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Osmolyte-Induced Perturbations of Hydrogen Bonding Between Hydration Layer Waters: Correlation with Protein Conformational Changes

Feng Guo and Joel M. Friedman*

Department of Biophysics and Physiology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, New York, U.S.A. 10461

Abstract

Gadolinium vibronic side band luminescent spectroscopy (GVSBS) is used to probe osmolyte-induced changes in the hydrogen bond strength between first and second shell waters on the surface of free Gd^{3+} and Gd^{3+} coordinated to EDTA and to structured calcium binding peptides in solution. In parallel, Raman is used to probe the corresponding impact of the same set of osmolytes on hydrogen bonding among waters in the bulk phase. Increasing concentration of added urea is observed to progressively weaken the hydrogen bonding within the hydration layer but has minimal observed impact on bulk water. In contrast polyols are observed to enhance hydrogen bonding in both the hydration layer and the bulk with the amplitude being polyol dependent with trehalose being more effective than sucrose, glucose or glycerol. The observed patterns indicate that size and properties of the osmolyte as well as the local architecture of the specific surface site of hydration impacts preferential exclusion effects and local hydrogen bond strength. Correlation of the vibronic spectra with CD measurements on the peptides as a function of added osmolytes shows an increase in secondary structure with added polyols and that the progressive weakening of the hydrogen bonding upon addition of urea first increases water occupancy within the peptide and only subsequently does the peptide unfold. The results support models in which the initial steps in the unfolding process involves osmolyte induced enhancement of water occupancy within the interior of the protein.

Introduction

Proteins occur and function in osmolyte-rich environments. Naturally occurring osmolytes are small organic or inorganic molecules that help maintain cellular volume, protect protein structure/function and stabilize macromolecules under extreme conditions^{1–4}. Sugars, polyols and urea are typical osmolytes that are known to perturb protein stability and dynamics^{3–10}. It is known that some microorganisms use sugars such as trehalose to survive anhydrobiosis and other stress conditions^{11,12}. Urea is frequently used to induce protein unfolding. In nature some organism or living cells are exposed to high concentration of urea. To counter urea denaturing effect on protein in vivo, cells utilize small organic molecules such as trimethylamine N-oxide (TMAO) which can stabilize protein structure and allow for retention of activity in the presence of urea^{1–4}.

Tel: 718-430-3591, jfriedma@aecom.yu.edu.

Supporting Information

The absent effect of PEG400 on OH VSB of free Gd^{3+} or EDTA- Gd^{3+}

This information is available free of charge via the Internet at <http://pubs.acs.org>.

Exposing the mechanisms through which osmolytes impact protein properties is necessary both to understand how proteins function in vivo and to optimize the utilization of proteins for biomedical and bioengineering applications. There are many proposed mechanisms for osmolyte effects^{13–23}. Preferential hydration is one of the most accepted models^{19–22}. It emphasizes how preferential exclusion of specific osmolytes from the hydration layer impacts protein stability. This thermodynamically based model, which indicates that the differences in distribution are due to differences in enthalpic rather than entropic factors, does not provide a detailed molecular level account of why there are different distributions of osmolytes in the hydration layers versus in the bulk. Recently there have been studies indicating that osmolytes^{24–33} can impact the organizational structure of water (weakening or strengthening of the hydrogen bonding) and through this process affect macromolecular stability. The influence of osmolytes on protein function can occur through this osmolyte-induced perturbation of water structure. Protein function is predicated upon the protein being dynamically active. Functionally important protein motions require hydration waters to act as a lubricant. The concept is that protein assumes a wide variety of isoenergetic but functionally distinct conformational states and substates that are organized hierarchically^{34,35}. Functionality requires that a given protein molecule undergo an entropic search to access the most reactive conformational states/substates. The water-lubricated protein motions facilitate this search by lowering the enthalpic barriers between substates. Under these conditions the protein dynamics associated with the entropic search are slaved to solvent fluctuations^{36–40} and can be grouped hierarchically based on which category of solvent motion to which they are slaved^{37–42}. Osmolyte-induced weakening or strengthening of the hydration bonding among hydration waters could therefore alter the properties of dynamics slaved to the motion of those waters.

These proposed linkages between osmolyte-induced hydration changes and protein perturbations are still unclear. It is therefore of great interest to experimentally probe hydration changes due to biologically relevant osmolytes and correlate these to osmolyte induced changes in such protein properties as structure, function, dynamics and stability. Experimental testing of these proposed linkages requires probing hydration shell and bulk waters as a function of added osmolytes and correlating the water effects with changes in protein properties.

Probing water interactions in the hydration layer of a protein is a challenge. Water hydrogen bonding can be readily probed spectroscopically by IR or Raman; however, these methods probe all waters within the probe volume. There is a need for a probe that is both specifically sensitive to local hydration water behavior and amenable to systematic variation of osmolyte species and concentration. This last feature precludes NMR and crystallography as a suitable probe. Ultra-fast spectroscopic techniques^{43–47} have been used to allegedly probe water dynamics on the surface of proteins as reflected in rapid dynamic Stokes shifts which may include rapid protein dynamics as well. These studies can not directly probe how osmolytes alter the hydrogen bonding among hydration shell waters.

