Molecular Level Probing of Preferential Hydration and Its Modulation by Osmolytes through the Use of Pyranine Complexed to Hemoglobin*

Camille J. Roche, Feng Guo, and Joel M. Friedman

From the Department of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, New York 10461

Two spectroscopic probes are used to expose molecular level changes in hydration shell water interactions that directly relate to such issues as preferential hydration and protein stability. The major focus of the present study is on the use of pyranine (HPT) fluorescence to probe as a function of added osmolytes (PEG, urea, trehalose, and magnesium), the extent to which glycerol is preferentially excluded from the hydration shell of free HPT and HPT localized in the diphosphoglycerate (DPG) binding site of hemoglobin in both solution and in Sol-Gel matrices. The pyranine study is complemented by the use of vibronic side band luminescence from the gadolinium cation that directly exposes the changes in hydrogen bonding between first and second shell waters as a function of added osmolytes. Together the results form the basis for a water partitioning model that can account for both preferential hydration and water/osmolyte-mediated conformational changes in protein structure.

It is becoming ever more apparent that water plays a major role both in stabilizing protein structures and in modulating protein dynamics (1–15). Many of the impressive advances in understanding the interplay between water and proteins and the resulting impact on protein stability have been derived from thermodynamic analyses and simulations (14, 16, 17). Molecular level probing of the hydration shell of proteins under conditions that either enhance or destabilize native structures has proven to be a more daunting task due to the relative dearth of suitable probes and probe techniques. In the present study we utilize pyranine (HPT)$^2$ as a site-specific fluorescent probe that provides direct spectroscopic detail relating to interactions within hydration layers. In addition, the hydration shell information provided by pyranine is supplemented with Gd$^{3+}$ vibronic side band data that expose how hydrogen bonding between first and second hydration shell waters is influenced by osmolytes. Together the results from these two probes directly relate to issues such as preferential hydration and osmolyte-induced changes in protein stability. The results are consistent with a relatively simple model that partitions waters and the associated water interactions into three domains: surface waters directly interacting with the hydrated molecule (e.g. protein, cation, chelate etc.), waters within the hydration layer that are impacted by these surface waters, and bulk solvent. The results also support the concept of at least two modes through which osmolytes can alter water interactions within the hydration layer: traditional preferential hydration mechanisms and alteration of hydrogen bonding patterns.

The fluorescence from pyranine (8-hydroxy-1,3,6-pyrene trisulfonate, referred to as HPT in the subsequent text) is highly responsive to the composition of its solvent shell. This sensitivity arises from the dissociation/recombination behavior of the single ionizable hydroxyl on the HPT fluorophore (18, 19). For the electronic ground state of HPT, the $pK_a$ of this proton is $\sim 7$; whereas, in the excited state, the $pK_a$ decreases to less than one (18). At pH 6.5, excitation at 355 nm accesses the low $pK_a$ excited state resulting in proton dissociation with subsequent transfer to surrounding water molecules. Under these conditions the 355-nm excitation yields a single emission band at 513 nm originating from the transfer of the dissociated proton to water and a slow recombination back to the HPT. In the presence of weak proton acceptors such as immobilized water, alcohols, or polyols in general, the extended proton transfer does not occur and the resulting fluorescence maximum shifts to 435 nm. As a result, the ratio of these two peaks in the fluorescence spectrum from HPT reflects the composition of the solvent shell of the HPT with respect to mobile waters and poor proton acceptors. This property has been previously exploited to probe the alcohol content of Sol-Gels during and after gelation (19, 20). In the present study this sensitivity is exploited to probe: (i) the extent to which added glycerol invades the hydration shell of both free and HbA-bound HPT, and (ii) modulation of this effect as a function of added osmolytes including PEG, urea, trehalose and Mg$^{2+}$.

The potential utility of the HPT fluorescence as a probe of hydration is further enhanced by the observation that HPT, a known fluorescent analog of DPG, binds stoichiometrically to the DPG binding site of human adult hemoglobin (HbA), which is located at the $\beta\beta$ terminus of the water-filled central cavity running through the central core of the $\alpha_1\beta_\alpha/\alpha_2\beta_\alpha$ HbA tetramer (21–25). This site is on the periphery of the protein, where the bound HPT is in contact with the hydration shell...
Spectroscopic Probing of Hydration Shell Phenomena

waters of HbA. Consequently, HPT fluorescence from HbA-bound HPT versus that from dilute HbA-free HPT samples can be compared as a function of added polyols. Such a comparison permits a direct evaluation of the degree of preferential hydration (reflected in the relative exclusion of the polyol from the hydration shell for protein-bound versus protein-free HPT) at the DPG binding site of HbA as a function of added glycerol in the presence and absence of osmolytes.

