Extended Law of Corresponding States Applied to Solvent Isotope Effect on a Globular Protein

Saskia Bucciarelli,† Najet Mahmoudi,*,† Lucía Casal-Dujat,† Marie Jéhannin,† Corinne Jud,‡ and Anna Stradner*†

†Physical Chemistry, Department of Chemistry, Lund University, SE-22100 Lund, Sweden
‡Adolphe Merkle Institute (AMI), University of Fribourg, CH-1700 Fribourg, Switzerland

ABSTRACT: Investigating proteins with techniques such as NMR or neutron scattering frequently requires the partial or complete substitution of D2O for H2O as a solvent, often tacitly assuming that such a solvent substitution does not significantly alter the properties of the protein. Here, we report a systematic investigation of the solvent isotope effect on the phase diagram of the lens protein γB-crystallin in aqueous solution as a model system exhibiting liquid−liquid phase separation. We demonstrate that the observed strong variation of the critical temperature Tc can be described by the extended law of corresponding states for all H2O/D2O ratios, where scaling of the temperature by Tc or the reduced second virial coefficient accurately reproduces the binodal, spinodal, and osmotic compressibility. These findings highlight the impact of H2O/D2O substitution on γB-crystallin properties and warrant further investigations into the universality of this phenomenon and its underlying mechanisms.

Since its first production by Lewis and MacDonald in 1933 and despite its low natural availability (at one part in about 6500 parts of light water),1 heavy water (D2O) and its biological effects have been investigated extensively.7 Studies included, for example, the cytotoxic and cytostatic activities of D2O against human cancer cells,1 its contradictory effects on cellular secretion,4 and the absence of retinal toxicity when D2O is used in bovine and human retina perfusion studies.8 Furthermore, solvent isotope effects in protein studies have contributed to a deeper understanding of protein structural stability. Indeed, secondary structure studies of disparate proteins have shown that the folded state is mostly stabilized in D2O.6−11 Additionally, D2O was shown to increase protein stability against thermal denaturation.12−14

Some experimental techniques, such as nuclear magnetic resonance (NMR), spectroscopic techniques,15,16 and neutron scattering,17,18 commonly used to investigate protein internal and solution structure and dynamics often rely on solvent isotope substitution, partially or completely replacing H2O by D2O. In coherent neutron scattering methods, deuterium is used instead of hydrogen in order to decrease the background arising from the incoherent scattering of the latter or to perform contrast variation studies on multicomponent systems.19 In infrared spectroscopy,15 vibrational circular dichroism20 and NMR,21 the signal from water absorption bands interferes considerably with that from protein molecules, thus making the use of a deuterated solvent necessary. In interpreting the results of such measurements, the effects of solvent isotope on protein colloidal stability are frequently left out and it is often assumed that proteins have essentially the same solution structure in H2O and D2O. Earlier work, however, has shown that lysozyme, a globular protein with a short-range attractive interaction potential, is less soluble in D2O than in H2O,22,23 which is linked to enhanced protein−protein attractions in D2O.24 Stradner et al.25 found that using D2O as solvent for lysozyme strongly affects its colloidal stability. Additionally, the effect of isotope substitution on solvent properties, and consequently on interactions and phase behavior in other colloidal systems such as polymer solutions and surfactant systems, is also well established.19 This clearly calls for an in-depth investigation of the effect of solvent isotope content on the phase behavior and interaction potential governing solutions of globular proteins, in order to help clarify to which extent results obtained in solvents with different isotope content can be compared and used to draw conclusions on living systems, such as cells or the eye lens, which contain only H2O. We have therefore performed a systematic study of the phase behavior and the solution structure of the globular protein γB-crystallin, one of the major components of the eye lens, in aqueous solutions of varying deuterium content.

γB-crystallin, the smallest of the eye lens proteins with a radius of about 1.8 nm is commonly used as a model system for colloidal particles interacting via a short-range attractive interaction potential.26 It displays a complex phase behavior that combines liquid−liquid phase separation and dynamical arrest27−29 with an arrest line that extends into the unstable region below the spinodal, a feature previously also observed in lysozyme.30 The equilibrium phase diagram of the latter was

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shown to follow the extended law of corresponding states (ELCS) of Noro and Frenkel,\textsuperscript{31} whereby the critical point and the connected coexistence curve at various ionic strengths are rescaled using the reduced second virial coefficient $b_2$, defined as the ratio of the second virial coefficient $B_2$ and $B_2^{0}=2\pi\sigma^3/3$, the second virial coefficient of a solution of hard spheres with the same diameter $\sigma$ as the protein. This demonstrates that the critical point and the coexistence curve of lysozyme depend on the integral features of its interaction potential and not on the details of the latter.\textsuperscript{32,33} Moreover, Gripón et al.\textsuperscript{22} reported that in a salt-concentration range of 0.3–0.6 M NaCl, increasing the concentration of NaCl by 0.1 M was found to have the same effect on the solubility of lysozyme as substituting $H_2O$ by $D_2O$.\textsuperscript{22} We may thus speculate that the ELCS applies not only to globular proteins in solutions with different ionic strength but also to solvents with varying hydrogen isotope content.