In the present study, Raman is used to probe the effect of added osmolytes on the hydrogen bonding within bulk water. Hydrogen bonding changes in the hydration layer as a function of added osmolytes are probed using Gd^{3+} vibronic side band luminescence spectroscopy (GVSBLs)^{48–55}. The specificity and utility of this technique is based on earlier findings that GVSBLs can provide an infrared-like vibrational spectrum derived exclusively from molecules surrounding Gd^{3+} , including those of coordinated first shell waters^{50–52}. Changes in the vibrational frequencies from the first shell water molecule reflect changes in their hydrogen bonding to outer sphere solvent molecules^{56–58}. As such, GVSBLs is a potent molecular-level probe of hydrogen bonding within the hydration shell of Gd^{3+} . GVSBLs is derived from the Gd^{3+} luminescence spectrum in which there are weak vibronic (vibration

plus electronic) side bands (VSBs) associated with pure electronic transition from the lowest excited electronic state to the ground electronic state^{49,50}. It can provide local specificity in detecting how osmolytes perturb hydrogen bonding between the “probe” waters on the surface of Gd^{3+} cation and those in the adjacent layer. This local environment specificity is particularly useful if one desires to probe the changes in water hydrogen bonding near the surface of a Ca^{2+} ion binding protein, as Gd^{3+} cation readily replaces the similarly sized Ca^{2+} cation in chelates and calcium binding sites in proteins and peptides without disruption of the native structure associated with the calcium coordinated species^{59–62}. The technique allows for probing of how a series of added osmolytes influence hydrogen bonding among hydration waters both for the free Gd^{3+} in solution and for Gd^{3+} coordinated to a series of biologically relevant calcium binding sites. Comparisons of patterns of hydrogen bonding switches are more straightforward by using two species of lanthanide binding tag (LBT) peptides, SE2 and mSE3, that have a single calcium binding site as gadolinium coordinating models^{63,64}. There is no water in the binding site of SE2 while there is one water in the binding site of mSE3, which is weakly hydrogen bonded to surface hydration water of the mSE3 peptide⁶⁴ (and from unpublished studies of Dr. Imperiali, MIT).

In addition to using the vibronic spectrum to probe the effect of osmolytes on the hydration waters of free and coordinated Gd^{3+} cation, this study also utilizes far-UV CD to follow the osmolyte induced changes in secondary structure of the Gd^{3+} coordinated peptides. By simultaneously monitoring the hydration waters and the secondary structure as a function of added osmolyte, it becomes possible to establish whether disruption of secondary structure is concomitant with or subsequent to osmolytes induced changes in the hydration shell waters.

The results show that osmolytes can perturb hydrogen bonding among waters in both the bulk layer and the hydration layer. The magnitude of the perturbation is dramatically different for the bulk and hydration layers and is highly dependent on the specific osmolytes. The hydrogen bonding changes are additive when multiple osmolytes are combined. It is found that there is a correlation between perturbations of local hydration water structure and peptide stability and secondary structure, strongly indicative of a key role for water in mediating osmolyte effects on properties of macromolecules.

Experimental Methods

Materials preparation

SE2 and mSE3 peptides were obtained as a generous gift from Dr. Imperiali (Dept of Chemistry, MIT). All other materials were purchased from Sigma - Aldrich and used without further purification.

Osmolytes were either added from a stock aqueous solution, or as a solid where appropriate. For peptide- or EDTA-free GdCl_3 samples osmolyte titrations were made by mixing GdCl_3 stock solution with suitable amount of osmolytes to achieve final concentration of 0.5 M GdCl_3 with a desired concentration of osmolytes.

Solutions of 100 mM EDTA, 1 mM apo-mSE3 and 1 mM apo-SE2 were prepared in 10mM HEPES buffer at pH 7.0 with 80 mM, 1 mM or 1 mM GdCl_3 , respectively. Osmolytes were added to these samples to compare their specific effects on hydration shell waters of coordinated Gd^{3+} . SE2 and mSE3 peptide samples for CD measurements were prepared to achieve the final concentration from 20 μM to 200 μM in 10mM HEPES buffer at pH 7.0. Osmolytes were titrated to induce secondary structural changes.

Circular dichroism and luminescence measurements

Far-UV CD spectra (200–250 nm) were recorded with a J-815 spectropolarimeter (Jasco Inc., Easton, MD) at room temperature. Samples were measured in a 0.1 cm path length cuvette. Multiple scans were averaged, and baselines were subtracted. CD spectra are presented as the molar CD absorption coefficient $\Delta\epsilon_M (= \epsilon_L - \epsilon_R, \text{ where } \epsilon_L \text{ and } \epsilon_R \text{ are the molar absorption coefficients for left and right handed circularly polarized light})$. This value is calculated on the mean residue weight basis of the molar concentration of peptide. The standard free energy of protein or peptide unfolding ΔG_{N-D} and m-value can be obtained by fitting CD spectra at 220 nm to a two-state model as described elsewhere⁶⁵. The peptide α -helix content was calculated from a mean residue weighted molar extinction coefficient $\Delta\epsilon_{MRW,222 \text{ nm}, M}$ at 222 nm, based on the following formula: $\Delta\epsilon_{MRW,222 \text{ nm}, M} = [\theta_{222 \text{ nm}}]/3300 = 100 \times \theta_{222 \text{ nm}}/cnl$, where $\theta_{222 \text{ nm}}$ is ellipticity in millidegrees at 222 nm, C is concentration of peptide in mM, n is the number of amino acid residues and l is the path-length in cm. Percent helicity was calculated assuming 100% $\Delta\epsilon_{MRW,222 \text{ nm}, M} = -9.545 \text{ M}^{-1}\text{cm}^{-1}$ and that only α -helical structure contributes to the intensity at 222 nm⁶⁶. This calculation gives a good estimation of the percentage of α -helix as previously reported for short peptides^{67,68}.

