spectra of Snase in the presence of 1000 mM sucrose at different pressures and (B) denaturation profiles of Snase in the presence of different concentrations of sucrose at pD 5.5 and 21 °C.

the asymptotic intensity values of the native (folded) and denatured (unfolded) states, respectively. The standard Gibbs free energy change $\Delta G°$ at 1 bar is obtained by extrapolation to 1 bar:

$$\Delta G° = \lim_{p \to 1\ \mathrm{bar}} \Delta G°(p) \tag{3}$$

Different experimental techniques (fluorescence and FT-IR spectroscopy and small-angle X-ray scattering) revealed that the pressure-induced unfolding of Snase can, at least within the accuracy of the experiment, be described as a fully reversible two-state process, and the secondary and tertiary structural changes occur on the same time scale (*11*, *18*, *53*, *55*). In our laboratory, the fractional intensities of the secondary structure elements are usually calculated from a band fitting procedure assuming Gaussian−Lorentzian line shape functions (*10*−*15*). In our case here, where the observed conformational changes are rather small and due to the uncertainties in baseline corrections in a DAC, we chose to use FT-IR difference spectra instead to visualize the secondary structural changes. For calculation of the FT-IR difference spectra, the FT-IR spectra of the amide I′ band were corrected with a linear baseline and the intensity was normalized to the maximum intensity at ∼1640 cm^{-1}. The FT-IR difference spectra were calculated using the spectra of the pressure-denatured state at ∼7 kbar and subtracting the spectra of the native state of the respective protein/cosolvent system. In several cases, the difference spectra are completely negative, suggesting a strong attenuation of oscillator strength at high pressure. This might also be due to small pressure-induced baseline shifts (which might also be nonlinear) and changes in path length when the gasket of the DAC is compressed, which leads to small errors in the normalization of intensities. In the discussion of the results, we are therefore careful and discuss only losses and gains of conformations which are significant (mostly in the β-strand region) and outside the range of error bars (±0.025) of the absorbance (A). Because the transition dipole moments of the different secondary structure elements are not known, only qualitative trends can be given.

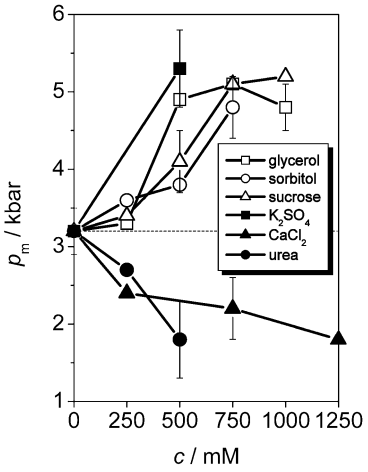

FIGURE 3: Denaturation pressure p_m of Snase at different concentrations (c) of cosolvents: (□) glycerol, (○) sorbitol, (△) sucrose, (■) potassium sulfate, (▲) calcium chloride, and (●) urea. For clarity, only selected error bars are given.

As high pressure causes compression of chemical bonds equivalent to changes in the force constants between the involved atoms, pressure-induced frequency shifts are frequently observed. While this effect is usually revealed by blue shifting IR bands, the hydrogen-bonded systems, such as proteins, exhibit red shifts (*45*). This is due to the strengthening of the O···H hydrogen bonds at the expense of the amide C═O bond. The frequency versus pressure plots generally feature a negative slope for inter- or intrachain hydrogen bond structures (such as α-helices and β-sheets), and a positive slope for water-bound random coil (*45*), but the slope is rather small (maximum of 0.2 cm^{-1} $kbar^{-1}$). These phenomena are clear evidence for the so-called elastic pressure effects on proteins. Deviations from this behavior can be attributed to pressure-induced conformational transitions.

Determination of m Values. The so-called m value is a measure of the degree of stabilization or destabilization of a protein at ambient pressure against unfolding in cosolvent systems and defined by

$$m = -\frac{\partial \Delta G°}{\partial c} \tag{4}$$

where c is the molar concentration of the corresponding cosolvent.

RESULTS

From the analysis of the denaturation profiles such as those shown in Figure 2B, the transition pressure of the pressure-induced unfolding (p_m) and the standard Gibbs free energy ($\Delta G°$) and volume ($\Delta V°$) change of unfolding of Snase have been obtained at 21 °C (room temperature). The p_m, $\Delta G°$, and $\Delta V°$ data for all cosolvents that have been investigated are plotted as a function of cosolvent concentration in Figures 3−5, respectively. The changes in the thermodynamic

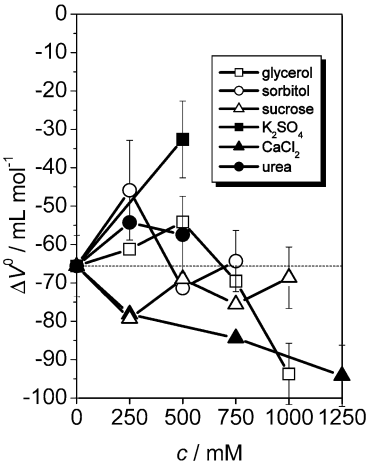

FIGURE 4: Volume change of pressure-induced unfolding, $\Delta V°$, of Snase at different cosolvent concentrations (c): (□) glycerol, (○) sorbitol, (△) sucrose, (■) potassium sulfate, (▲) calcium chloride, and (●) urea. For clarity, only selected error bars are given.

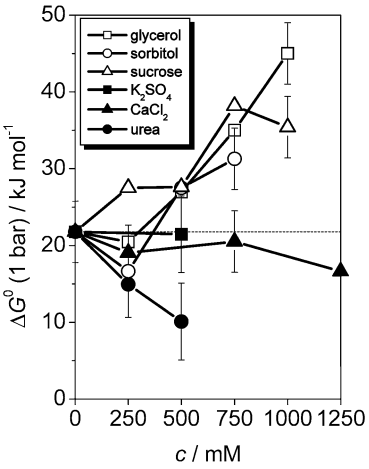

FIGURE 5: Standard Gibbs free energy change $\Delta G°$, extrapolated to 1 bar, for the pressure-induced unfolding of Snase at different molar concentrations (c) of cosolvents (□) glycerol, (○) sorbitol, (△) sucrose, (■) potassium sulfate, (▲) calcium chloride, and (●) urea. For clarity, only selected error bars are given.

parameters obtained with increasing cosolvent concentrations are summarized in Table 1.