The present pyanine-based study uses two experimental protocols. In the first, the fluorescence spectrum of HbA-bound and HbA-free HPT is generated in solution and in Sol-Gel matrices as a function of added glycerol. The Sol-Gel study is included because there are growing indications that the unusual Sol-Gel induced damping of protein dynamics (26–36) is attributed to altered hydration layers in which there is a decrease in the mobility of hydration shell waters and hence those protein dynamics that are slaved to water mobility. This component of the study seeks to compare the extent to which the hydration shell surrounding the HPT-occupied DPG binding site of HbA preferentially excludes glycerol over water compared with the hydration shell of the free HPT probe in solution (37). The second protocol entails having the free and HbA-bound HPT samples in both solution and in Sol-Gel exposed to an aqueous buffer solvent that is 50% glycerol by volume. The relative water/glycerol occupancy of the hydration shell of HPT for the different samples is then probed as a function of added osmolytes that are likely to either change the relative composition of the hydration layer through any of several possible mechanisms. Polyethylene glycol (PEG400 and PEG5000) is used as a prototype osmolyte that exerts influence through its preferential exclusion from the hydration layer. Smaller osmolytes such as urea, Mg\(^{2+}\), and trehalose are used as osmolytes that are anticipated to exert influence by either weakening (urea and magnesium) or enhancing (trehalose) the hydrogen bonding capacity of water (14, 15, 38–41). The working hypothesis that is supported by the experimental results is that preferential exclusion of glycerol from the hydration shell of proteins can be modulated by the addition of other osmolytes either by enhancing osmotic stress through additional preferential exclusion or by altering the hydrogen bonding properties of water molecules.

The second component of the study utilizes Gd\(^{3+}\) vibronic sideband luminescence spectroscopy (VSBSLS) (42–44) to expose how hydrogen bonding between the first and second shell waters within the hydration shell of the Gd\(^{3+}\) solvent shell is impacted by the osmolytes used to perturb the partitioning of glycerol in the HPT experiments. Gd\(^{3+}\) VSBSLS is based on the appearance within the luminescence spectrum of Gd\(^{3+}\) of weak but relatively sharp vibronic transitions that are induced by the vibrations of the molecular species contained primarily within the first solvation layer (45, 46). In the case of free aqueous phase Gd\(^{3+}\), the vibronic side bands originate from the waters within the first hydration shell, whereas Gd\(^{3+}\) bound to either chelators (e.g. EDTA) or calcium binding sites in calcium-binding proteins, the vibronic bands manifest the vibrational frequencies of both the coordinated waters and the coordinating ligands such as carboxyl groups. In the present study we focus on the OH stretching frequency of the hydration shell waters. This frequency is inversely correlated with the strength of hydrogen bonding between the first and second shell waters (42, 47). As such it provides a direct measure of how added osmolytes influence the hydrogen bonding capacity of water specifically within the hydration layer as opposed to the bulk water.

EXPERIMENTAL PROCEDURES

HbA—OxyHbA, generously provided by Drs. Manjula and Acharya, was prepared according to previous protocols (48, 49). HPT was purchased from Molecular Probes (Eugene, OR) and used without further purification. Stock solutions of the probe were prepared in 0.05 M BisTris OAc, pH 6.5. All other chemicals were reagent grade (Sigma). At the concentrations used in this study, the fluorescence intensity appeared roughly linear with concentration. Over this concentration range, the relative ratio of the polyol/water fluorescence peaks (see below) were independent of the HPT concentration. At higher concentrations of HPT, saturation effects occurred that were attributable to complex formation.

Deoxy–HbA was prepared by degassing a 2.0 mM (heme) in 0.05 M BisTris OAc, pH 6.5 solution of oxy-HbA, previously prepared in the same buffer, with nitrogen and adding sodium dithionite (2 × heme) if needed. HPT was then added to the resulting deoxy–HbA solution in a ratio of 1:1 (in tetramer, 0.25 heme concentration). This stock solution was used both for solution measurements and encapsulation in the Sol-Gel as described below. The solution samples were prepared directly in a buffer already containing glycerol (glycerol percentage is by volume), 0.05 M BisTris OAc, pH 6.5, 5% glycerol.

Integrity of HbA—The integrity of the protein was verified by careful examination of the HbA spectrum in the bands of interest before, during, and after experimental procedures to ensure that the ligation state of the heme did not change during the time course of the experiment. In addition, samples were prepared that were deliberately oxidized or partially ligated to identify what changes would occur in the emission spectrum of HPT in these circumstances. Pseudobands appear in the emission spectrum of HPT in both cases due to absorption in the range of the heme Q band. None of these bands were ever observed for any of the deoxy samples used in this study.

HbA samples were also periodically checked for denaturation in the tryptophan fluorescence emission region, particularly in the presence of urea. All spectral changes reported were recorded after assuring that the spectra were no longer changing with time and the samples were at equilibrium.

Osmolytes—Osmolytes were either added from a stock solution prepared in 0.05 M BisTris OAc, pH 6.5, or as a solid where appropriate. The pH was carefully controlled to avoid shifting by some osmolytes.

Sol-Gel—Sol-Gel-encapsulated HbA samples were prepared using a previously described protocol (31, 32). Basically a solution of protein–HPT in buffer (0.05 M BisTris OAc, pH 6.5) was added to TMOS (tetramethylylorthosilicate) (Sigma, ultrapure) and buffer (0.05 M BisTris OAc, pH 6.5, + 25% glycerol) in a 1:1:1 ratio. The resulting mixture (final concentration of the protein was 0.5 mM in heme, 0.125 mM in tetramer) was spun in
an NMR tube (1-cm diameter) under nitrogen atmosphere until gelation was complete. The bathing buffer of the Sol-Gel was 0.05 M BisTris OAc, pH 6.5, + 25% glycerol + HPT (1:1) tetramer. The bathing buffer was subsequently changed to 0% glycerol, or higher, in 0.05 M BisTris OAc, pH 6.5, + X% glycerol + HPT (1:1) tetramer. Osmylates were either added from a stock solution prepared in 0.05 M BisTris OAc, pH 6.5, or as a solid where appropriate.

