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Cation-Induced Hydration Effects Cause Lower Critical Solution Temperature Behavior in Protein Solutions

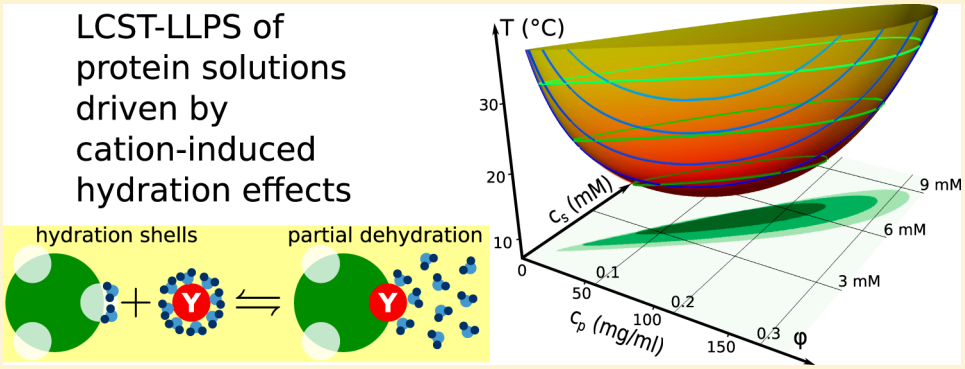
Olga Matsarskaia,[†] Michal K. Braun,[†] Felix Roosen-Runge,*[,‡] Marcell Wolf,[†] Fajun Zhang,*[,†] Roland Roth,[§] and Frank Schreiber[†]

[†]Institut für Angewandte Physik, Universität Tübingen, Auf der Morgenstelle 10, 72076 Tübingen, Germany

[‡]Institut Laue-Langevin, 71 Avenue des Martyrs, 38000 Grenoble, France

[§]Institut für Theoretische Physik, Universität Tübingen, Auf der Morgenstelle 14, 72076 Tübingen, Germany

Supporting Information



ABSTRACT: The phase behavior of protein solutions is important for numerous phenomena in biology and soft matter. We report a lower critical solution temperature (LCST) phase behavior of aqueous solutions of a globular protein induced by multivalent metal ions around physiological temperatures. The LCST behavior manifests itself via a liquid−liquid phase separation of the protein−salt solution upon heating. Isothermal titration calorimetry and zeta-potential measurements indicate that here cation−protein binding is an endothermic, entropy-driven process. We offer a mechanistic explanation of the LCST. First, cations bind to protein surface groups driven by entropy changes of hydration water. Second, the bound cations bridge to other protein molecules, inducing an entropy-driven attraction causing the LCST. Our findings have general implications for condensation, LCST, and hydration behavior of (bio)polymer solutions as well as the understanding of biological effects of (heavy) metal ions and their hydration.

INTRODUCTION

Understanding and tuning the phase behavior and phase transitions of proteins in solution are important goals in many areas of protein science, such as protein condensation diseases or formulation of antibody-based drugs, and the search for tunable pathways to protein crystallization. In particular, liquid−liquid phase separation (LLPS), that is, the separation of protein solutions into a dilute and dense phase, has attracted much attention due to its potential role in protein condensation diseases such as eye cataract and sickle cell anemia.[1−3] Moreover, a metastable LLPS can play an important role in the nucleation of protein crystals.[3−5]

The generic control parameter of LLPS is temperature (T). Intuitively, one expects a mixed system at a higher T due to the dominating entropic contributions and phase separation at a lower T. This so-called *upper critical solution temperature* (*UCST*) behavior occurs, for example, in aqueous solutions of several proteins such as crystallins,[1] lysozyme,[6−8] or β-lactoglobulin.[9] Interestingly, under certain conditions, systems feature a *lower critical solution temperature* (*LCST*), that is, the mixed state of a system occurs at a *lower* temperature than the phase-separated state. It is important to note that, for a given system, UCST and LCST typically form a closed-loop[10] diagram with phase separation between LCST and UCST and intermixing below and above the critical points, respectively. A LLPS with LCST behavior (further referred to as LCST−LLPS) has been frequently observed, for example, in solutions of synthetic polymers[11−14] and elastin-like peptides.[15,16] In these cases, this behavior is attributed to increasing polymer−polymer interactions and polymer contraction upon an increase in temperature. These are accompanied by the release of water molecules surrounding their hydrophobic regions (see, e.g., ref 14). However, in aqueous solutions of globular proteins, LCST−LLPS is a so-far unexplored phenomenon with potentially interesting general implications for underlying control mechanisms of phase behavior.

