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Identifying Chaotropic and Kosmotropic Agents by Nanorheology

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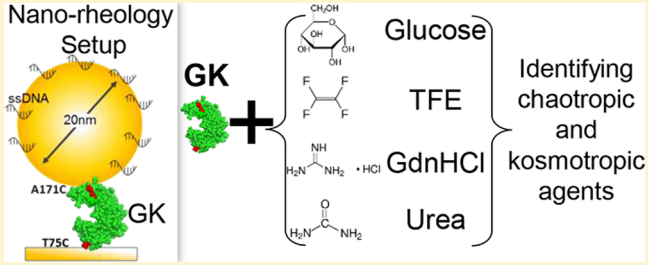
Supporting Information

ABSTRACT: Chemical agents are classified into chaotropes (disorder inducing) and kosmotropes (order inducing) based on their ability to destabilize or stabilize, respectively, the structure of hydrated macromolecules and their solutions. Here, we examine the effect of such agents on the mechanical stiffness of the hydration layer of proteins, measured by nanorheology. We examine four different agents and conclude that chaotropic substances induce the overall softening of the protein-hydration layer system, whereas the kosmotropic substances induce stiffening. Specifically, with glucose and trifluoroethanol, two known kosmotropic agents, we observe the stiffening of the hydration layer. In contrast, with guanidine hydrochloride and urea, known kaotropic agents, we observe softening. Thus, the viscoelastic mechanics of the folded, hydrated protein provides an experimental measure of the effect that chaotropes and kosmotropes have on the dynamics.



INTRODUCTION

The hydration shell of proteins must be considered as an integral part of these molecules.[1] It determines the structure, stability, and dynamics of the molecule, as much as the polypeptide chain. It plays a critical role in mediating interactions with ligands as well as the phase separation behavior of the molecules.[2] The hydration layer is inhomogeneous along the surface of the molecule,[3] reflecting the inhomogeneity of the protein's surface. Thus, the hydration layer is, on average, structured, even though individual water molecules exchange on picosecond time scales with bulk water. The structure and dynamics of the hydration layer evidently depends on the thermodynamic variables; temperature and pressure, leading to dramatically counterintuitive effects, such as the phenomenon of cold unfolding.[4] Other factors which may perturb the hydration layer are the presence of kosmotropic or chaotropic agents. These are substances which affect the stability of macromolecular structures in water, such as nucleic acids, proteins, and membranes. Chaotropes decrease and kosmotropes increase the stability of proteins and in general affect noncovalent interactions between macromolecules in water. However, these terms do not represent a well-defined thermodynamic function (such as temperature, pH, etc.), but rather they describe an overall effect on macromolecular stability. The perspective by Ball and Hallsworth[5] explains this point and also reviews the history of how the terms chaotropic and kosmotropic, as used by different authors, change from being descriptive of the overall effect on macromolecular stability to describe specific mechanisms underlying these effects. In any case, among the properties and effects of kosmotropic agents, they increase the stability of proteins, reduce the solubility of hydrophobic molecules, and form strong hydrogen bonds with water.[6−8] Chaotropes, on the contrary, decrease the protein stability and increase the solubility of hydrophobes.[8−11] These effects result from the interaction of these compounds with the surface of the protein, which includes the hydration layer.[5] Therefore, the addition of chaotropes and kosmotropes represents a "knob" that the experimentalist can turn to investigate how and to what extent the interface (hydration layer)−(polypeptide surface) determines the stability and eventually the dynamics of the protein.[12] To compare the experimental results and also to clarify the very concepts, an agreed-upon measure of chaotropicity and kosmotropicity would be very useful. One such measure has been proposed by Cray et al.[13] It is based on the shift in the temperature of the gelation point of a "standard" agar solution upon addition of the chaotropic or kosmotropic agent. Specifically, their measure is proportional to $-\mathrm{sign}(\Delta T)/[\mathrm{A}]$, where [A] is the concentration which causes a 1 C shift in the gelling temperature and (ΔT) is the sign of the temperature shift. Thus, chaotropes, which shift the gelling temperature downward, have positive values of this measure, whereas kosmotropes have negative values. They choose the as-proportionality constant for their scale (arbitrarily, evidently) and the specific heat of the agar solution, 4.15 kJ kg^{-1} C^{-1}, so that the complete expression for what we might call the