The Raman spectrum of the OH stretching mode of bulk water and the GVSBLs were acquired on a QuantaMaster Model QM-4/2000SE enhanced performance scanning spectrofluorometer (Photon Technology International, Lawrenceville, NJ). All of the aqueous samples were contained in quartz cuvettes and probed using 90° excitation geometry. The Raman spectra were generated using a 350 nm excitation. For GVSBLs the excitation wavelength was around 273 nm where Gd^{3+} has a relatively enhanced absorption cross section. Continuous wave excitation to generate GVSBLs was used for samples of unbound Gd^{3+} and of Gd^{3+} coordinated to EDTA. Time gated GVSBLs was used for Gd^{3+} coordinated to mSE3/SE2 samples to eliminate short lived fluorescence signals from the peptides. The gated GVSBLs were accumulated from a time point starting from 100 μs through 1000 μs after the pulsed excitation. The luminescence spectrum was scanned and recorded from 290 nm to 400 nm. To rule out the possibility that the observed weak signals were due to Raman scattering (for the un-gated signals) or non- Gd^{3+} -derived fluorescence, excitation profiles were routinely conducted for observed peaks in emission spectra. The emission monochromator slits were adjusted in order to obtain suitable signal to noise ratios. The luminescence spectra were plotted as intensity vs. frequency shift (cm^{-1}) from the electronic peak at 311 nm ($32,154 \text{ cm}^{-1}$) to represent the energy difference that is equal to the vibration frequency of coordinated ligands. The spectra were processed and analyzed using GRAMS/32 AI (6.00) for background subtraction, smoothing and peak analysis.

The accuracy of the high resolution fluorometer achieves $\pm 0.1 \text{ nm}$, which equals to $\pm 8 \text{ cm}^{-1}$ in the region of the vibronic side band by converting spectra from wavelength (nm) to wavenumber (cm^{-1}) ($\Delta\nu(\text{cm}^{-1}) = 10^7 \times (\Delta\lambda(\text{nm})/\lambda_1 \cdot \lambda_2)$). The vibronic side band is at around 346 nm. Therefore: $\Delta\lambda = \pm 0.1 \text{ nm}$, $\lambda_1 \approx \lambda_2 = 346 \text{ nm}$. $\Delta\nu \sim \pm 8 \text{ cm}^{-1}$. Another contributing uncertainty in the peak position assignment is the random error that arises from the difference from one trace to the next, which can be controlled by averaging multiple repeated traces. The number of averaged traces is based on the signal to noise associated with the individual trace. For protein-free samples where the signal to noise is very high, an average of three traces is typical whereas for samples with weak signals, averaging can consist of as many as one hundred scans. Ordering of small peak shifts are verified by directly overlapping the normalized average spectra and comparing vibronic bands from samples with different additives. The intense electronic band at $\sim 311 \text{ nm}$ which does not shift under these conditions serves as a wavelength/frequency reference point. Under these conditions the entire peak band profile contributes to the evaluation (as opposed to just comparing peak positions) permitting facile determination if an entire band has shifted

relative to another band given that the line shape/band width does not change. It is consequently possible to detect shifts smaller than the resolution associated with a given wavelength, similarly to difference spectra analysis. The ordering with respect to which samples shift more than others is therefore still valid even though the actual magnitude of the small shifts could be less than 10 cm^{-1} and claims of specific shifts in excess of 10 cm^{-1} are precisely accurate. Most of the measurements have been repeated on different samples on different occasions yielding consistent results and the trends are very clear and do not rest upon the exact values associated with the smallest shifts or small differences among many samples. The issues are similar to those associated with many FTIR and Raman studies

The frequency of the VSB arising from the water stretching mode ($\sim 3300\text{ cm}^{-1}$) measures hydrogen bonding between the first shell waters and outer group^{50,55}. A decrease or increase of this frequency is correlated with an increase or decrease in hydrogen bond strength respectively.

Results

Raman spectra: Osmolyte specific influences on bulk water

Figure 1 shows the Raman band associated with the OH stretching mode of water for aqueous samples containing free GdCl_3 and GdCl_3 coordinated to either EDTA or the mSE3 peptide as a function of different urea concentrations. The figure shows that the OH stretching frequency of water is not responsive to either the addition of urea or the presence of the added EDTA or peptides. Previous studies have also shown that the vibrational spectrum of bulk water is minimally influenced by the addition of urea⁶⁹.

Water Raman spectrum of samples containing free GdCl_3 and GdCl_3 coordinated to either EDTA or mSE3 were also generated as a function of added glycerol, trehalose and PEG400. It is noted that the OH stretch region provides only general information on the hydrogen bonding network as a whole since the individual contributions from the OH modes of polyols/sugars and water cannot be separated^{70,71}. The results here are presented as the difference spectra, resulting from the weighted subtraction (according to volume ratio used to make a polyol/water mixture) of the OH Raman spectra of the neat polyol sample from that of the osmolyte/water mixture. It was not possible to get the OH Raman from pure sugar (trehalose) powder; however, the low concentration of the added sugars compared to the water allows us to use the unprocessed Raman spectra of the sugar containing solutions with confidence that the contribution of OH stretch mode from sugars is relatively insignificant. As with the urea, the spectrum for a given addition of polyol is insensitive to the presence of peptide or EDTA at the concentrations used in the study. Shown in Figure 2 is a clear pattern where the progressive addition of PEG400, trehalose and glycerol all progressively decreases the OH water stretching frequency. The concentration dependent water Raman OH stretch frequencies are shown in Figure 3 for PEG400, trehalose and glycerol. For all osmolytes evaluated the magnitude of vibrational frequency shifts correlates with the concentration of added osmolyte. The possible complications due to the overlap between the OH stretching modes from water and polyols at the very high polyol concentrations can be overcome by using the OH bending mode at around 1650 cm^{-1} ⁷⁰. The band does not overlap with any modes of polyols. Shifts in the OH bend mode are inversely proportional to OH stretch and thus varies linearly with hydrogen bond strength⁵⁸. It has been shown⁷⁰ that as glycerol concentration increases the OH bend frequency shifts to a higher wavenumber, indicating stronger hydrogen bonding of water which is consistent with our presented results. In the presence of peptides and proteins it is difficult to use this band due to overlap from carbonyl and carboxyl stretching modes.