Glycerol. Glycerol and other polyolic cosolvents have been shown to enhance protein stability (*24*, *46*, *47*). It has been proposed that the mechanism of this phenomenon is essentially due to a preferential hydration of the protein, i.e., the exclusion of the cosolvent molecules from the protein surface. For sugars (e.g., sucrose), glycerol, and polyhydric alcohols (e.g., ethylene glycol and sorbitol), the preferential hydration was found also to induce protein stabilization. Glycerol, being a strongly hydrophilic cosolvent, interacts strongly with the water and has a weaker affinity for the polar residues on the protein surface, thus leading to preferential hydration of the protein. Furthermore, the steric exclusion principle should allow a distance of closest approach of a cosolvent with a molecular volume greater than that of water. As a result, the volume fraction occupied by the cosolvent at the surface of the protein should be lower than that in the bulk solvent. Thermodynamically, this thus also manifests itself as preferential hydration. Upon denaturation, the surface of the protein increases, thus leading to an enhanced preferential hydration of the denatured protein relative to the native protein (*22*). Since this is energetically costly, the native protein is stabilized relative to the denatured protein.

The 50% denaturation pressure (p_m) as a function of molar glycerol concentration (c) is depicted in Figure 3. The error bars as given in the figures have been obtained from at least two independent experiments. The magnitude of the error bar is largely due to the inaccuracy of the pressure determination in a diamond anvil cell (±200−300 bar). The protein in pure buffer solution unfolds at ∼3.2 kbar and 21 °C. In comparison to the pure protein buffer solution data, there is only a slight change in the transition pressure p_m up to a glycerol concentration 250 mM, which corresponds to an ∼2.5 wt % glycerol solution. When $c \geq 500$ mM, a drastic stabilizing effect of glycerol is observed as seen by the ∼1.7 kbar higher denaturation pressure. In fact, also for a significant stabilization of proteins by polyhydric alcohols against temperature-induced denaturation at ambient pressure, a minimum concentration of ∼0.3 M is reported (*25*). For comparison, a stabilizing effect of 1.5 °C M^{-1} by glycerol has been found by DSC studies of the temperature-induced denaturation of Snase (*48*). Here, the temperature of unfolding (T_m) is used as an indication of protein stability. These results are in agreement with the general finding of a stabilizing effect of glycerol against temperature denaturation of proteins, which has been found for many other proteins, such as BSA (*49*), lysozyme (*50*, *51*), α-chymotrypsinogen (*24*, *51*), and Rnase A (*24*, *50*). With increasing glycerol concentrations, the 33 kDa protein of spinach photosystem II (hereafter termed the 33 kDa protein) also exhibits an increasing transition pressure upon pressure denaturation (*52*).

Figure 4 shows the corresponding $\Delta V°$ values as obtained from the intensity versus p plots using eq 1 as a function of glycerol concentration. For the pure Snase buffer solution, a $\Delta V°$ value of -66 ± 8 mL mol^{-1} was determined. Within the experimental error bar, a similar $\Delta V°$ for the pressure-induced unfolding of Snase was found with other techniques (*11*, *18*, *53*, *55*). For the lower glycerol concentrations up to 500 mM, a slight decrease in the absolute value of $\Delta V°$ is observed only, whereas at high concentrations of up to 1000 mM glycerol, the $|\Delta V°|$ value increases drastically, at a rate of approximately −80 mL mol^{-1} M^{-1} between 500 and 1000 mM and reaches −94 mL mol^{-1} for the 1 M glycerol solution. This decrease in $\Delta V°$ leads to no further increase in denaturation pressure p_m, however (Figure 3). A decreasing $\Delta V°$ has also been found for the 33 kDa protein up to 1 M glycerol (*52*). In contrast, an increasing $\Delta V°$ with increasing glycerol concentrations is found for the Arc repressor by Oliveira *et al.* (*56*). Interestingly, an effect opposed to the pressure-dependent data on Snase is seen in the temperature-dependent PPC experiments of Ravindra *et al.* (*48*). The ambient pressure data reveal a slight increase in $\Delta V°$(1 bar) of 2 mL mol^{-1} M^{-1} with increasing glycerol concentrations upon temperature-induced unfolding and denaturation. The different behavior might be due to the temperature and pressure dependence of $\Delta V°$ and, in addition, due to different unfolded state structures or, to be more precise, different populations of unfolded state conformations in the temperature- and pressure-induced unfolded states, respectively. The observed, if any, small changes in $\Delta V°$ for low glycerol concentrations agree (up to ∼0.75 M) with the data of Frye

Table 1: Changes in p_m, $\Delta V°$, and $\Delta G°$ for the Pressure-Induced Denaturation of Snase with Cosolvent Concentrations at pD 5.5 and 21 °C[a]

cosolvent	dp_m/dc (kbar M^{-1})	$d\Delta V°/dc$ (mL mol^{-1} M^{-1})	$d\Delta G°/dc$ (kJ mol^{-1} M^{-1})	dT_m/dc (°C M^{-1})	$d\Delta V°_{PPC}/dc$ (mL mol^{-1} M^{-1})	$d\Delta G°_{PPC}/dc$ (kJ mol^{-1} M^{-1})
glycerol	$(+1.7 \pm 0.3)$	(-20 ± 7)	$(+24 \pm 5)$	+1.5	+2	+4
sorbitol	$+2.0 \pm 0.3$	~0	$+17 \pm 5$	+8	+10	+5
sucrose	$+2.3 \pm 0.3$	~0	$+15 \pm 5$			
K_2SO_4	$+4.1 \pm 0.4$	$+65 \pm 7$	0 ± 7	+16	+33	*b*
urea	-2.8 ± 0.3	$+10 \pm 7$	-23 ± 5	−8	−25	−2
$CaCl_2$	-1.0 ± 0.3	-21 ± 5	-4 ± 7			

[a] The glycerol data show no linear behavior; data were therefore calculated only for the concentration range of 250−500 mM. The other values were obtained by fits over the whole concentration range that was covered. Additionally, we show the effect of cosolvent concentration on T_m, $\Delta V°_{PPC}$, and $\Delta G°_{PPC}$(21 °C) for the temperature-induced denaturation process of Snase at pD 5.5 as revealed from PPC experiments (*48*). [b] No exact value can be given, because ΔC_p could not be determined with sufficient accuracy.

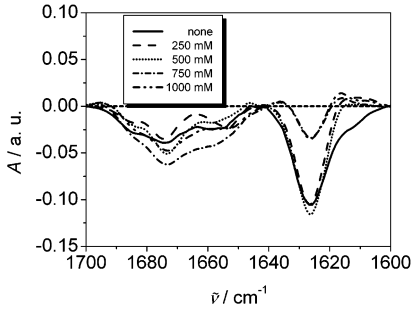

FIGURE 6: FT-IR difference spectra between the pressure-induced unfolded state and the native state of Snase upon addition of 0, 250, 500, 750, and 1000 mM glycerol (error bar in the absorbance scale of ±0.025).

et al. (*19*) for another stabilizing agent, xylose. They found that the $\Delta V°$ of pressure-induced unfolding of Snase is independent of the concentration of xylose up to ~1 M.