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Hallsworth scale H of chao—and kosmotropicity is $H = -4.15$ sign(ΔT)/[A], where [A] is expressed in molar and H has then units of kJ kg^{-1} C^{-1} mol^{-1}. Because the main interaction which solidifies the gel is the hydrogen bonding between the agar polymers, this scale must mainly reflect the influence of the chao- and kosmotropic agents on the free energy cost of breaking those hydrogen bonds. It is an equilibrium measure of chaotropicity. In contrast, here, we explore a nonequilibrium measure of the effect of chaotropes and kosmotropes based on direct measurements of the protein hydration layer stiffness. We propose that kosmotropic agents stiffen the hydration layer, whereas the chaotropic agents soften it. Stiff and soft here refer to both elastic and viscous mechanical responses: the two are coupled, because the structure is viscoelastic.[14,15] Because the effect assayed by nanorheology is dynamical rather than structural, there is new information content in comparing the Hallsworth measure of chaotropicity with the present assay. This is a rather preliminary report in which we describe the basic effect and look at just four substances. Further work by nanorheology may take a more systematic and quantitative approach. Note that chao- and kosmotropes are also discussed in terms of their effects on cellular systems;[16] however, here, we focus on molecular scale effects. In the experiments, we use nanorheology to measure changes in stiffness of the hydration layer of a folded functional enzyme caused by the addition of chemical agents. Nanorheology is an ensemble averaged, sub-Angstrom resolution method which measures the mechanical response of soft molecules in the frequency range of 10 Hz to 10 kHz.[17,18] With this technique, the viscoelastic transition of the folded state of proteins was discovered.[14,15] Previous work with this method also showed that modifying the hydration layer of an enzyme (while preserving enzyme's structure and function) has a dramatic effect on the mechanical response of the molecule. It was found that small (<1%) concentrations of DMSO increase the overall mechanical stiffness of the enzyme up to a factor of 2.[12] DMSO is a kosmotropic agent believed to increase the ordering of water molecules in the first hydration shell of water-dissolved substances.[8,12,19] On the Hallsworth scale, it has a value H(DMSO) = −9.72, similar to trehalose (−10.6).[13]

Inspired by this unexpected result, we selected a few suspected or confirmed kosmotropic and chaotropic agents: glucose, trifluoroethanol (TFE), urea, and guanidine hydrochloride (GdnHCl)[8,20] and investigated their effect on the mechanical stiffness of the enzyme guanylate kinase (GK). We use GK because it has been our model protein for establishing the nanorheology method, but similar measurements could be obtained for other globular proteins as well.

Glucose (chemical formula $C_6H_{12}O_6$) may be thought of as a kosmotropic agent, for instance, based on the effect on the melting temperature of DNA and the critical micelle concentration of SDS and Triton X-100.[10] Back et al. similarly report a (positive) shift in denaturation temperature T_m measured by differential scanning calorimetry (DSC) for four different proteins, which identifies glucose as a kosmotrope, as "effective" as sucrose, which they also assayed. A similar result on the T_m of ribonuclease A, measured by CD spectroscopy, is reported by Poddar et al.[21] In general, studies have shown that sugars enhance thermal stability of proteins.[22,23] However, on the Hallsworth scale, glucose is essentially neutral: H(glucose) = +1.19.[13]