Gd³⁺ Vibronic Spectra: Osmolyte-specific effects on the hydrogen bond strength between first and second shell waters of free and coordinated Gd³⁺

In contrast to the lack of sensitivity of the OH stretching mode in bulk water Raman spectra to the presence of added chelate or peptide, the OH stretching frequency derived from the vibronic spectrum of Gd³⁺, does show a dependence on the coordination environment of the Gd³⁺. There is a progression towards higher OH stretching frequencies (weaker hydrogen bonding) between first and second shell waters, in going from free Gd³⁺ (3270 cm⁻¹) to Gd³⁺ coordinated to Ca²⁺ binding sites of EDTA (3310 cm⁻¹), and from EDTA to Gd³⁺ tightly coordinated to the peptide of mSE3 (3391 cm⁻¹).

The vibronic spectra as a function of added urea are shown in Figure 4a for free GdCl₃ and in Figure 4b for EDTA coordinated Gd³⁺. Figure 4c compares the urea dependent vibronic OH stretching frequency increase for free GdCl₃ samples and EDTA coordinated Gd³⁺. Also included are the results using Raman showing no effect due to the added urea. In contrast to absence of an observable effect in the Raman, urea induces a large vibrational stretching frequency increase in the vibronic spectrum for both free and EDTA-coordinated Gd³⁺. For EDTA samples the vibronic band derive from the C=O (1600 cm⁻¹) or C-H (3300 cm⁻¹) stretching remains unchanged upon addition of urea as was observed previously for the addition of other osmolytes ⁷². The systematic increase of OH stretching vibration frequency for both free and EDTA coordinated Gd³⁺ scales with urea concentration. The frequency increase is greater for EDTA coordinated Gd³⁺ than for the free Gd³⁺.

Figure 5 shows the glycerol concentration dependent frequency shifts in the OH stretching frequency from the vibronic spectra Gd³⁺ either free or coordinated (EDTA and mSE3). Also shown is the glycerol dependent shift in the OH stretching frequency derived from the Raman spectrum. It can be seen that glycerol causes a decrease in the OH stretching frequency for both bulk water (Raman) and first hydration shell water (vibronic) but the patterns are distinctly different for the two categories of waters. The effect of glycerol on hydration water becomes noticeable only when the concentration of glycerol is above 50% (v/v) (6.852 M). No detectable shifts of OH vibronic spectrum is observed at low concentration (<50% (v/v)). Glycerol is more effective in influencing the bulk water OH stretching frequency than those of the hydration waters. It can also be seen that the magnitude of the vibronic frequency shifts is less for coordinated Gd³⁺ compared to free Gd³⁺. This point is especially evident for the peptide. Thus the decrease in frequency appears to scale with the degree to which the waters coordinated to the Gd³⁺ are constrained, with free Gd³⁺ < Gd³⁺-EDTA < Gd³⁺-peptide.

A summary of sugar concentration dependent decreases of the OH stretching frequencies from Gd³⁺ vibronic spectra by sugars is shown in Figure 6. Whereas the pattern is similar for all sugars, the slope of the curves is sugar specific with tagatose and trehalose being more effective than glucose and sucrose with respect to decreasing the frequency. The comparison between Figure 5 and Figure 6 indicate that sugars are more effective than glycerol in decreasing the hydration water OH stretching frequencies on a per mole basis for both free and coordinated Gd³⁺.

In contrast to urea, sugars and glycerol, the addition of PEG400 causes no change in the OH vibronic spectra for either free Gd³⁺ or EDTA-Gd³⁺ in solution. This lack of an effect in the vibronic spectrum is in marked contrast to the large PEG400-induced frequency decrease observed in the water Raman spectrum under comparable concentrations of added PEG (shown in supplemental figure 1).

Urea and trehalose have opposite effects with respect to shifting the OH vibronic peak position. The results shown in Figure 7a for free GdCl₃ and Figure 7b for EDTA

coordinated Gd^{3+} indicate that the effects are additive when both osmolytes are added. The introduction of 1.0 M trehalose and 2.0 M urea results in a shift the vibronic spectrum to a higher wavenumber by 10 cm^{-1} relative to osmolyte-free GdCl_3 sample. This magnitude equals the algebra sum of individual frequency shifts by 1.0 M trehalose (-27 cm^{-1} , a decrease in OH stretching) and 2.0 M urea (36 cm^{-1} increase in OH stretching), respectively. A similar result in the vibronic spectrum is observed for EDTA- Gd^{3+} samples (Figure 7b). In this case the introduction of 1.0 M trehalose (which alone causes a decrease $\sim 18\text{ cm}^{-1}$) and 1.0 M urea (which alone causes an increase of 23 cm^{-1}) produces an additive increase of OH stretching by ca. 3 cm^{-1} .

Osmolyte-induced changes in peptide secondary structure correlates with hydration modifications

Far UV circular dichroism in concert with Gd^{3+} vibronic spectroscopy were used to determine for the peptides how the osmolyte-induced changes in hydrogen bonding pattern correlate with osmolyte-induced changes in secondary structure of these ordered peptides. As shown in Figure 8 the Gd^{3+} bound mSE3 peptide in the absence of any osmolyte adopts a folded secondary structure in solution. The CD spectrum has a minimum at ca 222 nm, indicating the presence of α -helical structure⁷³. The estimate of α -helicity calculated on the basis of observed molar ellipticity at 222 nm is 15%. This is in agreement with the crystallographic structure prediction of 17.6%⁶⁴. CD measurements as a function of peptide concentration were used to show that this secondary structure was not the result of dimer formation or aggregation as seen (Figure 9) from the absence of a dependence of $\Delta\epsilon_{\text{MRW},222\text{ nm},\text{M}}$ on concentration over 10-fold range.

