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Three Stages of Lysozyme Thermal Stabilization by High and Medium Charge Density Anions

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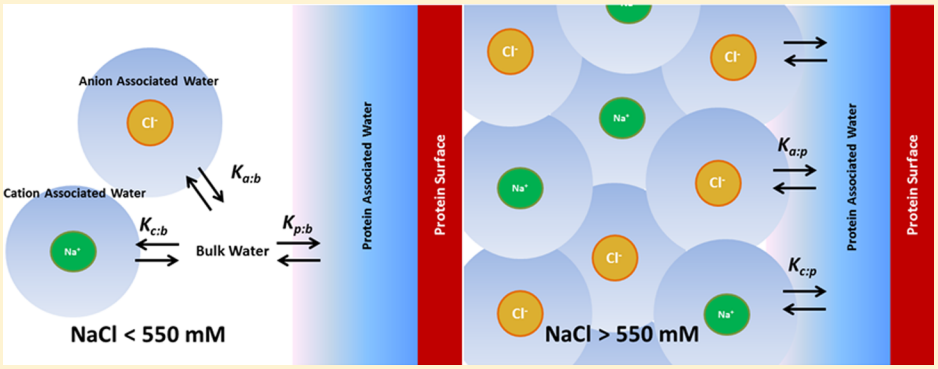
Supporting Information



ABSTRACT: Addition of high and medium charge density anions (phosphate, sulfate, and chloride) to lysozyme in pure water demonstrates three stages for stabilization of the protein structure. The first two stages have a minor impact on lysozyme stability and are probably associated with direct interaction of the ions with charged and partial charges on the protein's surface. There is a clear transition between the second and third stages; in the case of sodium chloride, disodium sulfate and disodium hydrogen phosphate this is at 550, 210, and 120 mM, respectively. Stabilization of lysozyme can be explained by the free energy required to hydrate the protein as it unfolds. At low ion concentrations, the protein's hydration layer is at equilibrium with the bulk water. After the transition, bulk water is depleted and the protein is competing for water with the ions. With competition for water between the protein and the ions at higher salt concentrations, the free energy required to hydrate the interior of the protein rises and it is this that stabilizes the protein structure.

INTRODUCTION

In 1888, Franz Hofmeister observed that salts influence protein solubility in a predictable manner. The series of salts listed from those that precipitate to those that solubilize proteins is referred to as the Hofmeister series.[1−3] In the 1960s, Peter Von Hippel and his co-workers found that the Hofmeister series also applied to protein thermal stability.[4] The salts that solubilize proteins also destabilize them, and salts that precipitate proteins also stabilize them.

Early theories explained the Hofmeister effect in terms of the alteration to the hydrogen bond population of the bulk water by the ions, kosmotropes enhancing the order of water, and chaotropes breaking the structure of water. This idea was supported by experimental work using phospholipids that suggested that "hydration forces" around the phosphate groups were responsible for holding lipid bilayers apart that could not be explained by electrostatics alone and suggested nanometer-scale range interactions between water and phosphate ions.[5,6] There are chemists who adhere to the theory that alteration of the water structure explains the Hofmeister effect.[7] Advances in analytical technology has resulted in the "structure making" and "structure breaking" theory being rigorously challenged. A range of techniques including terahertz spectroscopy and X-ray scattering[8−10] failed to detect perturbation of water beyond the first layer around ions. Currently it is widely believed that given the limited evidence for ions influencing water molecules beyond the first layer the hydrogen bond population of water has no bearing on the Hofmeister effect. Against this are limited studies using dielectric relaxation spectroscopy,[11] neutron scattering[12] and molecular dynamic simulation[13] that suggest a longer range interaction between ions and water. The "structure making" and "structure breaking" theory is open to challenge and has been shown to fail to explain the behavior of organic molecules on protein stability.[14]

There is evidence that ions interact electrostatically with proteins' oppositely charged side chains charge (negating electrostatic interaction) which has an effect of protein solubility[15] but has an unpredictable effect on protein stability.[16] This phenomenon occurs at relatively low salt concentrations. A simple model was proposed to explain the cloud point of lysozyme in monovalent anion containing solutions which explained the observed effect on solubility in

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terms of charge screening at low salt concentrations and surface tension effects at high salt concentrations.[15] Preferential interaction is a theory that explains the Hofmeister effect in terms of attraction or repulsion of the ions to the protein surface.[17] A development to the preferential interaction theory suggested that the effect was determined by a combination of preferential interaction and the excluded volume of the ions.[18,19] It is applied to explain both protein stability and solubility. These models are in agreement with the observation that low charge density anions (chaotropes) interact with apolar surfaces on the protein and are described as having preferred interaction with the protein surface.[20] High charge density anions (kosmotropes) are described as being excluded from the protein surface at higher ion concentrations, though the mechanism for anion exclusion is not apparent. The mechanism for stabilization of proteins by high density charge ions is also not apparent unless through an indirect mechanism such as surface tension. One theory is that the presence of the stabilizing molecules results in preferential hydration of the protein surface and it is this that stabilizes the structure.[21] At present, the mechanisms for protein stabilization and destabilization by salts are subject to active debate.