TFE (chemical formula $C_2H_3F_3O$) is known as a kosmotropic agent. This alcohol is commonly used to induce protein secondary structure, especially α-helix formation. This effect depends on concentration.[24] Alcohols in general affect the enzymatic activity.[25] Although low TFE concentrations can induce order, at higher concentrations, TFE can act as a denaturant.[26,27] More specifically, the detailed study by Carrotta et al.[29] addressed the effects of TFE both from the point of view of protein−protein interactions and protein conformation, using bovine serum albumin (BSA) and a variety of physical measurements. They measure the second virial coefficient B_2 from the Rayleigh scattering at different BSA concentrations and find that B_2 (which is positive without TFE) first goes up for increasing the TFE concentration and then goes down and eventually becomes negative beyond about 16% (v/v) concentration. $B_2 > 0$ reflects the overall repulsive interactions and $B_2 < 0$ reflects the overall attractive interactions between the molecules; therefore, their result is that small concentrations of TFE increase intermolecular repulsion and therefore stabilize the solution, thus acting kosmotropically, whereas large concentrations of TFE lead to intermolecular attraction, promoting aggregation and thus acting chaotropically. On the other hand, the hydrodynamic radius of the molecule, which they measure by dynamic light scattering, increases monotonically with increasing TFE, and this is certainly a hydration layer effect because the radius of gyration measured by small-angle X-ray scattering is constant (independent of TFE concentration). Using CD and fluorescence spectroscopy, they also show that the destabilization at the higher TFE concentrations is not because of unfolding. In summary: TFE acts as a kosmotrope at small (≤10%) concentrations and as a chaotrope at large (>16%) concentrations. At concentrations of about 16% and beyond, they have a metastable state, as the solution tends to slowly aggregate. In hindsight, this metastability seems to affect our experiment too (see later). They attribute it to an increased solvent exposure of hydrophobic pockets of the protein at the higher TFE concentrations, and this is probably an effect of increased fluctuations rather than reflecting a significant change in the average structure of the protein. Similarly, Povey et al.[27] studied the effect of TFE on the stability of lysozyme, using NMR, CD, and fluorescence spectroscopies. They report that low concentrations of TFE stabilize the tertiary structure of the protein, whereas high concentrations destabilize it. Their conclusion is that 10% TFE "tightens" structural contacts, which from the point of view of our measurements presumably should mean smaller fluctuations, that is, an overall "stiffer" molecule. Of note is that this state is not optimal for the enzymatic activity (they measure about 70% of enzymatic activity in the presence of 10% TFE). In general, their study reaffirms the important role of fluctuations in discussing the effects of this agent. Given the above, TFE is an interesting agent for our study.

On the other hand, urea (chemical formula $CO(NH_2)_2$) is a chaotropic agent. At high concentrations (6−10 M), urea can denature proteins by disrupting noncovalent bonds and directly binding to amide units via hydrogen bonds.[28] It has a Hallsworth index H(urea) = +16.6. Finally, guanidinium hydrochloride (GdnHCl) with the chemical formula CH_6ClN_3 is the hydrochloride salt of guanidine frequently used to denature proteins.[24] It is a strong chaotrope: H(GdnHC1) = +31.9.

Our results, though preliminary, show directly that chaotropes and kosmotropes indeed affect hydration layer stiffness in opposite ways. An operational classification of chemical agents

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based on this property seems possible, and it would be different from the Hallsworth scale, as is adumbrated by the case of glucose. This is not surprising because the gel assay probes equilibrium properties, whereas nanorheology probes dynamic response.

MATERIALS AND METHODS

To probe hydration layer stiffness, we employ the nanorheology setup of Figure 1. In the experiments, the enzyme GK

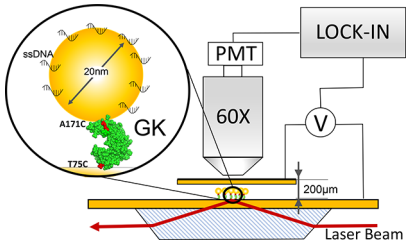

Figure 1. Nanorheology setup showing the flow chamber (20 μL total volume, not to scale) with enzyme-tethered GNPs, the parallel plate capacitor geometry used for mechanical excitation, and the evanescent wave-scattering optics used for read out. The inset shows the enzyme GK with the Cys substitutions (Thr-075-Cys; Arg-171-Cys) which provide "chemical handles" to attach the molecules to the gold bottom plate and GNPs through the S−Au bond. The ssDNA oligomers attached to the GNP have the purpose of increasing the surface charge.