Figure 5 exhibits the concentration profile of the standard Gibbs free energy change upon unfolding, $\Delta G°$, at 1 bar, as obtained with eqs 2 and 3. For the pure Snase buffer solution, a $\Delta G°$ value of 21 ± 5 kJ mol^{-1} is obtained, which is in good agreement with data obtained previously by other methods (*11*, *18*, *53*, *55*). It can be clearly seen that $\Delta G°$ increases drastically with increasing glycerol concentrations above 250 mM glycerol. If a linear increase is assumed, linear regression results in a large *m* value of -24 ± 5 kJ mol^{-1} M^{-1}. We note that Ravindra *et al*. (*48*) also found an increase in $\Delta G°$ ($m = -4$ kJ mol^{-1} M^{-1}) with increasing glycerol concentrations for the temperature-induced unfolding process. The increase in the Gibbs free energy of unfolding proves that glycerol acts as a stabilizing cosolvent. This is also reflected in the increase in the denaturation pressure p_m as shown in Figure 3.

The corresponding FT-IR difference spectra, namely, the spectra of the native state subtracted from the spectra of the denaturated state at ~7 kbar, are shown in Figure 6. If pressure-independent extinction coefficients of the secondary structure elements are assumed (which is in fact expected because of the small energies that are involved), the changes observed in the FT-IR difference spectra directly reflect changes in the secondary structure elements. The magnitude of the decrease in the level of a secondary structure element in the denaturated state thus corresponds to a negative amplitude of the intensity in the difference spectra at the corresponding band position of that secondary structure element (see Table 2). The pressure-denatured state of pure Snase without cosolvent contains less β-sheet, α-helical, and turn structures than the native state as seen in Figure 6, in

Table 2: FT-IR Band Maxima of the Different Secondary Structure Elements of Snase in the Amide I′ Region Band in D_2O (*11*)

secondary structure element	wavenumber (cm^{-1})
β-sheet	~1627
	~1674
disordered	~1641
α-helical	~1651
turns	~1659
	~1666
	~1684

which the most drastic change is visible in the β-sheet region. For glycerol concentrations of 250 and 500 mM, no significant changes in the difference spectra of Snase in a pure buffer solution (without cosolvent) are observed. The addition of 750 and 1000 mM glycerol induces a denaturated state, however, which is closer to the native state conformation. This can be clearly seen when focusing on the wavenumber region being characteristic of β-sheet structures that appear at ~1627 cm^{-1}. At this position, the change in intensity at high glycerol concentrations drastically decreases, indicating a higher level of β-sheet structures remaining in the denaturated state compared to that of Snase in a pure buffer solution. Within the experimental error, no major changes are observed in the other wavenumber regions, which are characteristic of α-helices (~1651 cm^{-1}) and turns (~1659, ~1666, and ~1684 cm^{-1}). Hence, although the absolute value of $\Delta V°$ increases at very high glycerol concentrations, according to the IR data, the denatured state structure seems to be less unfolded. A less unfolded state for Snase has also been revealed for the temperature-induced denatured state in the presence of glycerol (*48*). One has to keep in mind that the apparent volume change of unfolding ($\Delta V°$), in addition to contributions from the loss of cavities upon unfolding of the secondary and tertiary structures, also drastically depends on changes in hydration or solvation. These are known to contribute significantly to the apparent expansion coefficient of the protein and to $\Delta V°$, in particular also when preferential hydration or direct ligand binding effects occur (*48*). We thus presume that for the highest glycerol concentrations, the change in $\Delta V°$ by solvation effects exceeds the intrinsic volume change upon unfolding.

Sorbitol. Several reports state that stabilization of proteins in the presence of sorbitol, which is approximately twice as large as glycerol, is also mainly due to it being excluded from the surface of the protein; i.e., in their presence, proteins are preferentially hydrated (*22*, *51*). Figure 3 shows the denaturation pressure p_m of Snase at 21 °C as a function of sorbitol concentration. p_m shifts at a rate of ~2.0 ± 0.3 kbar

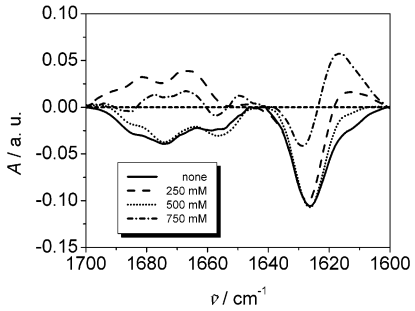

FIGURE 7: FT-IR difference spectra between the pressure-induced unfolded state and the native state of Snase upon addition of 0, 250, 500, and 750 mM sorbitol (error bar in the absorbance scale of ±0.025).

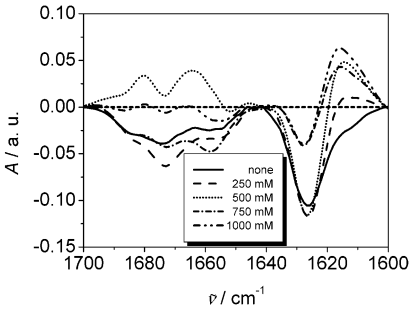

FIGURE 8: FT-IR difference spectra between the pressure-induced unfolded state and the native state of Snase upon addition of 0, 250, 500, 750, and 1000 mM sucrose (error bar in the absorbance scale of ±0.025).

M^{-1} to higher pressures. As expected, Snase has also been found to be markedly protected against thermal denaturation by sorbitol (*48*). In this case, the increase in unfolding temperature ($dT_m/dc = 8$ °C M^{-1}) is much larger than for glycerol ($dT_m/dc = 1.5$ °C M^{-1}). The increased thermostability is in accord with measurements on other proteins, such as Rnase A (*22*), α-chymotrypsinogen (*51*), and lysozyme (*51*).

Figure 4 depicts the sorbitol concentration dependence of the volume change of unfolding. Within the experimental error, $\Delta V°$ is found to be almost independent of the concentration of sorbitol up to 750 mM. We note that for the cosolvent xylose, $\Delta V°$ was found to be independent of the xylose concentration in pressure-dependent denaturation experiments (*19*). In contrast, the temperature-induced unfolding process reveals a drastic decrease in the absolute value of $\Delta V°$ determined by pressure perturbation calorimetry [$d\Delta V°(1\text{ bar})/dc = 10$ mL mol^{-1} M^{-1}]. However, this might largely be due to the temperature dependence of $\Delta V°(1\text{ bar})$ since an increasing sorbitol concentration increases the temperature of unfolding (*48*).

Figure 5 displays the standard Gibbs free energy of unfolding, $\Delta G°$, at 1 bar as a function of sorbitol concentration. Changes in $\Delta G°$ occur at sorbitol concentrations of 250 and 750 mM. If linear behavior is assumed for $\Delta G°(c)$, an m value of -17 ± 5 kJ mol^{-1} M^{-1} can be determined for c values of ≥250 mM. For comparison, DSC measurements yield an m value of -5 kJ mol^{-1} M^{-1} for the temperature-induced unfolding process. Hence, in contrast to the sorbitol concentration dependence of p_m and $\Delta G°$, we observe no major changes in $\Delta V°$.