In this paper, we describe differential scanning calorimetry analysis of lysozyme temperature induced unfolding and use a simple three-stage hypothesis to explain the mechanism behind anion stabilization of lysozyme.

MATERIALS AND METHODS

Lysozyme and the salts were all sourced from Sigma-Aldrich, Gillingham, UK. Lysozyme was dialyzed with HPLC grade water using a Mini 8 kDa membrane dialysis kit (GE Healthcare, Little Chalfont, U.K.) overnight at 4 °C. The concentration of each lysozyme stock was measured and adjusted using the absorbance at 280 nm on an Ultrospec 2100 pro UV spectrophotometer (Amersham Biosciences, Amersham, U.K.). Lysozyme and salts were adjusted to pH 7. The lysozyme concentration used for the differential scanning calorimeter (DSC) runs was 1 mg/mL lysozyme. The DSC was a Nano-DSC (TA Instruments, New Castle, DE, USA). The heating rate was 1.5 °C/min from 30 to 100 °C. Data evaluation used the software provided by the manufacturer. Buffer−buffer baselines were subtracted from sample data. The T_m value (the temperature with the maximum heat capacity), $\Delta H_{unfolding}$ (the change in enthalpy on unfolding), and ΔC_p^* (the apparent heat capacity change) were measured. $\Delta G_{unfolding}$ was calculated using the Gibbs−Helmholtz equation.

$$\Delta G_{unfolding} = \Delta H^{xM}_{unfolding}\left(1 - \frac{T^0_m}{T^{xM}_m}\right) - \Delta C_p^*\left[(T^{xM}_m - T^0_m) + T^0_m \ln\left(\frac{T^0_m}{T^{xM}_m}\right)\right] \quad (1)$$

where $\Delta H^{xM}_{unfolding}$ is the change in enthalpy in the presence of x M salt, T^0_m is the temperature of maximum unfolding in pure water, T^{xM}_m is the temperature of maximum unfolding in x M salt, and ΔC_p^* is the apparent heat capacity change in the presence of the salt.[10] $\Delta\Delta G_{unfolding}$ was plotted against the natural log of the concentration and a line fitted using partial least squares regression to the linear sections of the data to calculate the $\partial\Delta\Delta G_{unfolding}/\partial \ln C$. The number of water molecules around the ions at the second transition was estimated based on the molar ratio of the ions to water.

RESULTS AND DISCUSSION

DSC Results. The $\Delta G_{unfolding}$ values for lysozyme in aqueous solutions containing different concentrations of sodium chloride (Figure 1), disodium sulfate (Figure 2), and disodium hydrogen phosphate (Figure 3) at pH 7.0 were calculated using the Gibbs−Helmholtz equation[10] using the $\Delta H^{xM}_{unfolding}$, T^0_m, and T^{xM}_m values determined experimentally using a DSC. The ΔC_p^* used was an average over the experiments, as this value did not significantly change. The effect of salt concentration on $\Delta G_{unfolding}$ shows three distinct stages. The first transition is around 1 mM. Below this point, stabilization does not follow the Hofmeister series; see ref 16 for details. The second transition (Y) is at 550, 210, and 120 mM for sodium chloride, sodium sulfate, and disodium hydrogen phosphate, respectively.

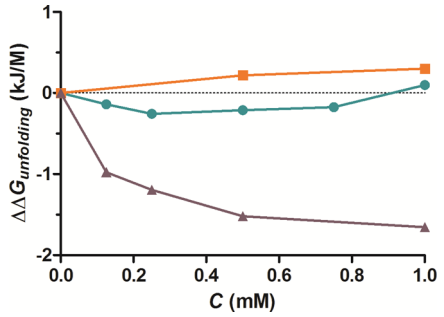

Figure 1. Change in free energy of unfolding ($\Delta\Delta G_{unfolding}$) values at low concentrations of the sodium chloride (circles), disodium sulfate (squares) and disodium hydrogen phosphate (triangles), pH 7.0.