tethers 20 nm diameter gold nanoparticles (GNPs, Nanocs) to a semitransparent gold-coated glass slide. GK is an ∼24 kDa, ∼4 nm size globular protein. This surface preparation of protein-tethered GNPs is in buffered aqueous solution in a flow chamber which is also an electrolytic capacitor. An ac electric field produced by applying a ∼500 mV sinusoidal voltage at the two electrodes drives the negatively charged GNPs. The GNP on the one side, and the gold film on the other side of the protein layer act as the two plates of a rheometer, applying an oscillatory stress on the enzyme plus hydration layers. There are actually two hydration layers: one at the surface of the protein and one at the gold surfaces. Both get periodically deformed at the driving frequency in the region of contact between the gold surfaces and the enzyme. Of course, the enzyme also gets deformed. From a mechanical (as well as a functional) point of view, the "protein" is the system of the folded polypeptide chain plus the hydration layer.[1,29] GK was prepared by mutagenesis with the internal Cys changed to arginine and threonine by Cys substituted at positions 171 and 75, respectively, as described by Wang and Zocchi,[18] for coupling to the gold surfaces.

The experiments are performed by first recording the frequency response of the system in the working buffer SSC/3 (50 mM sodium chloride and 5 mM trisodium citrate, pH 7.0, Invitrogen), in the range 10−200 Hz, using a fixed amplitude of the applied voltage such that the maximum amplitude of the response does not exceed 2−4 Å (in order not to damage the sample). Then, the buffer in the flow cell is exchanged with the same buffer containing a given concentration of kosmotropic or chaotropic agent. A new frequency response is recorded, all other conditions being exactly the same. The kosmotropic agents used are D-(+)-glucose (1 and 0.5 M, Sigma) and TFE 99.5% (10 and 33%, Alfa Aesar). The chaotropic agents used are urea (1 M and 100 mM, EMD) and GdnHCl (1 and 0.5 M, Sigma).

For fixed amplitude of the forcing, the frequency response of the enzyme (which includes the mechanical response of the hydration layer) is well-described by the Maxwell model of viscoelasticity, where the (one-dimensional) mechanics is summarized by an elastic parameter κ (dimensions of force/length), and a dissipation parameter γ (dimensions of mass/time).[14,15] In the context of our experiment, the corresponding deformation amplitude ($|z|$) versus frequency (ω) curve takes the form[12]

$$|z| = \frac{F_0}{\gamma\omega}\sqrt{1 + (\omega/\omega_c)^2} \tag{1}$$

where $\omega_c = \kappa/\gamma$ is the corner frequency below which the response changes from elastic to viscous and F_0 is the amplitude of the applied force. To quantify the effect of the different chemical agents on the system's mechanics, we fit the amplitude versus frequency response curves with this form, that is, $|z| = (A/\omega)\sqrt{1 + (\omega/B)^2}$, where $A = F_0/\gamma$ and $B = \kappa/\gamma$. For each sample, we determine the parameters A and B, for both frequency responses (with and without the added agent). Because F_0 is the same for the same sample, we can find the relative change in the parameters κ, γ of the viscoelastic description induced by the presence of kosmotropic or chaotropic agents. Namely, $A = F_0/\gamma$, $B = \kappa/\gamma$ and therefore $A_f/A_i = \gamma_i/\gamma_f$, $(B/A)_f/(B/A)_i = \kappa_f/\kappa_i$, where f ("final") and i ("initial") refer to the quantity in the presence or absence, respectively, of the chemical agent. The parameter ratios