Fluorescence Measurements—Spectra were obtained from either of two instruments: a Schimadzu RF-5301PC (Columbia, MD) or a modular PTI Fluorescence system (Photon Technology Inc., Lawrenceville, NJ). Spectra were generated using the following experimental settings: an excitation wavelength of 351 or 355 nm was used, 1-mm slit width for excitation and emission slits, and an integration time of 0.5 or 1 s at ambient temperature. For Sol-Gel samples in 1-cm NMR tubes, or solution phase deoxy-HbA + HPT samples in a 1-mm cell, the instrument was configured for front face emission (52). A range of samples of HbA bound to HPT in 50% glycerol were tested (62-134 μM in protein tetramer) to ensure that the ratio of the two bands in the emission spectrum of HPT did not change with the concentration of the protein and the measurements were within linear range. The solution phase HbA-free HPT samples were monitored using the standard 90° excitation/emission geometry.

Gadolinium Cation Vibronic Side Band Luminescence Spectra—The gadolinium cation concentration in all probed solutions was 100 mM. Luminescence spectra were measured on a QuantaMaster model QM-4/2000SE enhanced performance scanning spectrofluorometer (Photon Technology International, Lawrenceville, NJ). Samples were held in the quartz cuvette and placed in the sample chamber in the configuration for 90° excitation. The emission profiles were recorded with the excitation of 273 nm, where gadolinium has relatively strong absorption compared with the 311-nm absorption origin. The luminescence was recorded from 290 to 400 nm. The step size was set to be 0.5 nm. The luminescence spectra were plotted as intensity versus wavelength (nm). Each spectrum was repeated at least three times and averaged. The spectra were processed and analyzed using GRAMS/32 Al (6.00) for background subtraction, smoothing, and peak position analysis.

RESULTS

HPT Fluorescence as a Function of Added Glycerol: the Influence of Protein and Encapsulation—Fig. 1 shows the fluorescence of HPT as a function of added glycerol (0–75% glycerol by volume) for free HPT in an aqueous buffered solution. In the top panel is shown the progression of changes for the un-normalized data, whereas the bottom panel shows the changes on a plot where the spectra are normalized with respect to the 510 nm peak (set as unity). It can be seen that with added glycerol the polyol-associated 435-nm peak increases as the water-derived 510-nm peak decreases. The inset shows a plot of the relative intensity of the 435-nm polyol peak as a function of added glycerol. It can be seen that the increase is highly non-linear with a considerable enhancement for glycerol additions beyond the 50% value. The un-normalized plot reveals what appears to be two isosbestic points for two different concentration regimes.

Fig. 2 compares the fractional change in the relative intensities of the water and polyol peaks as a function of added glycerol (0, 50, and 75% glycerol by volume) for free-HPT (a), HPT bound to deoxy-HbA in solution (b) and HPT bound to deoxy-HPT encapsulated in a porous Sol-Gel (c). For the thin Sol-Gel samples lining the inner wall of the 1-cm diameter NMR tube, the bathing buffers (with different glycerol content) are present in large volume excess. A solution of Hb-free HPT in aqueous buffer excited at 355 nm yields a single fluorescence emission band at 513 nm representing the proton-dissociated form of the molecule with transfer of the proton to water. It can be seen in Fig. 2 (traces labeled a) that with increasing added glycerol, a band at 435 nm appears and increases relative to the 513-nm band as already demonstrated in Fig. 1. The 435-nm band is also observed when other alcohols or sugars are added to the solution (not shown).

Fig. 2 shows that for HbA-bound HPT in both solution (traces b) and Sol-Gel (traces c), the HPT fluorescence emission spectra shows changes with increasing glycerol similar to that seen for free-HPT in solution and in the Sol-Gel (not shown)
but with some significant differences. There are two clear differences between the Hb-free and Hb-bound HPT spectra. The first is that the peak positions for both the water-sensitive and the glycol-sensitive bands are responsive to coordination to HbA. With coordination to HbA, the water band shifts from 513 to ~510 nm and the glycerol band shifts from 435 to ~460 nm. This shift allows for an assessment of whether the HPT is free or bound to the HbA. Indeed the addition of CO to the solution phase deoxy-HbA-HPT complex, which causes the Thr to Arg quaternary structure switch, results in the appearance of fluorescence peaks indicative of release of HPT from the DPG binding site (not shown). The second major difference is that at high glycerol concentrations, the ratio of the glycerol to water peak is lower for HbA-coordinated HPT both in solution and in Sol-Gel than for the free HPT, with the Sol-Gel sample yielding the lowest ratio.