FT-IR difference spectra were recorded to characterize the changes in the secondary structure of Snase in the presence of sorbitol (Figure 7). As indicated by the figure, the addition of up to 500 mM sorbitol does not drastically change the structure of the denatured state in comparison to Snase in pure buffer solution. In the presence of 750 mM sorbitol, however, the amount of remaining β-sheet structures in the denatured state becomes significantly larger than in the denaturated state without sorbitol.

Sucrose. Figure 3 exhibits the plot of the denaturation pressure p_m of Snase as a function of sucrose concentration. At 250 mM sucrose, there is no significant change in p_m. A similar finding has been observed for the 33 kDa protein (*52*). At higher concentrations, up to a sucrose concentration of 1 M, p_m increases roughly linearly at a rate of 2.3 ± 0.3 kbar M^{-1} with increasing sucrose concentrations. The stabilizing effect is thus similar to that of glycerol and sorbitol. Also, the 33 kDa protein is protected significantly by sucrose at concentrations above 250 mM against pressure-induced denaturation (*52*). A similar strong stabilizing effect of sucrose has also been observed in thermal denaturation studies on α-chymotrypsinogen A (*57*), Rnase (*50*, *57*), BSA (*58*), and whey proteins (*59*).

As seen in Figure 4, the $\Delta V°$ value of Snase with increasing concentrations of sucrose appears to be only slightly smaller than that of Snase in a pure buffer solution, and is nearly independent of the concentration of sucrose. A similar weak, if any, dependence of $\Delta V°$ on cosolvent concentration has been found for the disaccharide xylose by Frye *et al*. (*19*). In contrast, for the 33 kDa protein, a different behavior is observed. With increasing sucrose concentrations, the $\Delta V°$ value of the 33 kDa protein increases upon pressure-induced denaturation (*52*).

Figure 5 shows the $\Delta G°$ value of Snase at different sucrose concentrations. As for the case of glycerol and sorbitol, $\Delta G°$ increases significantly with increasing sucrose concentrations. An m value of -15 ± 5 kJ mol^{-1} M^{-1} is found by linear regression. A similar behavior is found for the stabilization of the disaccharide xylose by Frye *et al*. (*19*).

The changes in secondary structure upon pressure-induced unfolding in the absence and presence of sucrose are represented by the difference spectra shown in Figure 8. Up to a sucrose concentration of 500 mM, no significant changes in the conformation of the denatured state are observed with respect to Snase in a pure buffer solution. For higher sucrose concentrations, however, more β-sheet structure elements are retained in the pressure-denatured state.

K_2SO_4. The mechanism of stabilization by salt anions is essentially a reduction of the net positive charge on the protein through screening and anion binding. Stabilization of proteins via ligand binding is more effective at lower pH than at neutral pH (*60*). At a reduced protein surface charge and at higher anion concentrations, the Hofmeister effect prevails. Sulfate, for example, is known to be a good protein stabilizer, because sulfate strongly salts out nonpolar compounds and only weakly interacts and thus salts in the peptide group (*60*). Here we looked at the role of the kosmotropic salt K_2SO_4 in a solvent environment, at pH 5.5, where Snase is not at its isoelectric point ($pI \approx 9.5$), at a pH, however, where the net charge of the protein is still low and the direct binding tendency of the salt is still weak.

Figure 3 shows the denaturation pressure (p_m) of Snase at 21 °C and pD 5.5 plotted against the molar concentration of

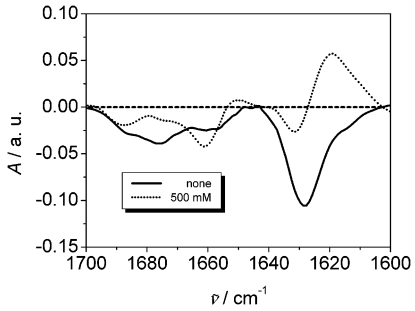

FIGURE 9: FT-IR difference spectra between the pressure-induced unfolded state and the native state of Snase upon addition of 0 and 500 mM K_2SO_4 (error bar in the absorbance scale of ±0.025).

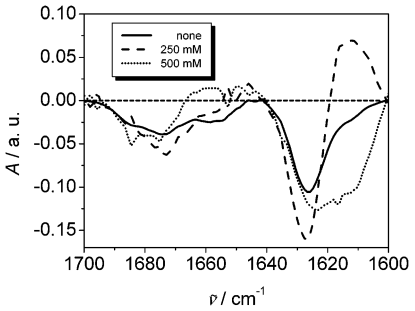

FIGURE 10: FT-IR difference spectra between the pressure-induced unfolded state and the native state upon addition of 0, 250, and 500 mM urea (error bar in the absorbance scale of ±0.025).

K_2SO_4. As can be clearly seen, addition of the salt decreases significantly the efficacy of pressure denaturation, at a dp_m/dc rate of 4.1 ± 0.4 kbar M^{-1}. An extraordinary large stabilizing effect of 16 °C M^{-1} by K_2SO_4 has been found for the temperature-induced denaturation of Snase by Ravindra *et al*. (*48*). Also, Nishimura *et al*. (*61*) found a significant protection against temperature-induced denaturation of Snase at pH 7.5, as well as a marked protection against urea-dependent unfolding by addition of Na_2SO_4. An extraordinary stabilizing effect of K_2SO_4 against temperature denaturation was also found for Rnase A (*20*, *31*).

Figure 4 shows that $|\Delta V°|$ is reduced by 33 mL mol^{-1} at 500 mM K_2SO_4. Ravindra *et al*. found a large increase in $\Delta V°$ of 38 mL mol^{-1} M^{-1} for the temperature denaturation of Snase that finally results in positive $\Delta V°$ values above 500 mM K_2SO_4 (*48*).

The $\Delta G°$ of Snase seems to be independent of the K_2SO_4 concentration up to 500 mM as seen in Figure 5 ($m = 0 \pm 7$ kJ mol^{-1} M^{-1}). In contrast, a large *m* value could be estimated for the temperature denaturation of Snase. Also, in urea-induced unfolding experiments, an increase in $\Delta G°$ is observed upon addition of 400 mM Na_2SO_4 (*61*). Our data indicate that, with regard to $\Delta G°$ values, Snase is not stabilized against pressure-induced unfolding in the presence of K_2SO_4; thus, the reason for the increased pressure stability, i.e., p_m value, is the smaller $|\Delta V°|$ value. A similar behavior is observed for the 33 kDa protein with the addition of 1 M NaCl (*20*).