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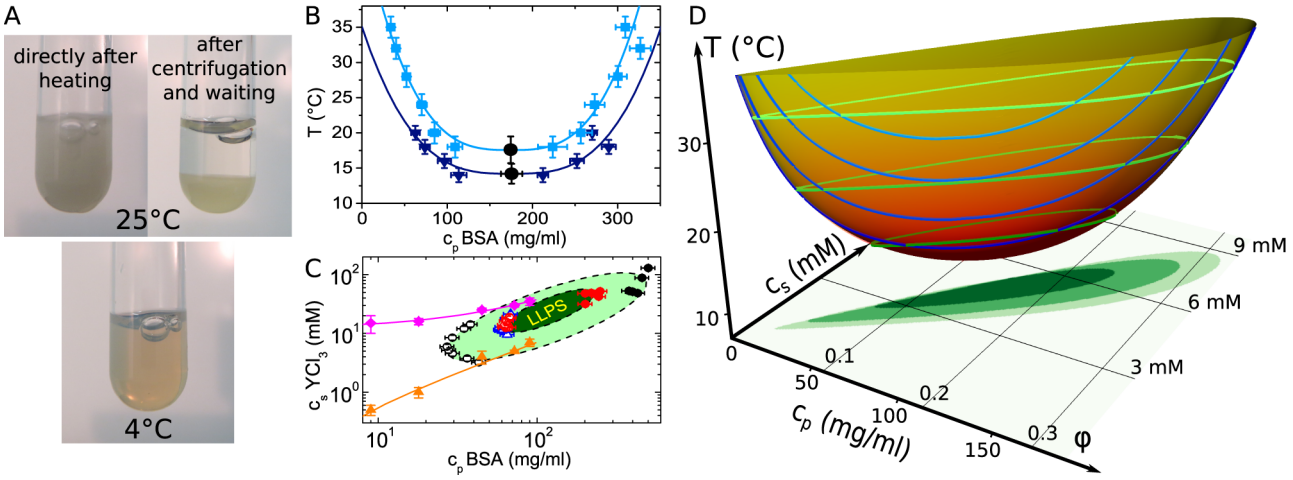

Figure 1. LCST−LLPS in solutions of BSA induced by the addition of YCl_3: (A) A solution with 150 mg/mL BSA and 30 mM YCl_3 is uniform at 4 °C (bottom) and phase-separates at 25 °C (top). (B) The LCST behavior is systematically reflected in the binodals for two system compositions: 150 mg/mL BSA, 30 mM YCl_3 (light blue squares) and 175 mg/mL BSA, 38 mM YCl_3 (dark blue triangles). The two points (c_c, T_c) (black circles) were calculated on the basis of the critical behavior $|c - c_c|/c_c = A((T - T_c)/T_c)^{\beta}$ with $\beta = 0.325$ corresponding to a 3D Ising system. (C) In the isothermal (c_p, c_s)-plane, LLPS of the protein solution into protein-poor and protein-rich phases occurs in a closed region (light and dark green ellipsoids). With an increase in temperature, the LLPS region broadens, reflecting the LCST behavior. Outside the LLPS region, a reentrant condensation is observed between the critical salt concentrations c* and c** (orange triangles and magenta diamonds; see refs 20, 21 for details). (D) LCST−LLPS coexistence surface calculated for a protein model with cation-activated attractive patches with a binding free energy based on the thermodynamical characterization of the cation binding (see Figure 2 and text for details).