The Effect of Added PEG on the Partitioning of Glycerol into the Hydration Sphere of HPT—Fig. 3 shows the influence of added PEG on the fluorescence spectrum of HPT in the presence of 50% glycerol. Panel A shows the progressive increase in the relative intensity of the polyol peak at 435 nm with added PEG5000 for a solution of HPT containing 50% glycerol by volume. A similar sequence occurs for the addition of PEG400 but higher concentrations of PEG are required to elicit a comparable effect as the PEG5000. This difference in the concentration dependence is seen in the inset in panel A that compares the relative increase of the 435-nm peak as a function of added PEG for two different PEG sizes. Addition of PEG in the absence of glycerol has a minimal effect on the fluorescence spectrum (not shown). Shown in panels B and C are the effects of added PEG5000 and PEG400 on the relative intensity of polyol peak for a solution of HbA-HPT in the presence of 50% glycerol, respectively. At the maximum concentration attainable for the added PEG5000 (10 mM) in the presence of HbA (before precipitation of protein is observed), there is no observable change in the relative intensity of the fluorescence peaks (panel B). In contrast, at the factor of 10 higher attainable concentration of PEG400 (100 mM), an increase in the relative intensity of the polyol peak at 460 nm is observed (panel C).

The Effect of Added Mg$^{2+}$, Urea, and Trehalose on Partitioning of Glycerol into the Hydration Sphere of HPT—Figs. 4–6 show the effect of added Mg$^{2+}$ (panel A) and urea (panel B) on the HPT fluorescence spectrum in the presence of 50% glycerol for free HPT, HPT bound to solution phase HbA, and HPT bound to Sol-Gel encapsulated HbA, respectively. Also shown in each figure is the effect of adding trehalose subsequent to the addition of urea (panel C). The fluorescence spectra are normalized at the 513/510-nm band in all cases. All three samples show similar responses upon addition of the osmolytes. It can be seen (top panels) that there is an increase in the ratio of the water peak at 513/510 nm to the glycerol peak at 435/460 nm upon the addition of 1 mM magnesium ion (as magnesium acetate), indicating a decrease in the partitioning of glycerol in the hydration sphere of HPT both unbound and bound to HbA. Similar but reduced changes (not shown) are seen for added sodium and potassium salts. Fig. 4A, inset, shows a plot of the change in the relative fluorescence intensity at 435 nm as a function of Mg$^{2+}$ concentration. As the concentration of the cation is increased the glycol band is preferentially decreased over that of the water-associated 513-nm band.
Panels B in Figs. 4–6 show that the addition of 1 M urea to the three different samples results in changes similar in direction but smaller in amplitude to those occurring with added magnesium ions. This concentration of urea, a known protein denaturant, had virtually no detectable effect, e.g. a red shift on the front face tryptophan emission spectrum of HbA indicating little if any unfolding at this concentration of urea. Fig. 4B, inset, shows the change in the relative fluorescence intensity at 435 nm as a function of increasing urea. The glycol band is preferentially decreased as the urea concentration is increased. The bottom panel (panel C) in all three figures shows how subsequent to the addition of urea, the addition of 0.5 M trehalose, a disaccharide known to stabilize native protein conformations (11, 17, 53–59), impacts the urea induced changes. It can be seen that in all three cases, the addition of trehalose reversed the effect of adding urea, resulting in enhancement of the peak associated with the absence of proton dissociation (435/460 nm). The trehalose-induced change in the intensity for the free HPT is included in Fig. 4B, inset. Similar sugar-induced trends are observed for other sugars such as glucose, sucrose, and tagatose. In the absence of glycerol, none of the non-sugar osmolytes when added to HPT at the concentrations used above result in the appearance of a polyol peak in the fluorescence. As discussed below, addition of trehalose does result in a small but insignificant contribution to the polyol peak.

Because trehalose like glycerol is a polyol, its presence in the hydration shell of HPT should generate the 435/460-nm emission band. As a consequence the results shown in the bottom panel of the Figs. 4–6 could be due exclusively to the additive effect of glycerol plus trehalose. Fig. 7 shows the spectroscopic consequences of the addition of trehalose (1 M) to glycerol-free (A) and glycerol containing (B–D) HPT solutions. For the glycerol-free sample, the addition of trehalose results in the appearance of a small amplitude change (relative to the water peak) 435-nm band. In marked contrast, the addition of trehalose in the presence of 25–50% glycerol results in an enhancement of the 435-nm peak, which in each case is far in excess of an additive effect anticipated based on the behavior shown in Fig. 7A. This result indicates that the spectroscopic changes occurring when adding the trehalose represent a synergistic effect with the glycerol. Similar results were obtained for the HbA-bound HPT samples both in solution and Sol-Gel (data not shown). Fig. 8 compares the change in the relative intensity of the 430-nm peak as a function of added glycerol for HPT-containing solutions with and without added trehalose (1 M). It can be seen that the effect of trehalose is to enhance the glycerol effect at all concentrations of the added glycerol. The trehalose effect becomes progressively larger with increased additions of glycerol. The solubility of the trehalose at the higher concentrations of glycerol limited the extent of the comparison (60% glycerol). Un-normalized spectra recorded upon serial dilutions of a solution of 50% glycerol/HPT in 1 M trehalose while maintaining the same concentration of glycerol and HPT show that the water-sensitive band of HPT increases linearly in response to the reduction of the sugar concentration, whereas the polyol band remains almost unchanged. The
The response of the water peak is linear with respect to the change in trehalose concentration.