The FT-IR difference spectra are shown in Figure 9. At 500 mM K_2SO_4, the amount of residual β-sheet structures in the denatured state is much higher than that in the denatured state of Snase without addition of the salt. The conservation of secondary structures is even more pronounced than for that observed at high concentrations of glycerol, sorbitol, and sucrose. Similar conclusions have been drawn for the effect of K_2SO_4 on the temperature-induced denatured state of Snase (*48*). The smaller changes in the secondary structure upon pressure denaturation should result in a greater compactness of the denatured state and hence a smaller $|\Delta V°|$ value (see Figure 4), which can indeed be deduced from the measured denaturation profiles.

Urea. It is well-known that the chaotropic agent urea causes destabilization of proteins by binding to the protein surface, in particular to the peptide bonds. Since the unfolded state presents many more sites for urea interaction, this state is stabilized relative to the folded state, and as a consequence, proteins unfold and attain a more or less random coil structure at high concentrations of urea (*27*, *28*). With increasing urea concentrations, the transition pressure decreases linearly, at a rate of −2.8 ± 0.4 kbar M^{-1} (Figure 3). A corresponding destabilizing effect of −8 °C M^{-1} is found for the temperature-induced denaturation of Snase by the DSC method (*48*). Many examples for the destabilizing effect of urea against temperature-induced denaturation can be found in the literature, such as for BSA (*49*), lysozyme (*31*), α-chymotrypsinogen (*31*), and Rnase T_1 (*23*). An extrapolation of p_m versus *c* to a p_m of 1 bar yields a urea concentration of 1.3 M for denaturation of Snase. This is in accord with the observation of the pH-induced unfolding of Snase in the presence of 1.5 M urea, where the denaturation sets in at pH ∼5.5 (*63*). In a urea-induced unfolding experiment at pH 7.5, Snase is denatured at ∼2 M urea (*61*).

Increasing the urea concentration to 500 mM leads to a slight increase in $\Delta V°$ of 10 ± 7 mL mol^{-1} M^{-1}. In contrast, for the thermal denaturation of Snase, $\Delta V°$ decreases with an increasing urea concentration, at a rate $d\Delta V°/dc$ of −25 mL mol^{-1} M^{-1} (*48*), likely largely due to the decrease in T_m as a function of urea and the large temperature dependence of $\Delta V°$.

The plot of $\Delta G°$ versus urea concentration is shown in Figure 5. With an increasing urea concentration, $\Delta G°$ deceases significantly, indicating a drastic decrease in the stability of the protein. A linear regression yields an *m* value of 23 ± 5 kJ mol^{-1} M^{-1}. For the temperature-induced unfolding transition, as revealed by DSC, an *m* value of 2 kJ mol^{-1} M^{-1} has been found (*48*).

The changes in the secondary structure in the presence of urea are revealed from the FT-IR difference spectra depicted in Figure 10. These spectra are rather noisy, because of the strong absorption bands of urea appearing at ∼1605, ∼1635, and 1685 cm^{-1}. Nevertheless, it is still clearly visible that the denatured state in the presence of 250 and 500 mM urea contains less β-sheet structure than that without urea. Hence, not only the temperature-denatured (*48*) but also the pressure-denatured state contains less ordered structures in the presence of urea.

$CaCl_2$. $CaCl_2$ is known as a rather potent protein destabilizer (*30*). From the view of the Hofmeister series, Cl^- lies close to the transition region between kosmotropic and chaotropic ions (*49*, *64*, *65*), whereas Ca^{2+} is a strong chaotropic ion. Thus, the overall chaotropic nature and destabilizing effect of $CaCl_2$ are a result of the strong chaotropic nature of the Ca^{2+} ion (*64*). $CaCl_2$ salts in the protein, but is not preferentially excluded from or bonded to the protein (*2*, *67*). On the other hand, Cl^- can act as a stabilizer at low pH (screening effect), where the net charge

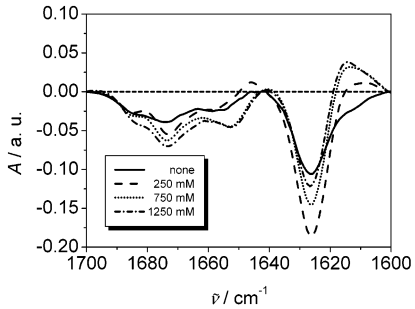

FIGURE 11: FT-IR difference spectra between the pressure-induced unfolded state and the native state of Snase with addition of 0, 250, 750, and 1250 mM $CaCl_2$ (error bar in the absorbance scale of ± 0.025).

of the protein is positive (*68*). As expected, the denaturation pressure (p_m) decreases with an increasing $CaCl_2$ concentration as shown in Figure 3 ($dp_m/dc = -1.0 \pm 0.3$ kbar M^{-1}). The destabilizing effect of $CaCl_2$ is thus roughly 3 times smaller than that of the destabilizing agent urea. Observations of a destabilizing effect ($dT_m/dc = -13$ °C M^{-1}) of $CaCl_2$ for the temperature stability are reported for Rnase A (*30*).

Figure 4 shows that, with increasing $CaCl_2$ concentrations, $\Delta V°$ decreases, at a rate of -21 ± 5 mL mol^{-1} M^{-1}. Contrary to the changes in denaturation pressure and volume, no major changes in $\Delta G°$ are observed ($m \approx 4$ kJ mol^{-1} M^{-1}) with an increasing $CaCl_2$ concentration (Figure 5), however. Consequently, the reason for the decrease in p_m is the increasing $|\Delta V°|$ value with an increasing $CaCl_2$ concentration. A similar behavior is also found for the 33 kDa protein by addition of 1 M NaCl (*20*).

The corresponding FT-IR difference spectra are shown in Figure 11. The data clearly show that the amount of remaining β-sheet structures in the denatured state of Snase decreases in the presence of $CaCl_2$, although in a highly nonlinear fashion. A large disruption of the residual β-sheet in the high-pressure unfolded state at 250 mM $CaCl_2$ is followed by an attenuation of this effect as the concentration of $CaCl_2$ is further increased. As only partial unfolding has been found for the pressure-induced denatured state of proteins such as Snase (*11*), this means that Snase becomes further unfolded in the presence of $CaCl_2$, whereby $|\Delta V°|$ is expected to increase, as has indeed been observed. The increasing $|\Delta V°|$ leads to a decrease in p_m with increasing $CaCl_2$ concentrations.

DISCUSSION

Our goal was to understand how high-pressure denaturation is affected in the presence of different cosolvents and how these data compare with the corresponding scenario for the temperature-induced unfolding process. In fact, the mechanisms of pressure- and temperature-induced unfolding of proteins seem to be different (penetration of water into a swollen, molten globule kind of state vs exposure of more hydrophopic residues, respectively), but as the balance of hydrophilic versus hydrophobic residues does not change very much upon unfolding, one might assume the difference between the Hofmeister series for pressure- and temperature-induced unfolding to be small. Minor differences being connected to the size of the cosolvent molecules could be expected, however.