Here, we report LCST−LLPS in solutions of globular, hydrophilic proteins in their native conformation, induced by the addition of trivalent cations. On the basis of the thermodynamics of the interaction between protein and metal ions, we present a picture of the underlying mechanism of the LCST−LLPS in our system. Our findings have implications for the general understanding of LCST behavior in protein and (bio)polymer systems. In addition, the thermodynamic characterization of the interaction of heavy or multivalent metal ions with proteins is also relevant for protein crystallization[9] and understanding the biological effects of metal ions,[17] for example, in cancer treatment[18] and bacterial metabolism.[19]

EXPERIMENTAL METHODS

Bovine serum albumin (BSA) and YCl_3 were obtained from Sigma-Aldrich. Protein and salt stock solutions were prepared in degassed ultrapure (18.2 MΩ) water (Merck Millipore). All samples were prepared from stock solutions. LLPS was facilitated via centrifugation. The partitioning of YCl_3 between the two phases (Figure 1C) was determined by anomalous X-ray absorption (ID02 at ESRF; for technical details, see ref 20).

Measurements of isothermal titration calorimetry (ITC) were performed using a MicroCal iTC200 calorimeter (Malvern). YCl_3 solution (3 mM) was titrated into BSA solution (1 mg/mL) at a stirring speed of 750 rpm and a temperature of 24 °C. The injection volumes were 0.4 μL for the first injection and 1 μL for the following ones. Each injection lasted 2 s with 180 s spacings between every two injections. A total of 30 injections were performed. A "low-gain" mode was used for the titration. The heat of dilution of the 3 mM YCl_3 solution was measured separately with the same parameters and subtracted from that of the YCl_3-protein titration as a background. The raw ITC data are shown in the Supporting Information (SI).

Zeta-potential measurements were performed at 15, 20, and 25 °C using a Zetasizer Nano (Malvern), employing phase analysis light scattering. Samples were prepared by mixing appropriate volumes of BSA, MilliQ water, and YCl_3 solution and filled into zeta-potential cuvettes (Malvern). Each sample contained 1 mg/mL BSA. The concentrations of YCl_3 used were from 0.1 to 1 mM. An average zeta-potential value from five independent measurements was calculated per sample.

RESULTS AND DISCUSSION

The LCST−LLPS behavior of BSA−YCl_3 systems can be directly observed by visual inspection: Figure 1A shows a sample with 150 mg/mL BSA and 30 mM YCl_3 at 4 and 25 °C. At low T, the sample is transparent and homogeneous. When heated up to 25 °C, the solution becomes turbid and over time or after centrifugation separates into two liquid phases, protein-rich and protein-poor, with a distinct interface between them. This transition is reversible and the LCST for these sample conditions is around 13 °C (see also the video in the SI). Note that BSA solutions without trivalent salt do not show LLPS, implying that the mechanism behind the phase behavior, and thus the LCST, is linked to ion-induced protein interactions.[22]

To investigate the LCST behavior in more detail, we have determined LLPS binodals for different aqueous BSA−YCl_3 mixtures (Figure 1B). Indeed, all binodals broaden in protein density with increasing T. We prepared several samples with a constant composition of 150 mg/mL BSA and 30 mM YCl_3 or 175 mg/mL BSA and 38 mM YCl_3 at different T's. After LLPS, the concentrations of the protein-poor phases were determined by UV absorption. The protein concentrations of the corresponding protein-rich phases were calculated according to mass conservation. This method allows the determination of the binodals for a fixed sample composition, which is essential because the salt-induced protein interactions might depend on the protein/salt ratio as well as the total concentration of the sample.

The isothermal phase behavior features a reentrant condensation with respect to salt concentration[21] and a LLPS in a closed region that is metastable with respect to

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crystallization.[20] Importantly, as expected for LCST behavior, the LLPS region shrinks with decreasing T (Figure 1C).

Conceptually, at a fixed T, these phenomena can be understood on the basis of a combination of two effects. First, the overall protein charge is reduced and finally inverted. Second, cation bridges between different protein molecules are formed,[9] representing a salt-induced interprotein attraction. This mechanism allows for a qualitative and semiquantitative description of the isothermal phase behavior.[23] However, this isothermal picture cannot explain the observed LCST behavior. A LCST implies an overall more attractive interaction with increasing temperature, which suggests an attraction of entropic origin. Thus, a thermodynamic investigation is needed to address the mechanism of the LCST behavior.