**Gd**³⁺ **Vibronic Side Band Spectra** (GVSBS)—Fig. 9A shows the 273-nm excited luminescence spectrum from **Gd**³⁺ in solution as has previously been reported (42, 46, 60). The intense band at 311 nm is derived from the pure electronic transition between the lowest excited electronic state and the ground state. The highlighted vibronic side band arises from the OH stretching frequency of the waters in the first hydration shell. The energy separation of the VSB from the electronic origin (at 311 nm) provides the frequency of the vibrational mode. The addition of either urea (panel B) or **Mg**²⁺ (panel C) causes no shift in the main luminescence peak but does cause the side band corresponding to the OH stretch to shift to redder wavelengths indicative of an increase in the frequency of this mode, which represents a weakening of hydrogen bonding between the first and second shell hydration waters. The very much weaker OH bending mode at ~1600 cm⁻¹ shifts in the opposite direction (not shown). Of the several cations examined (all chloride salts), **Mg**²⁺ proved to be the most effective in inducing the shift on a per mole basis. In contrast to the urea and cation-induced shift to higher frequencies, shifts to lower frequencies are observed for added sugars including trehalose, sucrose, tagatose, and glucose. The shift to lower OH stretching frequencies is indicative of a strengthening of the hydrogen bonding between the first and second hydration shell waters. The subsequent addition of a sugar to a gadolinium solution containing urea reverses the urea-induced shift to higher OH stretching frequencies as seen in panel B for sucrose added to a urea containing solution. The addition of PEG at the levels used in the HPT measurements yielded no change in frequency of the vibronic bands. A detailed account of the concentration dependence of the shifts as a function of added sugars,
Sample Integrity—A key assumption in this study is that the measurements are derived from samples where the hydration shell is being perturbed under conditions where the protein remains in its native state. Fig. 10 shows clear absence of a conformational perturbation in deoxy-HbA in going from the osmolyte-free buffer to a buffer containing 1 M urea with 50% by volume added glycerol, the most extreme potential denaturant condition employed in this series of measurements. The 

\[ C. J. Roche, F. Feng Guo, and J. M. Friedman, unpublished data. \]

panel shows the absorption spectra containing the Q band in the visible (555 nm) and very weak Band III in the near IR (756–758 nm) regions of the spectrum. Both absorption bands show minimal changes with the peak position consistent with the native deoxy T state conformations of HbA. Similarly the front face fluorescence spectrum shown in the bottom panel shows no indication of any unfolding as would be reflected in a red shift of the 290-nm excited tryptophan emission band. We
find that concentrations of 3 and 6 M urea are required to generate spectroscopic signatures of unfolding for solution and encapsulated samples, respectively. The spectra from the other samples used in the study also reflect the maintenance of the native conformation.

**DISCUSSION**

The present study builds on earlier work in which HPT fluorescence emission is used to probe the composition of its hydration shell as a function of environment (19, 61–63). The extension to proteins via binding to HbA is a significant advance that permits the probing of the hydration shell at a functionally important site of a well studied protein. This advance is possible by virtue of the DPG-like binding properties of HPT (21–25) and the current finding that the wavelength of the fluorescence emission spectrum of HPT is sensitive to whether the HPT is bound to HbA or free in solution. This latter feature is important in providing assurance that for the HbA containing samples, the reported changes in the polyol/water ratio originate from HbA-bound HPT and not HPT that was released subsequent to the addition of reagents. This combination of properties associated with HPT fluorescence is used both to evaluate preferential hydration for the free and HbA-bound HPT probe and to monitor changes in the hydration shell of the free and HbA-bound probe as a function of added osmolytes.

**Glycerol-induced Perturbations of the Hydration Shell of HPT: Protein Effects**—The progressive enhancement of the response of the HPT fluorescence spectrum to the addition of glycerol and the presence of two isosbestic points in the plot of spectra as a function of added glycerol are suggestive of two titration regimes: one at low glycerol concentration (<50% glycerol) and one at high glycerol levels (>50% glycerol). These findings are consistent with a previous conclusion that beyond a certain percentage of added glycerol (50% by weight), there is a significant depletion of intact water clusters manifesting the hydrogen bonding pattern of bulk water (64). The present results also suggest that somewhere around the onset of that depletion phenomenon, the glycerol starts to significantly invade the hydration shell of the HPT. A comparison of the spectral changes of free and HbA bound HPT provides direct spectroscopic support for enhanced preferential exclusion of glycerol from the hydration shell of HbA (16, 17, 37, 65). It can be seen in Fig. 2 that at the high glycerol levels (75% glycerol solvents), the glycerol distribution from HPT bound to HbA is substantially lower than for the free HPT with the Sol-Gel-encapsulated HbA showing greater exclusion of glycerol than the corresponding solution phase HbA samples.