Upon addition of the polyols glycerol, sorbitol, and sucrose to Snase solutions, an increase in the denaturation pressure p_m is observed. The protection by these cosolvents against pressure-induced denaturation is a consequence of the increased $\Delta G°$(1 bar) value with increasing cosolvent concentrations. Frye *et al*. (*19*) report on a similar stabilization mechanism and protection for the sugar xylose. The $\Delta V°$ seems to be almost independent of the concentration of these cosolvents, except for high glycerol concentrations, where $|\Delta V°|$ becomes larger, resulting in no further increase in p_m. It is likely that the lack of an effect of these polyols on the volume change of unfolding is due to two competing effects. With an increasing polyol concentration, the protein is preferentially hydrated; i.e., these agents are excluded from the protein with the result of an increase in $\Delta G°$. Since the unfolded state exposes more surface area than the folded state, the difference in preferential hydration between the two states is quite large, and this difference should become larger as the concentration of polyols increases. This in turn should lead to an increase in the absolute value of the volume change of unfolding, since $\Delta V°$ depends strongly on the degree of hydration. On the other hand, the FT-IR difference spectra demonstrate that the nature of the pressure-unfolded state changes significantly at very high polyol concentrations, and more for glycerol than the others. Indeed, the unfolded state retains significant β-sheet character at high polyol concentrations, indicating that the protein is less unfolded under these conditions. This should lead to a decrease in the absolute value of the volume change. Apparently, these two contradictory effects of polyols result in the near independence of the volume change of unfolding upon polyol concentration. With regard to temperature-induced denaturation, PPC investigations of the effect of polyols on the temperature-induced unfolding transition of Snase (*48*) revealed a different behavior of $\Delta V°(c)$, which might be largely due to the strong temperature dependence of $\Delta V°$, however.

Ruan *et al*. (*20*) pointed out that the change in $\Delta V°$ by cosolvents is an important contribution to the stabilization of the 33 kDa protein upon pressure denaturation. According to le Châtelier's principle, when pressure is applied, a reduction in the magnitude of the negative $\Delta V°$ will shift the equilibrium toward the native state, thus resulting in a protective effect. We observed a marked decrease in $|\Delta V°|$ in the presence of K_2SO_4, but without a significant change in $\Delta G°$. A similar behavior is found for the 33 kDa protein in the presence of NaCl (*20*). In the presence of K_2SO_4, the pressure-denatured state still contains a large population of ordered secondary structures, possibly resembling a molten globule kind of state. A similar structure is also expected for the corresponding temperature-induced unfolded state conformation (*48*). In the case of K_2SO_4, the decrease in the absolute value of the volume change of unfolding due to a more ordered or structured unfolded state, in contrast to the polyols, is not offset by the preferential hydration effect. Thus, the absolute value of $\Delta V°$ decreases significantly with this salt.

A more disordered structure in the pressure-denatured state, with regard to Snase without a cosolvent, is observed in the presence of $CaCl_2$, a strong chaotropic agent. Consequently, a linear increase in $|\Delta V°|$ and a pressure destabilization of Snase with an increasing $CaCl_2$ concentration are found, which leads to a slight reduction in $\Delta G°$, only.

Upon addition of urea, Snase unfolds at lower p_m values and the effect of increasing urea concentrations on $\Delta V°$ is small. As a result, $\Delta G°$ decreases markedly with increasing urea concentrations. Urea induces a more disordered structure; i.e., the denatured state contains fewer ordered secondary structure elements than Snase in a pure buffer solution, as seen from the infrared difference spectra.

These data thus indicate that polyhydric alcohols, sugars, and urea induce a significant change in the denaturation pressure (p_m) and $\Delta G°$ at 1 bar, whereas the $\Delta V°$, within experimental error, does not depend significantly on the cosolvent concentration, except for glycerol at 1 M. In contrast, the salts have almost no effect on $\Delta G°$ and the entire effect on the p_m is due to changes in $\Delta V°$.

From temperature- and urea-induced unfolding studies, it is known that at neutral pH the net charge of Snase is positive and repulsion destabilizes the structure of Snase. The addition of salt stabilizes the protein by acting as a counterion for the protein; thus, the net charge of the protein is reduced, in addition to the level of repulsion (screening effect). If the net charge is low or the salt concentration is high, the Hofmeister effect prevails (*61*). Then, the major effect results from changes in the water-bonded structure and preferential exclusion of the ions from the vicinity of the protein. From the Hofmeister series, ions K^+ and SO_4^{2-} are known as good and excellent water structure makers, respectively. K_2SO_4 is hence preferentially excluded from the protein surface accompanied by an increase in $\Delta G°$ as observed in temperature- and detergent-dependent as well as dialysis unfolding experiments (*2*, *64*, *67*). In contrast, in the pressure-dependent experiment, no significant changes in $\Delta G°$ are found, but a decrease in $|\Delta V°|$ is found. As a result of the stronger structured water-bonded network shell around the surface of Snase in the presence of K_2SO_4, a rather compact, molten globule kind of state is probably formed. Thus, the population of remaining secondary structure elements in the denatured state is higher, leading, consequently, to the reduced $|\Delta V°|$ values.

In contrast, $CaCl_2$, in particular because of the structure breaking properties of the Ca^{2+} ion, has the opposite effect. The water-bonded network becomes weaker in the presence of $CaCl_2$. No significant change in $\Delta G°$ is observed, but this salt leads to an increase in $|\Delta V°|$. In the presence of $CaCl_2$, which is probably not preferentially excluded from or bonded to the protein, the water-bonded network structure is weaker (*64*, *67*). As a result, the protein unfolds to a larger extent, resulting in a more disordered structure and larger $|\Delta V°|$, as seen in the difference spectra.