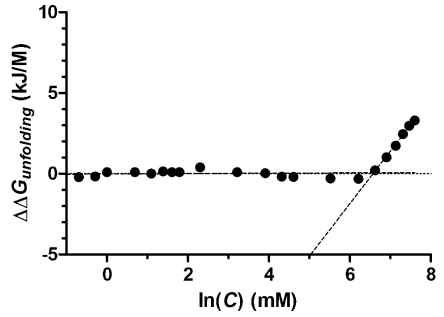

Figure 2. Change in free energy of unfolding ($\Delta\Delta G_{unfolding}$) values for varying concentrations of the sodium chloride, pH 7.0. The dotted lines are purely a guide to the eye.

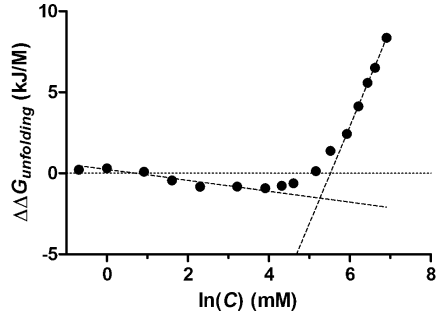

Figure 3. Change in free energy of unfolding ($\Delta\Delta G_{unfolding}$) values for varying concentrations of the disodium sulfate, pH 7.0. The dotted lines are purely a guide to the eye.

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At concentrations below the transition, the anion chloride had no effect on $\Delta G_{unfolding}$, while sulfate and phosphate reduced $\Delta G_{unfolding}$ slightly. Above the transition, $\Delta G_{unfolding}$ rose abruptly. The volume taken up by the protein (1 mg/mL lysozyme) in this experiment is only ∼0.1% (v/v), so it has little impact on the ratio of water molecules per ion in the solution at different salt concentrations. Importantly, the transition (Y) occurred when there was a ratio of ∼100 water molecules per sodium and chloride ions, ∼250 water molecules per two sodium ions and a sulfate ion, and ∼450 water molecules per two sodium ions and a phosphate ion.

To better understand the role of ions in protein unfolding, the unfolding reaction has to be considered. Protein unfolding involves the breaking of the noncovalent bonds that hold the protein's tertiary (and secondary structure) together. It also exposes the apolar core to water. From a thermodynamic perspective, the free energy required to unfold a protein ($\Delta G_{unfolding}$) can be expressed as a function of the free energy required to break the internal noncovalent bonds ($\Delta G_{noncovalent\ bonds}$) and the free energy required to hydrate the exposed core ($\Delta G_{hydration}$). In proteins with minimal salt bridging, the $\Delta G_{noncovalent\ bonds}$ values remain effectively independent of salt concentration in the solution around the protein. It is $\Delta G_{hydration}$ that is susceptible to being modulated by the presence of the salt. A change to $\Delta G_{hydration}$ by the presence of ions would affect a protein's structural stability. If the assumption is that $\Delta G_{noncovalent\ bonds}$ is constant, then $\Delta G_{unfolding} \approx \Delta G_{hydration}$.

For lysozyme at 1 mg/mL and pH 7.0, the following three stages of stabilization are proposed.

Stage 1: At salt concentrations below 1 mM ($X \approx 1$ mM), the stoichiometry suggests ion pairing with opposite charged side chains predominates. For thermal stability, this phenomenon is not related to the Hofmeister series and is probably side chain and ion specific, being dictated by local conditions on the protein surface; see ref 16 for details.

Stage 2: Between salt concentrations of X and Y (where Y is 550, 210, and 120 mM for sodium chloride, sodium sulfate, and disodium hydrogen phosphate, respectively), the authors suggest weak interactions between the ions and partial charges on the peptide backbone and polar side chains predominate. This interaction only has a weakly negative effect on the $\Delta\Delta G_{unfolding}$ value of lysozyme.

Stage 3: It is proposed that below the transition (Y) the water required to hydrate the apolar core of the protein as it unfolds is coming from the bulk water (water that is effectively unperturbed by the presence of cosolutes) but as the salt concentration increases the bulk water is depleted until the water needed to hydrate the protein's core has to come from water associated with the anions and cations. In other words, as the protein unfolds, it is competing for water with the ions' "hydration layers". This increases $\Delta\Delta G_{hydration}$ (the energy expended hydrating the apolar core of the protein as it unfolds) and thus $\Delta\Delta G_{unfolding}$. The relationship expressed in terms of the equilibrium constant for water in the protein's hydration layer switches from an equilibrium with the bulk water to an equilibrium with the water associated with the anions and cations; see Figure 4. The idea of competition between the protein and salts is not a new one and was originally raised by Franz Hofmeister in terms of the "water absorbing effect" of salts[2,3] and predates the water maker breaker theory by about 40 years.