**Preferential Hydration: Size Exclusion Effects**—The spectroscopic changes that occur when PEG is added to the glycerol containing HPT samples are consistent with the combination of two effects: PEG being excluded from the hydration shell of the HPT (by virtue of sterical considerations) and PEG, by virtue of its large water-excluding volume as well as being excluded from the surface of the HPT, generating osmotic stress at the site of the HPT. The scaling

![Figure 7](https://example.com/figure7.png)
of the effect with the size of the PEG chains is further support for this model.

Osmolyte-induced Repartitioning of Glycerol—The results showing the repartitioning of glycerol in response to added osmolytes are consistent with enhanced preferential exclusion of glycerol when either Mg\(^{2+}\) or urea is added to the solution. Unlike the PEG-induced effect, these effects are not easily explained by size exclusion of the osmolyte. Instead it appears likely that osmolyte-induced alterations of hydrogen bonding may be at work. The magnesium di-cation, due its high charge density has a tight hydration layer comprised of waters that are largely decoupled from the bulk hydrogen bonding network (38), and is therefore likely to disrupt the extended hydrogen bonding network associated with bulk water. Simulation (14) of water/urea mixtures show that urea creates an extended cloud of waters that only weakly participate in the hydrogen bonding network of bulk water. Both of these descriptions are supported by our finding using the vibronic side band spectroscopy (42, 43, 47) to show that hydrogen bonding between the first and second hydration shell waters surrounding aqueous Gd\(^{3+}\)/H\(^{+}\) is weakened by the addition of either urea or Mg\(^{2+}\). Furthermore, the trend for progressive weakening of the hydrogen bonding scales with increasing charge density for a series of mono and divalent cations.\(^4\) The absence of any detectable change in the VSB with the addition of PEG is consistent with PEG effects not arising from a direct impact on the hydrogen bonding.

In contrast to urea and Mg\(^{2+}\), added sugars either enhance the partitioning of glycerol into the hydration layer or diminish the concentration of mobile waters capable of sustaining a long lived dissociated proton from HPT. Sugars are known to enhance and strengthen extended hydrogen bonding networks involving water (11, 66) and as a result decrease the availability of mobile waters capable of retaining dissociated protons. In the presence of glycerol one can envision that the added trehalose would greatly facilitate the “locking up” of available mobile waters in a rigid hydrogen bonding network comprised of trehalose, glycerol, and water with the individual waters acting as linkers between polyols. Our results from the titration of trehalose into a solution of glycerol/HPT support this interpre-

\(^4\) F. Guo and J. M. Friedman, manuscript in preparation.
The water band decreased monotonically with progressive addition of trehalose to the glycerol/HPT solution, whereas the polyol band remained unchanged consistent with the removal of mobile waters from the hydration shell without further increasing the polyol concentration in the hydration shell.

A Model for How Small Sized Osmolytes Influence Hydration Shell Phenomena—The results presented here are consistent with a model in which the hydration shell is modulated by both surface phenomena and added osmolytes. Fig. 11 shows a schematic in which there are three categories of interconverting water layers associated with the environment surrounding a protein molecule (crystal structure (67)). First there are surface waters that are in very close contact with the surface residues of the protein. Next there are the waters that comprise the hydration layer of the protein. This layer of water is bounded on one side by the surface waters and on the other side by the third layer comprised of waters that form the bulk water phase.

We propose that the waters on the rough molecular surface of the protein are sterically or otherwise inhibited from fully participating in the hydrogen bonding network associated with bulk water. This surface effect is seen (43) when comparing the hydrogen bonding between the first and second shell waters on the surface of free Gd\(^{3+}\), EDTA coordinated Gd\(^{3+}\), and parvalbumin-coordinated Gd\(^{3+}\). The vibronic spectra show (43) that the EDTA chelate results in a weakening of hydrogen bonding between the first and second shell waters consistent with x-ray data (68) showing that the chelate distorts the orientation of surface waters on the bound lanthanide. For the parvalbumin-bound Gd\(^{3+}\) there is also a general weakening of the average hydrogen bonding among hydration waters as well as a large increase in the distribution of hydrogen bond strengths. This effect is probably enhanced for the confined hydration layer surrounding proteins encapsulated in Sol-Gels where both the spatial constraints and the roughness of the templated cage surrounding the protein are likely to further inhibit the formation of the optimum hydrogen bonding network that is associated with bulk water.