In this study, we investigate the effect of the gradual substitution of $D_2O$ for $H_2O$ in $γ$B-crystallin solutions on their phase behavior and the strength of their interaction potential. We show, through a combination of transmission measurements, static light scattering (SLS), and small-angle X-ray scattering (SAXS), that increasing the $D_2O$ content of the solvent has a large effect on both and that the validity of the ELCS can indeed be extended to $H_2O/D_2O$ substitution.

We located the metastable binodal (or coexistence curve for liquid–liquid phase separation) and the spinodal of $γ$B-crystallin solutions in five different solvents with varying $H_2O/D_2O$ content (cf. Table 1, cf. Supporting Information (SI) for details). The resulting data is shown in Figure 1a and b. The critical temperature $T_c$ is clearly strongly affected by the amount of deuterium in the solution, as summarized in the inset of Figure 1. Fitting the left ($\phi_c < \phi_r$) and right ($\phi_c > \phi_r$) branches of all binodals and spinodals with

$$T = T_c \left[1 - A \left|\frac{\phi - \phi_c}{\phi_r}\right|^{1/\beta}\right]$$

with individual fitting parameters $A$ and a critical exponent $\beta = 0.33$,\textsuperscript{29} we find that $\phi_r$ on the other hand remains unaffected by $D_2O$ and that the shape of the curves does not depend on solvent isotope content. In fact, the same fitting parameters $A_{\text{left}} = (0.06 \pm 0.02)$ and $A_{\text{right}} = (0.02 \pm 0.01)$ reproduce all the binodals and the same $A_{\text{left}} = (0.39 \pm 0.08)$ and $A_{\text{right}} = (0.08 \pm 0.04)$ reproduce all the spinodals (cf. Figure 1a and b). This becomes even more apparent when scaling the temperature by $T_*$, which causes the curves to superimpose, as shown in Figure 1c and d, reminiscent of the results reported by Gibaud et al.\textsuperscript{32} and by Platten et al.\textsuperscript{33} for lysozyme in solutions with varying ionic strength. These authors have shown that under these conditions, the Noro–Frenkel ELCS applies and that the reduced second virial coefficient $b_2$ can be used to predict the phase behavior of the protein. To test whether the isotope effect on the phase behavior of $γB$-crystallin can also be predicted based on the ELCS, we have experimentally determined $b_2$ from SLS on dilute solutions in $100/0$, $50/50$, and $0/100$ solvent, following the method described by Gibaud et al.\textsuperscript{32} (cf. SI for details). The resulting temperature-dependent $b_2$ values are shown in Figure 2a. At the critical temperature $T_*$, we find $b_2(T_*) = -2.7 \pm 0.5$ for all three solutions under investigation. When scaled to $T_c$, all $b_2$ indeed fall onto one master curve, as shown in Figure 2b. We discuss the following results in light of a simple square-well (SW) potential

$$U_{\text{SW}}(r) = \begin{cases} \infty & r < \sigma \\ -\epsilon & \sigma \leq r \leq \Lambda \sigma \\ 0 & r > \Lambda \sigma \end{cases}$$

(2)

describing the interactions as a combination of a hard core repulsion and a short-range attraction of strength (or well depth) $\epsilon$ and range $\Lambda$. In accordance with earlier results,\textsuperscript{29,30} we take $\Lambda = 1.20$, that is, a range of the attractive interaction potential corresponding to 20% of the protein diameter. $b_2$ is then given by

$$b_2(T^*) = 1 - (\Lambda^3 - 1) \left(\exp\frac{1}{T^*} - 1\right)$$

(3)

where $T^* = k_B T/\epsilon$.\textsuperscript{33} Following the approach of Platten et al.,\textsuperscript{33} we assume a linear dependence of the SW depth on temperature and fit $b_2(T^*)$ with

$$b_2\left(\frac{T}{T_c}\right) = 1 - (\Lambda^3 - 1) \left[\exp\left\{\frac{a T / T_c + b}{T / T_c}\right\} - 1\right]$$

(4)

The fitting parameters $a$ and $b$ were found to be $a = -3.3 \pm 0.5$ and $b = 5.0 \pm 0.5$. Combining eqs 3 and 4, we obtain the depth of the SW as a function of $T/T_c$

$$\frac{\epsilon}{k_B T} = \frac{a T / T_c + b}{T / T_c}$$

(5)

as shown in Figure 2c. Using eq 4, temperatures can universally be converted to $b_2$ and vice versa, as demonstrated in Figure 1c–f, where the binodals and spinodals obtained in all solvents superimpose when plotted in the $T/T^*$–$\phi$ plane, as well as in the $b_2$–$\phi$ plane. The ELCS proposed by Noro and Frenkel,\textsuperscript{31} thus, can indeed accurately reproduce the phase diagram of this globular protein in solutions with different hydrogen isotope content.