REFERENCES

1. Timasheff, S. N. (2002) Protein−solvent preferential interactions, protein hydration, and the modulation of biochemical reactions by solvent components, *Proc. Natl. Acad. Sci. U.S.A. 99*, 9721−9726.
2. Timasheff, S. N. (1993) The control of protein stability and association by weak interactions with water: How do solvents affect these processes? *Annu. Rev. Biophys. Biomol. Struct. 22*, 67−97.
3. Cacace, M. G., Landau, E. M., and Ramsden, J. J. (1997) The Hofmeister series: salt and solvent effects on interfacial phenomena, *Q. Rev. Biophys. 30*, 241−277.
4. Chalikian, T. V. (2003) Volumetric properties of proteins, *Annu. Rev. Biophys. Biomol. Struct. 32*, 207−235.
5. Heremans, K., and Smeller, L. (1998) Protein structure and dynamics at high pressure, *Biochim. Biophys. Acta 1386*, 353−370.
6. Winter, R. (2002) Synchrotron X-ray and neutron small-angle scattering of lyotropic lipid mesophases, model biomembranes and proteins in solution at high pressure, *Biochim. Biophys. Acta 1595*, 160−184.
7. Winter, R., and Jonas, J., Eds. (1999) *High-Pressure Molecular Science*, NATO ASI Series 358, Kluwer Academic Publishers, Dordrecht, The Netherlands.
8. Balny, C., Masson, P., and Heremans, K. (2002) High-pressure effects on biological macromolecules: from structural changes to alteration of cellular processes, *Biochim. Biophys. Acta 1595*, 3−10.
9. Winter, R., Ed. (2003) *Advances in High-Pressure Bioscience and Biotechnology II*, pp 490, Springer-Verlag, Heidelberg, Germany.
10. Panick, G., Malessa, R., Winter, R., Rapp, G., Frye, K. J., and Royer, C. A. (1998) Structure characterization of the pressure-denatured state and folding/refolding kinetics of staphylococcal nuclease by synchrotron small-angle X-ray scattering and Fourier-transform infrared spectroscopy, *J. Mol. Biol. 275*, 389−402.
11. Panick, G., Vidugiris, G. J. A., Malessa, R., Rapp, G., Winter, R., and Royer, C. A. (1999) Exploring the temperature−pressure phase diagram of staphylococcal nuclease, *Biochemistry 38*, 4157−4164.
12. Panick, G., and Winter, R. (2000) Pressure-induced unfolding/refolding of ribonuclease A: static and kinetic Fourier transform infrared spectroscopy study, *Biochemistry 39*, 1862−1869.
13. Desai, G., Panick, G., Zein, M., Winter, R., and Royer, C. A. (1999) Pressure-jump studies of the folding/unfolding of trp repressor, *J. Mol. Biol. 288*, 461−475.
14. Herberhold, H., and Winter, R. (2002) Temperature- and pressure-induced unfolding and refolding of ubiquitin: a static and kinetic Fourier transform infrared spectroscopy study, *Biochemistry 41*, 2396−2401.
15. Herberhold, H., Marchal, S., Lange, R., Scheyhing, C. H., Vogel, R. F., and Winter, R. (2003) Characterization of the pressure-induced intermediate and unfolded state of red-shifted green fluorescent protein: a static and kinetic FTIR, UV/VIS and fluorescence spectroscopy study, *J. Mol. Biol. 330*, 1153−1164.
16. Goossens, K., Smeller, L., Frank, J., and Heremans, K. (1996) Pressure-tuning the conformation of bovine pancreatic trypsin inhibitor studied by Fourier-transform infrared spectroscopy, *Eur. J. Biochem. 236*, 254−262.
17. Royer, C. A. (2002) Revisiting volume changes in pressure-induced protein unfolding, *Biochim. Biophys. Acta 1595*, 201−209.
18. Woenckhaus, J., Köhling, R., Thiyagarajan, P., Littrell, K. C., Seifert, S., Royer, C. A., and Winter, R. (2001) Pressure-jump small-angle X-ray scattering detected kinetics of staphylococcal nuclease folding, *Biophys. J. 80*, 1518−1523.
19. Frye, K. J., and Royer, C. A. (1997) The kinetic basis for the stabilization of staphylococcal nuclease by xylose, *Protein Sci. 6*, 789−793.
20. Ruan, K., Xu, C., Yu, Y., Li, J., Lange, R., Bec, N., and Balny, C. (2001) Pressure-exploration of the 33-kDa protein from the spinach photosystem II particle, *Eur. J. Biochem. 268*, 2742−2750.
21. Xie, G., and Timasheff, S. N. (1997) The thermodynamic mechanism of protein stabilization by trehalose, *Biophys. Chem. 64*, 25−43.
22. Xie, G., and Timasheff, S. N. (1997) Temperature dependence of the preferential interactions of ribonuclease A in aqueous co-solvent systems: thermodynamic analysis, *Protein Sci. 6*, 222−232.
23. Lin, T.-Y., and Timasheff, S. N. (1996) On the role of surface tension in the stabilization of globular proteins, *Science 5*, 372−381.
24. Gekko, K., and Timasheff, S. N. (1981) Mechanism of protein stabilization by glycerol: preferential hydration in glycerol-water mixtures, *Biochemistry 20*, 4667−4676.
25. Arakawa, T., and Timasheff, S. N. (1985) The stabilization of proteins by osmolytes, *Biophys. J. 47*, 411−414.
26. Timasheff, S. N., and Inoue, H. (1968) Preferential binding of solvent components to proteins in mixed water-organic solvent systems, *Biochemistry 7*, 2501−2513.
27. Herskovits, T. T. (1965) Conformation of proteins and Polypeptides. I. Extension of the solvent perturbation technique of difference spectroscopy to the study of proteins and polypeptides in organic solvents, *J. Mol. Chem. 240*, 628−638.