Factors that engender a disruption of an extended hydrogen bonding network within the hydration layer surrounding the
protocol will generate hydration shell waters that have a reduced capacity to form the strong inter-water hydrogen bonds associated with waters located in the bulk solvent. These hydration shell waters are therefore less polarizable. This less polarizable layer of hydration waters is expected to be less effective in solvating polar polyols compared with the bulk water layer. This difference between the hydration layer and bulk layer can therefore represent a molecular basis for preferential hydration, i.e. reduced concentration of polyols in the hydration layer compared with the bulk solvent that acts in parallel with the size or steric exclusion effects. That example is to be contrasted with introduction into the bulk phase of a nonpolar molecule or molecular species that disrupts hydrogen bonding (such as urea or Mg$^{2+}$). In this case there is an energy penalty associated with breaking up the water network to insert the introduced solute. It follows that if there are two water domains, one with strong hydrogen bonding network and the other, such as the hydration layer, with a weaker hydrogen bonding network, there will be partitioning of the introduced solute into the different layers based on the extent that the solute molecule disrupts or accommodates the hydrogen bonding network of the bulk water phase. The above argument predicts that urea and high charge density cations such as Mg$^{2+}$ should accumulate in the less polar hydration layers of the protein relative to the bulk solvent as previously concluded based on calculations. As a result, the hydration layer relative to the bulk should experience an enhanced impact of adding urea or Mg$^{2+}$. The enhanced impact results in a further increase in the concentration of “decoupled” waters in the hydration layer, which has two direct biophysical implications. The first is that it should enhance preferential exclusion of polar polyols due to a further decrease in the ability of the hydration layer waters to solvate the polar polyol as is observed in the present study. The second implication is that the energy penalty associated with moving hydration layer waters into the hydrophobic core of the protein is decreased due to the urea/magnesium-induced increase in the concentration of weakly hydrogen bonded waters in the hydration layer. The latter process has been seen for urea in simulations and is claimed to be the initial step in urea-induced unfolding where the introduction of waters in the protein interior facilitates the loosening of the structure and the subsequent invasion of urea, which further destabilizes the protein structure. This same phenomenon in which weakened hydrogen bonding among waters facilitates water penetration into the hydrophobic core of the protein can also be expected to contribute to pressure-induced unfolding and cold denaturation.

REFERENCES

1. Tanford, C. (1969) J. Mol. Biol. 39, 539–544
2. Austin, R. H., Xie, A., van der Meer, L., Redlich, B., Lindgard, P. A., Frauenfelder, H., and Fu, D. (2005) Phys. Rev. Lett. 94, 128101–128104
3. Fenimore, P. W., Frauenfelder, H., McMahon, B. H., and Young, R. D. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 14408–14413
4. Fenimore, P. W., Frauenfelder, H., McMahon, B. H., and Parak, F. G. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16047–16051
5. Frauenfelder, H., Fenimore, P. W., and McMahon, B. H. (2002) Biophys. Chem. 98, 35–48
6. Frauenfelder, H., and Gratton, E. (1986) Methods Enzymol. 127, 207–216
7. Doster, W., and Settles, M. (2005) Biochim. Biophys. Acta 1749, 173–186
8. Lichtenecker, H., Doster, W., Kleinert, T., Birk, A., Sepiol, B., and Vogl, G. (1999) Biophys. J. 76, 414–422
9. Diehl, M., Doster, W., Petry, W., and Schober, H. (1997) Biophys. J. 73, 2726–2732
10. Demmel, F., Doster, W., Petry, W., and Schulte, A. (1997) Eur. Biophys. J. 26, 327–335
11. Cordone, L., Cotton, G., Giuffrida, S., Palazzo, G., Venturioli, G., and Viappiani, C. (2005) Biochim. Biophys. Acta 1749, 252–281
12. Francia, F., Palazzo, G., Mallardi, A., Cordone, L., and Venturioli, G. (2003) Cell Biochem. Biophys. 43, 431–437
13. Bennion, B. J., and Daggett, V. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 5142–5147
14. Bennion, B. J., and Daggett, V. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 16047–16051
15. Timasheff, S. N. (2002) Biochemistry 41, 13473–13482
16. Parsegian, V. A., Rand, R. P., and Rau, D. C. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 3987–3992
17. Gutman, M., Nachtrieb, E., and Huppert, D. (1982) Eur. J. Biochem. 125, 175–181
18. Flora, K. T., Dabrowski, M. A., Mussen, S. P., and Brennan, J. D. (1999) Can. J. Chem.-Rev. Can. Chim. 77, 1617–1625
19. Keeling-Tucker, T., and Brennan, J. D. (2001) Chem. Materials 13, 3331–3350
20. MacQuarrie, R., and Gibson, Q. H. (1971) J. Biol. Chem. 246, 5832–5835
21. MacQuarrie, R., and Gibson, Q. H. (1972) J. Biol. Chem. 247, 5686–5694
22. Gottfried, D. S., Juszczak, L. I., Fatalev, N. A., Acharya, A. S., Hirsch, R. E., and Friedman, J. M. (1997) J. Biol. Chem. 272, 1571–1578
23. Marden, M. C., Hazard, E. S., and Gibson, Q. H. (1986) Biochemistry 25, 7591–7596
24. Chen, Q., Lalezari, I., Nagel, R. L., and Hirsch, R. E. (2005) Biophys. J. 88, 2057–2067
25. Abbruzzetti, S., Viappiani, C., Bruno, S., Bettati, S., Bonaccio, M., and Mozzarelli, A. (2001) J. Nanosci. Nanotechnol. 1, 407–413
26. Bettati, S., and Mozarrella, A. (1997) J. Biol. Chem. 272, 32050–32055
27. Das, T. K., Khan, I., Rouseau, D. L., and Friedman, J. M. (1999) Bipectroscopy 5, 64–70
28. Eggers, D. K., and Valentine, J. S. (2001) J. Mol. Biol. 314, 911–922
29. Eggers, D. K., and Valentine, J. S. (2001) Protein Sci. 10, 250–261
30. Khan, I., Shannon, C. F., Dantsker, D., Friedman, A. J., Perez-Gonzalez-de-Apodaca, J., and Friedman, J. M. (2000) Biochemistry 39, 16099–16109
31. Samuni, U., Dantsker, D., Khan, I., Friedman, J. M., and Petron, J., D., Friedman, J. M. (2002) J. Biol. Chem. 277, 25783–25790
32. Samuni, U., Dantsker, D., Juszczak, L. I., Bettati, S., Ronda, L., Mozzarelli, A., and Friedman, J. M. (2004) Biochemistry 43, 13674–13682
33. Shibayama, N., and Saigo, S. (1995) J. Mol. Biol. 251, 203–209
34. Shibayama, N., and Saigo, S. (2001) FEBS Lett. 492, 50–53
35. Samuni, U., Roche, C. J., Dantsker, D., Juszczak, L. I., and Friedman, J. M. (2006) Biochemistry 45, 2820–2835
36. Gekko, K., and Timasheff, S. N. (1981) Biochemistry 20, 4667–4676
37. Collins, K. D. (1997) Biophys. J. 72, 65–76
38. Kirukhin, M. Y., and Collins, K. D. (2002) Biophys. Chem. 99, 155–168
39. Collins, K. D. (2004) Methods 34, 300–311
40. Collins, M. D., Hummer, G., Quill, M. L., Matthews, B. W., and Gruner, S. M. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 16688–16671
41. Stavola, M., Friedman, A. J., Stepnonski, R. A., and, Sceats, M. G. (1981) Chem. Phys. Lett. 80, 192–194
42. Iben, I. E., Stavola, M., Macgregor, B. R., Zhang, X. Y., and Friedman, J. M. (1991) Biophys. J. 59, 1040–1049
43. Navati, M. S., Ray, A., Shaman, J., and Friedman, J. M. (2004) J. Phys. Chem. B 108, 1321–1327
44. Yatsis, E. S., Ehrenfreund, E., and El-Hamany, U. (1965) J. Chem. Phys. 42, 743–749
45. Yehuda Haas, G. S. (1971) Chem. Phys. Lett. 11, 143–145
46. Stavola, M., Isganistis, L., and Sceats, M. G. (1981) J. Chem. Phys. 74, 2726–2732
Spectroscopic Probing of Hydration Shell Phenomena