The addition of bioprotective osmolytes such as glycerol, tagatose and trehalose stabilizes the secondary structure as reflected in an increase in the α -helical content of the peptide as shown in Figure 8 where the minima at ca 222 nm become more negative upon the addition of glycerol or trehalose. The osmolyte-induced increase in α -helical content correlates with the amplitude of the decrease in the OH stretch frequency observed from the vibronic side band. Figure 10 displays the linear relationship between α -helical content and the OH stretch mode frequency shifts of the hydration water. The correlation plot holds for all of the tested bioprotective osmolyte species. It is noted that the α -helical content of the peptide is only correlated to hydrogen bonding within the hydration layers and not the bulk layer as reflected in the Raman spectrum. For instance glycerol at low concentration ($< 50\%$ (v/v)) dramatically decreases the bulk water OH stretch frequency while the secondary structure of the peptide is not noticeably perturbed.

Figure 8 shows that with the addition of urea the peptides adopt a secondary structure containing a more unstructured conformation consistent with unfolding. The unfolding processes of holo-mSE3 and SE2 peptides monitored by far-UV CD at 220 nm are shown in Figure 11. Holo-mSE3 and holo-SE2 have similarly high stability, as most other holo calcium binding peptides or proteins. The ΔG° and m -value are calculated to be 15.75 kcal/mol and -2.77 kcal/mol M for mSE3 and 15.07 kcal/mol and -2.70 kcal/mol M for SE2. These values are consistent with other reported results of similar calcium binding peptides^{74,75}.

The urea-induced OH stretching frequencies of the coordinated water to the bound Gd^{3+} in the binding site are compared in Figure 11 along with the urea-induced denaturing process for both peptides. Also shown is the OH stretching frequency of the hydration waters to the free Gd^{3+} and EDTA- Gd^{3+} as a function of added urea. For free Gd^{3+} and EDTA- Gd^{3+} the probed OH stretching frequencies keep increasing as urea is progressively added. In the range of urea concentrations up to 2.0 M there is also a progressive increase in OH stretch frequencies probed by the vibronic band for mSE3 peptide, consistent with results from free

or EDTA coordinated Gd^{3+} samples. It is noted that at 2 M urea concentration the frequency of OH stretching in the mSE3 is approximately 3450 cm^{-1} , close to that of 5-hour heated powder of GdCl_3 which exhibits the largest increase relative to the initial unheated hydrated powder. Continued heating beyond this point resulted in a loss of the OH VSB, indicative of complete loss of first shell hydration waters. The heating-induced increase in OH frequency is attributed to a progressive loss of second shell waters resulting in an absence of hydrogen bonding interactions for the first shell waters. The similar frequencies for the OH VSB for mSE3 peptide coordinated Gd^{3+} in the presence of 2 M urea and the heated powder indicates that under these conditions, there is very weak or no hydrogen bonding between the single first shell water of the peptide coordinated Gd^{3+} and surrounding waters.

This weakening effect of the hydrogen bonding by urea however does not correlate with the peptide secondary structure which remains unchanged. When the urea concentrations is increased from 3.0 M through 4.0 M the secondary structures of mSE3 and SE2 are still well maintained; however, the OH stretching frequency from the hydration waters of the mSE3 peptide no longer increases but instead manifests a clearly detectable decrease. Over this same concentration range of urea, the SE2 peptide now begins to display an OH stretching band in the vibronic spectrum which is indicative of water invading the heretofore water-free coordination sphere⁶⁴ of the SE2 bound Gd^{3+} . Thus when the urea concentration is between 3 and 4 M, there is no indication of unfolding however the SE2 peptide shows an onset of water occupancy in the coordination sphere of the Gd^{3+} and the mSE3 peptide shows a reversal of progressive urea-induced increase in the OH stretching frequency within the hydration shell. Under these conditions, the Gd^{3+} remains fully coordinated to the peptides. This conclusion stems from the absence in the vibronic spectrum of a change in the ratio of the area of the C=O to O-H bands as the Gd^{3+} concentration is systematically lowered from near equimolar with respect to the peptide when the urea concentration is 4 M (data not shown). It is also noted that at a urea concentration of 4 M, the OH stretching frequencies of water coordinated to EDTA, SE2 and mSE3 bound Gd^{3+} all fall in the same regime at around 3410 cm^{-1} , indicative of similar coordination environments. The figure shows that the peptide does unfold once the concentration of urea exceeds 4 M; however, the OH stretching frequency from the vibronic spectrum of Gd^{3+} plus peptide solutions remains constant while for both free Gd^{3+} and EDTA- Gd^{3+} the frequency continues to increase slightly before leveling off.

Discussion

Hydrogen bonding among hydration layer waters of Gd^{3+} is a function of coordination site

In going from free aqueous Gd^{3+} to EDTA coordinated Gd^{3+} to mSE3 peptide coordinated Gd^{3+} the number of waters in the first hydration layer decreases from 9 to 3 to 1⁵². In addition the X-ray crystallography is indicative of more highly sequestered hydration environment for the peptide compared to EDTA. Indeed the very slight sequence modification in going from the mSE3 peptide to the SE2 peptide is sufficient to preclude the binding of even one water to the coordinated Gd^{3+} . The vibronic spectra show a progressive weakening in the hydrogen bonding between first and second shell hydration waters of Gd^{3+} as the coordination environment becomes more constrained. This result is explainable in terms of constrained environments around the first layer waters of the Gd^{3+} sterically or possibly electrostatically limiting the formation of an optimum configuration between first and second shell waters that would maximize the hydrogen bond strength.

Urea induces strong weakening effects on hydrogen bond strength among hydration waters of Gd^{3+}

The presented results show that hydrogen bond weakening effect of urea is primarily restricted to the waters in the hydration layer of Gd^{3+} . The molecular origin for the site specific urea effect is uncertain. Simulation studies suggest that urea causes tight ordering of water near its polar groups and disrupts the continuous hydrogen bond network up to 6 Å³³. The site specificity could arise if urea is preferentially populating the hydration layer of Gd^{3+} .