To further investigate the validity of the ELCS, we now focus on $S^{-1}(0)$, the inverse static structure factor in the forward direction, related to the osmotic compressibility $\kappa_T$ of the solution. We extracted $S^{-1}(0)$ from SANS data, following the method described in an earlier publication.\textsuperscript{30} Examples of static structure factors $S(q)$ for samples close to $\phi_r$ in 100/0, 50/50, and 0/100 solvents are given in Figure 3a–c, respectively. The critical (low-$q$) parts of the structure factors over a wide range of volume fractions were fitted with the OZ equation

$$S(q, T, \phi) = \frac{S_{\text{crit}}(0)}{1 + q^2 \xi^2_{\text{c}} + S_{\text{non-crit}}}$$

(6)

which is the sum of a strongly temperature-dependent critical component $(S_{\text{crit}}(0)/(1 + q^2 \xi^2_{\text{c}})))$, associated with the short-range attractions, and a temperature-independent noncritical background $(S_{\text{non-crit}})$, assumed to be $q$-independent in the low-$q$ range ($q \ll q^*$) considered here.\textsuperscript{34} As shown in Figure 4c, the

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latter follows the Carnahan–Starling (CS) prediction for \( S(0) \) of hard spheres, that is, it is associated with the hard sphere interactions of the proteins. Moreover, all data points superimpose without further scaling, confirming that the hard core repulsion of the protein remains unaffected by solvent isotope substitution. \( S_{\text{crit}}(0) = S_{0}r^{-\gamma} \), shown in Figure 4b, is the critical component of the static structure factor in the forward direction and \( \xi_s = \xi_{s,0}r^{-\nu} \) is the static correlation length that is unaffected by solvent isotope substitution.

Figure 1. Binodal (left) and spinodal (right) of \( \gamma B \)-crystallin solutions in solvents with varying hydrogen isotope content. (a) and (b) Unscaled data. (c) and (d) Data from (a) and (b) scaled by \( T_c \). (e) and (f) Binodals and spinodals in the \( b_2-\phi \) plane, where \( b_2 \) is the reduced second virial coefficient. The solid lines show the fits to eq 1 and the dotted line marks \( \phi_c \). Right: filled symbols correspond to \( T_{sp} \) from Ornstein–Zernike (OZ) fits to SAXS structure factors (eq 6) and open symbols correspond to \( T_{sp} \) from SLS. Inset: \( T_{c} \) of \( \gamma B \)-crystallin solutions as a function of solvent D\(_2\)O content.

Figure 2. Reduced second virial coefficient \( b_2 \) of \( \gamma B \)-crystallin in solvents with varying deuterium content, (a) as a function of temperature and (b) scaled by \( T_c \). The solid black line shows the global fit (eq 4) to all data sets. (c) Well depth \( \epsilon/(k_B T) = 1/T^* \) of the SW potential as a function of reduced temperature. The dashed black line corresponds to eq 5.
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Figure 3. Critical part of the experimentally determined static structure factors $S(q)$ (data symbols) at $\phi = 0.16$ (close to $\phi_c$) in (a) 100/0, (b) 50/50, and (c) 0/100 solvents, together with the OZ fits to the critical parts of the static structure factors close to criticality (eq 6, lines) at varying $T/T_c$. We then simultaneously fit all data sets over a large range of solvent isotope content, scaled by $T/T_c$. We now focus on low $q$ values and thus length scales significantly larger than the protein size, that is, on the total forward structure factor $S(0) = S_{\text{crit}}(0) + S_{\text{non-crit}}$, shown in Figure 4a for the five hydrogen isotope contents investigated at two different temperatures scaled by $T_c$. $S^{-1}(0)$ exhibits a minimum around $\phi_c$ for a given temperature, due to the critical component, which also exhibits a minimum at the same $\phi$ (cf. Figure 4b). More importantly, the $T_{c}$-scaling causes $S^{-1}(0)$ and $S_{\text{non-crit}}(0)$ corresponding to the different solvents to superimpose, similarly to the phase diagram. The ELCS, using $T/T_c$ or $b_2$ as a scaling parameter thus also applies to the osmotic compressibility.