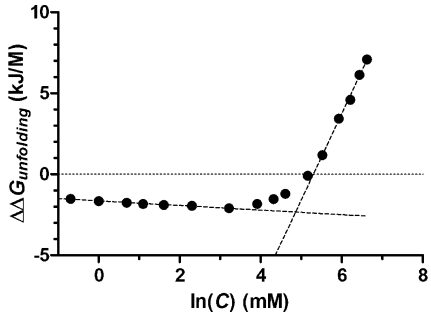

Figure 4. Change in free energy of unfolding ($\Delta\Delta G_{unfolding}$) values for varying concentrations of the disodium hydrogen phosphate, pH 7.0. The dotted lines are purely a guide to the eye.

It is worth noting that the X value is dictated by the stoichiometry of binding of the ions to the protein's charged side chains, which would suggest X is dependent on protein concentration. Y is likely to be independent of protein concentration at lower protein concentrations, but at higher protein concentrations, the water associated with the protein's hydration layer will have to be taken into account.

Comparison of Alternative Models. The hypothesis presented here differs significantly from the preferential interaction[17] and models combining preferential interaction with excluded volume[18,19] and does provide a mechanism for stabilization. The basic assumption behind the preferential interaction model is that the hydration layer around a protein is a single molecule thick and that the cosolute displaces this water and destabilizes the protein. Cosolutes that do not interact directly with the protein stabilize the protein by volume exclusion which sterically favors a compact, folded form to an open unfolded one.[22] Here we contend that it is not volume exclusion that drives protein stabilization but competition for water between salt and the protein's hydration layers and that it is the free energy of the hydration layer of the protein that is being modulated. As the free energy of the hydration layer is altered, this would be expected to affect both stability where water is required for hydration and solubility where water in the protein's hydration layer is displaced. This accounts for "kosmotrope" (or higher charge density anions) stabilization of proteins. The preferential hydration model assumes additional hydration of the protein surface[21] which is the opposite of the mechanism suggested by the authors.

If competition for water explains the activity of "kosmotropes", it opens the question of the ability of "chaotropes" (lower charge density anions) to destabilize proteins but increase the solubility. The "chaotropic" anions, iodide and perchlorate, both destabilize lysozyme starting from a low concentration.[16] If the assumption is made that the hydration layer around a protein is thicker than a single or double water layer and Kim Collins theory that low charge density ions associate with apolar surfaces on a protein is true,[20] then an apolar surface on the protein will be changed to a charged (albeit a low density charged) surface. The water interacting with an apolar and a charged surface will be different in its hydrogen bond population and would be expected to have different free energies. It is suggested that low density anions have a "detergent-like" effect; they turn apolar surfaces to charged surfaces and convert apolar surfaces that energetically favor association to like-charged surfaces that favor dispersion. This would account for a rise in solubility. Stability would also be reduced as the free energy required to hydrate the apolar

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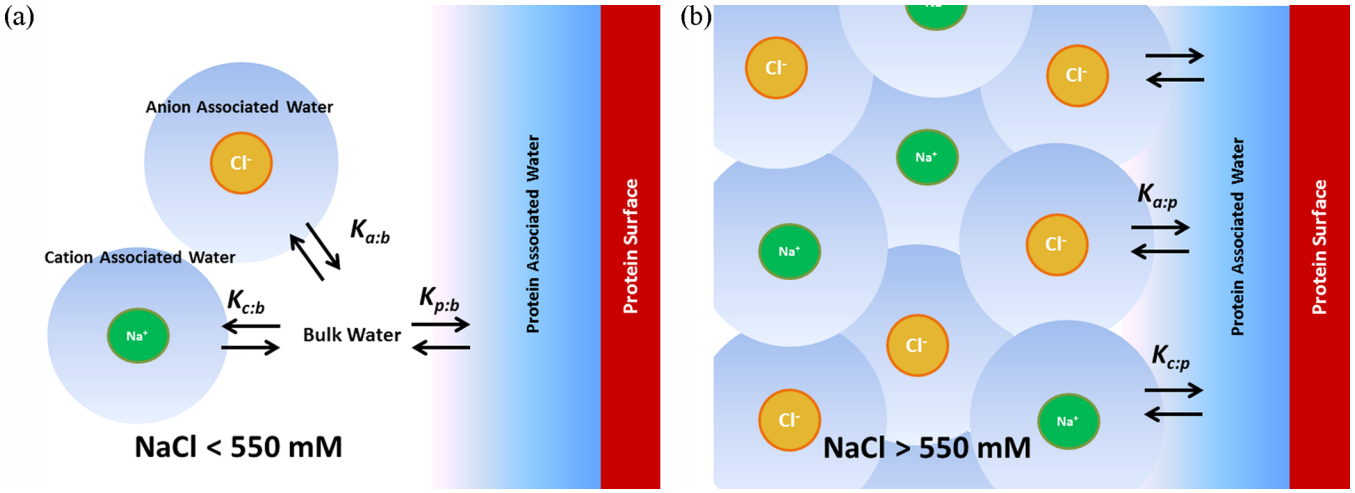