There is evidence that urea tends to preferentially accumulate in the hydration layers of the macromolecule rather than in the bulk³¹. This is so called preferential inclusion was explained in terms of the enthalpic penalty of localizing urea in the hydration layer versus the bulk solvent. Due to the high degree of ordering of waters around the urea, there is higher energy penalty associated with introducing the urea into a region with an optimized hydrogen bonding network. The hydrogen bonding among surface waters relative to the bulk is anticipated to be weakened due to either topological constraints or disruption of an extended network perturbed by polar or nonpolar groups. Under such conditions the urea would preferentially populate the hydration layer and thus have the larger impact on the waters in that layer. It follows that the preferential populating of the hydration layer should become more pronounced as the hydrogen bonding within the local environment becomes more disrupted. This leads to an expectation which is borne out in these results that urea has a progressively greater impact for the sequence: free- Gd^{3+} , EDTA-coordinated Gd^{3+} and peptide-coordinated Gd^{3+} . Consistent with this expectation is the observation that the magnitude of the urea-induced change in the vibronic spectrum of the different materials correlates with the frequency of the OH water stretching frequency in the vibronic spectrum of the urea-free samples. Those materials with the highest frequency (weakest hydrogen bonding among hydration shell waters) show the greatest change when urea is added.

Implication for the mechanism of urea-induced unfolding of proteins/peptides

There are many studies aimed at elucidating the mechanism of the denaturing action of urea and other denaturants^{20,22,23,33,76–94}. Three basic mechanisms are possible for urea: i) direct interactions of urea with the protein to stabilize the denatured unfolded state^{20,22,23,77–86}; ii) indirect influence of the urea through its effect on water^{33,88–93}; and iii) a combination of both direct and indirect mechanisms^{24,25,94}. Many studies^{20,22,23,77–86} implicate direct interactions of urea with protein internal sites as a major factor in the unfolding process. It has also been suggested that direct interaction between the urea and the internal protein backbone sites does not occur in the initial phase of the unfolding process and that the burst of urea-protein interactions occurs after an initial loosening of the protein structure by urea induced weakening of hydrogen bonding among waters^{24,33}.

In the present study, the secondary structure of both of the Gd^{3+} containing peptides is maintained as urea is titrated into the solution up to 4 M. In contrast, changes in the hydration waters are observed in the vibronic spectra over the same range of added urea. Initially, there is a urea induced weakening of the hydrogen bonding of hydration waters for the Gd^{3+} in the mSE3 peptide similar to what is seen for free Gd^{3+} and EDTA coordinated Gd^{3+} . Under the same conditions, the SE2 coordinated Gd^{3+} continues to be free of coordinated water. With increasing concentration of urea a second phase begins, which impacts only the hydration waters and their local environment without any detectable changes in the secondary structure. During this phase initiated by the continued addition of urea, the SE2 peptide, initially water-free during the previous phase, now manifests a vibronic spectrum indicating that water is now coordinated to the bound Gd^{3+} . For the mSE3 peptide, the new phase is associated with a reversal of the progressive weakening of

the hydrogen bonding with added urea that occurs with the urea added at lower concentrations. The progressive weakening of the hydrogen bonding during the first phase is seen not only for the mSE3 peptide but also for free and EDTA coordinated Gd^{3+} . The reversal of the progressive weakening of the hydrogen bonding with continued addition of urea for the mSE3 peptide is not seen for free or EDTA-coordinated Gd^{3+} both of which show continued weakening at the high urea levels. This mSE3 specific reversal of the urea induced weakening of the hydrogen bonding can be explained in terms of loosening of the highly constrained environment about peptide coordinated Gd^{3+} that allows greater access of water and greater ability of waters to adopt a more favorable hydrogen binding configuration relative to the peptide in the urea-free solution limit. This hypothesis is supported by the observation that when the reversal starts the vibronic OH stretching frequency for the peptide starts to approach the frequency for Gd^{3+} in the less constrained EDTA coordination site. It is only after this second phase that the melting of secondary structure occurs. This result is consistent with the concept that urea first facilitates the enhanced occupancy of water in proteins prior the actual unfolding process.

Polyols strengthen hydrogen bond strength

The presented results indicate that in general the addition of polyols enhances hydrogen bonding among waters. The hydrogen bonding enhancement in the bulk phase relative to that in the hydration layer is polyol specific. The pattern appears consistent with preferential exclusion playing a significant role. PEG appears to be totally excluded from the hydration layer even at high concentrations whereas glycerol appears excluded up to the 50% v/v limit beyond which it starts to invade the hydration layer. The smaller sugars all show a greater effect in the hydration layer consistent with less preferential exclusion. The preferential distribution of these osmolytes between the bulk and hydration is certainly influenced by of both steric exclusion of the larger osmolyte molecules and differential interactions, both protein-independent and protein-independent^{95,96}.

It has been previously reported that sugars and glycerol strongly interact with bulk water molecules through their large number of hydroxyl groups⁹⁷⁻⁹⁹. Among sugars of comparable size there are also differences in the interaction with waters that may originate from differences in the orientation of the OH groups on the sugars. The stereochemistry of the OH groups on a sugar is alleged to determine the degree to which the sugar can form strong interactions with waters²⁶. For comparably sized sugars, those capable of forming strong hydrogen bonding interactions with surrounding waters are likely to facilitate formation of hydrogen bonding networks that incorporate surface polar groups, surface waters and bulk waters. In contrast, those sugars that do not interact strongly with surrounding waters are not likely to create an extended hydrogen bonding network.

The vibronic spectra show that structuring effects of polyols and sugars can extend to the hydration shell waters of macromolecules. The concentration dependence is likely to have a contribution from the partitioning of the polyol between the bulk and hydration water layers based on the enthalpic penalty associated with the molecule being inserted in either layer. In general the penalty for the disruption is less for the hydration layer because of the weaker hydrogen bonding network in that layer allowing the insertion of the molecule without as much disruption as in the extended ordering within the bulk layer. Disruption of the strong network in the bulk layer can however be compensated for if the polyol can create a new comparably strong or stronger network in which it is included. For such osmolytes, we have the situation where if the osmolyte-induced hydrogen bonding network is much weaker in the hydration layer than in the bulk, the osmolyte will preferentially occupy the bulk layer. This process may account in part for the vibronic result that shows that only when the concentration of glycerol builds up beyond a certain level does the glycerol begin to alter hydration layer as previously reported by Dashnau et al⁷⁰.