In summary, the phase boundaries for liquid–liquid phase separation and the osmotic compressibility of aqueous solutions of the short-range attractive globular protein $\gamma$-crystallin scale with the reduced second virial coefficient $b_2$ quantifying protein–protein interactions, through the extended law of corresponding states (ELCS). This thermodynamic scaling confirms the applicability of the ELCS to the equilibrium properties of colloids with short-range attractions and provides an extension of its predictive power to systems with varying hydrogen isotope content. Using a simple square-well (SW) fluid analysis, we moreover show that the well depth, a measure of the strength of the attractions, increases by about 0.26–0.27$\kappa_B T$ when exchanging hydrogen for deuterium in the solvent. This proves that the isotope content of the solvent indeed has a dramatic effect on the protein interactions inducing a shift in critical temperature $T_c$ of 16 K between pure $\text{H}_2\text{O}$ and pure $\text{D}_2\text{O}$. This value is noticeably larger than the shift reported in the transition (or denaturation) temperature $T_n$ of globular proteins, which is 2–6 K when $\text{D}_2\text{O}$ is substituted for $\text{H}_2\text{O}$ in the solvent. Gripon et al. reported a shift in the value of the second virial coefficient, as well as in the solubility of $\text{lysozyme}$ in $\text{H}_2\text{O}$ compared to $\text{D}_2\text{O}$, corresponding to about 7.2 K. This value is equal to the difference between the temperature of maximum density of $\text{H}_2\text{O}$ and $\text{D}_2\text{O}$, again considerably smaller than the value found in our study. It has also been shown that near ambient temperature, $\text{H}_2\text{O}$ has a slightly more disordered structure than $\text{D}_2\text{O}$. The difference between $\text{H}_2\text{O}$ and $\text{D}_2\text{O}$ structure at a given temperature corresponds to the temperature-induced variation of either the $\text{H}_2\text{O}$ or $\text{D}_2\text{O}$ structure by a temperature shift of 5–10 K. Furthermore, at lower temperatures, quantum effects become more pronounced. Combined with the fact that hydrogen bonds in $\text{D}_2\text{O}$ are expected to be stronger than in $\text{H}_2\text{O}$ by 0.1–0.2 kcal mol$^{-1}$ and that their number generally increases with lowering temperature, this leads us to speculate that an improved understanding of the quantum mechanical nature of the hydrogen bond in a biologically relevant temperature regime (270–310 K) may help to elucidate the effects of heavy water on protein folding.
and phase behavior, as well as the toxicity of D2O and its potential as an anticancer agent. The mechanisms behind this large effect of solvent isotope content on the critical point for liquid–liquid phase separation of γB-crystallin reported here are still not fully understood at this moment. Further studies into the underlying causes, as well as to establish whether this is a generic phenomenon also applying to other globular proteins, are thus clearly necessary.

- EXPERIMENTAL METHODS

Following the method described by Thurston,41 γB-crystallin was isolated and purified from fresh calf lenses, purchased from a local slaughterhouse. Final solvents with different H2O/D2O mixing ratios were used in this study (cf. Table 1).

SLS experiments were performed on either a home-built multiangle light scattering instrument42 or on a commercial goniometer system (3D LS Spectrometer from LS Instruments AG3). SLS covers a range of scattering vectors q = [4πn(sin θ/2)]/λ, from 0.001 to 0.003 Å−1, with θ being the scattering angle, n the refractive index of the solvent, and λ the wavelength of the radiation.

Refractive index measurements were performed on an Abbe refractometer at three different λ and extrapolated to the wavelength under consideration.

B2 values were determined by SLS on dilute solutions using the commercial goniometer instrument at a scattering angle θ = 90°. In order to access q values much larger than those covered by SLS, we utilized the SASSX technique. Measurements were performed on a Ganesha 300 XL SASSX System from SASSXLAB with an accessible q range of 0.003–2.5 Å−1. Data was corrected for background radiation, transmission, solvent, and capillary and was normalized with the protein concentration. The c-normalized scattering intensity is expressed as I(q)/c ∼ P(q) S(q), where S(q) is the static structure factor and P(q) ∼ I0(q)/c0 is the form factor with P(q → 0) = 1. I0(q) is the measured intensity of a dilute solution (where S(q) ≈ 1) with concentration c0. Experimentally, the structure factors of concentrated samples are then given by S(q) = [I(q)/c]/[I0(q)/c0].

- ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpclett.6b00593.

Details of sample preparation. Details of binodal, spinodal, and second virial coefficient B2 determination. 3D plot of full data set of Figure 4a and b. (PDF)

- AUTHOR INFORMATION

Corresponding Authors
*E-mail: Najet.Mahmoudi@fkem1.lu.se. *E-mail: Anna.Stradner@fkem1.lu.se.

Present Addresses
(L.C.-D.) Aventura AB, SE-223 63 Lund, Sweden. (M.J.) Department of Applied Mathematics, Research School of Physical Sciences and Engineering, Australian National University, Canberra, Australian Capital Territory 2601, Australia. (C.J.) Agroscope, Institute for Livestock Sciences ILS, CH-1725 Posieux, Switzerland.

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