Figure 5. The competition for water hypothesis for protein hydration layer's interaction with water in the presence of high and medium charge density ions. Below the transition (for NaCl this is 550 mM) the protein's hydration layer is at equilibrium with bulk (negligibly perturbed) water but after 550 mM NaCl, the protein has to compete with the ions for water. This change increases the free energy to hydrate a protein as it unfolds increasing the overall energy required to unfold it and increasing its stability. Where the equilibrium constant between protein associated water and bulk water is $K_{p:b}$; between anion associated water and bulk water is $K_{a:b}$; between cation associated water and bulk water is $K_{c:b}$; between anion associated water and protein associated water is $K_{a:p}$; between cation associated water and protein associated water is $K_{c:p}$.

surface exposed during unfolding would decrease, reducing the overall energy required to unfold the protein.

Interaction between Water, Ions, and Protein. The hydration layer around both salts and proteins has been subject to debate, and the perspective taken by researchers is often dictated by the analytical technique that is applied to measure this phenomenon. For the phenomenological hypothesis proposed here to be correct, the number of water molecules around the ions is more than a single or double layer. The case against water around ions being modified beyond the first water layer is detailed in a review by Huib Bakker.[23] Extended perturbation of water beyond the single or double layer has been proposed before for noble gases and salts.[12,13] The authors contend that water molecules in the hydration layer around ions are dynamic with strong close range interactions but also with a weaker longer range electrostatic component observed in the molecular dynamic simulation of water around ions[13] and that it is the free energy associated with the water perturbed by the ions that is modulating the $\Delta\Delta G_{unfolding}$ value.

The results presented in this paper do not provide evidence of the extent of the hydration layer around proteins but it is worth noting this field is also contentious and currently the source of much debate. Protein functional studies,[24] oxygen-17 magnetic relaxation dispersion experiments,[25] molecular dynamic simulation,[26] and densitometry[27] concluded that the proteins' hydration layer is a monolayer of water with the possibility of weaker perturbation to the second layer of water, and this extends 3−8 Å into the bulk liquid. This has been contradicted by terahertz spectroscopy where an apparent extended hydration layer can be detected around proteins and peptides.[28−30] Extended frequency range depolarized light scattering experiments[31] and ultra-fast two-dimensional infrared spectroscopy[32] also suggest a population of water around lysozyme that extends further than the tightly bound water. To quote Serge Timasheff, "the fact is that there is no rigid shell of water around a protein molecule, but rather there is a fluctuating cloud of water molecules that are thermodynamically affected more or less strongly by the protein molecule". The authors suggest the same can be said for water around ions.

SUMMARY

The phenomenological hypothesis presented in this paper is a three-stage thermodynamic explanation for the effect of "kosmotropes" (higher charged anions) on the thermal stability of lysozyme. In the first stage pairing of ions with oppositely charged side chains had a small but unpredictable effect on stability, in the second stage ions interaction with partial charges on the peptide backbone had a small negative effect on stability and the third stage dictated by competition for water between the protein and the ions stabilized the protein. The hypothesis explains the third stage of stabilization in terms of the energy of hydration of the core of the protein as it unfolds, and it is this that is being modulated by the presence of the ions competing for water increasing $\Delta G_{unfolding}$. The idea of competition for water to hydrate the interior of the protein as it unfolds is related to the mechanism originally proposed by Franz Hofmeister in terms of the "water absorbing effect" of salts.[2]

ASSOCIATED CONTENT

Supporting Information

Figures showing T_m versus salt concentration are provided, as this is a format commonly seen in the literature in this field and is easier to understand for researchers familiar with DSC operation. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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