Figure 12 demonstrates that the efficacy with which different sugars decrease the OH stretching frequencies (calculated on a basis of per molarity of sugars) correlates linearly with their limiting diffusion coefficients. The correlation is independent of the different Gd^{3+} coordinating environments. Because the limiting diffusion coefficient of saccharides correlates well with the mean number of equatorial hydroxyl groups of the sugar¹⁰⁰, the results is supportive of the claim that the potential of different sugars to impact hydration structure is a related to the structure of the sugars as discussed above.

Trehalose is one of the most efficient disaccharides with respect to strengthening the hydrogen bond strength. (Here the reviewer 2 thought the following sentence is awkward??) A naturally occurring osmolyte remarkable protein stabilizing and cryoprotection properties, trehalose is found to be relatively highly hydrated^{101,102} and have decreased hydration layer mobility¹⁰³, lower compressibility¹⁰⁴, lower diffusion coefficient¹⁰⁰ and decreased tetrahedrality of the surrounding water structure^{105,106}. These properties are consistent with the low number of intramolecular hydrogen bonds and the larger flexibility of its glycosidic linkage¹⁰¹. These features allow trehalose to to strongly influence the surrounding waters and foster the formation of strong hydrogen bonding networks of water even with other sugars as sucrose^{28,105–107}. These results suggest that the efficacy of a given sugar or polysaccharide to enhance protein stability is a function not only of the enhancement of hydrogen bonding within the bulk layer but also the ability of the osmolyte to integrate the hydration waters (or the protein surface) into the extended hydrogen bonding network. It has been demonstrated that under extreme dry condition, trehalose can participate in a strengthened hydrogen bonding network with the protein surface⁷². These results support the claim that trehalose serves as a better biopreservative reagent due to its potentially stronger influence on hydration waters as well as its ability to integrate into hydrogen bonding networks favoring enhanced protein stability.

Sugars and polyols can stabilize compact protein structure through hydration waters

The present results imply that protein structural stability can be enhanced by sugars and polyols through their enhancement of hydrogen bonding in the hydration shell of the macromolecule. If this proposed mechanism is operational then different osmolytes having the same hydrogen bonding effect on the hydration layer waters should have the same effect in enhancing the protein secondary structure. The underlying mechanism is opposite to the above discussed urea induced destabilization of protein structure. The strengthened hydrogen bonding in the hydration shells enhances the enthalpic penalty for the transfer of a water molecule from the hydrogen bonding network into the apolar interior of the protein. A with urea, the proposed mechanism for this effect is through a shift in the balance between the entropic forces that favor water entering all accessible volumes within the protein and the enthalpic forces favoring maintaining waters in a hydrogen bonding network³¹.

The magnitude of the urea-induced weakening or sugar-induced strengthening of the hydrogen bonding among hydration waters can be estimated through the application of empirical Bauer-Badger rule which predicts an enthalpy shift of 0.16 kJ/cm^{-1} ^{108,109}. This relationship results in a 9 kJ/mol decrease in enthalpy for breaking the local hydrogen bonding in the hydration layer of mSE3- Gd^{3+} induced by 2 M urea, while 1 M trehalose causes a 3 kJ/mol increase of enthalpic barrier. Considering that most globular proteins are marginally stable^{110–114}, it is likely that the osmolyte dependent stability of the hydration shell waters will contribute to conformational properties as is seen for the osmolyte-induced quaternary structure transition in a dimeric hemoglobin¹¹⁵.

In summary the results indicate an indirect mechanism through which both denaturant and bioprotectant type osmolytes impact protein stability through modulation of water hydrogen bonds, especially among those in the hydration shell. The correlation between the efficacy

with which polyols alter hydrogen bonding within the hydration layer and the stereochemistry of hydrogen bonding side chains raises the prospect for designing new chemical compounds with enhanced capacity to stabilize proteins and peptides.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Water Raman of Gd^{3+} at different coordination as a function of urea

a) Water Raman for aqueous samples of 0.5 M GdCl_3 in the absence of urea (—) and presence of 2.0 M urea (—) and 8.0 M MgCl_2 (—); b) water Raman for aqueous samples of 0.5 M GdCl_3 in the absence of urea (—), 1 mM apo-mSE3 in 10mM HEPES buffer at pH 7.0 with 1 mM GdCl_3 in the absence of urea (—) and presence of 8.0 M urea (—); c) water Raman for aqueous samples of 0.5 M GdCl_3 in the absence of urea (—), 100 mM EDTA in 10mM HEPES buffer at pH 7.0 with 80 mM GdCl_3 in the absence of urea (—) and presence of 8.0 M urea (—).

Figure 2. Water Raman spectra of osmolyte solutions

a) Water Raman spectra for (—) water and trehalose solution with concentration of (—) 0.25 M, (—) 0.5 M and (—) 1.0 M; b) Difference water Raman spectra for (—) only water and glycerol solution with concentration (v/v) of (—) 10%, (—) 20%, (—) 30%, (—) 40%, (—) 50%, (—) 60%, (—) 70%, (—) 80% and (—) 90%; c) Difference water Raman spectra for (—) only water and PEG400 solution with concentration (v/v) of (—) 10%, (—) 20%, (—) 30%, (—) 40%, (—) 50%, (—) 60%, (—) 70%, (—) 80%.

a)

b)

c)

Figure 3. Water Raman OH stretching frequencies of PEG400, trehalose and glycerol aqueous solution

a) Bar graph of OH stretch vibration frequency from water Raman of free 0.5 M GdCl₃ aqueous samples as a function of PEG400 concentration (v/v); b) Bar graph of OH stretch vibration frequency from water Raman of 0.5 M GdCl₃ aqueous sample with trehalose; c) Bar graph of OH stretch vibration frequency from water Raman of 0.5 M GdCl₃ aqueous sample with glycerol.