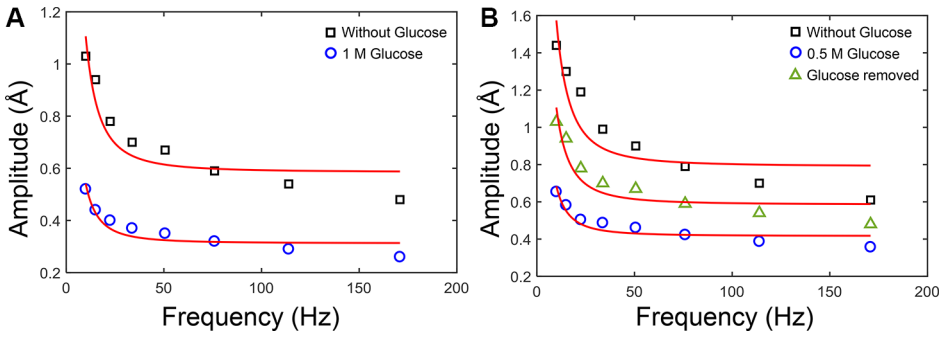

Figure 2. (A) rms deformation amplitude vs forcing frequency in the absence (squares) and presence (circles) of 1 M glucose for the same sample. Lines are fits with Maxwell model of viscoelasticity, as shown in eq 1. (B) Partial reversibility of the glucose induced changes in mechanical properties. The figure shows, for the same sample, the response amplitude vs frequency before adding glucose (squares), after adding 0.5 M glucose (circles), and after removing glucose (triangles). For both graphs each point is an average of 4−5 measurements. Typical standard deviation is 0.1 Å.

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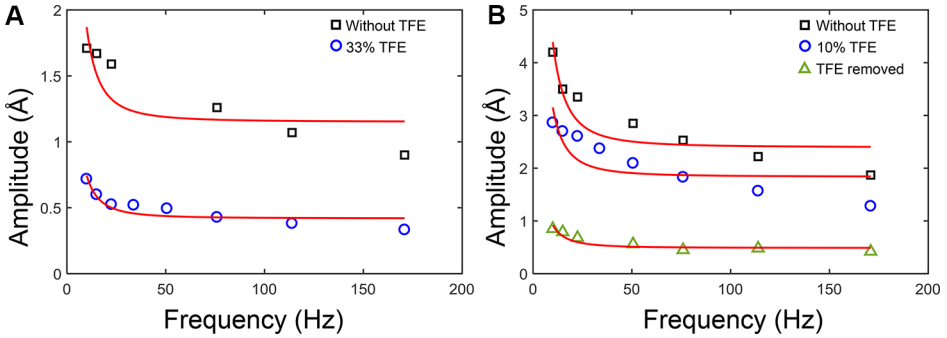

Figure 3. (A) Response amplitude vs forcing frequency in the absence (squares) and presence (circles) of 33% TFE, for the same sample. Lines are fits with the Maxwell model of viscoelasticity. (B) Nonreversibility of the TFE induced changes in mechanical properties. The figure shows, for the same sample, the response amplitude vs frequency before adding TFE (squares), after adding 10% TFE (circles) and after removing TFE (triangles). For both graphs, each point is an average of 4−5 measurements. Typical standard deviation is 0.1 Å

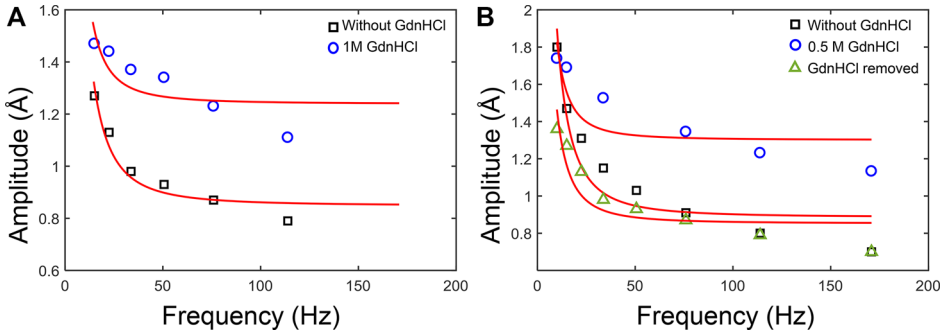

Figure 4. (A) Response amplitude vs forcing frequency in the absence (squares) and presence (circles) of 1 M GdnHCl, for the same sample. Lines are fits with Maxwell model of viscoelasticity. The effect of GdHCl on the viscoelastic dynamics is opposite to the effect of glucose, as it leads to softening of the hydration layer. (B) Reversibility of the GdnHCl induced changes in mechanical properties. The figure shows, for the same sample, the response amplitude vs frequency before adding GdnHCl (squares), after adding 0.5 M GdnHCl (circles), and after removing GdnHCl (triangles). For both graphs each point is an average of 4−5 measurements. Typical standard deviation is 0.1 Å.

reported in the Supporting Information were obtained in this manner (see Tables S1−S4).