28. Pace, C. N., Laurents, D. V., and Thomson, J. A. (1990) pH dependence of the urea and guanidine hydrochloride denaturation of ribonuclease A and ribonuclease T1, *Biochemistry 29*, 2564−2572.
29. Schmid, F. X., and Baldwin, R. L. (1979) The rate of interconversion between the two unfolded forms of ribonuclease A does not depend on guanidinium chloride concentration, *J. Mol. Biol. 133*, 285−287.
30. von Hippel, P. H., and Wong, K. Y. (1965) On the conformational stability of globular proteins. The effects of various electrolytes and nonelectrolytes on the thermal ribonuclease transition, *J. Biol. Chem. 240*, 3909−3023.
31. Ramos, C. H. I., and Baldwin, R. L. (2002) Sulfate anion stabilization of native ribonuclease A both by anion binding and by the Hofmeister effect, *Protein Sci. 11*, 1771−1778.
32. Hynes, T. R., and Fox, R. O. (1991) The crystal structure of staphylococcal nuclease refined at 1.7 Å resolution, *Proteins: Struct., Funct., Genet. 10*, 92−105.
33. Shortle, D., and Lin, B. (1985) Genetic analysis of staphylococcal nuclease: identification of three intragenic "global" suppressors of nuclease-minus mutations, *Genetics 110*, 539−555.
34. Shortle, D., Meeker, A. K., and Gerring, S. L. (1989) Effects of denaturants at low concentrations on the reversible denaturation of staphylococcal nuclease, *Arch. Biochem. Biophys. 272*, 103−113.
35. Shortle, D., and Meeker, A. K. (1986) Mutant forms of staphylococcal nuclease with altered patterns of guanidine hydrochloride and urea denaturation, *Proteins 1*, 81−89.
36. Frye, K. J., Perman, C. S., and Royer, C. A. (1996) Testing the correlation between ΔA and ΔV of protein unfolding using *m* value mutants of staphylococcal nuclease, *Biochemistry 35*, 10234−10239.
37. Covington, A. K., Paabo, M., Robinson, R. A., and Bates, R. G. (1968) Use of the glass electrode in deuterium oxide and the relation between the standardized pD (pa_D) scale and the operational pH in heavy water, *Anal. Chem. 40*, 700−706.
38. Mikkelsen, K., and Nielsen, S. O. (1960) Acidity measurements with the glass electrode in H_2O-D_2O mixtures, *J. Phys. Chem. 64*, 632−637.
39. Byler, D. M., and Susi, H. (1986) Examination of the secondary structure of proteins by deconvolved FT-IR spectra, *Biopolymers 25*, 469−487.
40. Surewicz, W. K., Mantsch, H. H., and Chapman, D. (1993) Determination of protein secondary structure by Fourier transform infrared spectroscopy: A critical assessment, *Biochemistry 32*, 389−394.
41. Jackson, M., and Mantsch, H. H. (1995) The use and misuse of FT-IR Spectroscopy in the determination of protein structure, *Crit. Rev. Biochem. Mol. Biol. 30*, 95−120.
42. Haris, P. I., and Chapman, D. (1995) The conformational analysis of peptides using Fourier transform IR Spectroscopy, *Biopolymers 37*, 251−263.
43. Siminovitch, D. J., Wong, P. T. T., and Mantsch, H. H. (1987) Effects of cis and trans unsaturation of the structure of phospholipid bilayers: a high-pressure infrared spectroscopic study, *Biochemistry 26*, 3277−3287.
44. Reis, O., Winter, R., and Zerda, T. W. (1996) The effect of high external pressure on DPPC-cholesterol multilamellar vesicles: a pressure-tuning Fourier transform infrared spectroscopic study, *Biochim. Biophys. Acta 1279*, 5−16.
45. Dzwolak, W., Kato, M., and Taniguchi, Y. (2002) Fourier transform infrared spectroscopy in high-pressure studies on proteins, *Biochim. Biophys. Acta 1595*, 131−144.
46. Priev, A., Almagor, A., Yedgar, S., and Gavish, B. (1996) Glycerol decreases the volume and compressibility of protein interior, *Biochemistry 35*, 2061−2066.
47. Gekko, K., and Morikawa, T. (1981) Preferential hydration of bovine serum albumin in polyhydric alcohol-water mixtures, *J. Biochem. 90*, 39−50.
48. Ravindra, R., Royer, C. A., and Winter, R. (2003) Pressure perturbation calorimetric studies of the solvation properties and the thermal unfolding of staphylococcal nuclease, *Phys. Chem. Chem. Phys*. (in press).
49. Courtenay, E. S., Capp, M. W., and Record, M. T., Jr. (2001) Thermodynamics of interactions of urea and guanidinium salts with protein surface: relationship between solute effects on protein processes and changes in water-accessible surface area, *Protein Sci. 10*, 2485−2497.
50. Hammou, H. O., Plaza del Pino, I. M., and Sanchez-Ruiz, J. M. (1998) Hydration changes upon protein unfolding: cosolvent effect analysis, *New J. Chem. 22*, 1453−1461.
51. Back, F. J., Oakenfull, D., and Smith, M. B. (1979) Increased thermal stability of proteins in the presence of sucrose and polyols, *Biochemistry 18*, 5191−5196.
52. Ruan, K., Xu, C., Li, T., Li, J., Lange, R., and Balny, C. (2003) The thermodynamic analysis of protein stabilization by sucrose and glycerol against pressure-induced unfolding, *Eur. J. Biochem. 270*, 1654−1661.
53. Vidugiris, G. J. A., Markley, J. L., and Royer, C. A. (1995) Evidence for a molten globule-like transition state in protein folding from determination of activation volumes, *Biochemistry 34*, 4909−4912.
54. Takeda, N., Kato, M., and Taniguchi, Y. (1995) Pressure- and thermally induced reversible changes in the secondary structure of ribonuclease A studied by FT-IR spectroscopy, *Biochemistry 34*, 5980−5987.
55. Lassalle, M. W., Yamada, H. J., and Akasaka, K. (2000) The pressure−temperature free energy-landscape of staphylococcal nuclease monitored by ^{1}H NMR, *J. Mol. Biol. 298*, 293−302.
56. Oliveira, A. C., Gaspar, L. P., Da Poian, A. T., and Silva, J. L. (1994) Arc repressor will not denature under pressure in the absence of water, *J. Mol. Biol. 240*, 184−187.
57. Lee, J. C., and Tiamasheff, S. N. (1981) The stabilization of proteins by sucrose, *J. Biol. Chem. 256*, 7193−7201.
58. Baier, S., and McClements, D. J., Jr. (2001) Impact of preferential interactions on thermal stability and gelation of bovine serum albumin in aqueous sucrose solutions, *J. Agric. Food Chem. 49*, 2600−2608.
59. Kulmyrzaev, A., Bryant, C., and McClements, D. J. (2000) Influence of sucrose on the thermal denaturation, gelation, and emulsion stabilization of whey proteins, *J. Agric. Food Chem. 48*, 1593−1597.
60. Matsue, S., Fujii, T., and Miyawaki, O. (2001) Effects of water activity and aqueous solvent ordering on thermal stability of lysozyme, α-chymotrypsinogen A, and alcohol dehydrogenase, *Int. J. Biol. Macromol. 28*, 343−349.
61. Nishimura, C., Uversky, V. N., and Fink, A. L. (2001) Effect of salts on the stability and folding of staphylococcal nuclease, *Biochemistry 40*, 2113−2128.
62. Ravindra, R., and Winter, R. (2003) Pressure perturbation calorimetric studies of the solvation properties and the thermal unfolding of proteins in solution, *Z. Phys. Chem. 217*, 1221−1243.
63. Filfil, R., and Chalikian, T. V. (2000) Volumetric and spectroscopic characterization of the native and acid-induced denatured states of staphylococcal nuclease, *J. Mol. Biol. 299*, 827−842.
64. Wiggins, P. M. (1997) Hydrophobic hydration, hydrophobic forces and protein folding, *Physica A 238*, 133−128.
65. Eggers, D. K., and Valentine, J. S. (2001) Molecular confinement influences protein structure and enhances thermal protein stability, *Protein Sci. 10*, 250−261.
66. Neagu, A., Neagu, M., and Der, A. (2001) Fluctuations and the Hofmeister effect, *Biophys. J. 81*, 1285−1294.
67. Arakawa, T., and Timasheff, S. N. (1984) Mechanism of protein salting in and salting out by divalent cation salts: balance between hydration and salt binding, *Biochemistry 23*, 5912−5923.
68. Makhatadze, G. I., Lopez, M. M., Richardson, R. M., III, and Thomas, S. T. (1998) Anion binding to the ubiquitin molecule, *Protein Sci. 7*, 689−697.

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