To elucidate the mechanism behind the cation-induced LCST−LLPS, we focus on the cation−protein interaction. As a first step, we measured the zeta potential, ζ, in solutions with 1 mg/mL BSA and varying YCl_3 concentration (Figure 2A).

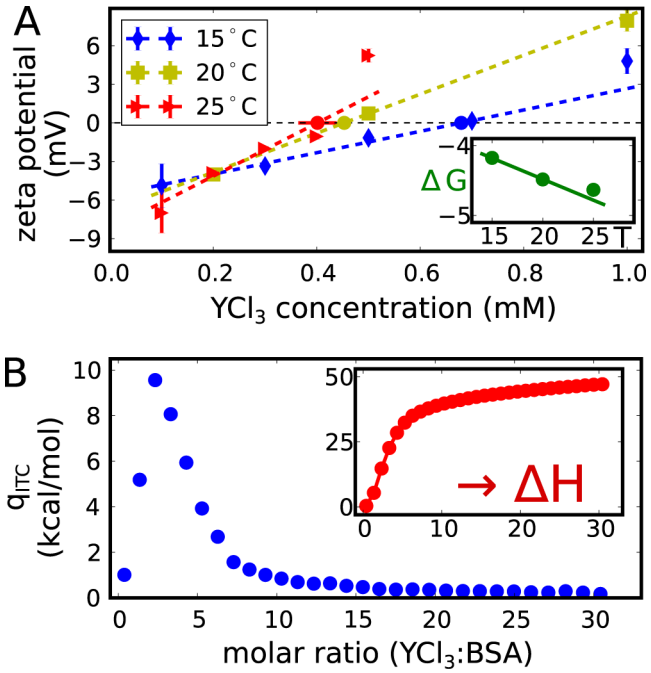

Figure 2. Thermodynamic characterization of cation binding to the protein: (A) Zeta-potential measurements provide an estimation of the average free energy of binding, $\Delta G_b = \Delta H_b - T\Delta S_b$ (inset, error-weighted fit), yielding entropic and enthalpic changes upon binding. Where not visible, error bars are smaller than the symbols. (B) The positive enthalpy change upon cation binding measured by ITC evidences an entropic driving force of the cation binding.

The ζ-profile is consistent with a binding of cations with a positive charge, $\nu_s = 3$, to N independent binding sites on a protein with an initial negative charge Q_0.[22] At a given salt concentration c_s, the net protein charge is

$$Q = Q_0 + N\nu_s \frac{c_s}{c_s + K} \quad (1)$$

The equilibrium constant, K, is linked to the binding free energy, $\Delta G_b' = G_{bound} - G_{free}$, via $K = \exp(\Delta G_b'/k_BT)$. Importantly, at the point of zero charge, $c_{s,0}$, electrostatic contributions to the binding are minimized and the non-electrostatic binding free energy, ΔG_b, can be estimated as

$$\Delta G_b = k_BT \log\left[-c_{s,0}\left(1 + \frac{N\nu_s}{Q_0}\right)\right] \quad (2)$$

Here, we use $N = 6$ and $Q_0 = -9$, which represent reasonable values for the present system.[22] Using values for ΔG_b at different temperatures, the error-weighted linear regression, $\Delta G_b = \Delta H_b - T\Delta S_b$, provides an estimation of the enthalpic and entropic changes upon binding (Figure 2A, inset), being fully equivalent to an error-weighted van't Hoff analysis. The estimated values are summarized in Table 1 and evidence directly the entropically driven character of cation binding.

In a second independent approach, we measured the heat, q_{ITC}, which accompanies the binding process using ITC. The resulting enthalpy curve (Figure 2B) shows the ion binding to be endothermic ($q_{ITC} > 0$). The enthalpy change per binding site, ΔH_b, can be estimated from the heat accumulated in the ITC measurements up to the molar ratio of zero charge divided by $-\nu_s/Q_0$.