RESULTS AND DISCUSSION

Our previous work with DMSO showed that the addition of this kosmotropic agent, even at small bulk concentrations (<1%), has the effect of increasing the overall stiffness of the enzyme, as measured by nanorheology, by a factor of ∼2.[12] The process is reversible.

With glucose, we observe a similar effect. Figure 2A shows the amplitude versus frequency response for the sample in the absence of glucose (squares), and, for the same sample and forcing amplitude, in the presence of 1 M glucose, all other conditions are identical. We see that the enzyme becomes more viscous and/or stiffer because the deformation amplitude is smaller, for the same force. Figure 2B shows the result of a similar experiment, where 0.5 M glucose is added (circles), and then removed (triangles). The process appears not quite reversible; however, it is also possible that the glucose was incompletely removed from the confined surface geometry by the washing steps. Also, complete reversibility may possibly be achieved by using smaller concentrations of the agent; this point should be investigated in the future work. For this and the following plots, each data point that corresponds to a specific frequency was obtained by a 50 s scan, with 250 Hz data acquisition rate, which results in 12 500 measurements. The graph for each agent is obtained by taking the average of four scans. To remove glucose and other agents, the fluid chamber was washed with 200 cell volumes over a time period of 10 min.

From the fits with the Maxwell model of viscoelasticity (solid lines), we find that in the presence of 1 or 0.5 M glucose, the parameter γ increases by a factor ∼3 and κ increases by a factor ∼2. Overall, the molecule becomes significantly stiffer/more viscous (the two properties are coupled because the system is viscoelastic). This result confirms the kosmotropic nature of glucose within our operational classification scheme. From an interaction point of view, glucose is believed to add structural order to the protein−solvent interface.[30]

With TFE, another kosmotropic agent, we also observe overall stiffening (Figure 3). However, here it is not clear whether the effect is mainly because of surface denaturation. Figure 3B shows a stiffening effect in the presence of 10% TFE, but this state appears metastable on the time scales of the experiment and evolves to a feature-less response (triangles in Figure 3B), even though TFE is (nominally) removed. In the absence of at least partial reversibility, we cannot conclude from the measurements of Figure 3 that we observe, transiently, a stiffening of the folded molecule because a similar response would result from an increasing fraction of surface-denatured molecules. For comparison, when the proteins are heat-denatured, the frequency response becomes similar to the circles in Figure 3A: feature-less and "stiff".[17]

The marked difference between glucose and TFE relating to reversibility is presumably related to the higher electro-negativity of TFE because of the fluorine groups, which causes stronger interactions with water molecules and polar groups.

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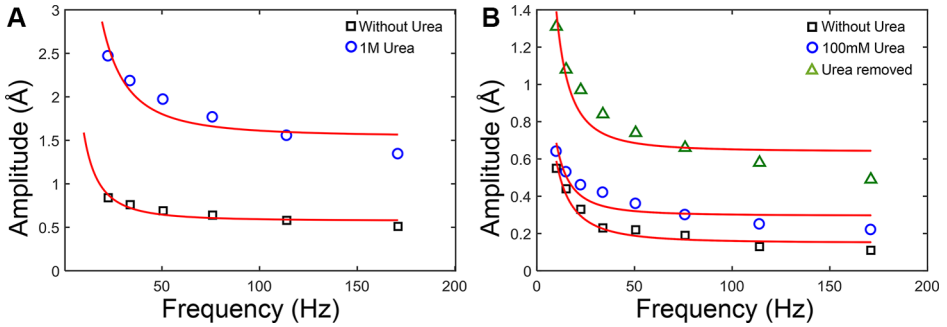

Figure 5. (A) Response amplitude vs forcing frequency in the absence (squares) and presence (circles) of 1 M urea, for the same sample. Lines are fits with Maxwell model of viscoelasticity. (B) Nonreversibility of the urea induced changes in mechanical properties. The figure shows, for the same sample, the response amplitude vs frequency before adding urea (squares), after adding 100 mM urea (circles), and after removing urea (triangles). For both graphs, each point is an average of 4−5 measurements. Typical standard deviation is 0.1 Å.