4228–4241
48. Manjula, B. N., Kumar, R., Sun, D. P., Ho, N. T., Ho, C., Rao, J. M., Malavalli, A., and Acharya, A. S. (1998) Protein Eng. 11, 583–588
49. Acharya, A. S., Sussman, L. G., and Manning, J. M. (1983) J. Biol. Chem. 258, 2296–2302
50. Shulgin, I. L., and Ruckenstein, E. (2005) J. Chem. Phys. 123, 054909
51. Arakawa, T., and Timasheff, S. N. (1985) Biophys. J. 47, 411–414
52. Hirsch, R. E. (1994) Methods Enzymol. 232, 231–246
53. Zou, Q., Bennion, B. J., Daggett, V., and Murphy, K. P. (2002) J. Am. Chem. Soc. 124, 1192–1202
54. Rosgen, J., Pettitt, B. M., and Bolen, D. W. (2005) Biophys. J. 89, 2988–2997
55. Lin, T. Y., and Timasheff, S. N. (1996) Protein Sci. 5, 372–381
56. Anchordoqui, T. J., Izutsu, K. I., Randolph, T. W., and Carpenter, J. F. (2001) Arch. Biochem. Biophys. 390, 35–41
57. Crowe, J. H., Carpenter, J. F., and Crowe, L. M. (1998) Annu. Rev. Physiol. 60, 73–103
58. Kreilgaard, L., Frokjaer, S., Flink, J. M., Randolph, T. W., and Carpenter, J. F. (1998) Arch. Biochem. Biophys. 360, 121–134
59. Allison, S. D., Manning, M. C., Randolph, T. W., Middleton, K., Davis, A., and Carpenter, J. F. (2000) J. Pharm. Sci. 89, 199–214
60. MacGregor, R. B., Jr. (1989) Arch. Biochem. Biophys. 274, 312–316
61. Nachliel, E., Pollak, N., Huppert, D., and Gutman, M. (2001) Biophys. J. 80, 1498–1506
62. Yam, R., Nachliel, E., Kiryati, S., Gutman, M., and Huppert, D. (1991) Biophys. J. 59, 4–11
63. Tleugabulova, D., Zhang, Z., Chen, Y., Brook, M. A., and Brennan, J. D. (2004) Langmuir 20, 848–854
64. Dashnau, J. L., Nucci, N. V., Sharp, K. A., and Vanderkooi, J. M. (2006) J. Phys. Chem. B Condens. Matter Mater. Surf. Interfaces Biophys. 110, 13670–13677
65. Timasheff, S. N. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 9721–9726
66. Cottone, G., Giuffrida, S., Ciccotti, G., and Cordone, L. (2005) Proteins 59, 291–302
67. Richard, V., Dodson, G. G., and Mauguen, Y. (1993) J. Mol. Biol. 233, 270–274
68. Hoard, J., Lee, B., and Lind, M. (1965) J. Am. Chem. Soc. 87, 1611–1612