The estimated values for ΔH_b in Table 1 from ITC and zeta-potential measurements agree well, which is not expected a priori. It is important to note that zeta-potential measurements are used to extract contributions from cation−protein binding, whereas ITC results encompass all contributions, including long-ranged electrostatics. Thus, the good agreement between these two complementary techniques provides a comprehensive thermodynamic characterization of cation−protein binding and suggests that the energy contributions from the binding dominate over long-ranged electrostatics. The endothermic character of Y^{3+} binding to BSA aligns well with literature data characterizing the complex formation of trivalent cations with amino acids as an endothermic reaction.[24,25]

CONCLUSIONS

The thermodynamic signatures of cation binding obtained from zeta potential and ITC measurements imply an entropy gain upon cation binding to the protein. Because the protein remains in its native compact conformation and no strong entropic changes are expected for its internal degrees of freedom, the entropy gain of the system is dominated by that of the solvent, that is, water.

Thus, focusing on hydration effects during cation binding reveals the entropic driving force (Figure 3): in the unbound state, both the trivalent cations and carboxylic binding sites are surrounded by stable hydration shells. Yttrium(III) ions in aqueous environments have been shown to be surrounded by

Table 1. Binding Parameters in kcal/mol from ITC and the Point of Zero Charge, $c_{s,0}$, from Zeta Potential (ζ) (see text)

	T (°C)	$c_{s,0}$ (mM)	ΔG_b	ΔH_b	$T\Delta S_b$
ζ	15	0.68 ± 0.01	−4.18 ± 0.01		17.3 ± 0.6
ζ	20	0.45 ± 0.01	−4.49 ± 0.01	13.2 ± 0.6	17.6 ± 0.6
ζ	25	0.40 ± 0.04	−4.63 ± 0.06		17.9 ± 0.6
ITC	25			15.7	

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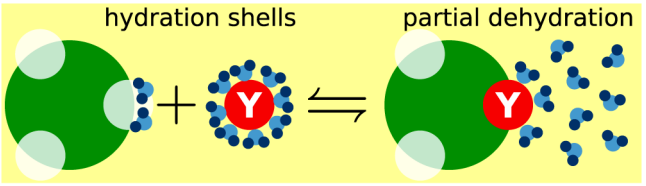

Figure 3. Mechanism of LCST behavior: In the unbound state, the cations and the hydrophilic protein sites are hydrated. Upon cation binding and cation bridging between proteins, water molecules are released and the system entropy is increased.

8−10 water molecules.[26,27] The protein hydration shell amounts to roughly two water molecules per hydrophilic residue.[28] In the cation-bound state, a part of the water molecules from the hydration shells is released and, thus, the system entropy increases. Given the entropy cost of 2 kcal/mol at 300 K to transfer a water molecule from solution into a tight hydration configuration,[29] the observed entropy change of ≈18 kcal/mol (Table 1) corresponds to a physically reasonable number of released water molecules, that is, ≈9. As apparent from the ITC measurements, the endothermic contribution of broken hydrogen bonds dominates the exothermic electrostatic and the coordinative cation−carboxyl interaction. Thus, the entropy effectively drives the cation binding. Similarly, cation bridges between proteins[9,23] formed after cation binding to the protein surface are expected to cause a release of water molecules, although presumably less pronounced. Thus, both cation binding and bridging cause an increase in entropy and, consequently, become more pronounced at a *higher* T. It is important to note that additional entropic contributions such as translational entropy of water should also lead to UCST behavior at higher T in our system. However, this behavior cannot be observed due to the fact that proteins denature above a certain T, thereby changing the system before the UCST is reached.

The thermodynamic characterization of the cation−protein interaction allows us to extend the theoretical modeling from isothermal conditions to a conclusive mechanistic picture of LCST−LLPS: Figure 1D displays the LCST−LLPS phase diagram depending on the two control parameters c_s and T, as calculated from a coarse-grained protein model with $N = 6$ cation-activated attractive patches (for theoretical background, see ref 23). We used the temperature-dependent binding free energy, $\varepsilon_b = \Delta H_b - T\Delta S_b$, derived from zeta-potential measurements (Table 1). For the cation-bridging free energy, $\varepsilon_{uo} = \Delta H_{bridge} - T\Delta S_{bridge}$, we choose $\Delta H_{bridge} = 8$ kcal/mol and $T\Delta S_{bridge} = 17.9$ kcal/mol (at 25 °C). The resulting coexistence surface reproduces the experimental binodals and thus enables a conceptual understanding of the LCST behavior.