Folding studies with these agents using DSC showed that TFE is a strong helix inducing solvent and can stabilize some folding intermediates, but it seems that with TFE the native structure is not thermodynamically stable. DMSO, which we used in a previous study, is also a structure-affecting solvent but it is not a strong denaturant thermodynamically. It leads to reversible stiffening of the hydration layer as measured by nanorheology.[12] The hydration layer becomes stiffer/more viscous in the presence of glucose because the deformation amplitude decreases for the same applied force. Glucose is not a denaturant but a protector.[31]

Turning now to chaotropic agents, Figure 4 shows the effect of GdHCl on mechanical response of the molecule, which is indeed opposite to that of glucose and DMSO: the enzyme becomes overall softer (for the same forcing amplitude, the deformation amplitude is larger). The shape of the response curve also changes, as seen by the large deviations from the Maxwell model curve. Figure 4B shows that the softening effect caused by adding 0.5 M GdHCl is reversible.

In terms of the parameters γ and κ, from the fits in Figure 4, we find that adding 0.5 M GdHCl does not essentially change γ, whereas κ is decreased by a factor of ∼0.6. However, these numbers must be taken only as estimates because the fits are poor. More work is needed to assess quantitatively the changes in the viscoelastic response. However, what is clear is that, compared to the kosmotropic agents glucose and DMSO, the chaotropic agent GdHCl leads to an effect on the mechanics of the opposite sign, namely a softening. This is the main point of the present study.

In Figure 5 we show the effect of similar concentrations of urea. Overall, the enzyme becomes softer. However, we were not able to show reversibility (Figure 5B). The parameter changes obtained from the fits appear different from the case of GdHCl: in the presence of 1 M urea, γ decreases by a factor of ∼0.3 and κ by a factor of ∼0.4.

We measured the effect of these concentrations of urea on the enzymatic activity of GK in solution, using a coupled enzymatic assay (NADH assay) and two different concentrations of urea, 100 mM, and 1 M. The reaction catalyzed by GK is GMP + ATP → GDP + ADP, and we chose substrate concentrations (2 mM ATP; 1 mM GMP) which maximize the speed.[32] The results show that the speed of the enzyme decreases by a factor of 2 in 100 mM urea and by a factor 4 in 1 M urea, but the overall GK is still active, as shown in Figure S1.

The urea−water combination acts as a protein denaturant when used at high concentrations such that urea molecules are readily available for hydrophobic solvation. However, at lower concentrations, there may be a window where the effect on the hydration layer is visible while the molecules are still folded and functional.

Even though both urea and GdnHCl operate by facilitating solvation of hydrophobic regions of proteins, molecular dynamics simulations show differences in the interaction of these agents with a hydrophobic polymer. Guanidinium, in particular, tends to interact with hydrophobic regions of a protein through its flat, nonpolar surface while exposing its polar/charged edges to the solvent. Urea, in contrast, exhibits a moderate tendency to aggregate, which may help it to solvate exposed protein backbone.[33] This could be one reason why the interaction between the enzyme and urea is less reversible.

CONCLUSIONS

We explore the effect of kosmotropic and chaotropic agents on the viscoelastic properties of folded, hydrated proteins, measured by nanorheology. We find that the kosmotropic agents make the hydration layer stiffer, whereas chaotropic agents make it softer. An operational classification of chaotropes and kosmotropes based on their effect on protein mechanical susceptibility measured by nanorheology appears possible and may be different from an existing classification based on thermodynamic equilibrium properties.[13] Exploring the relation between equilibrium and dynamic effects of these agents, as well as comparing the effects they have on the dynamics and interactions of different hydrated macromolecules, seem promising ways forward. Of the four agents studied here, the cases of glucose (kosmotropic) and GdHCl (chaotropic) are clear cut, as is the previously studied case of DMSO (kosmotropic). The cases of TFE and urea (both chaotropic) are less compelling because we could not show reversibility. Finally, if we ascribe the observed mechanical changes mostly to the surface of the protein molecule, which includes the hydration layer, then our results also reaffirm the central role of the hydration layer in determining protein dynamics.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpcb.7b12782.

Activity assay for GK in control condition and to different urea concentration and details about the

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parameter ratios (γ_i/γ_f, κ_f/κ_i) corresponding to four graphs (PDF)

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Notes

The authors declare no competing financial interest.

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