Figure 4. Urea induced VSB shifts of free Gd^{3+} or EDTA- Gd^{3+}

a) Normalized OH vibronic side band derived from GVSBLs of 0.5 M free GdCl_3 solution in the — absence of urea and presence of — 2.0 M urea and — 8.0 M urea. b) Normalized OH vibronic side band derived from GVSBLs of 100 mM EDTA with 80 mM Gd^{3+} in 10 mM HEPES buffer at pH 7.0 in the — absence and — presence of 1 M urea; c) Bar graph of —OH stretching mode vibration frequency shifts induced by additions of varied concentrations of urea derived from: — water Raman of free 0.5 M Gd^{3+} solution, — GVSBLs of free 0.5 M Gd^{3+} solution or — GVSBLs of 100 mM EDTA with 80 mM Gd^{3+} in 10 mM HEPES buffer at pH 7.0

Figure 5. Glycerol induced VSB shifts of Gd³⁺ at different coordination

Bar graph of -OH stretching mode vibration frequency shifts induced by addition of varied concentrations of glycerol derived from: — water Raman of 0.5 M GdCl₃ solution, — GVSBLs of 0.5 M GdCl₃ solution or — GVSBLs of 100 mM EDTA with 80 mM Gd³⁺ in 10 mM HEPES buffer at pH 7.0 and — GVSBLs of 1 mM apo-mSE3 in 10mM HEPES buffer at pH 7.0 with 1 mM GdCl₃.

Figure 6. OH VSB shifts of Gd^{3+} at different coordination as a function of tagatose, glucose, sucrose and trehalose

OH stretch mode frequency shifts of 0.5 M free $GdCl_3$ induced by addition of varied concentrations of ● tagatose, ● glucose, ● sucrose and ● trehalose. Also depicts the OH stretch mode frequency shifts of 100 mM EDTA with 80 mM Gd^{3+} in 10 mM HEPES buffer at pH 7.0 induced by addition of varied concentrations of ○ tagatose, ○ glucose, ○ sucrose and ○ trehalose. Also compares the OH stretch mode frequency shifts of 1 mM apo-mSE3 in 10mM HEPES buffer at pH 7.0 with 1 mM $GdCl_3$ induced by addition of varied concentrations of ■ tagatose and □ trehalose. *Inset:* enlarged area of the drawn box.

Figure 7. The synergy of urea and trehalose on VSB of free Gd³⁺ and EDTA-Gd³⁺

a) Normalized OH vibronic side band derived from GVSBLs of 0.5 M free GdCl₃ solution in the **—** absence of osmolytes and presence of **—** 2.0 M urea, **—** 1.0 M trehalose and **—** 2.0 M urea with 1.0 M trehalose. b) Normalized OH vibronic side band derived from GVSBLs of 100 mM EDTA with 80 mM Gd³⁺ in 10 mM HEPES buffer at pH 7.0 in the **—** absence of osmolytes and presence of **—** 1.0 M urea, **—** 1.0 M trehalose and **—** 1.0 M urea with 1.0 M trehalose.

Figure 8. Far-UV CD spectra of holo-mSE3 in the condition of urea, glycerol and trehalose
Far-UV CD spectra holo-mSE3 in 10mM HEPES buffer at pH 7.0 in the condition of: — alone; — 5.0 M urea; — 5.5 M urea; — 6.0 M urea; — 7.5 M urea; — 8.0 M urea; — 60% (v/v) glycerol and — 1.0 M trehalose.

Figure 9. Mean residue weighted molar extinction coefficient at 222 nm of far UV CD of the peptide mSE3 bound with Gd^{3+} as a function of concentration of the peptide with ● no osmolytes, ○ 50% glycerol, △ 0.5 M trehalose and □ 1.0 M tagatose.

Figure 10. The glycerol, trehalose and tagatose induced OH stretching frequency shifts of the first hydration layer waters coordinated to mSE3 bound with Gd^{3+} as a function of percentage of α -helix contents.

The ● glycerol, ● trehalose and ● tagatose induced OH stretching frequency shifts of the first hydration layer waters coordinated to mSE3 bound with Gd^{3+} as a function of percentage of α -helix contents calculated as described in method section.

Figure 11. The correlation between Far-UV CD at 220 nm monitored unfolding of mSE3 and SE2 peptides with the vibrational stretching frequencies probed by GVSBLs as a function of urea concentration

Urea denaturation of (○) mSE3 and (□) SE2 peptide. Unfolding was monitored at room temperature using mean residue weighted molar extinction coefficient of far-UV CD at 220 nm. The buffer is 10mM HEPES buffer at pH 7.0. Also depicted in the figure are the vibrational stretching frequencies probed by GVSBLs as a function of urea concentration for (○) mSE3 and (□) SE2 peptide, (△) free GdCl_3 and (▼) EDTA-Gd^{3+} solution. The OH stretching frequency of GdCl_3 powder heated for 5 hours is also marked (X).

Figure 12. OH stretching frequency shifts from VSB of free GdCl_3 solution and EDTA-Gd^{3+} by osmolytes of sucrose, glucose and trehalose on a basis of per molarity as a function of osmolytes limiting diffusion coefficients

OH stretching frequency shifts from VSB of 0.5 M free GdCl_3 solution (filled circles) or 100 mM EDTA with 80 mM Gd^{3+} in 10 mM HEPES buffer at pH 7.0 (unfilled circles) by osmolytes of ■ sucrose, ■ glucose and ■ trehalose on a basis of per molarity as a function of osmolytes limiting diffusion coefficients.