Summarizing the thermodynamic mechanism, cation binding to protein functional groups causes a partial dehydration and is driven by the entropy difference between hydration and bulk water. With increasing T, cation binding becomes more effective and, consequently, cation bridges between protein molecules represent a stronger attraction. Thus, the coexisting surface opens up at a higher T as observed in the T-dependent binodals (Figure 1B) and in the differences among different isothermal planes (Figure 1C).

Generally, hydration effects are well known to be important in biology[30] and to affect the phase behavior of polymer and protein solutions.[31,32] However, hydration effects do not per se induce LCST behavior. In fact, lysozyme solutions show UCST−LLPS even under extreme conditions, such as for partially collapsed hydration shells due to pressure[8] or hydration enhanced by glycerol.[7] Apart from the system discussed here, LCST−LLPS in protein solutions has only been reported for hemoglobin in the presence of poly(ethylene glycol).[2] LCST behavior of protein solubility, a so-called retrograde solubility, has been observed for hemoglobin[33] and equine serum albumin in concentrated ammonium sulfate solutions.[34] However, although expected from theoretical considerations, once significant entropic contributions of the solvent are present,[35−37] LCST behavior is still rather uncommon in protein solutions, whereas it is well known in polymer solutions where it is usually explained by effects of (hydrophobic) hydration,[10] accompanied by changes in molecular conformations.[16]

The different behaviors of proteins and (bio)polymers can be rationalized by differences in the surface patterns, in particular the regularity. Synthetic polymers and elastin-like peptides consist of periodically repeating units containing hydrophilic and hydrophobic parts. By contrast, proteins exhibit an irregular distribution of hydrophobic and hydrophilic patches on the solvent-exposed surface. Frequently, LCST is explained by a hydration shell that breaks up at a higher temperature and subsequently allows for polymers to condense. However, a breakup of the hydration shells alone does not explain condensation. A second requirement is an attraction between compatible surface patterns which in turn also stabilizes the dehydration. Thus, LCST behavior in (bio)polymer solutions is realized by a cooperative process of dehydration and surface-pattern-induced attraction, both of which are eventually driven by entropic contributions due to the release of water from a hydration shell into the bulk.

Thus, we present a simple rationalization of the difference between the LCST behaviors of proteins and polymers: the attraction of polymers can be more or less rationally designed through the choice of the repeating unit and the related hydrophobic pattern. By contrast, the conformation of globular proteins evolved over millions of years within the constraint of solubility. Consequently, hydrophobic and protein surface charge patterns generally ensure an overall repulsion of proteins within the biological temperature window. The addition of multivalent cations and thus attraction via interprotein cation bridges disturb the subtle interplay of protein interactions and eventually allow for LCST behavior.

In summary, we have observed a LCST phase behavior in aqueous BSA solutions induced by multivalent cations. The LCST is reflected in a LLPS that takes place above a certain temperature. We have identified the entropy-driven thermodynamic character of the cation−protein binding and protein−protein bridging. The reported LCST behavior can be explained as a result of cation binding and bridging between protein molecules. Importantly, the cation−protein interaction is driven by entropy changes of water molecules that are released from the hydration shells of both cation and protein upon binding. The thermodynamic characterization of the protein−cation interaction in this particular system is important for the general understanding of cation effects in biological and soft matter systems. The experimental and theoretical evidence of the LCST−LLPS presented here along with the picture of the underlying mechanism is promising for a better understanding and control of phase transitions in aqueous protein solutions and has general implications for hydration- and cation-mediated effects in soft matter systems.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpcb.6b04506.

Raw ITC data (PDF)

Movie of LCST−LLPS behavior and its reversibility (AVI)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: roosen-runge@ill.eu. Phone: +33 476207070 (F.R.-R.).

*E-mail: fajun.zhang@uni-tuebingen.de (F.Z.).

Notes

The authors declare no competing financial interest.

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